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REVIEW ARTICLE

Intracellular substrate cleavage: a novel dimension in the biochemistry, biology and pathology of matrix metalloproteinases

Bénédicte Cauwe, and Ghislain Opdenakker

Rega Institute for Medical Research, Laboratory of Immunobiology, University of Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium

Abstract

Matrix metalloproteinases (MMPs), originally discovered to function in the breakdown of extracellular matrix proteins, have gained the status of regulatory proteases in signaling events by liganding and processing hormones, cytokines, chemokines, adhesion molecules and other membrane receptors. However, MMPs also cleave intracellular substrates and have been demonstrated within cells in nuclear, mitochondrial, various vesicular and cytoplasmic compartments, including the cytoskeletal intracellular matrix. Unbiased high-throughput degradomics approaches have demonstrated that many intracellular proteins are cleaved by MMPs, including apoptotic regulators, signal transducers, molecular chaperones, cytoskeletal proteins, systemic autoantigens, enzymes in carbohydrate metabolism and protein biosynthesis, transcriptional and translational regulators, and proteins in charge of protein clearance such as lysosomal and ubiquitination enzymes. Besides proteolysis inside cells, intracellular proteins may also be modulated by MMPs in the extracellular milieu. Indeed, many intracellular proteins exit cells by non-classical secretion mechanisms or by various conditions of cell death by apoptosis, necrosis and NETosis, and become accessible to extracellular proteases. Intracellular substrate proteolysis by MMPs is involved in innate immune defense and apoptosis, and affects oncogenesis and pathology of cardiac, neurological, protein conformational and autoimmune diseases, including ischemia-reperfusion injury, cardiomyopathy, Parkinson's disease, cataract, multiple sclerosis and systemic lupus erythematosus. Since the same MMP may affect physiology and pathology in different and even opposite ways, depending on its extracellular or subcellular localization, an additional layer of complexity is added to therapeutic MMP inhibition. Hence, further elucidation of intracellular MMP localizations and intracellular substrate proteolysis is a new challenge in MMP research.

Keywords: *Oxidative stress; apoptosis; cancer; innate immune defense; cardiopathology; neurodegeneration; autoimmunity; chaperone*

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; AMPA-R, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ATP, adenosine triphosphate; BBB, blood–brain barrier; CNS, central nervous system; DCM, dilated cardiomyopathy; ECM, extracellular matrix; ER, endoplasmic reticulum; F-actin, filamentous actin; G-actin, globular actin; GSH, reduced glutathione; HMGB1/2, High-mobility group box 1/2 protein; hnRNP, heterogeneous nuclear ribonucleoprotein; HSP, heat shock protein; I/R, ischemia-reperfusion; IFN-γ, interferon-γ; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MI, myocardial infarct; MMP, matrix metalloproteinase; MS, mass spectrometry; MT-MMP, membrane-type MMP; NMDA-R1, N-methyl-D-aspartate receptor-1; O-phen, 1,10-phenantroline; PARP, poly (ADP-ribose) polymerase; PC, proprotein convertase; PD, Parkinson's disease; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocytes; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TGN, trans-Golgi network; TIMP, tissue inhibitor of metalloproteinases; TJ, tight junction; TNF-α, tumor necrosis factor-α.

Address for Correspondence: Ghislain Opdenakker, Rega Institute for Medical Research, Laboratory of Immunobiology, University of Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium. Tel: +32 16 337341. Fax: +32 16 337340. Email: ghislain.opdenakker@rega.kuleuven.be

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Introduction: from "nomen est omen" to "fortuna" or "how an ominous name may turn out fortunate"

The family of human matrix metalloproteinases (MMPs) currently consists of 24 different neutral endopeptidases. They play a role in many physiological processes such as reproduction, development, immune functions and tissue repair, but are also involved in pathological conditions such as cancer, inflammation, autoimmune diseases, vascular diseases and neurodegenerative disorders (Sternlicht and Werb, 2001; Egeblad and Werb, 2002; Parks *et al.*, 2004; Milner and Cawston, 2005; Hu *et al.*, 2007; Manicone and McGuire, 2008; Rosenberg, 2009).

Whether it is a good or bad attribute, the name of an enzyme needs to make any reader clairvoyant or interested in its activities. Originally, the enzymes that cleave extracellular matrix molecules (ECM) were named according to substrate conversion. For instance, collagenase was the name given to a collagen-degrading enzyme (Gross and Lapiere, 1962). When we purified a gelatin-degrading enzyme to homogeneity (Masure *et al.*, 1991), we were instructed by the IUPAC Enzyme Commission to name it gelatinase B, because of the used substrate and because a different gelatinase, henceforth named gelatinase A, was already defined (Figure 1). Along similar lines, the stromelysins, matrilysins and membrane-type enzymes were discovered and named. Stromelysin was, in fact, a better name given for a proteoglycan-degrading enzyme than proteoglycanase, because the latter name might wrongly suggest that the glycan structure (and not the protein) is cleaved in the matrix substrate molecule.

At a certain time point, it was decided to rename these enzymes matrix metalloproteinases. This nomenclature and abbreviation as MMP – with a suffix number – was based on the historic order of discovery. This renaming was not accomplished with perfect logic, since it was not decided to strictly adhere to one – e.g. human – species. This led to unfortunate outcomes. For example, today MMP-4, MMP-5 and MMP-6 are no longer on the list. The term metalloproteinase has remained a correct one, as all members have a catalytic Zn^{2+} ion in the active site. The addition of matrix to metalloproteinase was correct in the original context of ECM substrates, but is recently contested by findings that these enzymes contribute also to cytokine, hormone and chemokine processing (Sternlicht and Werb, 2001; Parks *et al.*, 2004) and process many membrane-bound substrates (Cauwe *et al.*, 2007). In a recent review, Butler and Overall (2009b) replaced matrix by multifaceted as a sign that the biology of these enzymes is gradually broadening. In fact, the ominous nature of ECM-eating enzymes has indeed transcended to a higher order of proteases regulating biological control processes. In other words, simple soldiers have become generals and MMPs are now considered multifunctional entities. Unless the whole nomenclature of these enzymes is reconsidered by means of delicate scientific diplomacy, the present adherence in more than 20,000 publications to this imperfect man-made classification has also fortunate side-effects. Indeed, as we will review here, MMPs cleave many "intracellular matrix" molecules (ICM). In fact, if we refer to matrix in its broadest sense, i.e. the organized grid or granular substance with multimolecular interactions, then the name given to these enzymes remains a tribute to many pioneers in the field and integrates, in an improved way, recent findings on the biology and pathology of MMPs.

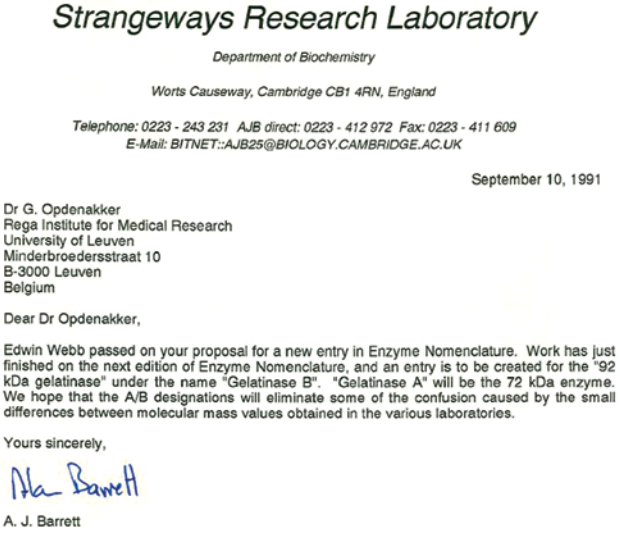
MMPs are multidomain enzymes characterized by a conserved three His Zn^{2+}-binding motif in the catalytic domain and a conserved Met turn following the active site (Bode *et al.*, 1993; Nagase and Woessner, 1999). The three His residues coordinate the Zn^{2+} ion, which interacts with a conserved Cys in the propeptide. This Cys–Zn^{2+} coordination confers latency to the MMPs which are synthesized as inactive pre-pro-enzymes (Visse and Nagase, 2003). The signal peptide is removed during translation, and activation of the pro-enzymes or zymogens requires disruption of the Cys–Zn^{2+} coordination (Figure 3). The active site, now freed of the propeptide, binds substrates and the Zn^{2+} ion becomes available for the binding of a hydrolytic water molecule that is essential for catalysis. Hence, the MMP activation mechanism was termed "cysteine switch mechanism" (Van Wart and Birkedal-Hansen, 1990).

In terms of structure, a typical MMP consists of a propeptide, a catalytical domain, a Zn^{2+}-binding domain, a linker or hinge region of variable length, and a hemopexin domain, which contributes to substrate specificity and to interactions with endogenous inhibitors and cargo receptors (Figure 3) (Piccard *et al.*, 2007). Exceptions to this multidomain organization rule are MMP-7/matrilysin-1, MMP-26/matrilysin-2 and MMP-23, which lack the hinge region and hemopexin domain. In addition, MMP-23/cysteine array (CA)-MMP has a unique Cys-rich domain and an immunoglobulin-like domain at the COOH-terminal side of the metalloproteinase domain. The structures of MMP-2/gelatinase A and MMP-9/gelatinase B contain three fibronectin type II repeats for the binding of gelatin, and MMP-9 is the only MMP to possess a Ser/Thr/Pro-rich O-glycosylated domain, which forms an anchorage site for multiple O-linked sugars (Van den Steen *et al.*, 2006). In addition to the secreted MMPs, six human membrane-bound MMPs (MT-MMPs) exist. These include four type I transmembrane proteins (MT1-MMP/MMP-14, MT2-MMP/MMP-15, MT3-MMP/MMP-16 and MT5-MMP/MMP-24) and two glycosyl phosphatidylinositol (GPI)-anchored proteins (MT4-MMP/MMP-17 and MT6-MMP/MMP-25) (Visse and Nagase, 2003; Nagase *et al.*, 2006).

As outlined in a number of pertinent review articles, MMP levels and activities are strictly controlled in a spatial and temporal fashion by genetic, epigenetic, transcriptional, post-transcriptional and post-translational mechanisms, as well as by zymogen activation, receptor-mediated endocytosis and inhibition by natural inhibitors (Emonard *et al.*, 2005; Nagase *et al.*, 2006; Ra and Parks, 2007; Clark *et al.*, 2008; Brew and Nagase, 2010; Kruger *et al.*, 2010). The general protease inhibitor, α_2-macroglobulin, is the principal MMP inhibitor in the circulation (Baker *et al.*, 2002), whereas the tissue inhibitors of metalloproteinases (TIMPs) are considered to be the key inhibitors in tissues (Brew and Nagase, 2010).

A new challenge in MMP research is to (re)assess substrate repertoires. Logical but infrequently used ways to perform such assessments are by comparing and defining specific activities towards substrates, by in-depth biological studies to define the topologies of MMPs *in vivo* (Olson *et al.*, 2009; Sela-Passwell *et al.*, 2010) and to discover the substrates that are cleaved *in situ* (Agrawal *et al.*, 2006). The first 25 years of MMP research succeeded due to tedious purifications and characterizations of additional MMPs (Nagase and Woessner, 1999; Nagase *et al.*, 1992; Brinckerhoff and Matrisian, 2002). The next milestone was the recognition that MMPs are not just massive ECM wreckers but also cleave many secreted molecules and membrane-associated substrates, which allows them to play sophisticated roles in the modulation of normal cellular behavior, cell–cell communication and tumor progression (McCawley and Matrisian, 2001; Sternlicht and Werb, 2001; Cauwe *et al.*, 2007). A dogma in MMP research has always been that they are secreted or membrane-associated proteases, acting in

the extracellular space or at the cell surface. Many skeptical readers will acknowledge that true secretory enzymes such as MMPs have no business, physiologically speaking, cleaving substrates in a particular intracellular location. However, the presence of identified and functional subcellular localization signals in specific MMPs goes against this skepticism (Si-Tayeb *et al.*, 2006; Eguchi *et al.*, 2008). At a completely different level, it is easily accepted that MMPs may act in the extracellular milieu on many intracellular proteins, once they are actively or passively released from cells. Accumulating evidence suggests that the extracellular and pericellular actions may be complemented by intracellular actions, as well as by cleavage of intracellular proteins in the extracellular space. By comparison of the evolution of literature on membrane-associated substrates versus intracellular substrates (Figure 2, top and middle graphs), it is quite obvious that studies on the cleavage of intracellular proteins by MMPs are lagging behind. Although steady numbers of articles have been published during the last 10 years, a spectacular rise, as observed for membrane-associated substrates, has not yet been observed (Figure 2). Perhaps the idea of obligatory extracellular MMP action still hinders active investigation in this field and prevents novel intracellular substrates from being reported. However, when complementing publications on intracellular substrates with literature on intracellular MMP localization, one can see a rising trend, possibly of an opening field (Figure 2, bottom graph). The rationale of the present review is aimed at summarizing the current data on intracellular MMP localization and activation mechanisms. Furthermore, we will discuss present knowledge on the proteolysis of intracellular substrates by MMPs, both inside cells or in the extracellular milieu, and the



Strangeways Research Laboratory

Department of Biochemistry

Worts Causeway, Cambridge CB1 4RN, England

Telephone: 0223 - 243 231 AJB direct: 0223 - 412 972 Fax: 0223 - 411 609
E-Mail: BITNET::AJB25@BIOLOGY.CAMBRIDGE.AC.UK

September 10, 1991

Dr G. Opdenakker
Rega Institute for Medical Research
University of Leuven
Minderbroedersstraat 10
B-3000 Leuven
Belgium

Dear Dr Opdenakker,

Edwin Webb passed on your proposal for a new entry in Enzyme Nomenclature. Work has just finished on the next edition of Enzyme Nomenclature, and an entry is to be created for the '92 kDa gelatinase" under the name "Gelatinase B". "Gelatinase A" will be the 72 kDa enzyme. We hope that the A/B designations will eliminate some of the confusion caused by the small differences between molecular mass values obtained in the various laboratories.

Yours sincerely,

A. J. Barrett

Figure 1. Instructions from the IUPAC Enzyme Comission.

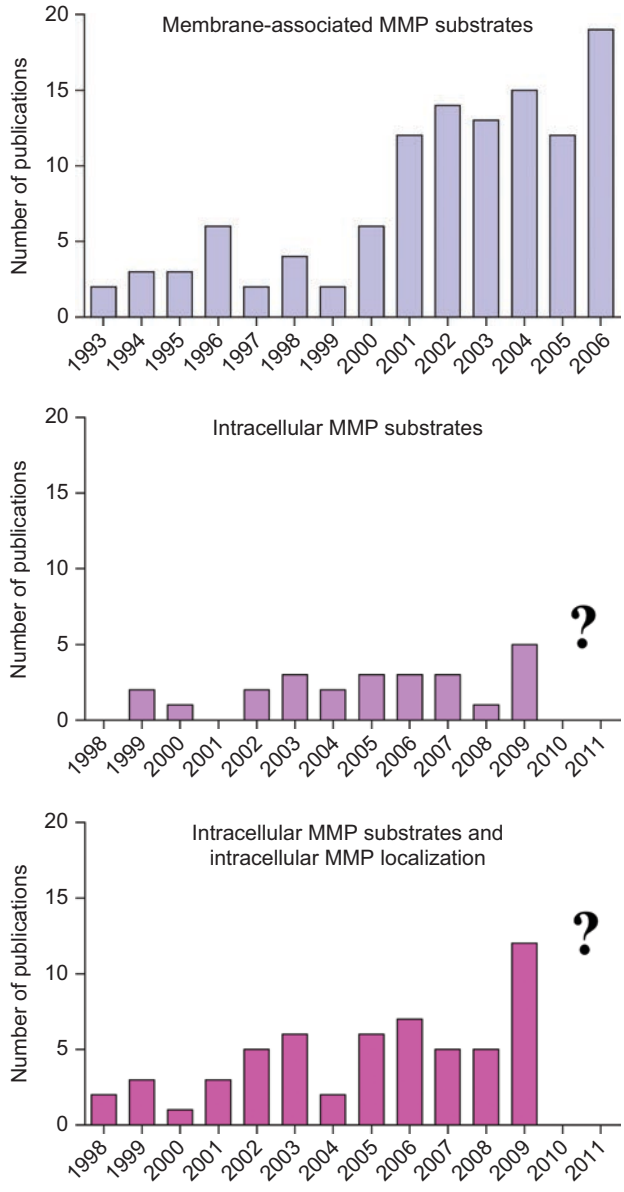


Figure 2. Comparison of the literature evolution for membrane-associated substrates versus intracellular substrates. The graph of membrane-associated substrates is based on a previous review (Cauwe *et al.*, 2007). For literature on intracellular substrates and intracellular MMP localization, the Pubmed database was searched for combinations of matrix metalloproteinase with intracellular, subcellular, intranuclear, nucleus, intracellular substrate, intracellular cleavage and intracellular action key words. By comparison of the number of articles during the same number of years (> 10 years after appearance of the first publication), it becomes clear that literature on intracellular substrates (middle graph) has not known the marked increase of membrane-associated substrate papers (top graph). However, an increase is noticed in the combination of data on intracellular MMP substrates and intracellular localization of MMPs. Hence, research on intracellular substrates and intracellular MMP actions is gaining interest and may catch up with other areas in MMP research.

concomitant physiopathological consequences of these cleavages. We hope that such an integrated view on intracellular substrate proteolysis will provide new insights and stimulate further studies in this novel and exciting field of MMP research.

1. Mechanisms of extracellular versus intracellular MMP activation

Disruption of the coordination between the catalytic Zn^{2+} ion and the conserved Cys in the propeptide is sufficient for latent MMPs to gain catalytic activity (Figure 3). The thiol–Zn^{2+} interaction may be broken by three mechanisms: (1) direct proteolysis of the propeptide by another MMP or protease; (2) modification of the free thiol by physiological reagents such as oxidants and disulfides, or by nonphysiological reagents such as alkylating agents and heavy metal ions; (3) distortion of the catalytic site by allosteric activation or by non-physiological reagents, including organomercurials (4-aminophenyl mercuric acetate or APMA), chaotropic agents (urea), and detergents (e.g. sodium dodecyl sulfate or SDS) (Ra and Parks, 2007; Sela-Passwell *et al.*, 2010; Springman *et al.*, 1990; Park *et al.*, 2010). The latter two mechanisms may allow MMPs to be activated by degradation of their own propeptide, which is called autocatalytic activation (Figure 3). Here, we discuss the physiological mechanisms that may lead to extracellular and intracellular MMP activation. Examples of intracellular activation mechanisms are summarized in Table 1.

1.1. Proteolytic cleavage

Historically, extracellular activation of pro-MMPs has been discovered mainly by *in vitro* studies with individual MMPs. The first examples were the activation of collagenase (MMP-1) by the serine protease plasmin

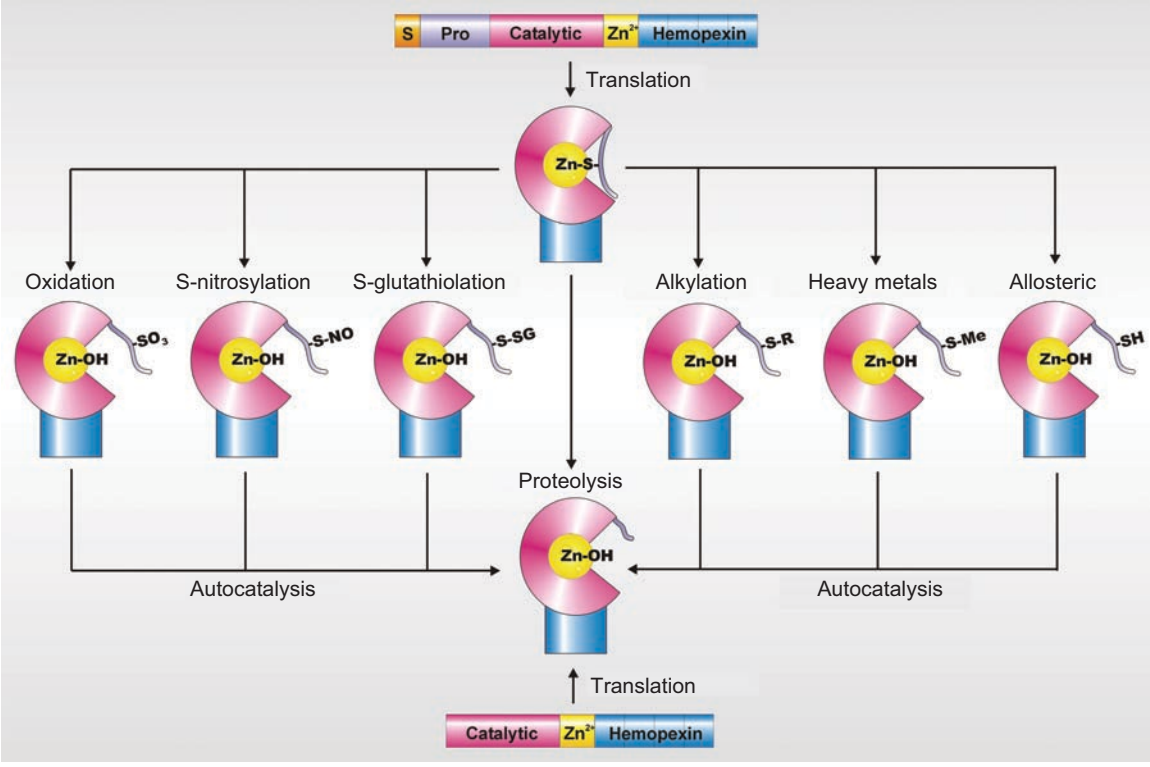


Figure 3. Activation mechanisms of MMPs. A typical MMP structure consists of a signal peptide (S), a pro-domain (Pro), a catalytic domain, a Zn^{2+}-binding domain (Zn^{2+}) and a hemopexin domain. MMPs gain catalytic activity by a mechanism called "cysteine switch activation", in which the coordination between the catalytic Zn^{2+} ion and the conserved Cys in the propeptide is broken to open up the catalytic site for the binding of a hydrolytic water molecule and a substrate. The most common mechanism for the disruption of the thiol-Zn^{2+} coordination is the direct proteolysis of the propeptide by another MMP or protease. Other physiological activation pathways include the modification of the free thiol by physiological reagents such as oxidants (e.g. ROS) and disulfides (e.g. RNS), leading to oxidation, S-nitrosylation or S-glutathiolation of the Cys in the propeptide. Finally, distortion of the catalytic site by the binding of a receptor or a substrate may lead to allosteric activation. Non-physiological activation *in vitro* can be achieved by the modification of the Cys by alkylating agents and heavy metal ions (Me), and by the distortion of the catalytic site by non-physiological reagents, such as organomercurials (APMA), chaotropic agents (urea), and detergents (e.g. SDS). Non-proteolytic activation may lead to subsequent autoproteolytic degradation of the propeptide, which is called autocatalytic activation. Besides posttranslational activation mechanisms, alternative splicing may yield MMP transcripts lacking the signal peptide and prodomain, which are translated into constitutively active enzymes.

Table 1. (Putative) intracellular activation mechanisms of MMPs.

Activation mode	Activator	pro-MMP	References
Proteolytic activation	Furin/proprotein convertases	MMP-11 MT1-MMP MT3-MMP	(Pei and Weiss, 1996; Pei and Weiss, 1995; Yana and Weiss, 2000)
	Serine protease	MMP-3	(Choi *et al.*, 2008)
	MMP-26 (autocatalysis)	MMP-26	(Marchenko *et al.*, 2002)
	MMP-26	MMP-9	(Uria and Lopez-Otin, 2000; Zhao *et al.*, 2003)
	MT1-MMP	MMP-2	(Ip *et al.*, 2007; Yang *et al.*, 2010)
	Caspases	MMP-2	(Yarbrough *et al.*, 2010)
Oxidative stress	Oxidation (HOCl)	MMP-1, -7, -8, -9	(Weiss *et al.*, 1985; Peppin and Weiss, 1986; Saari *et al.*, 1992; Fu *et al.*, 2001; Meli *et al.*, 2003)
	Oxidation (H_2O_2)	MMP-2, -8, -9	(Saari *et al.*, 1992; Burkhardt *et al.*, 1986; Fu *et al.*, 2001; Rajagopalan *et al.*, 1996; Paquette *et al.*, 2003)
Nitrosative stress	S-nitrosylation ($ONOO^-$, NO_2, NO)	MMP-1, -2, -8, -9	(Rajagopalan *et al.*, 1996; Maeda *et al.*, 1998; Okamoto *et al.*, 1997; Gu *et al.*, 2002; Viappiani *et al.*, 2009)
	S-glutathiolation ($ONOO^-$, GSH)	MMP-1, -2, -8, -9	(Okamoto *et al.*, 2001; Viappiani *et al.*, 2009)
Phosphorylation	Alkaline phosphatase	MMP-2	(Sariahmetoglu *et al.*, 2007)
Alternative splicing	No prodomain	MMP-11	(Luo *et al.*, 2002)

GSH, reduced glutathione; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; NO, nitric oxide; NO_2, nitrogen dioxide; $ONOO^-$, peroxynitrite

(Eeckhout and Vaes, 1977; Werb *et al.*, 1977). These were seminal studies because they established a clear link between serine proteases and MMPs and reinforced the concept of enzyme cascades, as these were known for the complement and the coagulation cascades. Many individual studies became linked in a chain, called the ECM protease cascade (Cuzner and Opdenakker, 1999). By a critical analysis of the interrelations between the plasminogen activation system and MMP activations, it became clear that different MMPs can also activate each other. Hence, it was necessary to denominate all the interrelations as a complex network of interactions (Van den Steen *et al.*, 2002). This image of an activation network has evolved into a maze of dynamic protease interactions with other proteases, with inhibitors and with their substrates, which was termed the "protease web" (Overall and Kleifeld, 2006) or the "proteolytic internet" (Kruger, 2009).

Pei and Weiss (1995) were the first to discover a mechanism for intracellular MMP activation. They identified an Arg-X-Arg-X-Lys-Arg recognition motif for the Golgi-associated pro-hormone convertase furin between the pro- and catalytic domain of MMP-11/stromelysin-3. Furin cleaves MMP-11 behind this motif, resulting in an activated form of MMP-11 in the *trans*-Golgi network (TGN), which is subsequently secreted (Santavicca *et al.*, 1996). Likewise, pro-MT1-MMP is efficiently processed by furin and related proprotein convertases into an active protease after recognition of two basic motifs in the enzyme's prodomain (Pei and Weiss, 1996; Yana and Weiss, 2000). Such Arg-X-Lys-Arg motifs are not only found in MMP-11 and MT1-MMP, but also in MMP-23 and in MT1-, MT2-, MT3-, MT4- and MT5-MMP. In addition, either Arg-X-X-Arg or Lys-X-X-Arg motifs are found in all MMPs except in MMP-12/metalloelastase and MMP-7. This suggests that a general mechanism exists for both intracellular and extracellular MMP activation by furin and proprotein convertases (Yana and Weiss, 2000). This was indeed confirmed for MT3-MMP, which co-localizes with furin in the TGN (Kang *et al.*, 2002). MMP-1, however, is not cleaved by furin, and MMP-2 is cleaved to an intermediate activation form, which is inactive. In contrast, MMP-3/stromelysin-1 cleavage by furin yields the anticipated molecular weight (MW) of active MMP-3, but its proteolytic activity was not verified (Cao *et al.*, 2005).

Furin-independent intracellular MMP activation modes also exist. For example, a serine protease different from furin generates an activated form of MMP-3 inside stressed dopaminergic cells (*vide infra*) (Choi *et al.*, 2008). Interestingly, in MMP-26 the latency motif containing the conserved Cys is inactive and MMP-26 is activated by autolytic cleavages (Marchenko *et al.*, 2002). In addition, the major fraction of synthesized MMP-26 remains intracellularly (Strongin, 2006) and may activate pro-MMP-9 in the cytoplasm (Uria and Lopez-Otin, 2000; Zhao *et al.*, 2003). Hence, the protease interaction network may also function inside cells. Indeed, MT1-MMP may be responsible for intracellular MMP-2 activation, since both MMPs were found to co-localize in the nuclei of aggressive hepatocellular carcinoma cells (Ip *et al.*, 2007) and in ischemic cell nuclei, which also contained co-localized furin (Yang *et al.*, 2010). A recent study showed that intracellular MMP activation may also be

performed by caspases. Administration of activated caspase-3 to heart homogenates resulted in increased MMP activity. In addition, a cocktail of activated caspases generated activated forms of MMP-2, as detected by substrate zymography (Yarbrough *et al.*, 2010). However, whereas increased MMP activity in the heart homogenates was confirmed by cleavage of a fluorogenic substrate, this was not verified for the activated forms of MMP-2. This is an essential control, since alkylating agents such as dithiothreitol (DTT) preserve caspase stability but may activate MMPs by catalytic site distortion and autocatalysis.

1.2. Oxidative stress and nitrosative stress

In aerobic organisms reactive oxygen species (ROS) are constantly generated during normal metabolism and in response to both internal and external stimuli. Oxidative stress is caused by imbalances in the production and removal of ROS and is implicated in many pathological settings such as cancer, chronic inflammation, premature labor and stillbirth, ischemia/reperfusion (I/R) injury, atherosclerosis, arthritis and neurodegenerative disorders (Nelson and Melendez, 2004; Roberts *et al.*, 2009). ROS include many different chemical oxidants such as hydrogen peroxide (H_2O_2), superoxide anion ($^{\bullet}O2^-$), singlet oxygen ($^1O_2$), and hydroxyl radicals ($^{\bullet}OH$). Inside the cells, reactive oxygen species are constantly generated as side-products of neutrophil-mediated phagocytosis, by cellular respiration, and by various NADPH oxidases. Similarly, free radical nitric oxide ($NO^{\bullet}$) is a ubiquitous intracellular messenger able to regulate physiological functions (Martinez and Andriantsitohaina, 2009). However, when a disequilibrium occurs between the levels of $^{\bullet}O2^-$ and $NO^{\bullet}$, they may react to form peroxynitrite ($ONOO^-$), a reactive nitrogen species (RNS). Other RNS include $NO^{\bullet}$, nitroxyl (HNO), nitrosonium cation (NO^+), S-nitrosothiols (RSNOs) and nitrogen dioxide (NO_2). By analogy with ROS and oxidative stress, when the generation of RNS in a system exceeds its ability to neutralize and eliminate them, nitrosative stress occurs. In addition, cross-talk between ROS and RNS can exacerbate their capacity to damage DNA, lipids and proteins (Martinez-Pomares *et al.*, 1998; Brandes *et al.*, 2009; Martinez and Andriantsitohaina, 2009).

ROS may play important roles as signaling molecules, regulating many genes, including MMP gene expression and activity (Nelson and Melendez, 2004). Indeed, ROS interact with the Cys thiol, which disrupts the interaction with the catalytic Zn^{2+} ion and leads to autocatalytic activation (Figure 3). Myeloperoxidase, a heme protein secreted by neutrophils, monocytes, and macrophages, uses hydrogen peroxide to generate hypochlorous acid (HOCl) (Hurst and Barrette, 1989). HOCl activates various pro-MMPs, including MMP-1, MMP-7, MMP-8, MMP-9 (Weiss *et al.*, 1985; Peppin and Weiss, 1986; Saari *et al.*, 1992; Fu *et al.*, 2001; Meli *et al.*, 2003). Whereas low levels of HOCl rapidly activate pro-MMP-7, higher concentrations or prolonged exposure inactivate the protease (Fu *et al.*, 2003). This biphasic effect was also observed in the activation of pro-MMP-2 by H_20_2, which also activates pro-MMP-8, pro-MMP-9, but not pro-MMP-1 and pro-MMP-7 (Burkhardt *et al.*, 1986; Saari *et al.*, 1992; Rajagopalan *et al.*, 1996; Fu *et al.*, 2001; Paquette *et al.*, 2003). Interestingly, 30% of the MMP-8 released by synovial-fluid neutrophils in rheumatoid arthritis was in an activated form compared to the pro-MMP-8 form in peripheral blood neutrophils of the same patients (Saari *et al.*, 1992). This suggests that activation of MMP-8 may occur intracellularly before and/or during degranulation.

RNS induce three main post-translational modifications in proteins: (1) S-nitrosylation or the covalent addition of an $NO^{\bullet}$ group to a Cys thiol; (2) S-glutathiolation or the addition of reduced glutathione (GSH) or other low-MW thiols to a Cys thiol; and (3) tyrosine nitration or the addition of an NO_2 group to Tyr residues (Martinez and Andriantsitohaina, 2009). Hence, similar to ROS-mediated activation, RNS modification causes activation by disrupting the latency-inducing Cys-Zn^{2+} coordination (cf. Figure 3) (Ali and Schulz, 2009). Various pro-MMPs can be activated by S-nitrosylation *in vitro*, including MMP-1, MMP-2, MMP-8, MMP-9 (Rajagopalan *et al.*, 1996; Okamoto *et al.*, 1997; Maeda *et al.*, 1998; Gu *et al.*, 2002; Viappiani *et al.*, 2009). So far, activation by S-nitrosylation *in vivo* was only shown for MMP-9 during cerebral ischemia (Gu *et al.*, 2002). In addition, the nitrosylated thiol was oxidized further to a sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid. This irreversible modification may cause prolonged activation of the enzyme under pathological conditions and this progression represents a graded transition from physiological signaling functions to pathological nitrosative and oxidative stress, and finally, toxicity (Hess *et al.*, 2005). Activation by S-glutathiolation (with $ONOO^-$ and GSH) *in vitro* was demonstrated for pro-MMP-1, pro-MMP-2, pro-MMP-8 and pro-MMP-9 and it was more pronounced than activation with $ONOO^-$ alone (Okamoto *et al.*, 2001; Viappiani *et al.*, 2009). The high cellular concentrations of GSH under physiological conditions suppress the activation of pro-MMPs, whereas prolonged and sustained inflammation will result in high levels of pro-MMPs as well as ROS and RNS, which may tip the balance to glutathiolation and activation of MMPs (Okamoto *et al.*, 2001). The biphasic effect observed with oxidative activation (*vide supra*) is also detected with S-glutathiolation, suggesting that ROS and RNS may exert temporal control over MMP activity (Viappiani *et al.*, 2009). Interestingly, RNS also inactivate TIMP-1, TIMP-2 and TIMP-4 (Frears *et al.*, 1996; Brown *et al.*, 2004; Chakraborti *et al.*, 2004; Donnini *et al.*, 2008),

which further enhances MMP action. Surprisingly, both the groups of Schulz and Okamoto report nitrosative activation without the shift to the lower-MW activation form, expected after removal of the propeptide. This suggests that autocatalytic propeptide proteolysis does not always occur and that higher MW forms may indeed be active. Hence, Schulz and coworkers state that the nomenclature of "latent pro-enzymes" incorrectly assumes that only the lower-MW species are active (Schulz, 2007; Ali and Schulz, 2009). Here, we will refer to the high MW forms of MMPs as the pro-forms and avoid using the terminology of "latent" or "inactive" forms, as these forms may be active in an oxidative context or even under physiological levels of ROS and RNS. The low MW forms (LMW) will be termed activated forms.

In conclusion, modification of the conserved Cys can activate MMPs both outside and inside cells. The conservation of the Cys residue implies that these mechanisms may be applicable to most MMPs. Severe oxidative damage will ultimately lead to apoptosis and cell death, which has been shown to accompany many pathological conditions, such as cancer, cardiovascular diseases and neurodegenerative disorders (Martinez and Andriantsitohaina, 2009; Brandes *et al.*, 2009). These are all conditions in which intracellular MMP activities affect disease, which will be discussed in §3.

1.3. Phosphorylation

Phosphorylation is a post-translational modification that typically regulates the activity of intracellular proteins and is of major importance for intracellular signal transduction and regulation of cellular function. The phosphorylation status of a protein is regulated by the balanced action of many protein kinases and phosphatases. Phosphorylation by protein kinase C diminishes MMP-2 activity *in vitro*, whereas dephosphorylation with alkaline phosphatase significantly enhances its activity (Sariahmetoglu *et al.*, 2007). MMP-2 contains 29 potential phosphorylation sites. All five sites that were phosphorylated in recombinant human MMP-2 purified from mammalian cells could be found on accessible residues at the surface of the protein. MMP-2 isolated from HT1080 cells showed phosphorylation of Thr, Ser and Tyr residues. Since most of the phosphorylation sites are within the fibronectin domain, which is essential for substrate binding, phosphorylation may also affect substrate affinity and specificity. Furthermore, phosphorylation may affect intracellular trafficking, protein stability and protein–protein interactions, as is the case for intracellular signaling and adaptor molecules (Sariahmetoglu *et al.*, 2007). Interestingly, MT1-MMP is also phosphorylated in its cytoplasmic domain, and impaired phosphorylation of Tyr573 inhibits tumor cell proliferation in three-dimensional matrices *in vitro* and tumor growth *in vivo* (Nyalendo *et al.*, 2008). Hence, investigation of the effects of phosphorylation on MMP activity and determination of the phosphorylation status of intracellular MMPs in homestatic conditions or under cellular stress may yield information on the regulation of intracellular MMP activity.

1.4. Alternative splicing

Besides post-translational regulation, MMP activity may also be regulated by post-transcriptional regulation. Indeed, for MMP-11 an additional gene promoter was found that is inducible and controls the expression of a novel MMP-11 transcript in cultured cells and in placenta (Luo *et al.*, 2002). This transcript encodes a 40 kDa isoform that lacks both the signal peptide for secretion and the prodomain (Figure 3). As a consequence, a constitutively active isoform of MMP-11 is present in the intracellular milieu. This isoform is as active as the secreted one but produced in 20-fold lower amounts. Surprisingly, human MMP-11 does not degrade the classical components of the ECM, unlike other MMPs and unlike mouse MMP-11 (Noel *et al.*, 1995). Remarkably, Luo *et al.* (2002) found a Met codon (for the initiation of a second transcript) within the DNA sequence of 10 other MMPs, suggesting that alternative splicing and promoter usage may be a characteristic of many MMPs. Indeed, splice variants lacking an NH_2-terminal secretory signal peptide have also been found for MMP-2 (e.g. accession number AL832088) (Sariahmetoglu *et al.*, 2007). In addition, a soluble versus transmembrane form of MT3-MMP was formed by alternative splicing (Matsumoto *et al.*, 1997) and because MMP-23 lacks an NH_2-terminal signal peptide, it was also suggested to function intracellularly (Velasco *et al.*, 1999). Alternative transcripts have been found for various other MMPs and characterization of the associated isoforms may shed new light on intracellular locations and activities of many MMPs (Luo *et al.*, 2002).

2. Intracellular substrate detection by degradomics

Whereas proteomics is the study of the entire ensemble of proteins produced in a biological system or by an organism, degradomics aims at the characterization of the complete set of substrates, i.e. the repertoire or "degradome" of a particular proteinase in a specific cell, tissue or organism (Lopez-Otin and Overall, 2002). During the last decade, many proteomic approaches have been adapted for degradomics and development of novel degradomics techniques has boomed. Since

Table 2. Degradomic approaches for the identification of MMP substrates and the determination of cleavage sites.

Method	Short description	Advantages	Disadvantages	References	MMP studies	MMP	Type[1]
GEL-BASED DEGRADOMICS							
2D-PAGE (two-dimensional polyacrylamide gel electrophoresis)	Proteolyzed and control protein mixtures are separated by two orthogonal electrophoresis steps: IEF according to pI and SDS-PAGE according to MW. Spots that disappear or decrease in abundance after cleavage are potential substrates. These may be picked out of the gel for identification by MS/MS after in-gel tryptic digest.	- decreases biological sample complexity *vs.* one-dimensional PAGE - identification of *in vivo* substrates - influence of PTMs on cleavage - inexpensive	- restricted to abundant and soluble proteins with average pI and MW - not quantitative: high intergel variability - labor intensive (many gels needed) - no cleavage sites identified	(O'Farrell, 1975; Friedman *et al.*, 2009)	(Hwang *et al.*, 2004; Descamps *et al.*, 2005; Hemers *et al.*, 2005; Sawicki *et al.*, 2005; Kim *et al.*, 2006)	MT1-MMP MMP-9 MMP-7 MMP-2	*In vitro* + *In vivo*
2D-DIGE (two-dimensional difference gel electrophoresis)	Cleaved and uncleaved protein mixtures are labeled with different fluorescent labels, mixed and analyzed by 2D-PAGE. Fluorescence ratios of individual spots quantify degradation. The use of a mix of both samples as an internal standard, labeled with a third dye, allows for inter-gel comparisons.	- quantitative: intergel variability reduced by internal standard + higher sensitivity of fluorescent signals - identification of *in vivo* substrates - influence of PTMs on cleavage	- restricted to abundant and soluble proteins with average pI and MW - labor intensive - no cleavage sites identified	(Minden *et al.*, 2009; Unlu *et al.*, 1997)	(Greenlee *et al.*, 2006)	MMP-2 MMP-9	*In vivo*
Diagonal gel electrophoresis	A protein mixture is first separated according to MW by SDS-PAGE. Gel lanes are renaturated separately and subjected to in-gel proteolysis by an exogenously added protease. Gel lane proteins are subsequently separated by a second SDS-PAGE, perpendicular to the first, yielding the intact proteins on a diagonal and fragment spots under the diagonal.	- no protein precipitation by IEF - decreases biological sample complexity vs. one-dimensional PAGE - technically simple and inexpensive	- incomplete or incorrect renaturation of substrates - limited access to substrates - restricted to abundant proteins - no cleavage sites nor *in vivo* substrates identified	(Nestler and Doseff, 1997)	ND	NA	NA
Shotgun proteomics	Proteolyzed and control protein mixtures are separated in adjacent SDS-PAGE lanes which are sliced into multiple bands. Proteins in each band are identified by LC-MS/MS after in-gel tryptic digests. Proteins are defined as substrates if the empirical MW displays a reduction by at least 20% compared with the theoretical MW, or if proteins are identified in clearly different gel slices.	- no protein precipitation by IEF - increased sensitivity compared to 2D-PAGE systems - identification of *in vivo* substrates	- complex bioinformatic and manual analysis of multiple MS/MS spectra - restricted to abundant proteins - only approximation of cleavage sites	(Thiede *et al.*, 2005)	ND	NA	NA

Table 2. continued on next page

Table 2. Continued.

Method	Short description	Advantages	Disadvantages	References	MMP studies	MMP	Type[1]
PROTOMAP (protein topography and migration analysis platform)	Shotgun proteomics with visualization of protein-specific peptide coverage from all the individual slices in peptographs, which show the sequence coverage for a given protein on the *x*-axis (NH_2 - to COOH-terminus) and the MW on the *y*-axis (high to low MW)	- advantages of shotgun proteomics - facilitated and more quantitative data analysis than shotgun proteomics - comparison of unlimited number of samples, allowing for kinetic analysis	- restricted to abundant proteins - only approximation of cleavage sites	(Dix *et al.*, 2008)	ND	NA	NA
2DD (two-dimensional degradomics)	A protein mixture is first separated by ion exchange chromatography (IEX) according to pI. All IEX fractions are concentrated and incubated in the absence or presence of a protease. Digested and undigested fractions are analyzed in adjacent SDS-PAGE lanes. Protein bands that disappear or decrease, and novel fragments that appear in the protease-digested fraction are potential substrates and identified by MS/MS after in-gel tryptic digests or by Edman degradation after electroblotting onto PVDF, which directly unveils the sequence of neo-NH_2-termini.	- reduced sample complexity by fractionation - no protein precipitation by IEF - lower abundance substrates identified compared with other gel-based approaches - technically simple and inexpensive	- no *in vivo* substrates identified - labor intensive (many gels needed)	(Cauwe *et al.*, 2009)	(Cauwe *et al.*, 2009)	MMP-9	*In vitro*
DEGRADOMICS APPROACHES BASED ON QUANTITATIVE LABELING							
Reductive dimethylation	The proteolyzed and control sample are differentially labeled by reductive dimethylation with heavy (deuterium) or light (H) formaldehyde. Relative quantification of differentially mass-tagged proteins by MS/MS after trypsinization.	- technically simple and inexpensive	- high sample complexity by labeling of all free amines - no cleavage sites identified - high-end mass spectrometer needed	(Hsu *et al.*, 2003)	(Kleifeld *et al.*, 2010)	MMP-2	*In vitro*
$^{18}O/^{16}O$-labeling	The proteolyzed and control protein mixtures are trypsinized in the presence of ^{18}O water or natural ^{16}O water, which leads to differential incorporation of two light or two heavy ^{18}O's in the peptide COOH-termini.	- technically simple and inexpensive	- only two samples can be compared - high sample complexity - no cleavage sites identified - high-end mass spectrometer needed	(Staes *et al.*, 2004)	ND	NA	*NA*

Table 2. continued on next page

Table 2. Continued.

Method	Short description	Advantages	Disadvantages	References	MMP studies	MMP	Type[1]
ICAT (isotope-coded affinity tagging)	Cleaved and control protein mixtures are labeled with biotin-tagged reagents that differ in isotopic composition ($C^{13/12}$ or deuterium), pooled and digested with trypsin. Biotinylated peptides are pulled out and quantified by analysis of peak pairs in the MS spectra.	- quantification of relative protein abundances - reduced sample complexity	- consumption of label by high abundance proteins - proteins/peptides with few or no Cys will be missed - no cleavage sites identified - high-end mass spectrometer needed	(Gygi *et al.*, 1999)	(Butler *et al.*, 2008; Dean and Overall, 2007; Dean *et al.*, 2007; Tam *et al.*, 2004)	MT1-MMP	*In vitro*
iTRAQ™ (isobaric tags for relative and absolute quantitation)	Cleaved and control protein mixtures are trypsinized and all amino groups are labeled with iTRAQ™ labels that are chemically identical but fractionate differently during MS/MS, generating different spectral peaks. Relative quantification is obtained by peak-height analysis.	- more identifications than ICAT without Cys bias - identification of *in vivo* substrates - multiplexed quantification	- consumption of label by high abundance proteins - expensive - no cleavage sites identified - no discrimination between cellular and serum proteins - high-end mass spectrometer needed	(Ross *et al.*, 2004)	(Dean and Overall, 2007)	MMP-2	*In vitro*
SILAC (stable isotope labeling by amino acids in cell culture)	Duplicate cell cultures are grown in media containing stable isotope-labeled amino acids (e.g. ^{12}C - or ^{13}C-labeled Leu, Arg, Lys), subjected to a (cellular or exogenous) protease and subsequently pooled for MS/MS analysis. Relative quantification is obtained by peak-height comparison.	- labeling more efficient (100%) compared with ICAT and iTRAQ™ - discrimination between cellular and serum proteins - less errors by early pooling of samples	- no *in vivo* substrates identified - no cleavage sites identified - high-end mass spectrometer needed	(Ong *et al.*, 2002)	ND	NA	NA
SILAC MICE	Complete SILAC labeling of mice with a diet containing natural or ^{13}C-Lys over four generations.	- identification of *in vivo* substrates - *in vivo* kinetic analysis of cleavage	- time-consuming (breeding) - expensive - no cleavage sites identified - high-end mass spectrometer needed	(Kruger *et al.*, 2008)	ND	NA	NA
LABEL-FREE DEGRADOMIC APPROACHES							
2D LC-MS/MS (two-dimensional liquid chromatography -MS/MS)	Proteolyzed and control protein samples are not pooled but analyzed separately by two independent rounds of liquid chromatography. Differences between cleaved and uncleaved samples are identified by spectral counting.	- reduced sample complexity - independent of labeling efficiency - inexpensive	- highly dependent on reproducibility because of separate sample analysis - time-consuming - favors high abundance proteins - no cleavage sites identified	(Stoll *et al.*, 2007)	(Vaisar *et al.*, 2009)	MMP-9	*In vitro*

Table 2. continued on next page

Table 2. Continued.

Method	Short description	Advantages	Disadvantages	References	MMP studies	MMP	Type[1]
nano-UPLC-MS^E	Proteolyzed and control protein samples are not pooled but analyzed separately by RP-UPLC separation and coupled to MS/MS. MS data acquisition is achieved by the application of MS^E technology, which is based on continuous switching between low and high collision energy to collect precursor ion masses (low collision) and fragment ions (high collision) at ultrashort time intervals. Fragment ions are assigned to their corresponding precursor ion according to their similar retention times on ion chromatographs, which allows the creation of MS/MS spectra to obtain sequence and protein identifications.	- powerful high-throughput comparison of samples - collection of more precursor ions and fragmentation data by MS^E - independent of labeling efficiency - inexpensive - multiplexed quantification	- highly dependent on reproducibility because of separate sample analysis - complex data analysis - no cleavage sites identified - high-end mass spectrometer needed	(Xu *et al.*, 2008)	(Xu *et al.*, 2008)	MMP-9	*In vitro*
IDENTIFICATION OF NEO-NH_2-TERMINI							
COFRADIC (combined fractional diagonal chromatography)	Negative selection is achieved by acetylating primary amines followed by trypsin digestion and capping of internal NH_2-peptides with TNBS, strongly increasing their hydrophobicity. Unmodified NH_2-terminal peptides elute earlier after RP-HPLC and are selected for MS analysis. Trypsin digestion in the presence of light ^{16}O or heavy ^{18}O water or SILAC labeling allows for quantification. By using trideutero-acetylation, the ϵ-amines of Lys and the *in vivo* free NH_2-terminal α-amines can be distinguished from *in vivo* acetylated NH_2-termini.	- powerful and quantitative high-throughput identification of substrates and cleavage sites - reduced sample complexity by neo-NH_2-termini re-enrichment - identification of *in vivo* substrates - cleavage sites identified	- inappropriate peptide lengths and poor ionizability limit the number of identifications - incomplete capture of internal peptides can lead to false positives - labor and equipment intensive - high-end mass spectrometer needed	(Gevaert *et al.*, 2003; Staes *et al.*, 2008; Van Damme *et al.*, 2005)	ND	NA	NA
Positional proteomics	Similar to COFRADIC but capture of internal peptides is achieved by incubation of the acetylated and trypsinized cell lysate with amine-reactive NHS-activated beads.	- fast and simple high-throughput identification of substrates and cleavage sites - reduced sample complexity by neo-NH_2-termini re-enrichment - identification of *in vivo* substrates - cleavage sites identified	- inappropriate peptide lengths and poor ionizability limit the number of identifications - incomplete capture of internal peptides can lead to false positives - not quantitative - high-end mass spectrometer needed	(McDonald *et al.*, 2005)	ND	NA	NA

Table 2. continued on next page

Table 2. Continued.

Method	Short description	Advantages	Disadvantages	References	MMP studies	MMP	Type[1]
TAILS (terminal amine isotopic labeling of substrates)	The proteolyzed and control sample are differentially labeled by reductive dimethylation with heavy or light formaldehyde, or by iTRAQ™ labeling, which labels all free NH_2-termini. Samples are mixed and trypsinized, generating internal peptides which are removed by a highly efficient high MW aldehyde-derivatized polymer. Remaining peptides are then analyzed by LC-MS/MS. May also be combined with SILAC labeling.	- powerful and fast high-throughput identification of substrates and cleavage sites - reduced sample complexity by neo-NH_2-termini re-enrichment - identification of cleavage sites and *in vivo* substrates - aldehyde polymer reduces false positives and improves sample recovery	- inappropriate peptide lengths and poor ionizability limit the number of identifications - incomplete capture of internal peptides can lead to false positives - aldehyde-derivatized polymer is not yet commercially available - high-end mass spectrometer needed	(Kleifeld *et al.*, 2010)	(Kleifeld *et al.*, 2010)	MMP-2	*In vitro*
Positive selection of neo-NH_2-termini	The use of O-methylisourea or subtiligase allows for selective biotinylation of NH_2-termini, which are captured by streptavidin beads and released chemically or enzymatically for LC-MS/MS analysis.	- increased sensitivity compared with negative selection methods by non-selection of endogenously acetylated NH_2-termini - reduced sample complexity by neo-NH_2-termini re-enrichment - identification of *in vivo* substrates - cleavage sites identified	- using O-methylisourea biotinylation of Ser, His, Thr can lead to false positives - subtiligase does not recognize Pro + low specificity for Glu and Asp - subtiligase labeling requires large amounts of material - no identification of cleavage before cyclized Gln, Cys and Glu - not quantitative - high-end mass spectrometer needed	(Timmer *et al.*, 2007; Mahrus *et al.*, 2008)	ND	NA	NA
INTERACTION-BASED DEGRADOMIC APPROACHES							
Exosite scanning	Recombinant MMP exosites are used to fish for interacting substrates in a proteome. This can be combined with a yeast two-hybrid screen, or alternatively domains that are used as 'bait' can be tagged to facilitate purification of the exosite and bound proteins for identification by MS/MS.	- greatly reduced sample complexity - additional information on non-catalytic domain functions	- not all interacting proteins are substrates - yeast two-hybrid is slow and yields many false positive and false negative results + only binary complexes identified - no cleavage sites or *in vivo* substrates identified	(Overall *et al.*, 2002)	(McQuibban *et al.*, 2001)	MMP-2	*In vitro*
ICDC (inactive catalytic domain capture)	A catalytically inactive domain mutant binds and captures substrates in a complex protein mixture. This can be combined with a yeast two-hybrid screen, or alternatively the inactive catalytic domains can be tagged to facilitate purification of the protein complex for identification by MS/MS.	- greatly reduced sample complexity	- yeast two-hybrid is slow and yields many false positive and false negative results + only binary complexes identified - no cleavage sites or *in vivo* substrates identified	(Overall *et al.*, 2004)	(Overall *et al.*, 2004)	MT1-MMP	*In vitro*

Table 2. continued on next page

Table 2. Continued.

Method	Short description	Advantages	Disadvantages	References	MMP studies	MMP	Type[1]
PEPTIDE LIBRARY-BASED CHARACTERIZATION OF SUBSTRATE AND CLEAVAGE SITE SPECIFICITIES							
Phage display substrate	Phages displaying random peptides are fused to a ligand and immobilized on an affinity support through a receptor. After treatment with a protease, substrate peptides are cleaved, releasing the concordant phages from the solid phase, which are then used to infect F-positive bacteria to be amplified for a next selection step. After several rounds of selection, phages are cloned and DNA of the individual phages is amplified to identify the cleavable peptides.	- systematic characterization of substrate cleavage	- no cleavage of the native substrate conformation - labor intensive - limited number of identifications - no cleavage sites or *in vivo* substrates identified	(Smith, 1985; Deperthes, 2002)	(Smith *et al.*, 1995; Deng *et al.*, 2000; Chen *et al.*, 2002; Kridel *et al.*, 2001; Ohkubo *et al.*, 2001)	MMP-3 MMP-7 MMP-13 MMP-2 MMP-9 MT1-MMP	*In vitro*
CLiPS (cellular libraries of peptide substrates)	Recombinant peptides bound to fluorescent-probe peptide ligand are displayed on the surface of bacterial cells. Substrate peptide cleavage results in a reduction of cellular fluorescence, which is detected by flow cytometry. Non-fluorescent cells are removed and the enrichment of clones with hydrolyzed substrates is repeated.	- systematic characterization of substrate cleavage - quantitative real-time measurement - influence of PTMs on cleavage	- no cleavage of the native substrate conformation - labor intensive - limited number of identifications - no cleavage sites or *in vivo* substrates identified	(Boulware and Daugherty, 2006)	ND	NA	NA
mRNA display	Starting from a cDNA library, an mRNA transcript is ligated at its 3′ with puromycin. Translation of the puromycin-linked mRNA results in an mRNA-puromycin-peptide fusion product, of which the mRNA is made double-stranded by RT-PCR. The peptide is biotinylated at its NH_2-terminus and immobilized on streptavidin beads. Cleavage by a protease releases the DNA-puromycin-peptide fusion complex and DNA is amplified by PCR, enriching the library with substrate sequences.	- systematic characterization of substrate cleavage	- no cleavage of the native substrate conformation - labor intensive - limited number of identifications - no cleavage sites or *in vivo* substrates identified	(Ju *et al.*, 2007)	ND	NA	NA

Table 2. continued on next page

Table 2. Continued.

Method	Short description	Advantages	Disadvantages	References	MMP studies	MMP	Type[1]
PS-SCL (positional scanning synthetic combinatorial libraries)	Positional scanning libraries are generated by keeping a single position in a peptide constant, while other peptides are diversified with all possible combinations of amino acids, typically excluding cysteine. Cleavage is detected by release of a fluorescent leaving group from the peptide or by removal of a quenching group.	- systematic characterization of substrate cleavage and cleavage sites	- no cleavage of the native substrate conformation - labor intensive - limited number of identifications - no *in vivo* substrates identified	(Lam and Lebl, 1998; Backes *et al.*, 2000; Diamond, 2007)	(McGeehan *et al.*, 1994)	MMP-1 MMP-9	*In vitro*
PICS (proteomic identification of protease cleavage sites)	Proteome-derived libraries are generated by digestion with trypsin, GluC or chymotrypsin. Sulfhydryl groups are then protected with iodacetamide and primary amines are blocked by reductive methylation into tertiary amines. The resulting peptide mixture is incubated with a protease and neo-NH_2-terminal peptides are biotinylated and pulled out by immobilized streptavidin. These peptides are sequenced by LC-MS/MS, yielding the prime-side[2] sequence of the cleavage site.	- fast and robust profiling of protease-active site specificities and subset cooperativity - cleavage sites identified	- no cleavage of the native substrate conformation - modification of all Cys and Lys residues may interfere with peptide-protease interaction - no *in vivo* substrates identified	(Schilling and Overall, 2008)	(Schilling and Overall, 2008)	MMP-2	*In vitro*

IEF, isoelectric focusing; LC-MS/MS, liquid chromatography followed by tandem mass spectrometry; MS, mass spectrometry; MSE, high/low collision energy MS; MW, molecular weight; NA, not applicable; ND, not defined; NHS, N-hydroxysuccinimide; pI, isoelectric point; PTM, post-translational modification; PVDF, polyvinylidene fluoride; RP-HPLC, reversed phase-high pressure liquid chromatography; RT-PCR, real-time PCR; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNBS, 2,4,6-trinitrobenzenesulfonyl chloride; UPLC, ultraperformance liquid chromatography.

[1]Substrate type: describes whether the MMP substrates found in the described degradomics screen are *in vitro* or *in vivo* substrates.

[2]The substrate amino acids surrounding a cleavage site are indicated as Pn … P3-P2-P1 ↓ P1′-P2′-P3′ … Pn′, with the cleavage occurring at the peptide bond between P1 and P1′ (Berger and Schechter, 1970).

excellent and up-to-date reviews exist on this subject (Diamond, 2007; Overall and Blobel, 2007; Doucet and Overall, 2008; Doucet *et al.*, 2008; Van Damme *et al.*, 2008; Agard and Wells, 2009; Butler and Overall, 2009b; Demon *et al.*, 2009; Morrison *et al.*, 2009; Rodriguez *et al.*, 2009; Impens *et al.*, 2010), we here describe briefly the various techniques along with their major advantages and drawbacks, and their application in MMP research (Table 2). In addition, we mention the major peptide-based techniques that were developed to characterize substrate cleavage sites, and provide additional references for the interested reader.

Various degradomics techniques were developed specifically for the identification of intracellular substrates and were applied for the study of particular caspase degradomes (Van Damme *et al.*, 2008; Agard and Wells, 2009; Demon *et al.*, 2009). These applications were intuitively developed on the basis that latent procaspases are activated and act within cells. Since the cascades of apoptosis, necrosis and NETosis may lead to intracellular proteases entering the extracellular milieu, it is rarely questioned that typical extracellular substrates may end up in caspase degradomes. In contrast, although intracellular substrates have been defined as substrates of MMPs, both *in vitro* and *in vivo* (*vide infra*), the physiological or pathological relevance of such cleavages have only started to emerge. From Table 2, it is quite clear that many degradomics methods have not yet been used for the complete identification of MMP substrates. In addition, degradomics screens that identify *in vivo* substrates by direct comparison of wildtype and MMP knockout samples are still under-represented, which is probably also due to the lower reproducibility and high complexity of biological tissues and organ extracts.

In MMP research, degradomic approaches were aimed at identifying extracellular and membrane-bound substrates, and were used mainly to analyze secreted proteins in a cellular system or in "secretomes" in the absence or presence of a particular MMP or MMP inhibitor. However, these diverging approaches always yielded many identifications of intracellular substrates, which were often not reported or discarded as "background" from dying cells (Overall *et al.*, 2004; Tam *et al.*, 2004; Dean and Overall, 2007). Only very recently, these intracellular proteins started to be appreciated as a specific and novel subset of the MMP degradome (Cauwe *et al.*, 2008; Butler and Overall, 2009a; 2009b; Morrison *et al.*, 2009). Intracellular substrates may be released from cells during necrosis or by non-classical secretion mechanisms, which are often poorly characterized. Hence, many intracellular proteins may indeed end up being cleaved by MMPs in the extracellular milieu. This setting will be discussed in detail in §4.

In contrast with most degradomics methods applied for extracellular MMP substrate identification, a two-dimensional degradomics (2DD) approach was the first degradomics screen targeted directly at the identification of intracellular substrates. As illustrated in Table 2, 2DD uses ion exchange chromatography (IEX) to separate proteins according to their isoelectric point (pI) before concentration and cleavage with a protease. Digested and undigested IEX fractions are then separated according to their molecular weight (MW) in adjacent SDS-PAGE lanes and differential protein bands are isolated for identification by MS/MS or electroblotted onto PVDF for identification by Edman degradation, which, in the case of a fragment band, results in direct identification of the cleavage site (Cauwe *et al.*, 2009). Using gelatinase B/MMP-9 as a model enzyme, we applied this method to THP-1 cytosol and isolated 100–200 differential proteins, of which about 70 intracellular proteins were identified as MMP-9 candidate substrates. Various advantages exist for this multidimensional degradomics method. First and foremost, this technically straightforward approach does not require complex and expensive equipment, making it broadly accessible and easy to start up in every laboratory. Although the generation of multiple IEX fractions and SDS-PAGE gels may be somewhat labor-intensive, this is counterbalanced by the fact that final results can be obtained within a few weeks from start-up, as there is no need for lengthy and extensive optimization procedures as in most complex degradomic approaches. Second, the high loading capacity of the IEX columns and the insertion of a concentration step increase the dynamic range. This allows for the identification of both high and low abundance class substrates. Third, as the protein pool is fractionated before proteolysis, less saturation of the protease by high abundance class proteins will occur during digestion. Finally, 2DD can be easily extended to any protease, protease inhibitors may be applied to block specific enzymes and the technique is applicable with any cell type, tissue extract or body fluid. A selection of 2DD candidate substrates was confirmed biochemically by *in vitro* cleavage of the purified or recombinant substrate and visualization of cleavage by classical SDS-PAGE analysis. Alternatively, THP-1 cytoplasm was digested with MMP-9 *in vitro* and cleavage of specific substrates was confirmed by Western blot analysis of cleaved and uncleaved cytoplasm with substrate-specific primary antibodies. The collection of identified cleavage sites allowed us to analyze the cleavage site preference of MMP-9 in intracellular substrates and to compare this with observed preferences for extracellular and membrane-bound molecules. This was achieved by the generation of a sequence logo, using the bioinformatics software WebLogo 3, which adjusts for small samples and corrects for natural amino acid abundances (Schneider and Stephens, 1990; Crooks *et al.*, 2004) (Figure 4). This sequence logo clearly shows

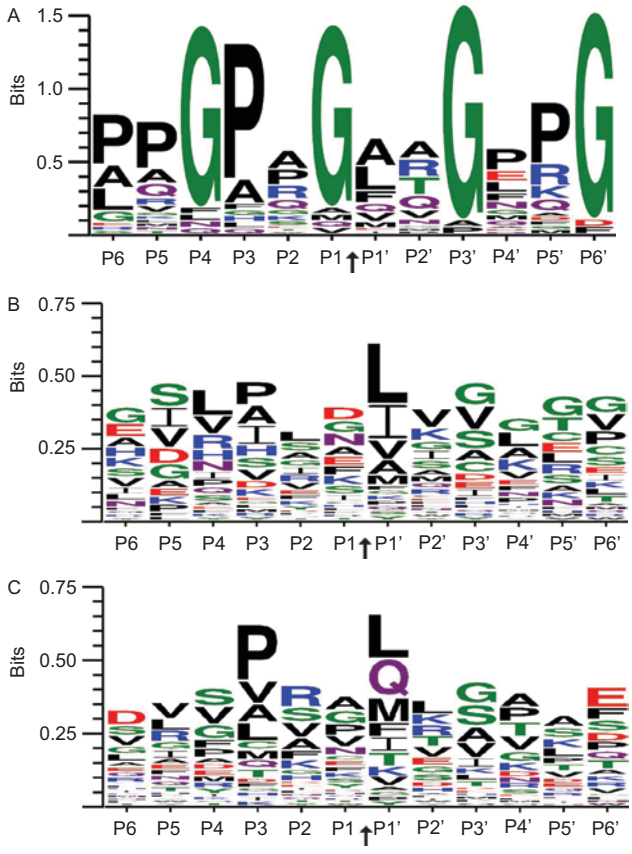


Figure 4. Comparison of the MMP-9-mediated cleavage sites in collagen and gelatin, in extracellular substrates and in intracellular substrates. Cleavage site specificities are visualized by sequence logos, created with the bio-informatics software WebLogo 3 and with the application of small sample adjustments and corrections for natural amino acid abundances (Crooks *et al.*, 2004; Schneider and Stephens, 1990). The overall height of the stack is a measure of sequence consensus, while the heights of the amino acid symbols within each stack indicate the relative frequency of each amino acid in that position. MMP-9 cleaves the peptide bonds between positions P1 and P1′ (arrow). (A) Cleavage preferences in gelatin type II, procollagen type II and collagen type V (Van den Steen *et al.*, 2002). (B) Cleavage specificities in extracellular substrates (secreted and membrane-associated) (Cauwe *et al.*, 2007; Van den Steen *et al.*, 2002; Lin *et al.*, 2008; Chow *et al.*, 2008; Xu *et al.*, 2008; Vaisar *et al.*, 2009). (C) Intracellular substrate specificities (Cauwe *et al.*, 2009; Descamps *et al.*, 2005; Starckx *et al.*, 2003; Levin *et al.*, 2009). Although differences exist between the three sequence logos, a general preference subsists in all substrates for hydrophobic amino acids at P1′, small amino acids at P1 (Gly, Ala) and a Pro residue at P3, as previously described (Van den Steen *et al.*, 2002).

similarities between extracellular and intracellular MMP substrates.

When analyzing the biological functions of the intracellular MMP-9 (candidate) substrates, we found that about 40% of these proteins were components of the cytoskeleton or intracellular matrix (ICM), or associated with ICM proteins (Cauwe *et al.*, 2009). In addition, two thirds of the candidates were autoantigens in cancer or in (multiple) autoimmune diseases. In order to gain insight into the diversity of intracellular proteins cleaved by all MMPs, we scrutinized the results of the MMP degradomics studies mentioned in Table 2 and compiled a general list of high-confidence degradomic candidate substrates (Table 3). Addition of a substrate to the list was based on the identification in various degradomics approaches, the (putative) cleavage by more than one MMP, the identification of a protein of the same subfamily or with a similar function as an MMP (candidate) substrate or the biochemical confirmation by *in vitro* cleavage or Western blot analysis (*vide supra*). This yielded a table of more than 120 proteins that were classified according to functional biological mechanisms. From Table 3 it is clear that cytoskeletal or ICM proteins constitute a considerable fraction of the intracellular degradome. This suggests that degradation of the ICM is a general MMP function. Other over-represented protein classes are carbohydrate metabolic and protein biosynthetic enzymes, proteins regulating transcription and translation, and molecular chaperones. The (putative) physiopathological contexts engendered by the cleavage of these intracellular proteins will be discussed throughout the manuscript. The leading theme is that such cleavages do take place and, irrespective of whether these are causes or consequences in physiological or pathological processes, they need to be considered if we critically (re)consider disease processes.

The fact that very diverging degradomics approaches, ranging from gel-based approaches to complex isotope- and MS-based methodologies, converge to a common list of substrates, is not only an internal validation of these methods, but also a confirmation that these biochemical identifications are not just "background noise" and need to be considered as meaningful in pathophysiological processes. Indeed, the major advantages of degradomics lie in its unbiased and high-throughput nature, which allows novel and unexpected views on the MMP degradome. Hence, expanding the application of MMP degradomics is of utmost importance and may yield many surprising insights and add novel dimensions to the kaleidoscope of MMP functions (Cauwe *et al.*, 2007; Butler and Overall, 2009b).

3. MMP action inside cells

Intracellular activation of MMPs and presence of activated MMPs in various intracellular compartments strongly suggests that MMPs may be responsible for proteolytic actions on intracellular substrates within the cells. In this chapter, we will first discuss how intracellular MMP activity can be detected. Next, we will summarize the present information concerning the presence of activated MMPs

Table 3. Confirmed and high-confidence* intracellular MMP substrates identified by degradomics.

Protein name	MMP	Method†	Confirm.‡	Subcellular localization	(Putative) function	Refs¶
CARBOHYDRATE METABOLISM						
Citrate synthase	MMP-2	iTRAQ™	ND	Mitochondrial matrix	Tricarboxylic acid cycle enzyme	5, 10
	MMP-9	2DD	IVC			
Enolase-α	MMP-2	iTRAQ™, TAILS	ND	Cytoplasm, cell membrane, myofibril, sarcomere, M-band, nucleus, extracellular.	Glycolytic enzyme; role in growth control, hypoxia tolerance and allergic responses; receptor and activator of plasminogen	5, 10, 11
	MMP-9	2DD				
Enolase-β	MMP-2	TAILS	ND	Cytoplasm, sarcomere, Z-band	Glycolytic enzyme; function in striated muscle development and regeneration; defects are the cause of glycogen storage disease type 13	8, 11
	MT1-MMP	ICAT				
Enolase-γ	MMP-2	ICAT, TAILS	IVC	Cytoplasm, cell membrane, extracellular	Glycolytic enzyme; has neurotrophic and neuroprotective properties	6, 8, 11
	MMP-1,-8,-9	ICAT				
	MT1-MMP					
Fructose-bisphophate aldolase A	MMP-2	iTRAQ™, ICAT, TAILS	ND	Cytoplasm, cytoskeleton, I-band, extracellular	Glycolytic enzyme that catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate; actin filament organization; defects are the cause of glycogen storage disease type 12	5, 6, 7, 8, 10, 11
	MT1-MMP	ICAT				
	MMP-9	UPLC-MS^E, 2DD				
Fructose-bisphophate aldolase C	MMP-2	ICAT	ND	Cytoplasm, cytoskeleton	Glycolytic enzyme that catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate	6
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	MMP-2	iTRAQ™, ICAT, TAILS	ND	Cytoplasm, cell membrane, extracellular	Glycolytic enzyme; independent of its glycolytic activity it is also involved in membrane trafficking in the early secretory pathway.	5, 6, 7, 8, 11
	MT1-MMP	ICAT				
	MMP-9	UPLC-MS^E				
Malate dehydrogenase	MMP-2	iTRAQ™, ICAT, TAILS	ND	Cytoplasm, extracellular	Tricarboxylic acid cycle enzyme	5, 6, 8, 11
	MT1-MMP	ICAT				
Phosphoglycerate Phosphokinase 1	MMP-2	ICAT, TAILS	ND	Cytoplasm, extracellular	Glycolytic enzyme; putative polymerase α cofactor protein; defects are associated with chronic hemolytic	5, 6, 7, 8, 10
	MT1-MMP	ICAT				
	MMP-9	UPLC-MS^E, 2DD				
Phosphoglycerate mutase 1	MMP-2	ICAT, TAILS	ND	Cytoplasm, extracellular	Glycolytic enzyme; interconversion of 3- and 2-phosphoglycerate with 2,3-bisphosphoglycerate as the primer of the reaction	6, 11
Triose phosphate isomerase	MMP-2	iTRAQ™, ICAT, TAILS	ND	Cytoplasm, extracellular	Enzyme of the glycolytic and gluconeogenesis pathways; defects are the cause of triosephosphate isomerase deficiency	5, 6, 7, 8, 11
	MT1-MMP	ICAT				
	MMP-9	UPLC-MS^E				
CYTOSKELETON						
Actin-β/γ	MMP-2	TAILS	WB	Cytoplasm, cytoskeleton, nucleus, cell projection, extracellular	Vital for cell morphogenesis and motility, endocytosis, phagocytosis and cytokinesis	7, 10, 11
	MMP-9	UPLC-MS^E, 2DD	IVC, WB			
	MMP-11	TAILS	ND			
	MMP-1,-8,-13	IVC	WB			
α-Actinin-1	MMP-2	ICAT, iTRAQ™	(Sung *et al.*, 2007)	Cytoskeleton, myofibril, sarcomere, Z-disc, nucleolus, extracellular	Connection of actin filaments of adjacent sarcomeres and transmission of the force generated by the actin–myosin complex	5, 6, 7, 8
	MT1-MMP	ICAT				
	MMP-9	UPLC-MS^E				
α-Actinin-4	MMP-2	TAILS	ND	cytoplasm, cytoskeleton, cell projection, nucleolus, extracellular	Connection of actin filaments of adjacent sarcomeres and transmission of the force generated by the actin-myosin complex; defects are the cause of focal segmental glomerulosclerosis	7, 8, 11
	MT1-MMP	ICAT				
	MMP-9	UPLC-MS^E				

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Table 3. Continued.

Protein name	MMP	Method†	Confirm.‡	Subcellular localization	(Putative) function	Refs¶
Actin regulatory protein CAP-G	MMP-2 MT1-MMP MMP-9	iTRAQ™, ICAT ICAT 2DD	ND	Cytoplasm, melanosome, nucleus, secreted	Reversible blocking of actin filament barbed ends without filament severing; may have important roles in macrophage function and regulation of cytoplasmic and/or nuclear structures through interactions with actin; potential DNA binding	6, 8, 10
Actin-related protein (Arp) 2	MMP-9 MT1-MMP	2DD ICAT	IVC ND	Cytoplasm, cytoskeleton, cell projection	ATP-binding component of the Arp2/3 complex which mediates the formation of branched F-actin networks	8, 10
Actin-related protein (Arp) 2/3 complex subunits	MMP-9 MMP-1, -2, -3, -8, -13	2DD IVC	IVC	Cytoplasm, cytoskeleton, Cell projection	Actin-binding component of the Arp2/3 complex which mediates the formation of branched F-actin networks	8, 10
Adenylyl cyclase-associated protein-1 (CAP1)	MMP-2,-8,-13 MMP-9	IVC 2DD	(Cauwe *et al.*, 2008)	Cytoplasm, cell membrane	Enhancement of actin filament turnover; roles in cell morphology, migration and endocytosis; promotion of cofilin-induced apoptosis by shuttling actin to mitochondria	10
Cofilin-1	MMP-2 MT1-MMP	ICAT, TAILS ICAT	ND	Cytoplasm, cytoskeleton, nuclear matrix, extracellular	Actin filament depolymerization and severing protein. Promotion of apoptosis by translocating to mitochondria and delivering actin	6, 8, 11
Desmin	MMP-2	TAILS	(Sung *et al.*, 2007)	Cytoplasm, cytoskeleton, Z-disk	Intermediate filament protein involved in cellular resistance to external stress	11
Ezrin	MMP-2 MT1-MMP MMP-9	iTRAQ™ ICAT 2DD	ND	Cytoplasm, cell membrane, cell projections, extracellular	Cross-linking of cortical actin filaments and plasma membranes; roles in ECM interactions, cell–cell communication, apoptosis, carcinogenesis and metastasis	5, 8, 10
Fascin	MMP-9	2DD	ND	Cytoplasm, cytoskeleton, cell membrane	Organization of F-actin into bundles with a minimum of 4.1:1 actin/fascin ratio	10
Filamin A	MMP-2 MT1-MMP	iTRAQ™, ICAT, TAILS ICAT	ND	Cytoplasm, cytoskeleton, nucleus, cell membrane, extracellular	Promotion of actin filament branching and connection of the actin cytoskeleton to various transmembrane proteins; scaffold for a wide range of cytoplasmic signaling proteins; tethers cell surface-localized furin, modulates its rate of internalization and directs its intracellular trafficking; defects are the cause of many developmental diseases	5, 6, 8, 11
Filamin B	MMP-2 MT1-MMP MMP-9	iTRAQ™, ICAT, TAILS ICAT 2DD	ND	Cytoplasm, cytoskeleton, sarcomere, Z-disc, cell membrane	Promotion of actin filament branching and connection of the actin cytoskeleton to various transmembrane proteins; scaffold for a wide range of cytoplasmic signaling proteins; defects are the cause of many developmental diseases	5, 6, 8, 10, 11
Filamin C	MMP-2 MT1-MMP	iTRAQ™, ICAT ICAT	ND	Cytoplasm, cytoskeleton, sarcomere, Z-disc, cell membrane	Muscle-specific filamin, putative roles in actin cross-linking, reorganization of the actin cytoskeleton in response to signaling events, and structural functions at the Z-disks in muscle cells. Critical for normal myogenesis and the structural integrity of the muscle fibers; defects are the cause of myopathy myofibrillar filamin C-related	5, 6, 8

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Table 3. Continued.

Protein name	MMP	Method†	Confirm.‡	Subcellular localization	(Putative) function	Refs¶
Gelsolin	MMP-1,-3 MMP-2 MT1-MMP MMP-9	IVC iTRAQ™, ICAT, TAILS 2D-PAGE 2DD	(Hwang *et al.*, 2004; Park *et al.*, 2006)	Cytoplasm, cytoskeleton, secreted	F-actin capping and severing; nucleation of F-actin assembly; scavenging of actin and pro-inflammatory components in the plasma; defects are the cause of amyloidosis type 5 or familial amyloidosis Finnish type	1, 5, 6, 10, 11
IQ motif containing GTPase activating protein 1 (IQGAP1)	MMP-9	2DD	ND	Cytoplasm, cytoskeleton, cell membrane, nucleus	Actin crosslinking/bundling; E-cadherin-mediated cell-cell contacts; microtubule capture/polarity; cell motility and invasion; phagocytosis	10
Microtubule-associated protein RP (MAPRE1)	MMP-2 MMP-9	TAILS 2DD	ND	Cytoplasm, microtubule network, centrosome	Microtubule formation and stabilization, promoting cell migration; binding and inhibition of the F-actin bundling and microtubule-associated protein APC	10, 11
Moesin	MMP-2 MT1-MMP MMP-9	iTRAQ™, ICAT ICAT 2DD, UPLC-MS^E	ND	Cytoplasm, cell membrane, cell projections, nucleolus, extracellular	Cross-linking of cortical actin filaments and plasma membranes; roles in ECM interactions, cell–cell communication, apoptosis, carcinogenesis and metastasis	5, 6, 7, 8, 10
Plectin-1	MMP-2 MT1-MMP	TAILS ICAT	ND	Cytoplasm, cytoskeleton, cell membrane	Interlinks intermediate filaments with microtubules and microfilaments and anchors intermediate filaments to desmosomes or hemidesmosomes; may bind actin to membrane complexes in muscle	6, 8
Profilin-1	MMP-2 MT1-MMP	iTRAQ™, ICAT ICAT	ND	Cytoplasm, cytoskeleton, extracellular	G-actin sequestering protein; catalyzes conversion from ADP-actin to ATP-actin; adds actin monomers to the growing end of the actin filament	5, 6, 8
Stathmin	MMP-9 MMP-1,-2,-8,-13	2DD IVC	WB	Cytoplasm, cytoskeleton	Regulation of microtubule dynamics by microtubule depolymerization and inhibition of polymerization	10
Trangelin 2	MMP-2 MMP-9	iTRAQ™, TAILS UPLC-MS^E	ND	Nuclear membrane, cell membrane	Putative actin cross-linking/gelling protein	5, 6, 7
Tubulin-α/β	MMP-2 MT1-MMP MMP-9 MMP-1,-3,-8,-13	ICAT, TAILS ICAT 2DD IVC	IVC ND IVC IVC	Cytoplasm, cytoskeleton, cell projection, extracellular	Heterodimers of α- and β-tubulin chains are the major constituents of microtubules	6, 8, 10, 11
Vimentin	MMP-2 MT1-MMP	iTRAQ™, ICAT, TAILS ICAT	ND	Cytoplasm, cytoskeleton, extracellular	Predominant subunit of intermediate filaments found in various non-epithelial cells, especially mesenchymal cells; organizer of critical proteins involved in attachment, migration, and cell signaling	5, 6, 8, 11
PROTEIN BIOSYNTHESIS						
Elongation factor 1-α1	MMP-2 MT1-MMP MMP-9	TAILS ICAT UPLC-MS^E, 2DD	ND	Cytoplasm, extracellular	Promotion of the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis	7, 8, 10, 11
Elongation factor 1-α2	MMP-2 MT1-MMP	TAILS ICAT	ND	Nucleus	Promotion of the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis	8, 11

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Table 3. Continued.

Protein name	MMP	Method†	Confirm.‡	Subcellular localization	(Putative) function	Refs¶
Elongation factor 2	MMP-2 MT1-MMP MMP-9	ICAT, TAILS ICAT UPLC-MS^E, 2DD	ND	Cytoplasm, extracellular	Promotion of the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome	6, 7, 8, 10, 11
Eukaryotic translation initiation factor 1	MMP-2	ICAT, TAILS	ND	Cytoplasm	Necessary for scanning and involved in initiation site selection. Promotes the assembly of 48S ribosomal complexes at the authentic initiation codon of a conventional capped mRNA.	6, 11
Eukaryotic translation initiation factor 5A	MMP-2 MT1-MMP MMP-9	ICAT ICAT 2DD	ND	Cytoplasm, extracellular	Promotion of the formation of the first peptide bond during protein biosynthesis	6, 8, 10
Alanyl-tRNA synthetase	MMP-2 MT1-MMP	ICAT ICAT	ND	Cytoplasm	Catalyzes the attachment of Ala to its cognate tRNA; edits incorrectly charged tRNA(Ala) *via* its editing domain	6, 8
Aspartyl-tRNA synthetase	MMP-2	iTRAQ™, TAILS	ND	Cytoplasm	Catalyzes the attachment of Asp to its cognate tRNA	5, 11
Cysteinyl-tRNA synthetase	MMP-2 MMP-9	iTRAQ™, ICAT 2DD	ND	Cytoplasm	Catalyzes the attachment of Cys to its cognate tRNA	5, 6, 10
Histidyl-tRNA synthetase	MMP-9 MMP-1,-2,-3,-8,-13	2DD, UPLC-MS^E IVC	IVC	Cytoplasm	Catalyzes the attachment of His to its cognate tRNA	7, 10
Threonyl-tRNA synthetase	MT1-MMP MMP-9	ICAT 2DD	ND	Cytoplasm	Catalyzes the attachment of Thr to its cognate tRNA	8, 10
Tryptophanyl-tRNA synthetase	MMP-2	iTRAQ™	ND	Cytoplasm	Catalyzes the attachment of Trp to its cognate tRNA; regulation of ERK, Akt, and eNOS activation pathways that are associated with angiogenesis, cytoskeletal reorganization and shear stress-responsive gene expression	5
Valyl-tRNA synthetase	MMP-9	UPLC-MS^E	ND	Cytoplasm	Catalyzes the attachment of Val to its cognate tRNA	7
REGULATION OF TRANSCRIPTION						
Hepatoma-derived growth factor (HDGF)	MMP-2 MMP-9	iTRAQ™ 2DD	ND	Cytoplasm, nucleus, extracellular	Heparin-binding protein with mitogenic activity for fibroblasts; acts as a transcriptional repressor; putative alarmin	6, 10
High-mobility group protein B1 (HMGB1)	MMP-2 MT1-MMP MMP-9	iTRAQ™, TAILS ICAT 2DD	ND WB	Nucleus, cytoplasm, extracellular	Intracellular function: stabilization of nucleosome formation and facilitation of transcription factor binding by bending DNA, role in DNA repair; cytosolic nucleic acid sensor; extracellular function: pro-inflammatory cytokine, alarmin	5, 8, 10, 11
High-mobility group protein B2 (HMGB2)	MMP-2 MT1-MMP MMP-9	iTRAQ™, TAILS ICAT 2DD	ND	Nucleus, cytoplasm, extracellular	Intracellular function: stabilization of nucleosome formation and facilitation of transcription factor binding by bending DNA, role in DNA repair; cytosolic nucleic acid sensor	5, 8, 10, 11

Table 3. continued on next page

Table 3. Continued.

Protein name	MMP	Method†	Confirm.‡	Subcellular localization	(Putative) function	Refs¶
High-mobility group protein B3 (HMGB3)	MMP-2	TAILS	ND	Nucleus	Intracellular function: stabilization of nucleosome formation and facilitation of transcription factor binding by bending DNA, role in DNA repair; cytosolic nucleic acid sensor; regulation of proliferation and differentiation of common lymphoid and myeloid progenitors	11
Histone H1.2	MMP-2	TAILS	ND	Nucleus	Histones H1 are necessary for the condensation of nucleosome chains into higher order structures.	11
Histone H1.3	MMP-2	iTRAQ™	ND	Nucleus	Histones H1 are necessary for the condensation of nucleosome chains into higher order structures.	5
Histone H2A	MMP-2	TAILS	ND	Nucleus, extracellular	Core component of nucleosome; central role in transcription regulation, DNA repair, DNA replication and chromosomal stability	11
Histone H2B	MMP-2	iTRAQ™	ND	Nucleus, extracellular	Core component of nucleosome; central role in transcription regulation, DNA repair, DNA replication and chromosomal stability	5
Histone H4	MMP-2	iTRAQ™	ND	Nucleus, extracellular	Core component of nucleosome; central role in transcription regulation, DNA repair, DNA replication and chromosomal stability	5
Lupus La protein	MMP-2 MT1-MMP	ICAT ICAT	ND	Nucleus, cell surface	Plays a role in the transcription of RNA polymerase III, most probably as a transcription termination factor as it binds to the 3′ termini of virtually all nascent polymerase III transcripts; major autoantigen in SLE	6, 8
Nucleobindin-1	MMP-2	iTRAQ™, TAILS	ND	Golgi apparatus, cell membrane, extracellular	Ca^{2+}- and DNA-binding protein; B-cell growth and differentiation factor; control of the unfolded protein response; regulation of receptor trafficking	5, 11
Nucleolin	MMP-2 MMP-9 MMP-1,-3,-8,-13	iTRAQ™, ICAT 2DD IVC	WB	Nucleus, cytoplasm, cell surface	Regulation of RNA polymerase I transcription; folding and maturation of pre-ribosomal RNA; ribosome assembly; nucleo-cytoplasmic transport; histone chaperone activity; interaction with viruses at the cell membrane	5, 6, 10
Nucleoside diphosphate kinase B	MMP-2 MMP-9	ICAT 2DD	ND	Cytoplasm, cytoskeleton, nucleus	Major role in the synthesis of nucleoside triphosphates other than ATP; negative regulation of Rho activity; transcriptional activator of the *Myc* gene	6, 10
REGULATION OF TRANSLATION						
hnRNP A1	MMP-2 MT1-MMP	TAILS ICAT	ND	Cytoplasm, nucleus, spliceosome	Involved in the packaging of pre-mRNA into hnRNP particles, transport of poly(A) mRNA from the nucleus to the cytoplasm and modulation of splice site selection	8, 11
hnRNP A/B	MMP-2	TAILS	ND	Cytoplasm, nucleus, ribonucleoprotein complex	Binds ssRNA; high affinity for G-rich and U-rich regions of heterogeneous nuclear RNA.	11

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Table 3. Continued.

Protein name	MMP	Method[†]	Confirm.[‡]	Subcellular localization	(Putative) function	Refs[¶]
hnRNP A2/B1	MMP-2	TAILS	ND	Cytoplasm, nucleus, spliceosome	Involved with pre-mRNA processing; forms complexes (ribonucleosomes) with at least 20 other different hnRNP and heterogeneous nuclear RNA in the nucleus.	11
hnRNP A3	MMP-2	TAILS	ND	Cytoplasm, nucleus, spliceosome	Plays a role in cytoplasmic trafficking of RNA; binds to the *cis*-acting response element, A2RE; may be involved in pre-mRNA splicing	11
hnRNP D0	MMP-2	ICAT	ND	Cytoplasm, nucleus, ribonucleoprotein complex	Binds with high affinity to RNA molecules that contain AU-rich elements (AREs) found within the 3′-UTR of many proto-oncogenes and cytokine mRNAs; binds to ds- and ssDNA sequences in a specific manner and functions as a transcription factor; putative roles in telomere elongation and translationally coupled mRNA turnover	6
hnRNP K	MMP-2 MT1-MMP	iTRAQ™, TAILS ICAT	ND	Cytoplasm, nucleus, spliceosome	Major pre-mRNA-binding protein; binds tenaciously to poly(C) sequences; probable role in the nuclear metabolism of hnRNAs, particularly for pre-mRNAs that contain cytidine-rich sequences; binds poly(C) single-stranded DNA.	5, 6, 8
hnRNP Q	MMP-2 MT1-MMP	ICAT, TAILS ICAT	ND	Cytoplasm, microsome, ER, nucleus	Involved with pre-mRNA processing; interacts preferentially with poly(A) and poly(U) RNA sequences; putative roles in translationally coupled mRNA turnover and cytoplasmic vesicle-based mRNA transport through interaction with synaptotagmins	5, 6, 8
hnRNP U	MMP-2	TAILS	ND	Cytoplasm, nucleus, spliceosome, cell surface	Binds to ds- and ssDNA and RNA; has high affinity for scaffold-attached region (SAR) DNA; inhibits elongation of transcription by polymerase II	11
Nucleophosmin	MMP-2 MMP-9	TAILS UPLC-MS[E]	ND	Nucleus	Involved in diverse cellular processes such as ribosome biogenesis, centrosome duplication, protein chaperoning, histone assembly, cell proliferation, and regulation of tumor suppressors; associated with nucleolar ribonucleoprotein structures and binds ss nucleic acids	7, 11
Small nuclear ribonucleo-protein Sm D3	MT1-MMP MMP-9	ICAT 2DD	ND	Nucleus, cytoplasm	Component of the spliceosome, involved in the nuclear processing of pre-mRNA	8, 10
PROTEIN CHAPERONING						
Calreticulin	MMP-2 MT1-MMP	iTRAQ™, TAILS ICAT	ND	Cytoplasm, ER lumen, nucleus, cell surface, secreted, ECM	Molecular calcium binding chaperone promoting folding, oligomeric assembly and quality control in the ER via the calreticulin/calnexin cycle; extracellular modulation of cell motility and promotion of tumor progression and metastasis	5, 8, 11
βB1-crystallin	MMP-9	2D-PAGE	IVC	Cytoplasm	Prevention of protein unfolding or aggregation; structural component of the eye lens; defects are the cause of cataract	2

Table 3. continued on next page

Table 3. Continued.

Protein name	MMP	Method†	Confirm.‡	Subcellular localization	(Putative) function	Refs¶
Cyclophilin A	MMP-2	iTRAQ™, ICAT, TAILS	IVC	Cytoplasm, nucleus, extracellular	Acceleration of protein folding, catalysis of *cis-trans* isomerization of peptidyl-prolyl bonds in oligopeptides; pro-inflammatory functions after secretion	5, 6, 7, 8, 10, 11
	MT1-MMP	ICAT	ND			
	MMP-9	UPLC-MS^E, 2DD				
	MMP-11	TAILS				
Cyclophilin B	MMP-2	iTRAQ™	ND	ER lumen, melanosome, extracellular	Acceleration of protein folding, catalysis of *cis-trans* isomerization of peptidyl-prolyl bonds in oligopeptides	5, 7, 8
	MT1-MMP	ICAT				
	MMP-9	UPLC-MS^E				
Cyclophilin D	MMP-2	TAILS	ND	ER lumen, melanosome	Acceleration of protein folding, catalysis of *cis-trans* isomerization of peptidyl-prolyl bonds in oligopeptides, RNA-binding, putative role in pre-mRNA splicing	8, 11
	MT1-MMP	ICAT				
Cyclophilin E	MMP-2	iTRAQ™	ND	Nucleus	Acceleration of protein folding, catalysis of *cis-trans* isomerization of peptidyl-prolyl bonds in oligopeptides; binding of RNA with potential role in pre-mRNA splicing	5, 10
	MMP-9	2DD				
Endoplasmin	MMP-2	TAILS	ND	ER lumen, cytoplasm, melanosome	Molecular chaperone that functions in the processing and transport of secreted proteins; functions in ER-associated degradation	6, 7, 11
	MMP-9	UPLC-MS^E				
	MMP-11	TAILS	IVC			
Heat shock protein 27	MMP-9	2DD	ND	Cytoplasm, cytoskeleton, nucleus, nucleus	Molecular chaperone involved in stress resistance and actin organization (actin capping); regulation of metastasis	10, 11
	MMP-11	TAILS				
Heat shock cognate protein 70	MMP-2	iTRAQ™, ICAT	ND	Cytoplasm, cytoskeleton, nucleus, cell surface	Stabilization of pre-existing proteins against aggregation; mediation of the folding of newly translated polypeptides in the cytosol and within organelles	5, 6, 7, 8, 10
	MT1-MMP	ICAT				
	MMP-9	UPLC-MS^E, 2DD				
Heat shock protein 70	MMP-2	ICAT, TAILS	ND	Cytoplasm, nucleus, extracellular	Molecular chaperone involved in stress resistance; protection of the centrosome during heat shock	6, 8, 11
	MT1-MMP	ICAT				
Heat shock protein 75	MMP-2	TAILS	ND	Mitochondrion	Molecular chaperone involved in stress resistance; binds to the intracellular domain of tumor necrosis factor type 1 receptor	11
Heat shock protein 90α	MMP-2	iTRAQ™, TAILS	ND	Cytoplasm, cytoskeleton, nucleus, ER lumen, mitochondria, cell surface	Molecular chaperone involved in stress resistance; Role in F-actin bundling and cross-linking; promotion of tumor cell invasion and metastasis	5, 6, 7, 8, 10, 11
	MT1-MMP	ICAT	IVC			
	MMP-9	UPLC-MS^E, 2DD	ND			
	MMP-11	TAILS	ND			
Heat shock protein 90β	MMP-2	TAILS	ND	Cytoplasm, melanosome, mitochondrion, extracellular	Molecular chaperone involved in stress resistance	6, 7, 8, 10, 11
	MT1-MMP	ICAT				
	MMP-9	UPLC-MS^E, 2DD				
	MMP-11	TAILS				
Heat shock protein 105	MMP-2	ICAT	ND	Cytoplasm, extracellular	Prevents the aggregation of denatured proteins in cells under severe stress, on which the ATP levels decrease markedly. Inhibits heat shock cognate protein 70 ATPase and chaperone activities	6
Oxygen-regulated protein 150	MT1-MMP	ICAT	ND	ER lumen, mitochondria	Molecular chaperone, cytoprotective role after oxygen deprivation	8, 10
	MMP-9	2DD	ND			

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Table 3. Continued.

Protein name	MMP	Method†	Confirm.‡	Subcellular localization	(Putative) function	Refs¶
REDOX REGULATION						
Protein DJ-1	MMP-2 MMP-1,-8,-9 MT1-MMP	iTRAQ™, ICAT, TAILS IVC ICAT	IVC	Cytoplasm, mitochondria, nucleus, extracellular	Redox-reactive signaling intermediate controlling oxidative stress in the brain, protects neurons against oxidative stress and cell death	5, 6, 8, 11
Peroxiredoxin 1	MMP-2	iTRAQ™, TAILS	ND	Cytoplasm, extracellular	Antioxidant by peroxidase activity, control of cytokine-induced peroxide levels which mediate signal transduction	5, 11
Peroxiredoxin 2	MMP-2 MMP-9	iTRAQ™ UPLC-MS^E, 2DD	ND	Cytoplasm, cell membrane	Antioxidant by peroxidase activity, control of cytokine-induced peroxide levels which mediate signal transduction	5, 7, 10
Peroxiredoxin 4	MMP-9	2D LC-MS/MS	ND	Cytoplasm, secreted	Putative antioxidant by peroxidase activity; regulation of the activation of NF-κB in the cytosol by a modulation of IκB-α phosphorylation	9
Peroxiredoxin 5	MMP-2	iTRAQ™	ND	Mitochondrion, cytoplasm, peroxisome	Antioxidant by peroxidase activity; involved in intracellular redox signaling	5
Peroxiredoxin 6	MMP-2 MMP-9	iTRAQ™, TAILS 2D-DIGE, 2DD	ND	Cytoplasm, lysosome, mitochondrion	Involved in redox regulation of the cell by reduction of H_2O_2 and short chain organic, fatty acid, and phospholipid hydroperoxides; putative roles in the regulation of phospholipid turnover and protection against oxidative injury	4, 5, 6, 10
SIGNAL TRANSDUCTION						
GDP dissociation inhibitor (GDI) 2	MMP-9	2DD	ND	Cytoplasm, cell membrane, extracellular	ND, putative Rab GDP dissociation inhibitor activity	10
Rab GDP dissociation inhibitor (GDI) β	MMP-2 MT1-MMP	ICAT, TAILS ICAT	ND	Cytoplasm, cell membrane, cell surface	Regulates the GDP/GTP exchange reaction of most Rab proteins by inhibiting the dissociation of GDP from them, and the subsequent binding of GTP to them	6, 8, 11
Rho GDP dissociation inhibitor (GDI) α	MMP-2 MMP-9	TAILS 2DD	ND	Cytoplasm, cytoskeleton, extracellular	Regulates the GDP/GTP exchange reaction of the Rho proteins by inhibiting the dissociation of GDP, and the subsequent binding of GTP	6, 10
Rho GDP dissociation inhibitor (GDI) β	MMP-2 MMP-9	ICAT 2DD	ND	Cytoplasm, cytoskeleton, cell membrane, extracellular	Regulates the GDP/GTP exchange reaction of the Rho proteins by inhibiting the dissociation of GDP, and the subsequent binding of GTP	6, 10
14-3-3 protein β/α	MMP-2	TAILS	ND	Cytoplasm, melanosome	Adapter protein implicated in the regulation of a large spectrum of signaling pathways by binding and modulating the activity of a large number of partners, usually by recognition of a phospho-Ser or phospho-Thr motif	11
14-3-3 protein η	MMP-2 MT1-MMP	TAILS ICAT	ND	Cytoplasm, nucleus, extracellular	Adapter protein implicated in the regulation of a large spectrum of signaling pathways by binding and modulating the activity of a large number of partners, usually by recognition of a phospho-Ser or phospho-Thr motif	8, 11

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Table 3. Continued.

Protein name	MMP	Method†	Confirm.‡	Subcellular localization	(Putative) function	Refs¶
14-3-3 protein γ	MMP-2	iTRAQ™, TAILS	ND	Cytoplasm, extracellular	Adapter protein implicated in the regulation of a large spectrum of signaling pathways by binding and modulating the activity of a large number of partners, usually by recognition of a phospho-Ser or phospho-Thr motif	5, 11
14-3-3 protein θ	MMP-2 MT1-MMP	iTRAQ™, TAILS ICAT	ND	Cytoplasm	Adapter protein implicated in the regulation of a large spectrum of signaling pathways by binding and modulating the activity of a large number of partners, usually by recognition of a phospho-Ser or phospho-Thr motif	5, 8, 11
14-3-3 protein ζ/δ	MMP-2 MT1-MMP	TAILS ICAT	ND	Cytoplasm, mitochondrion, nucleus, extracellular	Adapter protein implicated in the regulation of a large spectrum of signaling pathways by binding and modulating the activity of a large number of partners, usually by recognition of a phospho-Ser or phospho-Thr motif	8, 11
APOPTOSIS						
Apoptosis-linked-gene-2-interacting-protein X (Alix)	MMP-9	2DD	ND	Cytoplasm, melanosome, centrosome, extracellular	Control of the production of and trafficking through endosomes called multivesicular bodies; regulation of caspase-dependent and caspase-independent cell death	10
BH3 interacting domain death agonist (BID)	MMP-9	2DD	ND	Cytoplasm, mitochondrial membrane	Major proteolytic product p15 BID: release of cytochrome c; induction of ICE-like proteases and apoptosis; countering the protective effect of Bcl-2	10
Calpain 2	MMP-2 MT1-MMP	ICAT ICAT	ND ND	Cytoplasm, cell membrane	Ca^{2+}-regulated non-lysosomal thiol-protease which cleaves substrates involved in cytoskeletal remodeling, apoptosis and signal transduction.	6, 8
Calpastatin	MT1-MMP	ICAT	ND	Cytoplasm	Specific inhibitor of calpains	8
Cytochrome c	MMP-11	TAILS	ND	Mitochondrial matrix, cytosol, nucleus	Transfer of electrons to the cytochrome oxidase complex, the final protein carrier in the mitochondrial electron-transport chain; released in cytosol during apoptosis resulting in activation of caspase-9	11
LYSOSOMAL DEGRADATION						
β-glucuronidase	MMP-9	2D LC-MS/MS, 2DD	ND	Lysosome	Important role in lysosomal degradation of dermatan and keratan sulfates; defects are the cause of mucopolysaccharidosis type 7 or Sly syndrome	9, 10
Cathepsin A (lysosomal carboxypeptidase A)	MMP-2	ICAT	ND	Lysosome, ER, extracellular	Protective protein appears to be essential for both the activity of β-galactosidase and neuraminidase, it associates with these enzymes and exerts a protective function necessary for their stability and activity.	6
Cathepsin B	MMP-2 MT1-MMP	iTRAQ™, TAILS ICAT	ND	Lysosome, mitochondrion, melanosome	Thiol protease which is believed to participate in intracellular degradation and turnover of proteins. Has also been implicated in tumor invasion and metastasis.	5, 8, 11
Cathepsin D	MMP-9	UPLC-MS[E]	ND	Lysosome, melanosome, extracellular	Acid protease active in intracellular protein breakdown. Involved in the pathogenesis of several diseases such as breast cancer and possibly Alzheimer disease; defects are the cause of neuronal ceroid lipofuscinosis type 10	7

Table 3. continued on next page

Table 3. Continued.

Protein name	MMP	Method†	Confirm.‡	Subcellular localization	(Putative) function	Refs¶
Cathepsin E	MMP-9	2D LC-MS/MS	ND	Endosome, ER, Golgi	May have a role in immune function. Probably involved in the processing of antigenic peptides during MHC class II-mediated antigen presentation. May play a role in activation-induced lymphocyte depletion in the thymus, and in neuronal degeneration and glial cell activation in the brain.	9
Cathepsin L1	MMP-2 MT1-MMP	iTRAQ™, ICAT, TAILS ICAT	ND	Lysosome, extracellular	Important for the overall degradation of proteins in lysosomes.	5, 6, 8, 11
Cathepsin Z (or X)	MMP-9	UPLC-MS[E]	ND	Lysosome, ER	Implicated in the promotion of cancer progression	7
Iduronate-2-sulfatase (IDS)	MMP-2,-8,-9 MT1-MMP	ICAT	IVC	Lysosome	Required for the lysosomal degradation of heparan sulfate and dermatan sulfate; defects are the cause of mucopolysaccharidosis type 2 or Hunter disease	8
Niemann-Pick, type C2 (NPC2)	MT1-MMP	ICAT	IVC	Lysosome, secreted	Key role in cellular cholesterol homeostasis by participating in intracellular cholesterol trafficking and the production of low-density lipoprotein cholesterol-derived oxysterols; defects cause the lysosomal storage disease Niemann-Pick disease type C2	8
UBIQUITINATION						
Ubiquitin-activating enzyme E1	MMP-2	TAILS	ND	Cytoplasm, nucleus	Activates ubiquitin by first adenylating its C-terminal glycine residue with ATP, and thereafter linking this residue to the side chain of a cysteine residue in E1, yielding an ubiquitin-E1 thioester and free AMP.	11
Ubiquitin-conjugating enzyme E2 variant 1	MMP-9	2DD	ND	Cytoplasm, nucleus	Catalysis of non-canonical polyubiquitin chain synthesis; role in the activation of NF-κB; transcriptional activation of target genes; role in the control of cell cycle and differentiation; role in error-free DNA repair pathway; contribution to cell survival after DNA damage	1
Ubiquitin-conjugating enzyme E2 L3	MMP-2	ICAT	ND	Cytoplasm, nucleus	Catalyzes the covalent attachment of ubiquitin to other proteins, mediating the selective degradation of short-lived and abnormal proteins	6
Ubiquitin-conjugating enzyme E2N-like	MMP-9	2DD	ND	Nucleus	ND, belongs to the ubiquitin-conjugating enzyme family	10
MISCELLANEOUS						
Annexin I	MMP-2	iTRAQ™	ND	Cytoplasm, cell projection	Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis; regulation of phospholipase A2 activity; inhibition of neutrophil extravasation and induction of neutrophil apoptosis	5, 10
	MMP-9	2DD	WB	cell membrane, nucleus, extracellular		
Carbonic anhydrase II	MMP-9 MMP-13	2DD IVC	NIVC IVC	Cytoplasm, nucleus, extracellular	Reversible hydration of carbon dioxide; essential for bone resorption and osteoclast differentiation; defects are the cause of renal tubular acidosis or Guibaud-Vainsel syndrome	10

Table 3. continued on next page

Table 3. Continued.

Protein name	MMP	Method[†]	Confirm.[‡]	Subcellular localization	(Putative) function	Refs[¶]
Ferritin light chain	MMP-2 MMP-9	iTRAQ™ 2DD	ND	Cytoplasm, extracellular	Storage of iron in a soluble, non-toxic, readily available form; defects are the cause of hereditary hyper-ferritinemia-cataract syndrome and neuroferritinopathy	5, 10
Galectin-1	MMP-2 MT1-MMP MMP-9 MMP-11	iTRAQ™, TAILS ICAT UPLC-MS[E] TAILS	ND IVC ND IVC	Cytoplasm, ECM	May regulate apoptosis, cell proliferation and cell differentiation. Binds a wide array of complex carbohydrates. Affects T cell homeostatis by inhibiting CD45 protein phosphatase activity; putative alarmin	5, 7, 8, 11
Galectin-3	MMP-2,-9	iTRAQ™	(Ochieng *et al.*, 1994; Ochieng *et al.*, 1998)	Nucleus, cytoplasm, mitochondrion, cell membrane, secreted, cell surface	Galactose-specific lectin which binds IgE.; may mediate integrin-dependent stimulation of endothelial cell migration.	5
Myosin light chain	MMP-2	2D-PAGE	IVC	Cytoplasm, cytoskeleton, sarcomere, extracellular	Mechanical protein that generates force during muscle contraction	3
Progranulin	MT1-MMP	ICAT	IVC	Cytoplasm, extracellular	Pluripotent growth factor with roles in development, wound repair, inflammation and tumorigenesis; defects cause ubiquitin-positive frontotemporal dementia	8

ADP, adenosine diphosphate; APC, adenomatous polyposis coli; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CMP, cytidine monophosphate; dsDNA, double-stranded DNA; ECM, extracellular matrix; E-cadherin, epithelial cadherin; ER, endoplasmic reticulum; F-actin, filamentous actin; G-actin, globular actin; hnRNP, heterogeneous nuclear ribonucleoprotein; ICE, interleukin-1β-converting enzyme; IgE, immunoglobulin E; IκB-α, inhibitor of NFκB-α; IVC, *in vitro* cleavage; ND, not defined; NFκB, nuclear factor κB; ssDNA, single-stranded DNA; UMP, uridine monophosphate; SLE, systemic lupus erythematosus.

[*]Addition of a substrate to the list of high confidence candidate substrates was based on the identification in various degradomics approaches, the (putative) cleavage by more than one MMP, the identification of a protein of the same subfamily or with a similar function as an MMP (candidate) substrate or the biochemical confirmation by *in vitro* cleavage (IVC) or Western blot analysis (WB). Literature references in this column inducate full papers on the indidivual substrate.

[†]Degradomics method used for the identification of the (candidate) substrate. The meaning of the acronyms as well as explanations of the methods can be found in Table 1.

[‡]Confirmation; candidate substrates may be confirmed biochemically by cleavage of the recombinant/purified substrate *in vitro* (IVC), or by Western blot analysis (WB) of digested cytoplasm with substrate-specific primary antibodies. NIVC, not cleaved *in vitro*.

[¶]References are coded as follows: 1, Hwang *et al.* (2004); 2, Descamps *et al.* (2005); 3, Sawicki *et al.* (2005); 4, Greenlee *et al.*, (2006); 5, Dean and Overall (2007); 6, Dean *et al.* (2007); 7, Xu *et al.* (2008); 8, Butler *et al.* (2008); 9, Vaisar *et al.* (2009); 10, Cauwe *et al.* (2009); 11, Kleifeld *et al.* (2010).

in various subcellular locations and discuss the mechanisms by which MMPs (may) enter cells and translocate to specific cellular compartments. Finally, we will discuss the physiological and pathological effects of intracellular cleavage by MMPs in innate immune defense, cancer, cardiac and brain disease, cataract development and in the process of apoptosis.

3.1. Intracellular MMP detection: methodological considerations

Classical SDS-PAGE-based gelatin zymographic analysis, henceforth called "substrate zymography", used to detect activated forms of MMP-9 and MMP-2 does not reveal the actual *in situ* activity of the proteases. During SDS-PAGE analysis, MMPs undergo an artificial nonproteolytic activation by the detergent SDS, which denatures the proteases and deviates the propeptide out of the catalytic side (*vide supra*). After SDS-PAGE, SDS is washed away using Triton X-100 and the proteases refold, but the propeptide remains detached from the catalytic site, resulting in gelatin degradation by artefactually active pro-MMP-9 and pro-MMP-2. In contrast, nitrosative-stress induced activation of MMPs results in a size modification that is too small to be detected by SDS-PAGE (cf. §1.2). In the latter case, the pro-MMP may be a biologically active enzyme. In addition, SDS separates the MMPs from their natural inhibitors, i.e. the TIMPs. As a consequence, in the presence of TIMP-1/MMP-9 or TIMP-2/MMP-2 ratios in excess of 1, substrate zymographic analysis erroneously suggests MMP activities, although in the biological samples or eventually in the *in situ* situation they may be kept inactive by TIMP binding. In addition, one needs to keep in mind that not all fragments of pro-MMPs are active, because intermediate activation forms exist and that *ex vivo* active forms may be under endogenous inhibition *in vivo*.

Detection of MMPs by substrate zymography of cell lysates excludes determination of subcellular localization

and may result in artefactual activation of the enzymes by detergents in the cell lysis buffer. Detergent-soluble fractions of cellular lysates also contain membrane-associated proteins and as a consequence do not reflect intracellular localization per se. Hence, native, i.e. non-denaturing, subcellular fractionation into membrane, cytosolic, mitochondrial, nuclear and other organelle fractions, prior to the addition of detergents, is a prerequisite to demonstrating intracellular presence of activated MMPs by substrate zymography. Ideally, fractionation efficiency needs to be checked by assessing the distinctive presence of fraction-specific proteins, e.g. histone H3, actin and ICAM-1 as markers for nuclear, cytoskeletal and membrane fractions, respectively. Co-immunoprecipitation of the MMP and its substrate from the specific cell fraction gives an additional confirmation of subcellular colocalization of target and protease. To gain information on actual subcellular activity, the gel-based zymographic assay was adapted to assess net proteolytic activity *in situ* (Galis *et al.*, 1995). A substrate, e.g. gelatin or a peptide, is fluorescently labeled and applied to tissue sections. *In situ* zymography preserves the fine morphological details of the tissue or cell monolayer. However, whereas MMP-2 and MMP-9 are the most active gelatin degraders (Mackay *et al.*, 1990), gelatin is not an exclusive substrate of the gelatinases, but is also digested by other MMPs such as MMP-1, MMP-3, MMP-7, MMP-13/collagenase-3 and MT-MMPs (Sang and Douglas, 1996; Pavlaki and Zucker, 2003). Possible candidates able to digest both gelatin and type IV collagen are MMP-3 and MMP-7 (Sang and Douglas, 1996), MMP-26 (Park *et al.*, 2000) and serine proteases such as cathepsins (Bailey, 2000). A critical reader will take into consideration that the key messages here are the relative amounts of the various substrate-cleaving enzymes and their specific activities in substrate conversions. For example, a protease with a 100-fold lower activity on gelatin compared with, for example, MMP-9, will need a 100-fold higher concentration to be detected by *in situ* zymography with the same intensity as MMP-9. Likewise, activity assays based on fluorigenic peptides are rarely specific and must be combined with the use of specific neutralizing antibodies or cells of wildtype and MMP knockout mice.

Many proteases act in cascades (also called the "protease web" or the "proteolytic internet", cf. §1.1). Hence, proteases (and inhibitors) may be the substrates of other MMPs. As a consequence, it will often be difficult to define a direct cleavage, because the substrate conversion may be indirect, i.e. caused by an upstream protease. Such situations may be resolved *in vitro* (e.g. with the use of ultrapure substrates and proteases). However, to discriminate between direct and indirect substrates *in vivo*, highly specific inhibitors need to be developed first.

At present, the only safe approach to establish a causal relationship between a protease and cleavage of a specific substrate *in vivo* is by comparing wildtype mice with mice rendered genetically deficient in the particular protease. Of course, this approach has drawbacks and limitations, i.e. the knockout mice might be subfertile or not viable, pushing an already tedious procedure to its technical limits. The comparisons need to be done ideally in backcrossed animals and, to avoid genetic confounding, these backcrosses need to be performed ideally for more than 13 generations. In addition, one needs to keep in mind that differences, genetic and compensatory ones, will always exist between humans and mice (Hu *et al.*, 2007). The question remains whether any of the established MMP knockout mice provide credence for intracellular MMP activities. The answer to this question is not simple. First, spontaneous phenotypes in MMP knockouts are limited and, to our knowledge, no intracellular substrates have been detected so far in healthy animals, except for pro-cryptdins (Wilson *et al.*, 1999). Second, in induced phenotypes of infection and inflammation, various intracellular substrates have been defined *in vivo*, e.g. βB1-crystallin (Descamps *et al.*, 2005), connexin-43 (Lindsey *et al.*, 2006), zona occludens-1 (Asahi *et al.*, 2001) and X-ray cross-complementary factor 1 (Yang *et al.*, 2010). It may be, however, that in some of these substrates, the cleavage occurs extracellularly.

An alternative approach is the use of specific protease inhibitors. However, small-molecule inhibitors inhibiting only a single MMP have not yet been discovered and the only means of specific inhibition is the use of highly specific neutralizing antibodies (Hu *et al.*, 2007). A third associative method may also be used with patient samples. Indeed, significant inverse correlations between intact substrate and MMP levels in large groups of biological (patient) samples are strongly suggestive of *in vivo* cleavage. However, it is important to keep in mind that such correlations may be indicative of other associations than pure causal relationships.

As will be outlined, many substrates are cleaved by MMPs. It remains a methodological challenge to verify and validate whether such substrates are biologically relevant. With the use of unbiased methods to define new substrates, it is possible that biologically irrelevant substrates are detected. However, it is equally possible that by such approaches unanticipated relevant new functions of MMPs and substrates are discovered in physiology and pathology.

Substrate cleavage sites *in vitro* may be safely determined in two ways. After cleavage of the substrate in solution, the intact molecule and the fragments are separated by high performance liquid chromatography (HPLC). Mass spectrometry (MS) analysis of the elution fractions determines the mass and thus the sequence of the peptide fragments, as compared with database searches

Table 4. Intracellular localization of MMPs and TIMPs.

MMP	Subcellular localization	Cell type	Localization mechanism	Activation mode	Substrates*	Pathophysiological effect	Reference
MMP-1	Mitochondria, nucleus	Glial Müller cells, Tenon's fibroblasts, RPE cells	ND	ND	ND	Resistance to apoptosis	(Limb *et al.*, 2005)
MMP-2	Caveolae	Endothelial cells, cardiomyocytes	CAV-endocytosis	ND	ND	ND, putative regulation of intracellular localization and function of MMP-2	(Puyraimond *et al.*, 2001; Chow *et al.*, 2007a)
	Sarcomere	Cardiomyocytes	ND	Oxidative stress	TnI, MLC-1, MHC	Contributes to mechanical dysfunction of the heart after ischemia-reperfusion injury, cytokine-induced myocardial dysfunction and in dilated cardiomyopathy	(Rouet-Benzineb *et al.*, 1999; Gao *et al.*, 2003; Sawicki *et al.*, 2005; Wang *et al.*, 2002b)
	Cytoskeleton	Cardiomyocytes	ND	Oxidative stress (putative)	α-actinin, desmin	ND, may contribute to cardiac dysfunction induced by peroxynitrite	(Sung *et al.*, 2007)
	Cytoplasmic vesicles, cytoskeleton, nucleus	Astrocytes	ND	ND	ND	ND	(Sbai *et al.*, 2010)
	Secretory vesicles	Hippocampal neurons	secretion pathway	ND	ND	ND	(Sbai *et al.*, 2008)
	Mitochondria	Cardiomyocytes	ND	ND	ND	ND	(Kwan *et al.*, 2004; Wang *et al.*, 2002a)
	Cytosol	Cardiomyoblasts	ND	Oxidative stress	GSK-3β	Augmentation of GSK-3β kinase activity, which may contribute to cardiac injury resulting from enhanced oxidative stress	(Kandasamy and Schulz, 2009)
	Cytosol, nucleus	Endothelial cells	ND	Oxidative stress (putative)	ND	ND, putative function in the degradation of nuclear matrix in cigarette smoke-induced apoptosis	(Ruta *et al.*, 2009)
	Nucleus	Cardiomyocytes, hepatocytes	NLS	Oxidative stress (putative)	PARP-1	ND, putative removal of excess PARP-1 during oxidative stress, sparing the cell from ATP depletion	(Kwan *et al.*, 2004)
	Nucleus	Neurons	ND	MT1-MMP (putative)	PARP-1, XRCC1	Contribution to oxidative DNA damage in neurons early after ischemic insult, protective effect in later stages	(Yang *et al.*, 2010)
	Nucleus	Hepatocellular carcinoma cells	ND	ND	ND	ND, correlation with poor overall survival and large tumor size in hepatocellular carcinoma	(Ip *et al.*, 2007)
MMP-3	Cytosol	Dopaminergic neurons	ND	Serine protease	ND	Induction of neuronal apoptosis upstream of caspase-3	(Choi *et al.*, 2008)
	Cytoplasmic granules	Dopaminergic neuroblastoma cells	ND	Oxidative stress	α-syn	Increased fragment-induced aggregation and augmented toxicity of fragment-induced aggregates on cell viability	(Sung *et al.*, 2005)
	Cytoplasm, nucleus	Chondrosarcoma cells	CL- endocytosis, NLS, RAN-BP	ND	ND	Transcription of the *CTGF* gene, possibly affecting development, tissue remodeling and regeneration, arthritic and fibrotic diseases, and cancer progression	(Eguchi *et al.*, 2008)
	Cytosol, nucleus	Hepatocellular carcinoma cells, myofibroblasts, CHO cells	NLS	ND	ND	Induction of apoptosis	(Si-Tayeb *et al.*, 2006)

Table 4. continued on next page

Table 4. Continued.

MMP	Subcellular localization	Cell type	Localization mechanism	Activation mode	Substrates*	Pathophysiological effect	Reference
MMP-7	Cytoplasmic granules	Paneth cells	ND	ND	pro-Crps, pro-CRSC	Antibacterial activity, contributing to clearance of intestinal infections	(Wilson *et al.*, 1999; Ayabe *et al.*, 2002; Shirafuji *et al.*, 2003; Shanahan *et al.*, 2010)
	Golgi apparatus	Vaginal-cervical epithelial cells	secretion pathway	ND	occludin	Decrease of tight junction resistance	(Gorodeski, 2007)
	Cytosol	Cardiomyocytes	ND	ND	Cx43	Associated with adverse electrical remodeling and decreased survival after myocardial infarct	(Lindsey *et al.*, 2006)
	Cytosol	Neurons	CL-endocytosis	ND	SNAP-25	ND, possible perturbation of neurotransmitter exocytosis during brain inflammation	(Szklarczyk *et al.*, 2007b)
MMP-9	Secretory vesicles	Endothelial cells	secretion pathway	ND	ND	ND	(Nguyen *et al.*, 1998)
	Secretory lysosomes, cytoskeleton, nucleus	Astrocytes	endocytosis and ND	ND	ND	ND	(Sbai *et al.*, 2010)
	Cytosol	Lens fiber cells	ND	ND	βB1-, βB3- and γC-crys	Aggregation and precipitation leading to opacification of the eye lens and cataract	(Descamps *et al.*, 2005)
	Sarcomere	Cardiomyocytes	ND	Oxidative stress	MHC	May contribute to the disorganization of the contractile apparatus in dilated cardiomyopathy	(Rouet-Benzineb *et al.*, 1999)
	Mitochondria	Cardiomyocytes	ND	ND	ND	May contribute to the exacerbation of myocyte mechanical dysfunction in hyperhomocysteinemia	(Moshal *et al.*, 2008)
	Nucleus	Neurons	ND	ND	PARP-1, XRCC1	Contribution to oxidative DNA damage in neurons after ischemic insult	(Yang *et al.*, 2010)
MMP-11	TGN	Breast cancer cells	secretion pathway	Furin	ND	ND	(Pei and Weiss, 1995; Santavicca *et al.*, 1996)
	ND		ND	Alternative splicing	ND	ND	(Luo *et al.*, 2002)
MMP-12	Phagolysosome	Macrophages	ND	Catalytic activity not required	/	Direct antimicrobial activity by disrupting bacterial cell membranes	(Houghton *et al.*, 2009)
MMP-13	Cytoplasm, nucleus	Neurons, astrocytes, oligodendrocytes	ND	ND	ND	ND, early consequence of cerebral ischemia	(Cuadrado *et al.*, 2009)
MMP-26	Cytoplasm	Breast and endometrial carcinoma cells	ND	Autocatalysis	ERβ	May contribute to the disorganization of focal adhesions and actin filaments, resulting in changes in cell shape, adhesion and migration	(Savinov *et al.*, 2006)
	Cytoplasm	Prostate cancer cells	ND	Autocatalysis	pro-MMP-9 pro-MMP-26	Putative intracellular activation, leading to enhanced invasiveness of prostate cancer cells	(Zhao *et al.*, 2003)
	Cytoplasm, nucleus	Cytotrophoblasts and choriocarcinoma cells	ND	Autocatalysis	ND	ND, putative role in tissue-remodeling processes associated with placentation and tumor progression	(Zhang *et al.*, 2002)

Table 4. continued on next page

Table 4. Continued.

MMP	Subcellular localization	Cell type	Localization mechanism	Activation mode	Substrates*	Pathophysiological effect	Reference
MT1-MMP	Caveolae	Fibrosarcoma and endothelial cells	CAV-endocytosis	ND	ND	Mechanism of cell entry and/or relocalization to the leading edge of a migrating cell	(Puyraimond *et al.*, 2001; Remacle *et al.*, 2003; Galvez *et al.*, 2004)
	Endosome, centrosome, pericentrosome	Breast and colon carcinoma and glioma cells	endocytosis	Furin, autocatalysis	PCNT	Induction of chromosome instability and aneuploidy promoting malignant transformation	(Golubkov *et al.*, 2006; Golubkov *et al.*, 2005b; 2005a; Remacle *et al.*, 2005)
	Cytosol, cytoskeleton	Smooth muscle cells	ND	ND	FAK	May contribute to the disorganization of focal adhesions and actin filaments, resulting in changes in cell shape, adhesion and migration	(Shofuda *et al.*, 2004)
	Nucleus	Neurons	ND	Furin	pro-MMP-2	Putative activation of MMP-2 which contributes to oxidative DNA damage in neurons after ischemic insult	(Yang *et al.*, 2010)
	Nucleus	Hepatocellular carcinoma cells	CAV-endocytosis	ND	ND	ND, correlation with poor overall survival and large tumor size in hepatocellular carcinoma	(Ip *et al.*, 2007)
MT3-MMP	Cytosol, cytoskeleton	Smooth muscle cells	ND	ND	FAK	May contribute to the disorganization of focal adhesions and actin filaments, resulting in changes in cell shape, adhesion and migration	(Shofuda *et al.*, 2004)
	TGN	Madin-Darby canine kidney cells	Secretion pathway	Furin	ND	ND	(Kang *et al.*, 2002)
MT6-MMP	PMN granules and secretory vesicles	PMNs	Secretion pathway	Furin	ND	ND	(Kang *et al.*, 2001)
TIMP-1	Cytoplasm	Fibroblasts	ND	/	/	ND, continuous accumulation during cell cycle	(Zhao *et al.*, 1998)
	Nucleus	Fibroblasts	ND	/	/	ND, cell cycle-dependent accumulation reaching a maximum in the S phase	(Zhao *et al.*, 1998)
	Nucleus	Breast carcinoma cells	ND	/	/	ND	(Ritter *et al.*, 1999)
	Nucleus	Chondrosarcoma cells	ND	/	/	ND, putative control of nuclear MMP-3 proteolytic activity to enable proper transcription factor activity	(Si-Tayeb *et al.*, 2006)
TIMP-2	Cytoplasm	Fibroblasts	ND	/	/	ND	(Zhao *et al.*, 1998)
TIMP-3	Cytoplasm	Fibroblasts	ND	/	/	ND	(Zhao *et al.*, 1998)
TIMP-4	Sarcomere	Cardiomyocytes	ND	/	/	ND, putative regulatory role in cardiomyocyte homeostasis	(Schulze *et al.*, 2003)
	Nucleus	Cytotrophoblasts and choriocarcinoma cells	ND	/	/	ND, putative regulation of MMP-26 in tissue-remodeling processes associated with placentation and tumor progression	(Zhang *et al.*, 2002)

CAV-endocytosis, caveolae-dependent endocytosis; CHO, Chinese hamster ovary; CL-endocytosis, clathrin-dependent endocytosis; ND, not defined; NLS, nuclear localization signal; PMN, polymorphonuclear leukocytes; RAN-BP, RAN-binding protein; RPE, retinal pigment epithelial; TGN, *trans*-Golgi network.
*The meaning of the acronyms can be found in Table 5.

of theoretical peptide masses (Lopez-Otin and Overall, 2002). Alternatively, the fragments may be separated by SDS-PAGE analysis and electroblotted onto PVDF for identification of the NH_2-termini fragments by Edman degradation (Edman, 1970). Excision of fragment bands out of SDS-PAGE gels and subsequent MS analysis after in-gel trypsin digests will not yield cleavage sites and may only be indicative of cleavage regions. Indeed, unless the method is extremely well calibrated and sensitive, not all peptides may be captured and this will lead to false results. It is always advised to use complementary techniques to corroborate identified *in vitro* cleavage sites. In addition, with presently available tools for high-throughput substrate and cleavage site identification (cf. Table 1), it will be even more challenging to demonstrate that such cleavages are biologically meaningful. Since MMPs do not cleave at stringent substrate recognition sites, but only possess general preferences, *in silico* predicted cleavage sites are theoretical and need to be confirmed by one of the above methods or by generation and analysis of substrates with mutated cleavage sites.

3.2. Subcellular localization mechanisms of MMPs

As discussed in §1, MMPs may be activated in the extracellular milieu or inside cells. In this chapter we will summarize the present data on localization of MMPs in various subcellular compartments and the concomitant pathophysiological consequences. In addition, we will discuss the information about the mechanisms used by specific MMPs to enter cells and to translocate to different intracellular locales. These data are summarized in Table 4, showing for each MMP the various known subcellular localizations and translocation mechanisms in specific cell types, the (putative) intracellular activation modes, the intracellular substrates and the pathophysiological effects of intracellular translocation.

3.2.1. Cytosolic MMP activity

The term cytoplasm refers to the portion of the cell that is enclosed by the plasma membrane and is a collective term for the cytosol plus the organelles suspended in the cytosol. When intracellular localization of MMPs is determined solely on the basis of immunostainings, and not with additional confirmation by subcellular fractionation (cf. §3.1), discrimination between cytosolic and/or organelle staining is difficult. Hence, in these cases we use the more general term, cytoplasm. In addition, in the cytoplasm we only consider activated MMPs, since presence of pro-MMPs in the cytoplasm is mostly associated with protein synthesis in the endoplasmic reticulum (ER) and the Golgi apparatus, and reflects MMPs that are prepared for secretion (*vide infra*).

Many proteins have inefficient signal sequences, which may result in a variable fraction (5–20%) that is not translocated across the ER membrane but instead remains in the cytosol (Hegde and Bernstein, 2006). An example of this mechanism is the ER chaperone protein calreticulin, which has additional functions in the cytosol and nucleus (Shaffer *et al.*, 2005). Likewise, the signal sequence at the NH_2-terminus of MMP-3 contains a Pro residue at the fifth position, which was proposed to work as a helix breaker, and results in an inefficient signal peptide and sorting of MMP-3 to both the cytoplasm and the ER–Golgi pathway for further secretion (Eguchi *et al.*, 2008). Since a Pro at the start of the signal sequence is found in many MMPs, for example MMP-1,-8,-9,-13 and MT1-MMP, it would be interesting to investigate whether signal sequence inefficiency may be a general mechanism for dual sorting of MMPs to the cytosol and to the extracellular space.

Conversely, some MMPs have been found to enter cells by endocytosis. Endocytotic uptake occurs by various mechanisms, which can be roughly divided into those that are clathrin-dependent and those that are clathrin-independent. In clathrin-dependent endocytosis, the cytoplasmic domains of membrane-associated receptors or "cargo proteins" are specifically recognized by adaptor proteins and packaged into clathrin-coated vesicles that are brought into the cell (Traub, 2009; Grant and Donaldson, 2009). Clathrin-independent endocytosis is less well studied and encompasses many different endocytic mechanisms, such as caveolae- and flotilin-dependent endocytosis, uptake in GPI-enriched early endosomal compartments (GEEC) and actin-driven pathways such as macropinocytosis and phagocytosis (Doherty and McMahon, 2009; Grant and Donaldson, 2009).

MMP-7 may be internalized by clathrin-dependent endocytosis, since two inhibitors of clathrin-dependent endocytosis (phenylarsine oxide and chlorpromazine) inhibit the ability of exogenously added MMP-7 to cleave the intraneuronal protein synaptosomal-associated protein of 25 kDa (SNAP-25) (Szklarczyk *et al.*, 2007b). In support of this, immunostaining for MMP-7 showed translocation of exogenously added MMP-7 to the cytoplasmic compartment. Exogenous MMP-3 was shown to enter the cytoplasm of chondrosarcoma cells and to further translocate to the nucleus (Eguchi *et al.*, 2008). Laser confocal microscopy analysis showed colocalization of MMP-3 with low-density lipoprotein-related protein 1 (LRP1), suggesting that MMP-3 may enter cells by clathrin-dependent endocytosis (Traub, 2009). Other MMPs are also internalized by LRP-mediated endocytosis, including MMP-2 (Yang *et al.*, 2001; Emonard *et al.*, 2004), MMP-9 (Hahn-Dantona *et al.*, 2001; Van den Steen *et al.*, 2006) and MMP-13 (Barmina *et al.*, 1999; Raggatt *et al.*, 2006).

Regardless of the mode of entry, endocytosed cargo proteins and their loads are usually delivered to the

early endosome. The lumen of the early endosome is mildly acidic, which facilitates ligand release from the cargo receptors. The cargo proteins can be routed to the late endosomes and lysosomes for degradation, to the *trans*-Golgi network (TGN), or to recycling endosomal carriers that recycle the cargo proteins back to the plasma membrane (Grant and Donaldson, 2009). However, the internalized ligand proteins remain in the endosomal lumen and will be transported to lysosomes for degradation. Indeed, LRP-mediated endocytosis was described as a clearance mechanism to eliminate excessive MMP activity (Emonard *et al.*, 2005) and it is not clear how MMPs may escape endosomal entrapment and enter the cytosol. Some bacterial toxins and viruses are able to leave the endosomes by pH-dependent conformational changes (Cho *et al.*, 2003) or by pH-independent mechanisms (Matsuzawa *et al.*, 2004). Inhibition of endosomal acidification does not prevent SNAP-25 cleavage by exogenous MMP-7. Hence, MMP-7 uses an undetermined pH-independent mechanism to escape endosomal entrapment.

MT1-MMP is internalized by both clathrin-dependent (Jiang, A. *et al.*, 2001; Uekita *et al.*, 2001) and clathrin-independent caveolae-dependent mechanisms (Remacle *et al.*, 2003; Galvez *et al.*, 2004) and is recycled to the cell surface, which may be a mechanism to relocalize active MT1-MMP at the leading edge during cell migration (cf. §3.3.2). Caveolae are small membrane invaginations formed by the polymerization of caveolins and contain a subset of lipid-raft components, such as sphingolipids and cholesterol. Caveolae bud off the plasma membrane to form endocytic caveolar carriers that fuse with the caveosome or with the early endosome, or can fuse back to the plasma membrane (Parton and Simons, 2007). MMP-2 was found to colocalize with MT1-MMP, TIMP-2 and caveolin on the surface of endothelial cells (Puyraimond *et al.*, 2001) and cardiomyocytes (Chow *et al.*, 2007a). Again, the question is raised about the mechanisms that MT1-MMP and MMP-2 may use to access their cytoplasmic targets (*vide supra*). Indeed, after endocytosis, the cytoplasmic tail of MT1-MMP faces the cytoplasm, whereas the catalytical domain resides in the endosomal lumen. Hence, it would be interesting to investigate whether MT1-MMP could flip-flop, i.e. flip its orientation from inwards (as it is after endocytosis) to outwards (with its catalytic site in the cytosol and its tail in the endosome). Mechanisms for lipid flip-flop in cellular membranes have been described and are important in various biological processes such as membrane biogenesis, vesicle formation and the biosynthesis of glycolipid precursors of cell surface glycoconjugates (Pomorski and Menon, 2006). The same mechanism may apply for MMP-2 as it associates with the endocytic membrane through the binding of the caveolin scaffolding domain of caveolin-1 and caveolin-3 (Chow *et al.*, 2007a) or by association with TIMP-2 and MT1-MMP (Puyraimond *et al.*, 2001).

3.2.2. MMP activity in the secretory pathway

Classically, vesicles carrying secretory proteins are transported from the endoplasmic reticulum (ER) to the Golgi and subsequently to the TGN. As discussed in §1.1, MMP-11 and MT3-MMP are activated by furin within the TGN (Pei and Weiss, 1995; Santavicca *et al.*, 1996; Kang *et al.*, 2002). Likewise, treatment of vaginal-cervical epithelial cells with estrogen stimulates intracellular activation of MMP-7 in the Golgi apparatus (Gorodeski, 2007). Furthermore, resting neutrophils contain pro- and activated forms of MT6-MMP, which is distributed among specific granules, gelatinase B granules, secretory vesicles and the plasma membrane (Kang *et al.*, 2001). It is at present unknown whether MMP-8/neutrophil collagenase and MMP-9/gelatinase B may be activated intracellularly by MT6-MMP in neutrophils. However, the classical view is that these enzymes are rapidly activated after neutrophil degranulation, e.g. by HOCl (Weiss *et al.*, 1985; Peppin and Weiss, 1986) (cf. §1.2). Upon treatment with phorbol 12-myristate 13-acetate (PMA), microvascular endothelial cells accumulate activated forms of MMP-9. In addition, both pro- and activated forms of MMP-2 were found in secretory vesicles of neuroblastoma and primary neurons and reactive astrocytes (Sbai *et al.*, 2008; 2010). In reactive astrocytes and melanoma cells, MMP-2 and MMP-9 were found in different vesicle populations aligned along microfilaments and/or microtubules (Schnaeker *et al.*, 2004; Sbai *et al.*, 2010). In the context of the dogma of extracellular action of MMPs, the presence of intracellularly activated MMPs in the secretory pathway and in secretory vesicles was considered as a means for rapid secretion of MMP activity in the extracellular milieu. However, some MMP-2- and MMP-9-containing vesicles in neurons and melanoma cells also undergo retrograde transport, suggesting that these vesicles are not intended for secretion (Schnaeker *et al.*, 2004; Sbai *et al.*, 2008). In addition, since communication exists between the Golgi network and the endosomal pathway for the recycling of cargo proteins (Grant and Donaldson, 2009), it would be interesting to investigate whether proteins within the Golgi apparatus may be exchanged and taken up into the endosomal pathway, from where they may enter the cytosol by previously suggested mechanisms (cf. §3.2.1). Alternatively, flip-flop mechanisms may orient vesicular MT-MMPs to the cytosolic space, as was suggested for endosomal escape.

3.2.3. MMP activity associated with the cytoskeleton

The intracellular matrix (ICM) consists of the actin cytoskeleton and the microtubular network. The ICM preserves the cellular shape, enables cell migration and intracellular transport and plays major roles in cell

division. MMP-2 cleaves the cytoskeletal proteins desmin and α-actinin and colocalizes with α-actinin in cardiomyocytes (Sung *et al.*, 2007). As previously mentioned, MMP-2- and MMP-9-containing vesicles are aligned with the cytoskeleton in neurons and reactive astrocytes, and both gelatinases are found in cytoskeletal fractions from these cells (Sbai *et al.*, 2008; 2010). Disruption of the actin cytoskeleton with cytochalasin D in astrocytes results in the partial recruitment of MMP-2 and gelatinolytic activity into actin aggregates, which further indicates a connection between gelatinases and the actin cytoskeleton (Ogier *et al.*, 2006). Both MT1-MMP and MT3-MMP are detected in cytoskeletal fractions of smooth muscle cells, where they cleave the cytoskeletal protein focal adhesion kinase (FAK) (*vide infra*) (Shofuda *et al.*, 2004). In addition, MT1-MMP is trafficked along the microtubule to the centrosomal compartment, where it induces malignant transformation by cleaving the integral centrosomal protein pericentrin (*vide infra*) (Golubkov *et al.*, 2005a; 2005b; 2006). Moreover, cytoskeletal proteins constitute an important fraction of the intracellular degradomes of MMP-2, MMP-9 and MT1-MMP, as determined by various degradomics methods (cf. Table 3). Hence, the functional analysis of the effect(s) of ICM cleavage by MMPs may lead to novel insights, some of which will be suggested throughout the manuscript.

3.2.4. MMP activity in the sarcomere

The cardiac myocyte is a specialized striated muscle cell that is composed of bundles of myofibrils that contain myofilaments. The myofibrils have distinct, repeating units called sarcomeres, which represent the basic contractile units of the myocyte. MMP-2 was localized to the sarcomere of cardiac myocytes by various methods including immunogold electron microscopy, confocal microscopy, substrate zymography analysis of highly purified thin myofilament preparations and coimmunoprecipitation with sarcomere proteins that are cleaved by MMP-2 during cardiac injury, causing myocardial dysfunction (*vide infra*) (Wang *et al.*, 2002a; Gao *et al.*, 2003; Sawicki *et al.*, 2005; Kandasamy *et al.*, 2010). In addition, in dilated cardiomyopathy, both MMP-2 and MMP-9 are localized to the sarcomere, where they cleave myosin heavy chain (Rouet-Benzineb *et al.*, 1999). Under physiological circumstances, these MMP activities may be kept in check by sarcomeric TIMP-4 (Schulze *et al.*, 2003).

3.2.5. Mitochondrial MMP activity

Mitochondria may be considered as the power plants of the cell and are crucial for numerous cellular processes, including apoptosis, signaling, and metabolic pathways involving lipids, amino acids and iron. Both pro- and activated MMP-1 are associated with the mitochondrial membrane in glial Müller cells, Tenon's capsule fibroblasts, corneal fibroblasts and retinal pigment epithelial cells (Limb *et al.*, 2005). Since the mitochondrial localization of MMP-1 is found in resting cells, it suggests a physiological role for MMP-1 in cellular homeostasis. In addition, mitochondrial MMP-1 confers resistance to apoptosis by uncharacterized mechanisms. Both MMP-2 (Wang *et al.*, 2002b; Kwan *et al.*, 2004) and MMP-9 (Moshal *et al.*, 2008) are detected in cardiac mitochondria during cardiac injury and increased levels of mitochondrial MMP-9 are associated with exacerbated mechanical dysfunction.

3.2.6. MMP activity in the nucleus

Surprisingly, many studies report nuclear localization of MMPs, including MMP-1,-2,-3,-9,-13,-26 and MT1-MMP (cf. Table 4), and cleavage of nuclear matrix proteins (cf. Tables 3 and 5). The nuclear matrix binds more than 200 nuclear proteins and supports their assembly into functional macromolecular complexes involved in important nuclear processes, such as transcription, RNA splicing and DNA replication (Zink *et al.*, 2004). The nuclear matrix is surrounded by a nuclear lamina and a double-membrane nuclear envelope. Hence, nuclear import and export mechanisms are needed for the movement of large macromolecules into and out of the nucleus (Quimby and Corbett, 2001). As a consequence, to enter or exit the nucleus, MMPs need internal signals, termed nuclear localization signals (NLSs) or nuclear export signals (NESs). These signals are recognized by soluble receptors that mediate macromolecular transport through the nuclear pore complex. Two types of human NLSs exist, i.e. classical basic Lys-rich and M9-type, which are recognized by importins/karyopherins-α and transportin (importin/karyopherin-β2), respectively. Classical NESs are characterized by the presence of Leu residues and are recognized by the exportin CRM1 (Benmerah *et al.*, 2003; Quimby and Corbett, 2001).

Interestingly, Si-Tayeb *et al.* (2006) found two activated forms of MMP-3 in the nucleus of hepatocellular carcinoma cells and myofibroblasts, whereas pro-MMP-3 was not found in the nucleus. Using the bioinformatics software PSORT (Nakai and Horton, 1999), they detected a putative NLS (PKWRKTH) at position 107 to 113 in the catalytical domain, which was well conserved between the human, mouse and rat sequences of MMP-3. Nuclear entry was shown to be dependent on this NLS, as deletion of two amino acids led to a large decrease in nuclear localization. Since pro-MMP-3 was not found in the nucleus, the NLS may be shielded by the propeptide, much like the catalytical cleft is protected in the latent pro-MMPs. In contrast, pro-MMP-2 carries a putative NLS on its COOH-terminus that should be accessible without propeptide removal, thus allowing transfer of the full-length protein to the nucleus. Nuclear translocation of MMP-3 was confirmed by Eguchi *et al.* (2008), who showed that extracellular MMP-3 is taken up into chondrosarcoma

cells and subsequently translocates to the nucleus where it induces transcription of the *connective tissue growth factor* (*CTGF*) gene (*vide infra*). Five additional NLSs were found, three in the pro-domain of MMP-3 and two in the hemopexin domain, that were all driving an enhanced green fluorescent protein (EGFP)-NLS construct to the nucleus. In addition, MMP-3 was associated with a RAN-binding protein, which was involved in the nuclear import. Hence, nuclear entry of MMP-3 is regulated by two independent pathways. Loss of nuclear MMP-3 was detected 30 min after nuclear entry, suggesting degradation or nuclear export to the cytoplasm by a Leu-rich NES sequence in the hemopexin domain.

Besides MMP-2 and MMP-3, Si-Tayeb *et al.* (2006) detected putative NLSs in the sequences of MMP-1,-8,-10,-13,-19,-20,-23 and MT1-, MT3-, MT4- and MT5-MMP, which suggests that nuclear entry may be a feature of many MMPs. MMPs that do not contain an NLS may enter the nucleus by binding to cargoes, such as RAN-binding proteins, other proteins with an NLS, various types of RNA and complexes of RNA plus proteins (ribonucleoproteins or RNPs) (Quimby and Corbett, 2001). For example, MMP-9 may enter the nucleus bound to the nuclear protein Ku, an interaction that was detected at the cell surface (Monferran *et al.*, 2004). In addition, some proteins without NLS but associated with clathrin-coated pits or caveolae are also able to shuttle in and out of the nucleus, although the precise mechanisms involved in these processes are poorly understood (Benmerah *et al.*, 2003; Lee, KW *et al.*, 2004). Such nuclear translocation via caveolae-mediated endocytosis was proposed for MT1-MMP in aggressive hepatocellular carcinoma cells (Ip *et al.*, 2007).

To avoid excessive proteolysis of nuclear proteins during cellular homeostasis, these nuclear MMPs may be under inhibition by TIMP-1 and TIMP-4, which are also present in the nucleus (cf. Table 4) (Zhao *et al.*, 1998; Ritter *et al.*, 1999; Zhang *et al.*, 2002; Si-Tayeb *et al.*, 2006)

In conclusion, a systematic investigation of the localization mechanisms and the presence of MMPs in the various subcellular compartments – under conditions of cellular stress and homeostasis – may provide more insight into the present fragmented image. In addition, this may contribute to the elucidation of intracellular functions of MMPs, which may be of great importance when considering MMP inhibition therapy.

3.3. Intracellular proteolysis by MMPs in physiology and pathology

It has been clearly demonstrated in the previous chapters that MMPs may be activated intracellularly or enter cells and various cellular compartments after extracellular activation. This leads to questions about the physiological and pathological implications of intracellular MMP activity. Intracellular proteins, which are the subject of intracellular MMP proteolysis, will be discussed in the context of the physiological or pathological pathways affected by their cleavage. We will review well-established, confirmed intracellular substrates, which are summarized in Table 5 with the (putative) location of their cleavages. This table also includes the biological roles of the substrates, the studied MMPs, the identified cleavage sites, the context of the cleavage (*in vitro*, *ex vivo* or *in vivo*) and the (possible) effects of the cleavages on physiopathology. Cleavage of substrates identified by degradomics (cf. Table 3) will be mentioned in the relevant sections.

3.3.1. Intracellular proteolysis in innate immune defense

The efficacy of innate host defense can be attributed to the ability of the immune system to recognize and neutralize microbial invaders quickly and efficiently. In mammals, inflammatory leukocytes and affected epithelia synthesize and/or mobilize anti-microbial peptides that are capable of directly killing a variety of pathogens, i.e. cathelicidins and defensins. The α- and β-defensins comprise a family of cationic trisulfide peptides that kill bacteria by membrane disruption. α-defensins were identified as antimicrobial proteins purified from extracts of cytoplasmic granules of polymorphonuclear (PMN) leukocytes. Mice, however, lack leukocytic defensins. Nevertheless, Paneth cells that populate the crypts of Lieberkühn throughout the mouse small intestine express many antimicrobial α-defensins called cryptdins (crypt defensins). Cryptdin peptides, packaged in apically oriented granules of Paneth cells, are secreted into the crypt by degranulation that is both constitutive at a base-line level and inducible by infection. Biosynthesis of pre-pro-α-defensins (~10 kDa) involves the rapid cleavage of the signal peptide producing pro-α-defensins (~8.5 kDa) that have little or no microbicidal activity *in vitro*. Activation of defensin peptides requires proteolytic removal of the NH_2-terminal acidic pro-region, yielding a mature peptide of ~3.5 kDa (Selsted and Ouellette, 2005).

MMP-7 colocalizes with cryptdins (Crps) in mouse Paneth cells and mediates the processing and activation of various Crps *in vitro* (Wilson *et al.*, 1999). MMP-7 cleaves pro-Crp-1, -6 and -15 at Ser43-Val44; Ser53-Leu54 and Ser58-Leu59. The latter cleavage generates the NH_2-terminal consensus sequence in all related pro-Crps (pro-Crp-1 to -16) except for pro-Crp-4 and -5, which do not show sequence identity for the above mentioned cleavage sites (Putsep *et al.*, 2000). However, they are cleaved at the same positions, i.e. Ser43-Ile44, Ala53-Leu54 and Ser58-Leu59 (Ayabe *et al.*, 2002; Shirafuji *et al.*, 2003). When analyzing the processing intermediates of Crp4 ($Crp4^{44–92}$, $Crp4^{54–92}$ and $Crp4^{59–92}$), the most bactericidal of

Table 5. Confirmed intracellular MMP substrates and the (putative) physiopathological effects of their cleavage.

Substrate symbol	Substrate name	Subcellular localization	Biological role substrate	MMP	Cleavage sites*	Physiological/pathological effect of the cleavage	References
Cytosol							
βB1-crys	βB1-crystallin	Cytosol	Prevention of protein unfolding or aggregation, structural component of the eye lens	MMP-9	Ala47-Lys48^{m} *In vitro + ex vivo + in vivo*	Aggregation and precipitation leading to opacification of the eye lens and cataract	(Descamps *et al.*, 2005)
βB3-crys	βB3-crystallin	Cytosol	Prevention of protein unfolding or aggregation, structural component of the eye lens	MMP-9	*Ex vivo*	Aggregation and precipitation leading to opacification of the eye lens and cataract	(Descamps *et al.*, 2005)
γC-crys	γC-crystallin	Cytosol	Prevention of protein unfolding or aggregation, structural component of the eye lens	MMP-9	*In vitro*	ND, putative contribution to opacification of the eye lens and cataract	(Descamps *et al.*, 2005)
Cx43	Connexin-43	Cytosol, mitochondria	Gap junction protein	MMP-7	*In vitro + in vivo*	Associated with adverse electrical remodeling and decreased survival after myocardial infarction	(Lindsey *et al.*, 2006)
ERβ	Estrogen receptor-β	Cytosol, nucleus	Transmission of 17β-estradiol signaling	MMP-26	*In vitro + in vivo*	ND, associated with a favorable prognosis and longer overall survival of breast cancer patients	(Savinov *et al.*, 2006)
GSK-3β	Glycogen synthase kinase-3β	Cytosol, mitochondria, nucleus	Multifunctional Ser/Thr kinase regulating cellular functions such as apoptosis, cell cycle, cell migration and gene expression	MMP-2	*In vitro*	Augmentation of GSK-3β kinase activity, which may contribute to apoptosis and cardiac injury resulting from enhanced oxidative stress	(Kandasamy and Schulz, 2009)
SNAP-25	Synaptosomal-associated protein of 25 kDa	Cytosol	Neurovesicular fusion and neurotransmitter release	MMP-7	Ala128-Ile129 *In vitro*	ND, possible perturbation of neurotransmitter exocytosis during brain inflammation	(Szklarczyk *et al.*, 2007b)
ZO-1	Zona occludens-1	Cytosol, nucleus	Tight junction scaffolding protein mediating cytoskeletal anchorage of the tight junction	MMP-9 MMP-13	*In vitro + in vivo*	BBB disruption in cerebral ischemia	(Harkness *et al.*, 2000; Asahi *et al.*, 2001; Lu *et al.*, 2009)
Cytoplasmic granules							
pro-Crp-1-3	pro-Cryptdin-1 to -3	Cytoplasmic Paneth granules	Bactericidal action by bacterial membrane disruption	MMP-7	Ser43-Val44^{m} Ser53-Leu54^{m} Ser58-Leu59^{m} *In vitro + in vivo*	Antibacterial activity, contributing to clearance of intestinal infections	(Ayabe *et al.*, 2002; Shirafuji *et al.*, 2003; Wilson *et al.*, 1999)
pro-Crp-4-5	pro-Cryptdin-4,-5	Cytoplasmic Paneth granules	Bactericidal action by bacterial membrane disruption	MMP-7	Ser43-Ile44^{m} Ala53-Leu54^{m} Ser58-Leu59^{m} *In vitro + in vivo*	Antibacterial activity, contributing to clearance of intestinal infections	(Ayabe *et al.*, 2002; Shirafuji *et al.*, 2003; Wilson *et al.*, 1999)
pro-Crp-6-16	pro-Cryptdin-6 to -16	Cytoplasmic Paneth granules	Bactericidal action by bacterial membrane disruption	MMP-7	Ser43-Val44^{m} Ser53-Leu54^{m} Ser58-Leu59^{m} *In vitro + in vivo*	Antibacterial activity, contributing to clearance of intestinal infections	(Ayabe *et al.*, 2002; Shirafuji *et al.*, 2003; Wilson *et al.*, 1999)
pro-CRS4C	Cysteine-rich sequence 4C	Cytoplasmic Paneth granules	Bactericidal action by bacterial membrane disruption	MMP-7	Ser43-Val44^{m} Ala53-Leu54^{m} Ala58-Leu59^{m} *In vitro + in vivo*	Antibacterial activity, contributing to clearance of intestinal infections	(Shanahan *et al.*, 2010)

Table 5. continued on next page

Table 5. Continued.

Substrate symbol	Substrate name	Subcellular localization	Biological role substrate	MMP	Cleavage sites*	Physiological/pathological effect of the cleavage	References
α-syn	α-synuclein	Cytoplasm, mitochondria, nucleus	Regulation of neurotransmitter release, pathogenic accumulation and aggregation in Lewy bodies in Parkinson's disease	MMP-3	Thr54-Val55 Ala90-Ala91 Glu57-Lys58 Ala91-Thr92 Ala78-Gln79 Thr92-Gly93 Gln79-Lys80 Gly93-Phe94 Lys80-Thr81 Phe94-Val95 Ser87-Ile88 Lys102-Asn103 *In vitro*	Increased fragment-induced aggregation and augmented toxicity of fragment-induced aggregates on cell viability; inhibition of aggregation with high MMP concentrations	(Sung *et al.*, 2005; Levin *et al.*, 2009)
				MMP-1	Gly7-Leu8 Val71-Thr72 Ala18-Ala19 Ala78-Gln79 Lys21-Thr22 Ala90-Ala91 Gly41-Ser42 Lys97-Asp98 Gly47-Val48 Tyr33-Gln134 Val70-Val71 Gln134-Asp135 *In vitro*	Increased aggregation after limited proteolysis, inhibition of aggregation with high MMP concentrations	(Sung *et al.*, 2005; Levin *et al.*, 2009)
				MMP-9	Phe4-Met5 Val71-Thr72 Gly7-Leu8 Gly73-Val74 Ala18-Ala19 Val74-Thr75 Val66-Gly67 Ala78-Gln79 Val70-Val71 Asp98-Gln99 *In vitro*	No significant increase in aggregation after limited proteolysis, inhibition of aggregation with high MMP concentrations	(Sung *et al.*, 2005; Levin *et al.*, 2009)
				MT1-MMP MMP-2	*In vitro*	ND	(Sung *et al.*, 2005)
Cytoskeleton							
α-actinin	α-actinin	Cytoskeleton, myofibril, sarcomere, Z-disc, nucleolus, extracellular	Connection of actin filaments of adjacent sarcomeres and transmission of the force generated by the actin-myosin complex	MMP-2	*In vitro* + *ex vivo*	ND, may contribute to cardiac dysfunction induced by peroxynitrite	(Sung *et al.*, 2007)
Desmin	Desmin	Cytoplasm, cytoskeleton, Z-disk	Intermediate filament protein involved in cellular resistance to external stress	MMP-2	*In vitro*	ND	(Sung *et al.*, 2007)
FAK	125 kDa focal adhesion kinase	Cytoplasm, cytoskeleton	Tyrosine kinase involved in cytoskeleton remodeling, formation and disassembly of cell adhesion structures and regulation of Rho-family GTPases	MT1-MMP MT3-MMP	*In vitro*	May contribute to the disorganization of focal adhesions and actin filaments, resulting in changes in cell shape, adhesion and migration	(Shofuda *et al.*, 2004)
Pericentrin	Pericentrin	Centrosome	Integral centrosomal protein required for normal centrosome functioning and mitotic spindle formation	MT1-MMP	Gly1156-Leu1157 Gly672-Leu673 Ser2068-Leu2069[c] *In vitro*	Induction of chromosome instability and aneuploidy promoting malignant transformation	(Golubkov *et al.*, 2005b; 2006)
Sarcomere							
MHC	Myosin heavy chain	Sarcomere	Mechanical protein that generates force during muscle contraction	MMP-9 MMP-2	*In vitro*	May contribute to the disorganization of the contractile apparatus in dilated cardiomyopathy	(Rouet-Benzineb *et al.*, 1999)

Table 5. continued on next page

Table 5. Continued.

Substrate symbol	Substrate name	Subcellular localization	Biological role substrate	MMP	Cleavage sites*	Physiological/pathological effect of the cleavage	References
MLC-1	Myosin light chain-1	Cytoplasm, cytoskeleton, sarcomere	Mechanical protein that generates force during muscle contraction	MMP-2	*In vitro* + *ex vivo*	Contributes to mechanical dysfunction of the heart after ischemia-reperfusion injury	(Sawicki *et al.*, 2005)
TnI	Troponin I	Sarcomere	Binds to actin in thin filaments to hold the troponin-tropomyosin complex in place and inhibits myocardial force generation in the resting phase	MMP-2	*In vitro* + *ex vivo*	Contributes to mechanical dysfunction of the heart after ischemia-reperfusion injury and cytokine-induced myocardial dysfunction	(Wang *et al.*, 2002a; Gao *et al.*, 2003)
Nucleus							
PARP-1	Poly (ADP-ribose) polymerase-1	Nucleus	ATP-dependent DNA repair enzyme	MMP-2	*In vitro*	Contribution to oxidative DNA damage in neurons early after ischemia, putative removal of excess PARP-1 during later stages of cardiac and cerebral ischemia, sparing the cell from ATP depletion	(Kwan *et al.*, 2004)
XRCC1	X-ray cross-complementary factor 1	Nucleus	Central role in the DNA base excision repair pathway by interaction with the DNA repair enzymes	MMP-2 MMP-9	*In vitro* + *in vivo*	Contribution to oxidative DNA damage in neurons after ischemic insult	(Yang *et al.*, 2010)
Extracellular milieu							
αB-crys	αB-crystallin	Cytosol, cytoskeleton, nucleus	Prevention of protein unfolding or aggregation, structural component of the eye lens	MMP-9	*Cf.* Table 8	Generation of immunodominant T cell epitopes and removal of the neuroprotective effects of αB-crys in multiple sclerosis	(Starckx *et al.*, 2003)
CAP1	Adenylyl cyclase-associated protein 1	Cytoskeleton	Enhancement of actin filament turnover; roles in cell morphology, migration and endocytosis; promotion of apoptosis; autoantigen in SLE and RA	MMP-9 MMP-2,-8, -13	*In vitro* + *in vivo*	ND, putative anti-apoptotic effect	(Cauwe *et al.*, 2008)
Gelsolin	Gelsolin	Cytosol, cytoskeleton, secreted	F-actin capping and severing; nucleation of F-actin assembly; scavenging of actin and pro-inflammatory components in the plasma; defects are the cause of amyloidosis type 5 or familial amyloidosis Finnish type	MMP-3 MT1-MMP MMP-1,-2, -7,-9	Asn416-Val417 Ser51-Met52 Ala435-Gln436 *In vitro* Ala242-Met243 *In vitro* *In vitro*	May cause actin toxicity by weakening the actin scavenger system; generation of amyloidogenic peptides that cause familial amyloidosis Finnish type; may abolish the anti-apoptotic and Aβ-reducing function of gelsolin in Alzheimer's disease	(Hwang *et al.*, 2004; Park *et al.*, 2006; Page *et al.*, 2005; Antequera *et al.*, 2009; Ray *et al.*, 2000)

*Cleavage sites are indicated in the human sequence. The suffix in superscript indicates an alternative organism: m, murine; c, canine.
NB, MMPs that were described to be activated intracellularly by other MMPs are considered as intracellular substrates but are not included in this table.

the known mouse α-defensins, Weeks *et al.* (2006) found that their *in vitro* antibacterial effects are very similar, contrasting with the lack of bactericidal and membrane-disruptive capacity of pro-Crp-4$^{20-92}$. Hence, cleavage by MMP-7 at Ser43-Ile44 is sufficient to activate bactericidal activity, and the amino acids between 20 and 43 of the pro-region maintain the precursor in an inactive state, which may be important to prevent deleterious effects to the host cell during pro-α-defensin synthesis, folding and packaging into granules (Weeks *et al.*, 2006). Why this processing occurs intracellularly is an enigma, but intragranular colocalization of bactericidal peptides and MMP-7 might enhance their cleavage, when compared to cleavage in the diluted extracellular milieu. In addition, these and other observations (*vide infra*) do not exclude the possibility of additional MMP-7-mediated extracellular Crp activation.

MMP-7 gene deletion ablates pro-Crp processing, resulting in accumulation of cryptdin precursors in Paneth cell granules and the absence of activated mature Crp peptides in the small intestine. Since Crps constitute about 70% of the bactericidal activity released by Paneth cells (Ayabe *et al.*, 2000), MMP-7 knockout mice without functional Crp peptides, have a functional defect in clearance of intestinal infections, and they succumb more rapidly and to lower doses of virulent *Salmonella typhimurium* compared with control mice (Wilson *et al.*, 1999). Thus, defective pro-Crp activation is associated with a deficit in mucosal immunity and increased risk of systemic disease.

Whereas murine leukocytes do not contain defensin peptides, in humans, α-defensins are primarily found in neutrophils (human neutrophil peptides 1-4 or HNP-1 to -4) and small intestinal Paneth cells (human defensins 5 and 6 or HD-5 and -6), the latter being activated by trypsin (Ghosh *et al.*, 2002). MMP-7 cleaves within the prodomain of pro-HNP-1, which does not produce the sequence of the mature peptide, but of a processing intermediate with antimicrobial activity (Wilson *et al.*, 2009). However, since MMP-7 is not present in neutrophils, and the neutrophilic MMPs (MMP-8, MMP-9 and MT6-MMP) are compartmentalized separately from α-defensins, proteolytic activation of pro-HNPs within maturing human neutrophil granules does not seem to be an MMP-dependent process.

In the mouse, α-defensin genes duplicated and diversified further to give rise to the *Defcr-rs* gene subfamily that codes for numerous cysteine-rich sequence 4C (CRS4C) peptides that are unique to mice. Remarkably, pre-pro-regions of CRSC4 and Crp peptides are nearly identical but the mature CRSC4 peptides are cysteine-rich peptides very different from Crps (Ouellette and Lualdi, 1990). Native CRSC4 peptides purified from mouse small intestine exist as disulfide-stabilized homodimers and heterodimers with antibacterial activity (Huttner and Ouellette, 1994). As expected from the high sequence identity of the pro-regions, inactive pro-CRSC4-1 (cryptdin-related protein 4C-1, defensin-related cryptdin-related sequence 2) was found to be converted to mature bactericidal CRSC4-1 by MMP-7-mediated *in vitro* proteolysis of the precursor pro-region and at the same residue positions as determined for Crp activation, i.e. Ser43-Val44, Ala53-Leu54 and Ala58-Leu59. The absence of processed CRSC4 peptides in MMP-7 knockout mice demonstrates the *in vivo* activation of CRS4C by MMP-7 (Shanahan *et al.*, 2010).

Besides activation of antimicrobial peptides, it was recently discovered that MMPs may function as antimicrobial agents themselves. A recent study showed that MMP-12 possesses antimicrobial activities that do not require the catalytical domain, but reside in the COOH-terminal hemopexin domain. The hemopexin domain of MMP-12 inhibited the growth of various bacterial strains *in vitro*. The origin of bactericidal activity was narrowed down to a unique four amino acid sequence of acidic amino acids flanked by basic residues (e.g. Lys348-Asp-Asp-Lys in human MMP-12) present on an exposed β loop of the protein. This sequence is homologous in rabbit, rat, mouse and human MMP-12, but is not present in any other MMP. MMP-12-deficient mice exhibited impaired bacterial clearance and increased mortality when challenged with both Gram-negative and Gram-positive bacteria at macrophage-rich portals of entry, such as the peritoneum and the lung. After engulfment of bacteria by macrophages, intracellular stores of MMP-12 were mobilized to macrophage phagolysosomes, as determined by immunogold electron microscopy. Once inside phagolysosomes, MMP-12 adhered to bacterial cell walls and used its antimicrobial peptide to kill the bacteria by disruption of the cell membranes (Houghton *et al.*, 2009). *Strictu sensu*, in this case no evidence is provided for a host cell substrate conversion, but instead MMP-12 hemopexin domain binds to and kills micro-organisms by a non-catalytical function.

In conclusion, both catalytical and non-catalytical actions of MMPs inside cells may contribute to the host's innate immune defense against microorganisms.

3.3.2. Intracellular proteolysis in cancer

Extracellular proteolysis by MMPs has conflicting effects on cancer cell differentiation, proliferation, escape from apoptosis and immune surveillance, migration and invasion, and tumor angiogenesis. Depending on the substrates, as well as on the specific stage of cancer cell progression, the same MMP may exert a cancer-promoting or -limiting function (Egeblad and Werb, 2002; Cauwe *et al.*, 2007). The stepwise progression from an early dysplastic lesion to full-blown metastatic malignancy is associated with increases in genomic instability (Vogelstein and Kinzler, 2004). Chromosome

instability, the inability to correctly segregate sister chromatids during mitosis (leading to an abnormal chromosome number termed aneuploidy), is thought to initiate and propagate malignant transformation (Schvartzman *et al.*, 2010). Disruption of centrosome structure is an integral aspect of the origin of chromosomal instability in many cancers. Centrosomes nucleate and organize microtubules and form spindle poles during mitosis. In normal cells, the centrosome is usually composed of a pair of barrel-shaped structures, the centrioles, which are embedded in a lattice-like pericentriolar material (Zyss and Gergely, 2009). As discussed before, cell surface-associated MT1-MMP is internalized by endocytosis, and microtubular trafficking causes the protease to accumulate in the centrosomal and pericentrosomal compartment (Remacle *et al.*, 2003; 2005). MT1-MMP expression is associated with tumor progression and metastasis and is implicated both in the breaching of basement membranes by tumor cells and invasion of the ECM. In addition, MT1-MMP activates soluble MMPs, cleaves adhesion and signaling receptors and acts as a tumor growth factor (Poincloux *et al.*, 2009). Besides these extracellular invasion-promoting functions, MT1-MMP was shown to have an intracellular oncogenic function by cleaving the integral centrosomal protein, pericentrin, at Gly672-Leu673 and Gly1156-Leu1157 (Golubkov *et al.*, 2005a; 2005b). Pericentrin and pericentrin-2 (pericentrin-B or kendrin) are derived from splice variants of the same gene and are known to be essential for normal centrosome function by the anchorage of the γ-tubulin ring complex, which initiates microtubule nucleation, to the centrosome (Takahashi *et al.*, 2002). Normal epithelial cells transfected with MT1-MMP acquired the ability to activate MMP-2, to cleave pericentrin and to invade a Matrigel matrix. These events were associated with aberrations in chromosome segregation, up-regulation of gene expression of multiple oncogenic genes, aneuploidy and transformation of non-malignant human mammary epithelial cells. Indeed, MT1-MMP-expressing human epithelial cells were efficient at generating tumors in the orthotopic xenograft model in immunodeficient mice. However, the tumors regressed because of insufficient host angiogenic response and inadequate neovascularization, also in line with the lack of upregulation of pro-angiogenic gene expression. Both human and canine pericentrin peptides are cleaved by MT1-MMP *in vitro*, the latter at the Ser2068-Leu2069 peptide bond. However, murine pericentrin is resistant to cleavage because of an Asp residue at the P1 position (cf. Figure 4) instead of Ser or Gly. In addition, tumor biopsies showed proteolytic pericentrin fragments in association with high levels of activated MT1-MMP, whereas normal tissues contained intact pericentrin. Hence, the intracellular proteolysis of pericentrin by MT1-MMP may have an important role in the generation of chromosome instability and malignant transformation (Golubkov *et al.*, 2005a; 2005b; 2006).

Once tumor cells have acquired invasive capacities, polarized motility is governed by the organization of a leading edge in the direction of cell movement. Cell motility requires molecular processes at the cell surface in which contacts between the invading tumor cell and the surrounding cells and stroma are repeatedly broken (anti-adhesion) and new contacts are established as the tumor cell moves forward (adhesion). Hence, the leading edge is stabilized by the formation of new focal adhesions or cell–ECM contact sites. Besides its pericellular proteolytic functions, MT1-MMP also seems to affect tumor cell migration by perturbation of focal adhesions. Indeed, in vascular smooth muscle cells, overexpression of MT1-MMP (and MT3-MMP) results in cell rounding, decreased adherence and increased migration (Shofuda *et al.*, 2001). These events were associated with a decreased number of focal contacts with integrin-mediated adhesion, whereas the cell surface expression of integrin subunits remained unchanged, excluding their cleavage by MT1-MMP. However, MT1-MMP overexpression resulted in the cleavage of the 125 kDa focal adhesion kinase (FAK, pp125FAK, protein-tyrosine kinase 2) into a 90 kDa NH_2-terminal fragment (Shofuda *et al.*, 2004). FAK is a cytoplasmic protein-tyrosine kinase recruited to and activated at focal adhesion sites and is especially important for the connection of ECM-integrin complexes with downstream signaling molecules and actin stress fibers (Tomar and Schlaepfer, 2009). FAK cleavage was associated with partial dissociation of paxillin from the integrin-FAK complex and both events were inhibited by the metalloproteinase inhibitor BB94. Paxillin is another important cytoskeletal and scaffolding protein recruited early to focal adhesions (Tomar and Schlaepfer, 2009). Loss of the FAK/paxillin interaction may be a major contributing factor in the reduced organization of focal adhesions and actin filaments, resulting in decreased integrin-mediated cell adhesion observed in cells overexpressing MT1-MMP. Cleavage of FAK may be induced by MT1-MMP through an indirect mechanism, since calpains, caspases and granzyme B were also shown to proteolyse FAK (Wen *et al.*, 1997; Gervais *et al.*, 1998; Carragher *et al.*, 1999). However, as MT1-MMP, as already mentioned, has an intracellular cleavage function in tumor cells, and both MT1-MMP and MT3-MMP were found in the cytoskeletal fraction of vascular smooth muscle cells (Shofuda *et al.*, 2004), it is very likely that FAK is a direct intracellular target of MT1-MMP. Interestingly, viral Src kinase-transformed cells activate a FAK-dependent mechanism that attenuates endocytosis of MT1-MMP (cf. §3.2.1). This in turn increases cell-surface expression of MT1-MMP and pericellular degradation of the ECM (Wu *et al.*, 2005). Hence, intracellular cleavage of FAK by MT1-MMP may be a way to loosen focal adhesions, permitting the cell

to detach for further migration, and at the same time it may enhance endocytosis of MT1-MMP at the rear end for recycling and relocalization at the leading edge. In this context, a membrane flip-flop of MT1-MMP, as suggested in §3.2.1, would allow MT1-MMP to cleave FAK and other cytoskeletal substrates at the focal adhesion (cf. Tables 3 and 5).

Altogether, this may suggest a bimodal role for MT1-MMP in cancer progression. The early function of MT1-MMP takes place inside cells and promotes malignant transformation. When the cells have acquired invasive capacity, MT1-MMP is delivered to the surface of invadipodia where it assists in the proteolysis of a path for the migrating cell. After migration the intracellular cleavage of FAK may loosen focal adhesions at the rear end and recycle MT1-MMP to the novel invadipodia. Hence, MT1-MMP is not just a protease that supports tumor growth and metastasis, but it also functions as an oncogene that promotes chromosome instability and malignant transformation of normal cells at the early stages of the transition to malignancy (Golubkov and Strongin, 2007). Clinical trials with MMP inhibitors for the treatment of metastatic cancer have generally failed (Coussens *et al.*, 2002). Indeed, the beneficial effects of MT1-MMP inhibition in animal models of cancer may be explained in part by the absence of these oncogenic functions, which will not be the case for MMP inhibition in late-stage cancer trials.

Besides its actions in the centrosomal compartment and at focal adhesions, activated MT1-MMP is also detected in the nuclei of hepatocellular carcinoma cells (cf. §3.2.6). Interestingly, cancer patients with nuclear MT1-MMP (and co-localized MMP-2) have a poor overall survival and large tumor size, whereas MT1-MMP is not found in nuclei of the normal paralleled liver tissues and normal control livers (Ip *et al.*, 2007). Since the protein composition of the nuclear matrix is altered in cancer cells (Zink *et al.*, 2004), it is tempting to speculate that MT1-MMP, MMP-2 and other nuclear MMPs (cf. §3.2.6) may contribute to the degradation of nuclear matrix proteins. Firm proof-of-principle is not available but specific proteins in the nuclear matrix, e.g. fibronectin, are known extracellular substrates of MMPs.

Another MMP that may affect cancer progression by its nuclear function is MMP-3. As mentioned in §3.2.6 and Table 4, activated MMP-3 was found in the nuclei of cultured chondrocytes, in normal and osteoarthritic chondrocytes (Eguchi *et al.*, 2008), in the nuclei of dopaminergic neurons (Choi *et al.*, 2008) and hepatocytes (Si-Tayeb *et al.*, 2006). In the latter two studies, nuclear MMP-3 was found to promote apoptosis, which was discussed in §1.4.5. In chondrocytes, nuclear MMP-3 interacts with the transcription enhancer dominant in chondrocytes (TRENDIC) in the *connective tissue growth factor* (*CTGF*) gene promoter and activates *CTGF* gene transcription. Interestingly, both the catalytical domain and the hemopexin domain can activate the *CTGF* promoter independently (Eguchi *et al.*, 2008). CTGF (hypertrophic chondrocyte-specific protein 24, insulin-like growth factor-binding protein 8, CCN family member 2) is a member of the CCN family of secreted, matrix-associated proteins encoded by immediate early genes that play various roles in angiogenesis and tumor growth (Chu *et al.*, 2008). CCN stands for **c**ysteine-rich 61, **c**onnective tissue growth factor and **n**ephroblastoma overexpressed. CTGF is a multifunctional signaling modulator involved in a wide variety of biological and pathological processes such as angiogenesis, osteogenesis, renal disease, skin disorders and tumor development. Hence, MMP-3-induced transcription of *CTGF* in chondrocytes may be involved in matrix diseases, e.g. osteoarthritis and rheumatoid arthritis, and in fibrotic diseases, such as systemic sclerosis and atherosclerosis (Eguchi *et al.*, 2008). CTGF regulates cancer cell migration, invasion, angiogenesis and anoikis. Although CTGF expression is mostly associated with progression of many kinds of cancers, its role may vary considerably, depending on the tissue involved (Chu *et al.*, 2008). CTGF in turn induces increased transcription of the *MMP-1*, *-2*, *-3*, *-7*, *-9* and *MT1-MMP* genes, but reduces transcription of *TIMP-1* and *TIMP-2* (Kondo *et al.*, 2002; Chen, CC *et al.*, 2001), which may then result in a positive feedback loop in angiogenesis and tumor progression. In addition, CTGF is cleaved by MMP-1,-2,-3,-7,-13 and MT1-MMP (Hashimoto *et al.*, 2002; Dean and Overall, 2007; Dean *et al.*, 2007; Butler *et al.*, 2008), which may terminate the amplification loop.

Angiogenesis is an important mechanism in tumor biology. MMPs, and MMP-9 in particular, are involved in various aspects of the angiogenic switch (Hanahan and Weinberg, 2000) and in the angiogenic process (Ardi *et al.*, 2007; Deryugina and Quigley, 2010). So far, most validated substrates, e.g. CTGF, are extracellular proteins. A considerable amount of putative intracellular substrates of MMPs are linked to endothelial cell function (cf. Tables 3 and 5). However, the biological relevance of the cleavage of these intracellular proteins in the process of angiogenesis needs further exploration.

In contrast with these tumor-promoting functions of intracellular MMPs, MMP-26 may have antitumor properties inside breast cancer cells. MMP-26 is characterized by an unorthodox, autolytic activation mechanism (cf. §1.1) and accumulates primarily inside cells, as only a small fraction of the enzyme is secreted into the extracellular milieu (Strongin, 2006). In contrast to other MMPs, the promoter of the *MMP-26* gene includes the estrogen-response element (ERE). The estrogen-estrogen receptor (ER) complex stimulates the transcriptional activity of the *MMP-26* gene promoter in hormone-regulated carcinomas via binding of the ERE motif (Li *et al.*, 2004).

Interestingly, estrogen receptor β (ERβ, nuclear receptor subfamily 3 group A member 2) is susceptible to proteolysis by MMP-26, whereas ERα is resistant. MMP-26 targets the NH_2-terminal region of ERβ coding for the divergent NH_2-terminal A/B domain that is responsible for the ligand-independent transactivation function *in vitro*. In breast carcinoma specimens, the ERα-dependent expression of MMP-26 correlated inversely with the residual levels of intact ERβ in the cytoplasm, as determined by immunohistochemical analysis. The levels of MMP-26 are low in normal mammary epithelium but are strongly upregulated in ductal carcinoma *in situ* (DCIS). However, during further disease progression the expression of MMP-26 decreases again. In contrast with many tumor-promoting MMPs, the expression of MMP-26 in DCIS is correlated with a longer overall patient survival (Savinov *et al.*, 2006).

Conversely, activation of pro-MMP-9 by MMP-26 promotes invasion of human prostate cancer cells (Zhao *et al.*, 2003). Activated forms of MMP-9 were shown to accumulate in the cytosol of basal and phorbol PMA-stimulated human endothelial cells. Whereas MMP-9 was found complexed to TIMP-1 in the conditioned medium, it existed as a free enzyme in membrane-bound vesicles that were especially prominent at the invadipodia (as determined with immunogold electron microscopy). In contrast, TIMP-1 was spread more diffusely throughout the cytoplasm, occasionally present in small vesicles but never in the MMP-9-containing vesicles (Nguyen *et al.*, 1998). Both MMP-3, which was also detected in the cytoplasm, and self-activated MMP-26 may act as activators for the intracellular pro-MMP-9 (cf. §1.1). Storage of intracellularly activated MMP-9, ready for rapid release, may facilitate invasion and migration of cancer cells.

Finally, MMP-1 was found to be strongly associated with mitochondrial membranes (cf. §3.2.5) and nuclei and accumulated within cells during the mitotic phase of the cell cycle. The intracellular association of MMP-1 to mitochondria and nuclei conferred resistance to apoptosis (*vide infra*), which may be a mechanism for tumor cells to escape from apoptosis (Limb *et al.*, 2005).

3.3.3. Intracellular proteolysis in cardiac disease

Heart failure is a common cause of morbidity and mortality with a high incidence in developed countries. Approximately every 25 seconds, an American will have a cardiac event, and approximately every minute, someone will die of one (Lloyd-Jones *et al.*, 2010). Following cardiovascular stress, a cascade of compensatory structural events occurs within the myocardium and contributes to eventual left ventricular dysfunction and the manifestation of heart failure syndrome. MMPs have been extensively studied in various cardiac pathologies such as ischemia-reperfusion (I/R), dilated cardiomyopathy (DCM) and myocardial infarct (MI) (Creemers *et al.*, 2001; Brauer, 2006; Janssens and Lijnen, 2006; Spinale, 2007). Myocardial ischemia either with or without reperfusion induces ROS and pro-inflammatory cytokines. These reactive molecules are cardiodepressant through impairment of Ca^{2+} homeostasis. ROS can induce intracellular Ca^{2+} overload during oxidative stress. In addition, ROS directly injure the cell membrane and disturb the integrity of proteins, lipids and DNA, causing cell death (Hori and Nishida, 2009). However, low concentrations of specific ROS, such as peroxynitrite, may directly activate the zymogen forms of MMPs, as detailed in §1.2. In addition, MMPs may be activated by caspases after myocardial infarction (cf. §1.1).

During the last decade, Schulz and colleagues have accumulated a considerable amount of evidence on the intracellular localization and acute actions of MMPs, particularly MMP-2, in the heart. In contrast with most researchers, who have focused on the long term proteolytic effects of MMPs on ECM remodeling, they showed that MMP-2 contributes to acute cardiac mechanical dysfunction before the development of changes in the collagen matrix (Chow *et al.*, 2007b; Schulz, 2007; Ali and Schulz, 2009; Kandasamy *et al.*, 2010). Indeed, already after 20 minutes of global no-flow ischemia in the rat, a marked increase in pro-MMP-2 was observed in the coronary effluent that peaked within the first minute of reperfusion. The levels of pro-MMP-2 correlated positively with the duration of ischemia and negatively with the recovery of mechanical function. Contractile dysfunction associated with the induced I/R improved with a neutralizing MMP-2 antibody and with general MMP inhibitors (doxycycline and 1,10-phenantroline (O-phen)) (Cheung *et al.*, 2000).

Heart failure is characterized by a general decline in pump function caused by a decline in contractile properties of the cardiac myocytes. Myocardial contraction is initiated upon the release of Ca^{2+} into the cytosol from the sarcoplasmic reticulum following membrane depolarization. Thin filament activation and relaxation dynamics have emerged as a pivotal regulatory system tuning myofilament function to the beat-to-beat regulation of cardiac output. Perturbation of thin filament dynamics is now recognized as an important cellular mechanism causing reduced cardiac pump function in a variety of cardiac diseases (Kobayashi *et al.*, 2008). I/R injury in the heart is associated with degradation or loss of myofilament regulatory proteins as well as structural and cytoskeletal proteins (Hein *et al.*, 1995; Matsumura *et al.*, 1996).

By immunogold electron microscopy, confocal microscopy, immunoprecipitation experiments and zymographic analysis of highly purified thin myofilament preparations, MMP-2 was localized to sarcomeres in close association with the thin myofilaments in hearts subjected to I/R (Wang *et al.*, 2002b). The

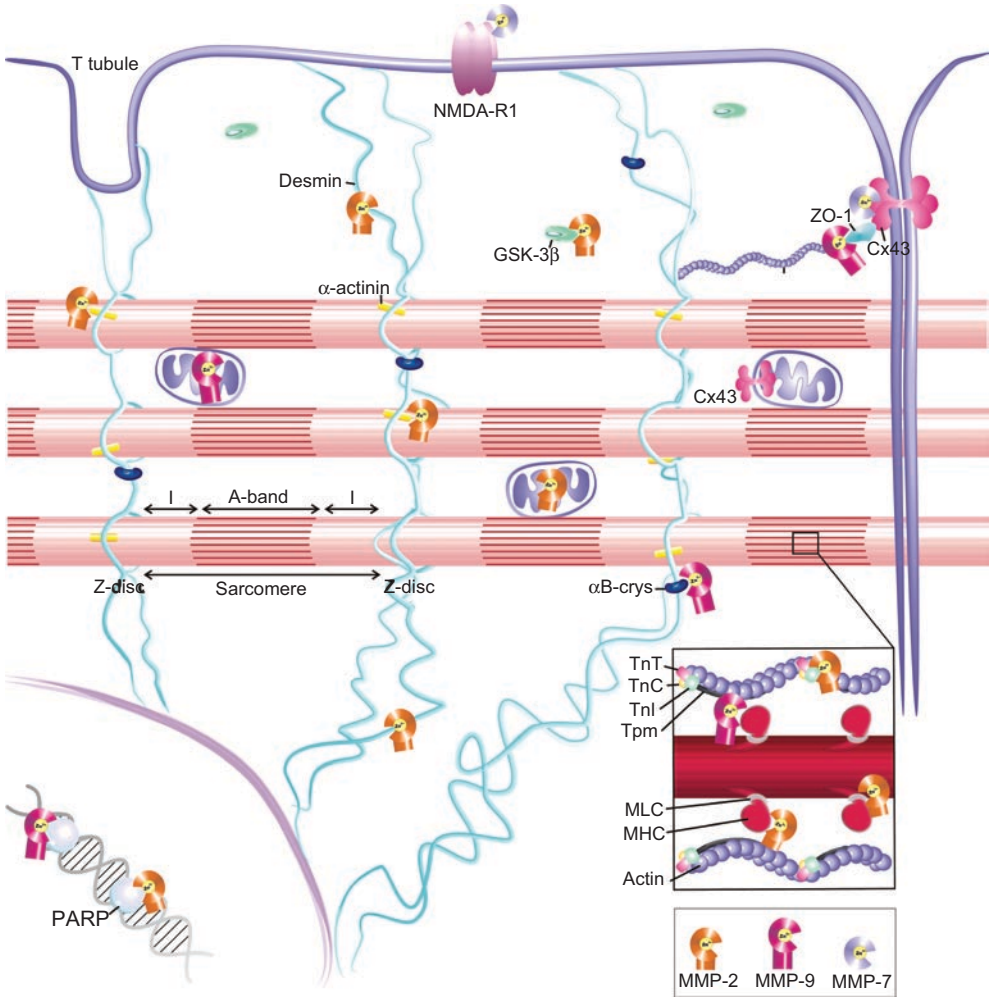

Figure 5. Intracellular proteolysis by MMPs in cardiac disease. MMPs may cause direct contractile dysfunction by the proteolysis of major sarcomeric proteins such as troponin I (TnI), myosin light chain (MLC) and myosin heavy chain (MHC). Intracardiomyocyte proteolysis by MMPs in cardiac injury also results in loss of cytoskeletal proteins, including α-actinin and desmin, and its chaperone, αB-crystallin (αB-crys). Proteolysis of connexin-43 (Cx43) leads to adverse electrical changes, probably by perturbation of the Cx43/zona occludens-1 (ZO-1) interaction. Under nitrosative stress, Cx43 translocates to the mitochondria, where its degradation leads to mitophagy and cell death, which may be ameliorated by cleavage of the N-methyl-D-aspartate receptor-1 (NMDAR1). Proteolysis of glycogen synthase kinase-3β (GSK-3β) enhances its pro-apoptotic capacities and degradation of PARP in the nucleus also leads to enhanced apoptosis and cardiomyocyte death.

thin filament is composed of F-actin together with two tropomyosin strands, each binding a troponin (Tn) complex (Figure 5). Tropomyosin and the Tn complex, composed of three subunits: troponin C (TnC), I (TnI) and T (TnT), regulate the affinity of F-actin towards myosin. In resting conditions (absence of Ca^{2+}), TnI inhibits myosin cross-bridge formation to actin, and concomitant generation of myocardial force, via interactions with TnT and tropomyosin (Kobayashi *et al.*, 2008). Activated MMP-2 was found to co-localize with TnI (troponin I) and to rapidly degrade TnI *in vitro*. Inhibition of MMP-2 activity with doxycycline and O-phen prevented I/R-induced TnI degradation and improved the recovery of the mechanical function of isolated rat hearts. Importantly, no significant myocardial necrosis was observed, as TnI and its degradation products were not found in the coronary effluent, an additional proof of the intracellular localization of MMP-2 action (Wang *et al.*, 2002b). This was confirmed in another model of myocardial contractile failure, in which the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-γ (IFN-γ) depressed myocardial contractile function by enhancing peroxynitrite production. MMP-2 activity was increased before the decline in myocardial mechanical function and this was followed by decreased levels of TnI in the cytokine-treated hearts, which was prevented by a neutralizing anti-MMP-2 antibody (Gao *et al.*, 2003). However, these neutralization experiments imply that the anti-MMP-2 antibody enters the intracellular milieu, for example by endocytosis, or that the binding and neutralization of

MMP-2 occurs extracellularly and impedes the entry of MMP-2 in the myocyte.

Using 2D-PAGE, Schulz and colleagues found that myosin light chain (MLC-1) was degraded in hearts subjected to I/R injury and that this proteolytic process was inhibited with MMP inhibitors (doxycycline and O-phen). Activated forms of MMP-2 were found in rat heart preparations of thick myofilaments, and by immunogold microscopy analysis, MMP-2 was localized in the sarcomere in a pattern consistent with the known distribution of MLC-1 (cf. Figure 5). In addition, purified MLC-1 was susceptible to MMP-2 (but not MMP-9) proteolysis in *vitro* (Sawicki *et al.*, 2005). Interestingly, a different localization of the two gelatinases, MMP-2 and MMP-9, was observed in hearts of patients with dilated cardiomyopathy (DCM) compared to control hearts. In DCM hearts the gelatinases were localized exclusively within the cardiomyocytes in close association with the sarcomeric structure, whereas localization was mainly around the myocytes in control hearts. MMP-9, and to a lesser extent MMP-2, cleaved purified myosin heavy chain (MHC) *in vitro* (cf. Figure 5) (Rouet-Benzineb *et al.*, 1999). Hence, MMPs may cleave both regulatory and structural elements that generate myocardial force, leading to mechanical dysfunction and heart failure.

This phenomenon may be enhanced by an imbalance between MMPs and TIMPs during cardiac injury. Indeed, during I/R in isolated rat hearts, TIMP-4, the most abundant TIMP in the heart, was also rapidly released in the coronary effluent, as shown by reverse zymography and Western blot analysis. By immunogold microscopy analysis a close association of TIMP-4 with sarcomeres was demonstrated in aerobically perfused rat hearts, whereas this pattern of positive staining was reduced in I/R hearts. Although both MMP-2 and TIMP-4 were released into the extracellular milieu during reperfusion, a net proteolytic balance in hearts exposed to I/R was shown by *in situ* zymography of heart sections. These data suggest that TIMP-4 plays an important homeostatic role in the normal myocardium and that its release from the cardiomyocytes contributes to myocardial I/R injury (Schulze *et al.*, 2003). Likewise, all TIMPs were downregulated in DCM hearts, especially TIMP-1 (Rouet-Benzineb *et al.*, 1999), further pointing to the participation of MMP/TIMP imbalances in cardiac diseases.

As mentioned before, I/R injury is also associated with the degradation of cytoskeletal proteins such as α-actinin, desmin and spectrin (Matsumura *et al.*, 1996). This may constitute an additional intracellular function of MMP-2, as α-actinin and desmin (but not spectrin) were found to be *in vitro* substrates of MMP-2 (cf. Figure 5). α-actinin (α-actinin cytoskeletal isoform, non-muscle α-actinin-1, F-actin cross-linking protein) is an actin-binding protein. It forms an anti-parallel rodshaped dimer with one actin-binding domain at each end of the rod and bundles actin filaments in multiple cytoskeletal frameworks. Besides binding to actin filaments, α-actinin associates with a number of cytoskeletal and signaling molecules, for instance, cytoplasmic domains of transmembrane receptors and ion channels. Thus, α-actinin exerts major structural and regulatory roles in cytoskeletal organization and muscle contraction (Sjoblom *et al.*, 2008). Desmin is the main intermediate filament protein expressed in cardiac, skeletal, and smooth muscle cells. It interacts with other proteins to form a continuous cytoskeletal network that maintains a spatial relationship between the contractile apparatus and other structural elements of the cell, thus providing maintenance of cellular integrity, force transmission, and mechanochemical signaling. Mutations in desmin and in αB-crystallin (αB-crys), a chaperone for desmin and another MMP substrate (*vide infra*), lead to desminopathy, a myofibrillar myopathy (Goldfarb and Dalakas, 2009). Peroxynitrite infusion into isolated rat hearts caused a decline in mechanical function and activation of MMP-2 with concomitant loss of intact α-actinin (but not desmin), which was prevented by an MMP inhibitor (PD-166793) or the peroxynitrite scavenger reduced glutathione (GSH). In addition, MMP-2 was found to co-localize with α-actinin in cardiomyocytes. Hence, these results suggest yet another contribution of MMP-2 to cardiac dysfunction by mediating cytoskeletal disorganization (Sung *et al.*, 2007).

In the heart, electrical stimulation readily spreads via direct cell-to-cell communication mediated by low resistance gap junction channels composed of proteins termed connexins, such that all myocytes contract in an ultra-fast wave at each beat. Hence, connexin channels ensure propagation of molecular and electrical signals and are crucial in myocardial synchronization and heart function (Tribulova *et al.*, 2008). Induction of myocardial infarct (MI) in MMP-7 knockout mice resulted in increased early survival and favorable alterations in electrical conduction patterns compared with wildtype mice. In wildtype mice, slower conduction velocity correlated with a 53% reduction in the gap junction protein connexin-43 (Cx43, gap junction 43 kDa heart protein, gap junction α-1 protein), which is the predominant connexin isoform in atrial as well as ventricular tissues. Because myocardial conduction patterns were altered, along with changes in gap junction proteins, the contributing factor for differences in survival was likely an alteration in electrical conduction due to MMP-7 genetic deletion. Surface plasmon resonance experiments revealed that MMP-7 binds to Cx43, and further *in vitro* analyses determined that MMP-7 cleaves Cx43 in the cytoplasmic COOH-terminal tail (cf. Figure 5). Infusion of MMP-7 induced Cx43 degradation and resulted in arrhythmias and heart block *in vivo* (Lindsey *et al.*, 2006). COOH-terminal truncation of Cx43 will abolish interaction with other proteins, such as zona

occludens-1 (ZO-1), which was reported to have striking effects on gap junction size and distribution (Hunter *et al.*, 2005) and may contribute to the negative influence of MMP-7 on electrical conduction. Interestingly, ZO-1 is an MMP-9 substrate (*vide infra*), which suggests that both MMPs may synergize in the perturbation of the Cx43/ZO-1 interaction, leading to adverse electrical changes and decreased survival after MI.

The mitochondria constitute yet another subcellular location wherefrom MMPs may disturb cardiac contractility. Both MMP-2 (Wang *et al.*, 2002b; Kwan *et al.*, 2004) and MMP-9 (Moshal *et al.*, 2008) are found in cardiac mitochondria. Elevated levels of blood homocysteine (HCY) (termed hyperhomocysteinemia) induced expression of MMP-9 in the mitochondrial compartment and induced mitochondrial permeability transition and contractile dysfunction, which was reversed by various compounds, including an inhibitor of the cardiomyocyte *N*-methyl-*D*-aspartate receptor-1 (NMDA-R1) (MK-801), a general MMP inhibitor (GM6001) and cyclosporine A. The intramitochondrial nitrosative stress induced by HCY in cardiomyocytes was associated with an increased translocation of Cx43 to the mitochondria and degradation of Cx43, and led to "mitophagy" (mitochondrial autophagy) and cell death. This suggests that MMP-9 or another MMP may degrade Cx43 in the mitochondria and cause contractile dysfunction in hyperhomocysteinemia. These effects were ameliorated by cardiac-specific deletion of NMDA-R1 (Tyagi *et al.*, 2010). Interestingly, MMP-7 cleaves neuronal NMDA-R1 in the extracellular ligand-binding domain and this impairs NMDA-stimulated Ca^{2+} flux (Szklarczyk *et al.*, 2008). Hence, extracellular action of MMP-7 may be cardioprotective, whereas intracardiomyocyte cleavage of Cx43 by MMP-7 may cause heart failure, complicating the use of MMP inhibitors in cardiac disease.

In abnormal conditions, cardiomyocytes mount an adaptive response that attempts to normalize ventricular wall stress and maintain cardiac output. Prolonged stress overwhelms this protective response and leads to cardiomyocyte apoptosis and heart failure. Glycogen synthase kinase-3β (GSK-3β) is a multifunctional Ser/Thr kinase that plays important roles in necrosis and apoptosis of cardiomyocytes. A major mechanism of cell necrosis is the opening of the mitochondrial permeability transition pore (mPTP). The threshold for mPTP opening is elevated by phosphorylation of GSK-3β at Ser9, which reduces activity of this kinase. In addition, inhibition of GSK-3β suppresses ATP hydrolysis by reducing ATP transport from the cytosol to the mitochondria. This prevents both ATP depletion and accumulation of inorganic phosphate, two factors promoting mPTP opening. Although the role of GSK-3β in apoptosis of cardiomyocytes has not been fully clarified, evidence to date supports its significant contribution to apoptosis induced by I/R, hypoxia/reoxygenation, β-adrenoreceptor activation and pressure overload (Miura and Miki, 2009). GSK-3β was cleaved upon incubation with MMP-2 and this proteolytic cleavage of GSK-3β increased its activity. H_2O_2 challenge of H9c2 cardiomyoblasts increased the activity and level of MMP-2, reduced the level of GSK-3β and increased GSK-3β kinase activity. Both effects on GSK-3β were reduced by MMP inhibitors (GM6001 and ONO-4187). In addition, MMP-2 pull-down assays from H9c2 cell lysates showed the binding of MMP-2 with GSK-3β (Kandasamy and Schulz, 2009). Since inhibition of GSK-3β is cardioprotective for many reasons (Miura and Miki, 2009), augmentation of its activity may be an additional way of MMP-2 to contribute to cardiac injury resulting from enhanced oxidative stress. Interestingly, MMP-2 cleaves another molecule that causes ATP depletion during oxidative stress, nuclear poly(ADP-ribose)polymerase (PARP, NAD^+ ADP-ribosyltransferase 1, poly(ADP-ribose) synthetase 1). PARP is a DNA repair enzyme. It is activated by DNA strand breaks, which may be caused by oxidative stress (Ahmad *et al.*, 2009). Both MMP-2 and MMP-9 were found in nuclear extracts of cardiomyocytes and hepatocytes (cf. Figure 5). MMP-2 was immunoprecipitated from nuclear extracts of heart cells with anti-PARP antibody and degraded PARP *in vitro* (Kwan *et al.*, 2004). Inhibitors of PARP have been shown to have a protective effect in cardiac injury (Pacher *et al.*, 2005). Mild damage to DNA activates the DNA repair machinery. In contrast, once excessive oxidative and nitrosative stress-induced DNA damage occurs, as in various forms of myocardial reperfusion injury and heart failure, overactivated PARP initiates an energy-consuming cycle by transferring ADP-ribose units from NAD^+ to nuclear proteins, resulting in rapid depletion of the intracellular NAD^+ and ATP pools, slowing the rate of glycolysis and mitochondrial oxidative phosphorylation (the generation of ATP), and eventually leading to cellular dysfunction and death. In addition, PARP also regulates the expression of a variety of pro-inflammatory mediators, which might facilitate the progression to heart failure. Hence, Schulz (2007) suggested that nuclear MMP-2 may play a protective role during oxidative stress by proteolytic removal of the activated PARP overload. This means that besides contrasting extracellular versus intracellular functions (cf. MMP-7, *vide supra*), MMPs may also have opposed roles depending on their subcellular location.

In conclusion, MMP activities are detected in all subcellular compartments of the cardiomyocyte (cf. Figure 5) and generally contribute to dysfunction of the contractile apparatus and adverse electrical conduction after cardiac injury. Therapeutic intervention by MMP inhibition thus seems promising. However, as some subcellular MMP functions may be cardioprotective, adverse effects may be

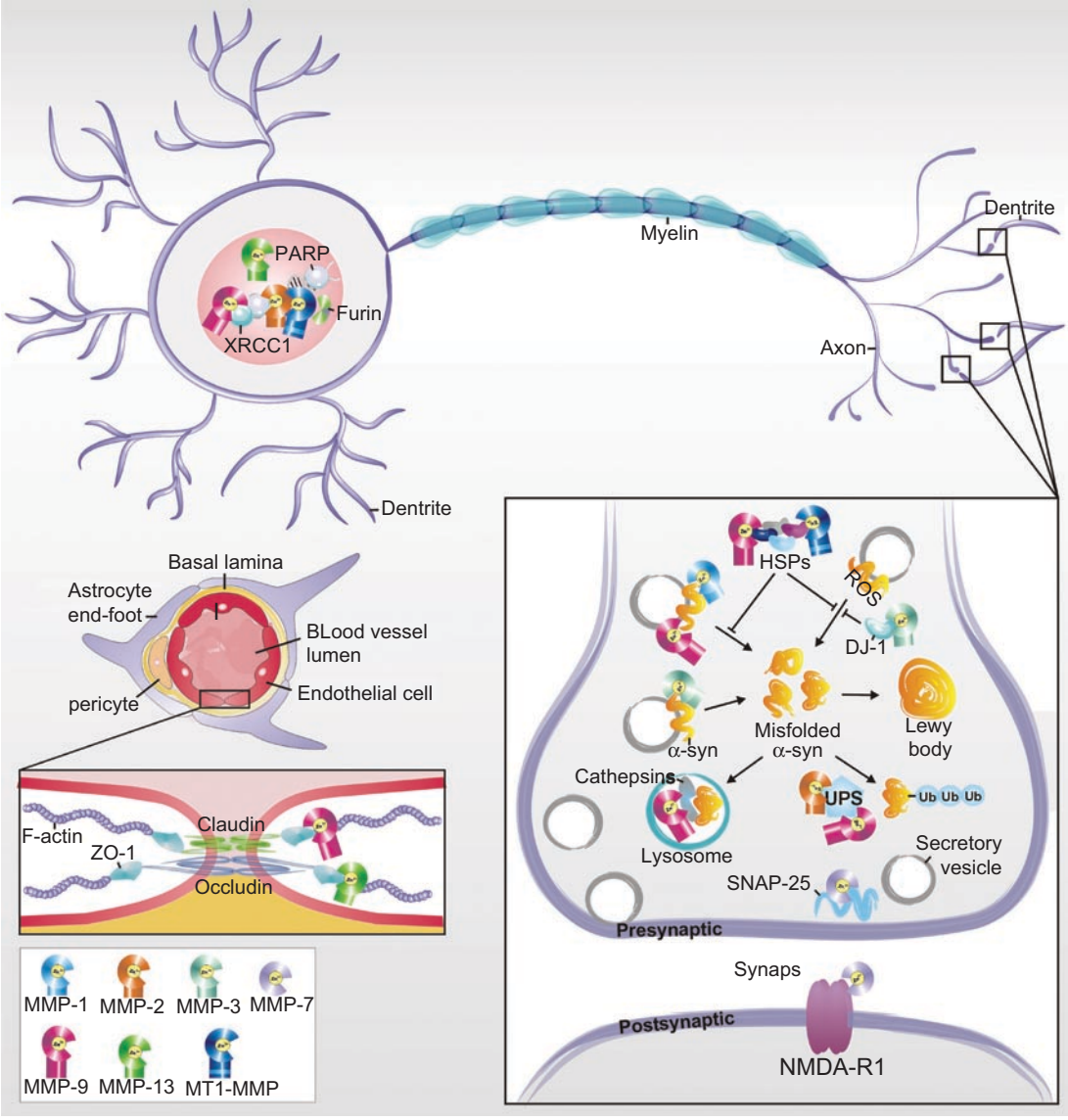

Figure 6. Intracellular proteolysis by MMPs in acute and chronic neurodegeneration. In acute cerebral ischemia, MMPs contribute to blood brain barrier disruption by the extracellular degradation of claudin and occludin, and by the intracellular proteolysis of zona occludens-1 (ZO-1). Furthermore, activated MMP-2 and MMP-9 in the nucleus may cleave poly (ADP-ribose) polymerase (PARP) and X-ray cross-complementary factor 1 (XRCC1), which causes apoptosis and neuronal cell death early after ischemia as it impairs DNA repair after oxidative DNA damage. However, in later disease stages the clearance of the overactivated PARP overload may contribute to recovery. MMP-2 may be activated by MT1-MMP, which in turn may be activated by furin, since all three proteases were found to colocalize in the nuclei of ischemic neurons. In presynaptic terminals, proteolysis of synaptosomal-associated protein of 25 kDa (SNAP-25) may perturb synaptic vesicle exocytosis during neuroinflammation. Intracellular proteolysis by MMPs also has pathogenic effects in chronic neurodegenerative diseases, such as Parkinson's disease. MMPs cleave α-synuclein and increase its aggregation, which may lead to enhanced accumulation of toxic Lewy bodies. By degrading protective chaperones (e.g. heat shock proteins or HSPs) and anti-oxidant molecules (e.g. DJ-1), MMPs also contribute indirectly to toxic protein precipitation and aggregation. Finally, MMPs may also perturb the clearance of misfolded proteins by inactivating many proteins of the ubiquitin-proteasomal system (UPS) and lysosomal proteins.

inevitable without the use of subcellular compartment-specific inhibitors.

3.3.4. Intracellular proteolysis in acute and chronic neurodegenerative diseases

Within seconds to minutes after the loss of blood flow to a region of the brain, the ischemic cascade is rapidly initiated and comprises a series of successive biochemical events. Hypoperfusion of a brain area leads to oxidative damage and excitotoxicity, which is the process by which neurons are damaged and killed *via* the overactivation of receptors for the excitatory neurotransmitter glutamate, such as the *N*-methyl-*D*-aspartate receptor-1 (NMDA-R1) and the α-amino-3-hydroxy-5-methyl-4-

isoxazolepropionic acid receptor (AMPA-R) receptor. Oxidative stress and excitotoxicity in turn cause microvascular injury, blood–brain barrier (BBB) dysfunction and post-ischemic inflammation, ultimately resulting in cell death of neurons, glia and endothelial cells. These events exacerbate the initial injury and may lead to permanent cerebral damage, the extent of which is dependent on the degree and duration of ischemia (Lee, SR *et al.*, 2004; Lakhan *et al.*, 2009). The extracellular participation of MMPs in cerebral ischemia has been well documented. MMPs cause the increase in permeability of the BBB by attacking the endothelial cell tight junctions (TJs) and the basal laminae, i.e. the subendothelial and the parenchymal basement membranes (Agrawal *et al.*, 2006), resulting in the final common pathway downstream of acute neuroinflammatory damage. When acute hypoxia–ischemia initiates the cellular damage, MMPs target the matrix proteins around blood vessels and brain cells, resulting in cytotoxic and vasogenic edema, hemorrhagic transformation, and apoptosis of neurons and oligodendrocytes (Cauwe *et al.*, 2007; Candelario-Jalil *et al.*, 2009; Rosenberg, 2009).

The BBB is a diffusion barrier, consisting of an interdependent network of cells designed to segregate the central nervous system (CNS) from the systemic circulation. One of the primary functions of the BBB is the strict regulation of paracellular permeability, which is mediated by the endothelial tight junctions (TJs) of the capillary that limit paracellular movement of solutes, ions, and water. The preservation of the TJs is governed by three essential transmembrane proteins: claudins, occludin, and junction adhesion molecules (Figure 6). The cytoplasmic regions of these transmembrane proteins are attached to intracellular scaffolding proteins, such as zona occludens-1,-2 and -3 and cingulin, which in turn are anchored to the actin cytoskeleton. Treatment of brain endothelial cells with activated MMP-9 resulted in an increased frequency of discontinuities in the immunohistological staining of zona occludens-1 (ZO-1, tight junction protein 1) and a consistent decrease in the intensity of ZO-1 expression (Harkness *et al.*, 2000). ZO-1 was degraded *in vivo* during transient and permanent focal ischemia and this was attenuated with MMP inhibitors (BB-94 and KB-R7785) (Asahi *et al.*, 2000; Jiang, X. *et al.*, 2001; Bauer *et al.*, 2010) and in MMP-9 knockout mice, with concomitant reduction of BBB disruption and infarct size (Asahi *et al.*, 2001). Degradation of ZO-1 was also observed after treatment of rat brain endothelial cells with activated MMP-13 (Lu *et al.*, 2009). However, since ZO-1 is a cytoplasmic protein, degradation must occur intracellularly by cytoplasmic proteases. Indeed, cerebral ischemia and hypoxia increased both MMP-9 expression and activation in brain endothelial cells (Asahi *et al.*, 2000; Bauer *et al.*, 2010), suggesting that endogenous intracellular MMP-9 cleaves ZO-1. However, hypoxic conditioned medium of astrocytes (with upregulated levels of MMP-2, -9 and -13) also induced ZO-1 cleavage in brain endothelial cells, which was reversed with neutralizing antibodies against MMP-9 and MMP-13. Hence, alternative explanations are that extracellular MMPs trigger ZO-1 cleavage indirectly, or that MMPs are internalized, for example by endocytic mechanisms, as discussed in §3.2.1.

The aberrant, excessive activity of extracellular MMPs contributes directly to neuronal cell death in multiple ways, including modulation of anoikis (Gu *et al.*, 2002) and calpain activity (Copin *et al.*, 2005). Interestingly, S-nitrosylated MMP-9 (cf. §1.2) may not only induce neuronal apoptosis by anoikis, but possibly also by the cleavage of actin. Indeed, increased pro- and activated MMP-9 levels in ischemic brains were associated with slightly decreased levels of actin (Gu *et al.*, 2002), and cleavage of actin and other cytoskeletal proteins is pro-apoptotic (*vide infra*). In addition, MMPs may also contribute to neuronal apoptosis by an intranuclear function. Indeed, various studies show early upregulation of gelatinolytic activity in the nucleus after an ischemic insult (Gasche *et al.*, 2001; Gu *et al.*, 2005; Yang *et al.*, 2007; Amantea *et al.*, 2008). The MMPs identified in the nucleus were MMP-2, MMP-9, MMP-13 and MT1-MMP (cf. Figure 6) (Cuadrado *et al.*, 2009; Yang *et al.*, 2010). In addition, furin was found co-localized with MT1-MMP and MMP-2 in ischemic cell nuclei, which may trigger the MMP activation cascade (cf. §1.1). MMP inhibition (CH6631) reduced cerebral infarction as well as cerebral ischemia-induced apoptosis, as evidenced by reduced DNA fragmentation and cytochrome c release and increased intact PARP (Copin *et al.*, 2005). Purified PARP was cleaved *in vitro* by MMP-2 and MMP-9, and by less defined gelatinase preparations and total nuclear extracts from ischemic brains. In addition, this cleavage was inhibited by an MMP-2/-9 inhibitor. PARP activity was significantly reduced in ischemic brains compared with those treated with BB1101. Activated PARP recruits X-ray cross-complementary factor 1 (DNA repair protein XRCC1) which is also cleaved by MMP-2 and MMP-9 in *vitro*, as well as by gelatinase preparations and total nuclear extracts. Nuclear gelatinolytic activity co-localized with PARP and XRCC1 staining in ischemic brains and their *in vivo* degradation during ischemia was reduced by the MMP inhibitor BB1101. In addition, accumulation of oxidative DNA damage was also reduced by BB1101 (Yang *et al.*, 2010). As discussed above (cf. §3.3.3), PARP is activated by DNA strand breaks caused by oxidative stress. XRCC1 is a substrate of PARP and is recruited by PARP to sites of DNA damage. XRCC1 functions as a scaffold protein able to coordinate and facilitate the steps of various DNA repair pathways by interacting with major DNA repair enzymes (Horton *et al.*, 2008). In response to cellular damage by oxygen radicals or excitotoxicity, a rapid and strong activation of PARP occurs in neurons and excessive PARP activation

has been implicated in cerebral ischemia (Skaper, 2003). Hence, Yang *et al.* (2010) suggest that in mild damage of early stage I/R injury, proteolysis of PARP1 and XRCC1 may contribute to oxidative DNA damage and neuronal apoptosis by impairing their protective action in the DNA repair pathway. However, in later disease stages, intranuclear MMP activity may contribute to recovery by clearing the burden of over-activated PARP1 that causes neuronal necrosis. Hence, the beneficial effects of MMP inhibitors in the early stages would need to be balanced with later interference in recovery. This scheme also implies specific time windows for the treatment of brain ischemia with MMP inhibitors (Hu *et al.*, 2007).

Intracellular proteolysis during brain inflammation not only affects neuronal survival but may also adversely influence synaptic function. Application of recombinant MMP-7 to cultured rat neurons induced long-lasting inhibition of vesicular recycling as well as reduced local abundance of vesicular and active zone proteins within synaptic terminals. Chronic application of MMP-7 resulted in synaptic atrophy, including smaller terminals and fewer synaptic vesicles (Szklarczyk *et al.*, 2007a). On one hand, this effect may be explained by extracellular proteolysis of ECM, adhesion molecules and receptors such as NMDAR-1 (Szklarczyk *et al.*, 2008). On the other hand, MMP-7 was evidenced to proteolyse the presynaptic protein synaptosomal-associated protein of 25 kDa (SNAP-25) (cf. Figure 6). SNAP-25 is a cytosolic soluble N-sensitive factor attachment protein receptor (SNARE) complex protein that participates in the regulation of synaptic vesicle exocytosis (Matteoli *et al.*, 2009). MMP-7 generates a long-lasting (7 days) 15 kDa SNAP-25 fragment by cleavage at the Ala128-Ile129 peptide bond (Szklarczyk *et al.*, 2007b). MMP-7-mediated proteolysis of SNAP-25 was inhibited by inhibitors of clathrin-dependent endocytosis (cf. §3.2.1). MMP-7 may thus share a particular function with clostridial toxins, which enter neuronal cells via receptor-mediated endocytosis and cleave SNARE proteins, generating long-lasting fragments and perturbing neurotransmitter exocytosis (Bajohrs *et al.*, 2004). Hence, exogenous MMP-7 is able to access and cleave an intraneuronal substrate and may impair neurotransmission during brain inflammation.

Besides their roles in neuronal apoptosis after acute cerebral I/R injury, intranuclear MMPs may also affect chronic neurodegenerative diseases such as Parkinson's disease (PD). PD results from neurodegeneration of dopaminergic neurons in the *substantia nigra*, which is associated with activation of microglia and accumulation of aggregated α-synuclein in specific brain stem, spinal cord, and cortical regions (Lees *et al.*, 2009). Recent studies have implicated MMPs in the death of dopaminergic neurons in this disease. *In vitro*, apoptotic dopaminergic neurons under cellular stress released activated MMP-3, but not the pro-form, which acted as a microglia activating molecule. Both released activated MMP-3 and the catalytical domain of MMP-3 led to the production of superoxide and microglial inflammatory cytokines such as TNF-α, which in turn exacerbated neuronal apoptosis and necrosis. In the N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-injected animal model of PD, neuronal degeneration, microglial activation, and superoxide generation were largely attenuated in MMP-3 knockout mice (Kim *et al.*, 2005; 2007). An activated form of MMP-3 was generated inside stressed dopaminergic cells by a serine protease different from furin (cf. §1.1). This intracellular activated form of MMP-3 (but not the extracellular MMP-3) was found to participate in apoptotic signaling, upstream of caspase-3. Hence, these results suggest that MMP-3 catalytic activity seems to play different synergistic roles in dopaminergic neuron degeneration, both intracellularly by promoting neuronal apoptosis and extracellularly by triggering neuroinflammation (Choi *et al.*, 2008).

In PD, dopaminergic neurons of the *substantia nigra* show toxic intracellular filamentous inclusions termed Lewy bodies, which consist mainly of an abnormal, post-translationally modified, and aggregated form of α-synuclein (α-syn, non-Aβ component of AD amyloid, non-A4 component of amyloid precursor, NACP) (cf. Figure 6). α-syn is a cytoplasmic (presynaptic) protein that normally regulates neurotransmitter release, possibly via interaction with lipid domains on secretory vesicles (Sulzer, 2010). Interestingly, MMP-3 and MMP-9, but not MMP-2, were found to co-localize with α-syn in Lewy bodies (personal communications from Johannes Levin and Stefan Lorenzl, University of Munich, Germany and from Yoon-Seong Kim, Weill Cornell Medical College, New York). Moreover, dopaminergic neuroblastoma cells under oxidative stress showed an upregulation of intracellular and secreted activated forms of MMP-3 and cleavage of α-syn, which was inhibited by an MMP inhibitor (MMP inhibitor II). Purified α-synuclein is cleaved by MMP-3 most efficiently, but also by MT1-MMP, MMP-2, MMP-1 and MMP-9 (ordered by decreasing efficiency) (Sung *et al.*, 2005). Many non-overlapping cleavage sites were found for the cleavage of α-syn by MMP-3, -1 and -9 (cf. Table 3) (Levin *et al.*, 2009). The tested MMPs cleaved preferentially in the COOH-terminal part of α-syn in and around the non-Aβ component of Alzheimer plaques (NAC) domain. The NAC domain is a hydrophobic region that is highly prone to aggregation. Indeed, limited proteolysis by MMP-1 and MMP-3, but not by MMP-9, increased α-syn aggregation. In addition, α-syn fragments produced by MMP-3 facilitated aggregation of intact α-syn and had a more toxic effect on cell viability. However, high MMP concentrations, which resulted in pronounced degradation of α-syn, blocked subsequent aggregation. Hence, it seems that, under oxidative stress, MMPs, especially MMP-3, may regulate

α-syn aggregation and toxicity in dopaminergic neurons, thereby affecting onset and progression of neurodegeneration in PD. In addition, MMP-3 was suggested to cleave protein DJ-1 (DJ-1, oncogene DJ1, Parkinson disease protein 7) (personal communication from Yoon-Seong Kim), and various degradomics screens identified DJ-1 as an *in vitro* substrate of MMP-1,-2,-8,-9 and MT1-MMP (cf. Table 2) (Dean and Overall, 2007; Dean *et al.*, 2007; Butler *et al.*, 2008). DJ-1 is an important redox-reactive signaling intermediate controlling oxidative stress after ischemia, upon neuroinflammation, and during age-related neurodegenerative processes such as PD (Kahle *et al.*, 2009). In conclusion, inhibition of these pathogenic actions of MMP(-3) may have a neuroprotective effect, as was already shown *in vitro* and *in vivo* with doxycycline in the MPTP animal model of PD (Cho *et al.*, 2009).

Besides causing direct aggregation of substrates by proteolysis, MMPs may also contribute indirectly to protein aggregation in neurodegenerative diseases such as PD by the inactivation of protective proteins such as chaperones, proteins from the ubiquitin-proteasomal system (UPS) and proteins that function in lysosomal degradation. Indeed, heat shock proteins (HSPs) play a substantive role in PD pathology and chaperone therapy was recently proposed as a novel treatment strategy (Bandopadhyay and de, 2010; Luo and Le, 2010). Indeed, extensive localization of several HSPs with α-syn in Lewy bodies has been demonstrated, including the (candidate) MMP substrates HSP27, HSP70 and HSP90. HSPs inhibit α-syn toxicity and aggregation. This was shown for another small HSP, αB-crystallin, which has protective functions in the heart (cf. §3.3.3) and in multiple sclerosis brain (cf. §4.2.1) and is also an MMP substrate. In addition, heat shock cognate protein 70 (HSC70) and HSP90 facilitate the transfer of α-syn from the cytosol to the lysosomes for degradation (Bandopadhyay and de, 2010). Furthermore, failure of the UPS has recently emerged as an additional pathogenic factor that underlies development of familial and sporadic PD (McNaught *et al.*, 2001). Hence, inactivation of UPS proteins by MMPs may contribute to the neurotoxic protein accumulation and aggregation observed when the UPS fails. Finally, additional defects in protein clearance may be caused by MMP-mediated degradation of lysosomal proteins (cf. Table 3). In conclusion, inhibition of MMPs in PD may not only avoid α-syn truncation and aggregation, but may also preserve chaperone function, and maintain effective protein degradation and clearance.

Neuronal survival requires continuous lysosomal turnover of cellular constituents delivered by autophagy and endocytosis. Hence, the lysosomal system is a convergence point for a surprising number of genetic mutations that cause neurodegenerative diseases (Nixon *et al.*, 2008). Interestingly, various MMP (candidate) substrates are lysosomal proteins that contain mutations in neurodegenerative disorders, including cathepsins D and E, Niemann-Pick, type C2 (NPC2), proganulin and triose phosphate isomerase (cf. Table 5). Intracellular modification of these proteins by MMPs may constitute an unexplored pathogenic factor contributing to neurodegeneration in these rare monogenetic diseases. Likewise, other proteins with mutations causing storage diseases are (potential) targets of MMPs, such as enolase-β, aldolase A, β-glucuronidase, iduronate-2-sulfatase, and filamin C. This is certainly an unexpected but interesting link that needs further clinical research.

3.3.5. Intracellular proteolysis and cataract

The human lens is suspended in a complex environment. The front side is constantly rinsed by the aqueous fluid, while the gel-like vitreous acts as a shock breaker. The lens is composed of three main types of proteins, α-, β- and γ-crystallins. Crystallins focus light on the retina by maintaining the necessary refractive characteristics and transparency of the lens. Besides this structural role, crystallins may also function as chaperones and suppress the aggregation of proteins denatured by oxidation, heat, and other stressors. Crystallins are constantly subjected to changes such as oxidation, deamidation, truncation, glycation, and methylation that accumulate with time. Such age-related modifications affect crystallin structure and function. With time, the modified crystallins aggregate, causing the lens to increasingly scatter light instead of focusing light on the retina, and causing the lens to gradually lose its transparency and become opaque. This age-related lens opacity, or cataract, is the major cause of blindness worldwide. Calpains have been implicated in the proteolytic modification of crystallins but their role in human cataractogenesis is yet to be established, primarily because of the presence of a calpain inhibitor in the lens at a several-fold higher concentration than calpain itself (Sharma and Santhoshkumar, 2009). *Ex vivo* incubation of mouse lenses with recombinant activated MMP-9 was shown to cause opacification of the lens within 15 min (Descamps *et al.*, 2005). 2D-PAGE analysis of extracts from these lenses showed the disappearance of intact βB1-crystallin (βB1-crys) and the appearance of a βB1-crys fragment, as well as a minor βB3-crys fragment. Truncation of βB1-crys by MMP-9 was confirmed *in vitro* in crude eye extracts and the scissile bond was identified at Ala47-Lys48 by Edman degradation. *In vitro* degradation of eye extracts also identified a fragment of γC-crys. However, as this fragment was not found in the *ex vivo* opacificated lenses, it is not likely to be an *in vivo* target of MMP-9 in murine cataract. Injection into the vitreous chamber of activated MMP-9 or the mouse CXC chemokine, granulocyte chemotactic protein-2 (GCP-2), to attract neutrophils as an endogenous source of MMP-9, resulted in βB1-crys degradation *in vivo*, whereas such degradation was not observed in the lenses of MMP-9 knockout

mice. *Ex vivo* development of cataract in the presence of activated MMP-9 was accompanied by enzymatic loosening of the lens capsule, which is an uninterrupted basement membrane enclosing the lens (Descamps *et al.*, 2005). Since the lens capsule is composed of the same molecules as most basement membranes (Danysh and Duncan, 2009), it is not surprising that MMP-9 or other MMPs may penetrate it and subsequently enter the lens fiber cells by an undefined mechanism. Interestingly, αB-crys, a more prominent crystallin in the eye, is also cleaved by MMP-9 and other MMPs (*vide infra*) (Starckx *et al.*, 2003; Shiryaev *et al.*, 2009). Although extensive αB-crys fragmentation was observed in human aged lenses (Sharma and Santhoshkumar, 2009), it was not shown to be an MMP target in the above-mentioned mouse study. However, this may be accounted for by interspecies differences and the animal model used, and does not exclude αB-crys as an (MMP) target in human cataract.

In conclusion, lens crystallins were among the first intracellular MMP substrates discovered by *in vivo* studies with knockout mice and with direct pathological implications in cataract development.

3.3.6. Intracellular proteolysis and apoptosis

Extracellular MMPs may affect cellular apoptosis in multiple ways with both pro- and anti-apoptotic outcomes (Mannello *et al.*, 2005). Many effects of intracellular proteolysis by MMPs on cell death have been described in previous sections, such as the anti-apoptotic effect of mitochondrial MMP-1 (cf. §3.3.2) (Limb *et al.*, 2005) and cleavage of GSK-3β by MMP-2, which enhances its pro-apoptotic and other pathogenic activities in the heart (Kandasamy and Schulz, 2009). Intranuclear activities of MMP-9 and MMP-2 after cerebral ischemia may inactivate the nuclear matrix proteins PARP-1 and XRCC1, causing apoptosis and necrosis early after ischemia, but protecting neuronal cells from death at later stages in the evolution of ischemic lesions (Yang *et al.*, 2010). Activated MMP-3 inside stressed dopaminergic neurons contributes to pro-apoptotic signaling upstream of caspase-3 (Choi *et al.*, 2008). The active form of MMP-3 (but not the pro-form) was already mentioned to be efficiently transported to the nucleus by means of a nuclear localization signal (cf. §3.2.6). Cell populations expressing a nuclear activated form of MMP-3 (by transfection) contained higher percentages of apoptotic cells than the control populations transfected with pro-MMP-3 (remaining cytosolic), as determined by immunofluorescent staining for activated caspase-3. This induction of apoptosis was dependent on the catalytic activity of MMP-3, as transfection of an inactive mutant or treatment with the broad spectrum MMP inhibitor GM6001 significantly reduced the apoptotic index. Hence, nuclear MMP-3 induces apoptosis by its proteolytic activity (Si-Tayeb *et al.*, 2006). In completely different settings, cigarette smoke-induced apoptosis was associated with gelatinolytic activity in the nucleus, increased pro- and activated MMP-2 levels in nuclear and cytosolic fractions and early PARP-1 fragmentation before the time point of caspase-3 activation, again suggesting a role for MMP-2 activity in nuclear matrix proteolysis during apoptosis (Ruta *et al.*, 2009). Interestingly, MMP-2 expression and activation were reduced by inhibitors of two major apoptotic pathways in endothelial cells, caspase- and p38 mitogen-activated protein kinase (MAPK)-induced apoptosis, i.e. the pan caspase inhibitor Z-VAD and the p38 inhibitor SB203580. Curiously, Z-VAD increased, whereas SB203580 decreased MT1-MMP expression and activity. These results would suggest that alteration of MMP-2 activity is rather effect than cause in the apoptotic cascade. However, addition of pro- and activated MMP-2 to endothelial cell cultures showed a dose-dependent induction of apoptosis. Elevation of p38 and its phosphorylation were observed following the addition of active MMP-2, pointing to a positive regulatory loop for MMP-2 in the p38 MAPK apoptotic pathway. However, neither pro-MMP-2 nor activated MMP-2 influenced the levels of active caspase-3, suggesting that MMP-2 is downstream of caspases (Shapiro *et al.*, 2010). Indeed, a recent study by Yarbrough *et al.* (2010) showed increased MMP activity after treatment of heart homogenates with active caspase-3 and generation of activated forms of MMP-2 by an active caspase cocktail, suggesting direct proteolytic activation of MMPs by active caspases (as discussed in §1.1).

If one analyzes critically the apoptotic cascade by evaluation of intracellular substrates, rather than by scrutinizing the enzymes, it becomes factual knowledge that most of the well-defined intracellular MMP substrates (cf. Table 5) are also cleaved by pro-apoptotic proteases such as calpains, caspases and granzymes, as summarized in Table 6. Strikingly, as can be deduced from Table 6, cleavage of these proteins by MMPs or (other) apoptotic proteases seems to have a general pro-apoptotic effect (Figure 7). In addition, from degradomics substrate analysis (Table 3), it is clear that other important apoptosis regulators may be modulated by MMPs. These regulators include BH3-interacting domain death antagonist (BID), apoptosis-linked-gene-2-interacting-protein X (ALIX or programmed cell death protein 6) and cytochrome c. Granzyme A induces apoptosis by multiple cleavages, including the cleavage of histones, a process that opens up chromatin to DNases, and proteolysis of high mobility group B2 (HMGB2), which liberates the DNase activity of nucleoside diphosphate kinase A (Lieberman and Fan, 2003). Both histones and HMGB2 are high-confidence candidate substrates of MMPs (cf. Table 3).

As mentioned in §2, MMPs seem to degrade a whole array of intracellular matrix (ICM) proteins. Cleavage

Table 6. Cleavage of intracellular MMP substrates by pro-apoptotic proteases and effect of substrate cleavage on the apoptotic pathway.

Substrate*	MMP	Calpain	Caspase	Granzyme	Effect of cleavage	References
INTERCELLULAR JUNCTION PROTEINS						
Cx43	MMP-7	ND	ND	ND	Pro-apoptotic	(Giardina *et al.*, 2007)
ZO-1	MMP-9	ND	caspase X	ND	Pro-apoptotic	(Bojarski *et al.*, 2004)
CYTOSKELETAL PROTEINS						
α-actinin	MMP-2	Calpain X	capase-3	NC	Pro-apoptotic	(Selliah *et al.*, 1996; Communal *et al.*, 2002; Nakamura *et al.*, 1993; Triplett and Pavalko, 2006)
CAP1	MMP-9 MMP-2,-8,-13	ND	ND	ND	Anti-apoptotic	(Wang *et al.*, 2008)
Desmin	MMP-2	Calpain 1 Calpain 2	Caspase-6	ND	Pro-apoptotic	(Whipple and Koohmaraie, 1991; Chen *et al.*, 2003; Papp *et al.*, 2000)
FAK	MT1-MMP MT3-MMP	Calpain 1 Calpain 2	Caspase-3,-7 Capase-6,-8	Granzyme B	Pro-apoptotic	(Carragher *et al.*, 1999; Wen *et al.*, 1997; Gervais *et al.*, 1998; Cance and Golubovskaya, 2008)
Gelsolin	MMP-1,-2,-3, -7,-9	Calpain X	Caspase-3	ND	Pro-apoptotic	(Fujita *et al.*, 1999; Geng *et al.*, 1998; Kothakota *et al.*, 1997; Wolf *et al.*, 1999)
Pericentrin	MT1-MMP	NC	NC	NC	Pro-apoptotic	(Zimmerman *et al.*, 2004)
SARCOMERIC PROTEINS						
MLC-1	MMP-2	Calpain 3	Caspase-3	Granzyme A	Pro-apoptotic	(Cohen *et al.*, 2006; Moretti *et al.*, 2002; Nakamura *et al.*, 1993)
MHC	MMP-2 MMP-9	Calpain 2	Caspase X	Granzyme A	Pro-apoptotic	(Nakamura *et al.*, 1993; Gerner *et al.*, 2000; Azarian *et al.*, 1993)
TnI	MMP-2	Calpain 1 Calpain 2	NC	ND	Pro-apoptotic	(Gao *et al.*, 1997; Ruetten *et al.*, 2001; Di Lisa *et al.*, 1995)
MOLECULAR CHAPERONES						
αB-crys	MMP-9	Calpain 2	ND	ND	Pro-apoptotic	(Kelley *et al.*, 1993; Kamradt *et al.*, 2001)
βB1-crys	MMP-9	Calpain 2 CSS2	ND	ND	ND	(Ma *et al.*, 2004; David *et al.*, 1993)
βB3-crys	MMP-9	Calpain 2	ND	ND	ND	(David *et al.*, 1993)
γC-crys	MMP-9	Calpain 2 Calpain 3	ND	ND	ND	(Baruch *et al.*, 2001; Tang *et al.*, 2007)
PRESYNAPTIC PROTEINS						
α-syn	MMP-1,-2,-3,-9 MT1-MMP	Calpain 1 Calpain 2	ND	ND	Anti-apoptotic	(Mishizen-Eberz *et al.*, 2003; Kim *et al.*, 2004)
SNAP-25	MMP-7	Calpain 2	ND	ND	ND	(Ando *et al.*, 2005)
NUCLEAR MATRIX PROTEINS						
PARP-1	MMP-2	Calpain 1	Caspase-1,-3	Granzyme A Granzyme B	Pro-apoptotic	(Buki *et al.*, 1997; Tewari *et al.*, 1995; Gu *et al.*, 1995; Froelich *et al.*, 1996; Zhu *et al.*, 2009)
XRCC1	MMP-2 MMP-9	ND	ND	ND	Pro-apoptotic	(Yang *et al.*, 2010)
ANTIBACTERIAL PEPTIDES						
pro-Crps	MMP-7	ND	ND	ND	ND	/
pro-CRS4C	MMP-7	ND	ND	ND	ND	/
SIGNALING PROTEINS						
ER-β	MMP-26	ND	ND	ND	Anti-apoptotic	(Lazennec, 2006)
GSK-3β	MMP-2	Calpain 1	ND	ND	Pro-apoptotic	(Goni-Oliver *et al.*, 2007)

*The meaning of the acronyms can be found in Table 5.
NC, not cleaved; ND, not defined.

and reorganization of cytoskeletal proteins is essential in apoptosis progression as it ensures the systematic dismantling of the dying cell and probably contributes to the early cell rounding and retraction, and partly to apoptotic membrane blebbing (Ndozangue-Touriguine *et al.*, 2008; Taylor *et al.*, 2008). Indeed, MMPs degrade many focal adhesion proteins, including FAK, α-actinin and the filamins (cf. Tables 3 and 5 and Figure 7). MMPs may also participate in the degradation of the actin meshwork surrounding the nuclear lamina, which disrupts the attachment between the actin cytoskeleton and the nuclear envelope, tearing the nucleus apart during apoptosis. Interestingly, apoptosis in ischemic brains was associated with increased levels of pro- and

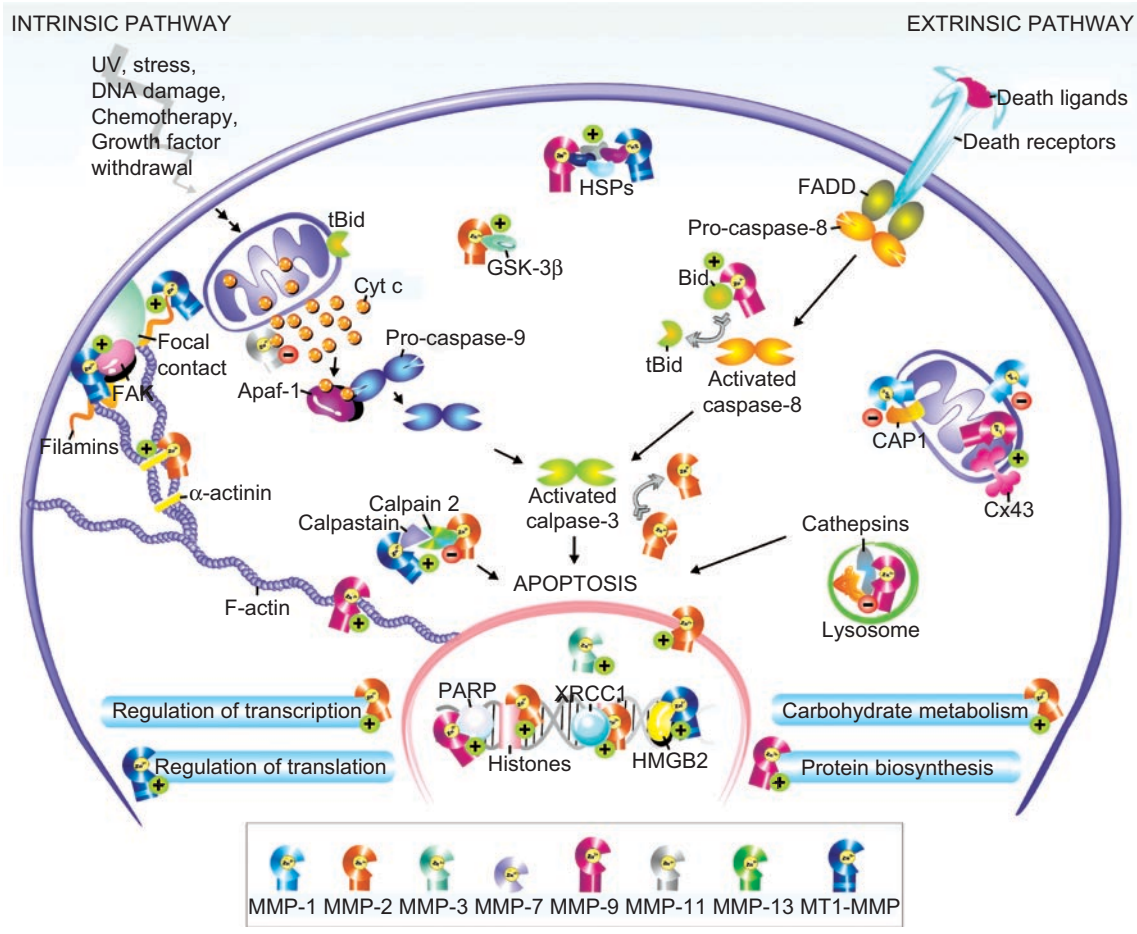

Figure 7. Intracellular proteolysis by MMPs in apoptosis. Cleavage of intracellular substrates by MMPs has many pro-apoptotic effects (+), but may also inhibit apoptotic pathways (–). Degradation of cytoskeletal proteins (e.g. focal adhesion kinase (FAK), α-actinin, actin and the filamins) contributes to the systematic dismantling of the dying cell, as well as to the disruption of the actin meshwork surrounding the nuclear lamina. Inactivation of housekeeping proteins such as the enzymes that regulate transcription and translation, carbohydrate metabolism and protein biosynthesis, may shut off life-support systems of the cell. Cleavage of heat shock proteins (HSPs) might abolish their multiple anti-apopototic functions. Truncation of the pro-apoptotic protein BH3– interacting death domain antagonist (Bid) may contribute to the intrinsic apoptotic pathway, in contrast with cytochrome c degradation. Translocation and degradation of connexin-43 (Cx43) in the mitochondria leads to mitophagy and cell death. Cleavage of glycogen synthase kinase-3β (GSK-3β) enhances its pro-apoptotic capacities. Inactivation of poly (ADP-ribose) polymerase (PARP) and X-ray cross-complementary factor 1 (XRCC1) causes apoptosis early after ischemia. Cleavage of histones may open up the chromatin to DNases and proteolysis of high mobility group B2 (HMGB2) liberates DNase activity. Degradation of the calpain inhibitor calpastatin may stimulate apoptosis, whereas inactivation of the pro-apoptotic proteases calpain 2 and various cathepsins may have anti-apoptotic effects. Translocation of CAP1 to the mitochondria is proapoptotic and its degradation may result in defective apoptosis. The presence of activated MMP-3 in the nucleus is pro-apoptotic, whereas mitochondrial MMP-1 confers resistance to apoptosis. Apaf- 1, apoptotic protease activating factor-1; FADD, Fas-associated death domain-containing protein; tBid, truncated Bid.

S-nitrosylated active MMP-9 and decreased levels of actin (cf. §3.3.4) (Gu *et al.*, 2002). In addition, and again similarly to caspases, MMPs seem to target many proteins that are involved in essential housekeeping functions in the cell, such as regulation of transcription and translation, protein biosynthesis and carbohydrate metabolism (cf. Table 3). This is in line with the early shutdown of transcription and translation observed during apoptosis and may shut off the life-support systems of the cell (Taylor *et al.*, 2008). Furthermore, Tables 3 and 6 show that MMPs also target many heat shock proteins and other molecular chaperones, which are known to have multiple anti-apoptotic effects (Beere, 2004; Lanneau *et al.*, 2008). In conclusion, MMPs have many substrates in common with pro-apoptotic proteases and most MMP effects on substrates are pro-apoptotic (cf. Figure 7). The observation that exceptions to this rule exist, strengthens the fact that by such proteolysis a regulatory role is played in the apoptotic process, and this is in line with the well-known duality of pro- and anti-apoptotic roles of MMPs (Mannello *et al.*, 2005).

Both the calpains and caspases are families of cysteine proteases that have important roles in the initiation, regulation and execution of cell death (Harwood

et al., 2005). For these enzymes it is generally accepted that they act intracellularly. Interestingly, Schulz and coworkers discovered that the general calpain inhibitor calpastatin inhibits MMP-2 *in vitro*. Since much of the evidence for calpain degradation of substrates is based on the use of calpain inhibitors, calpains may have been incorrectly identified as the proteases responsible for some intracellular proteolytic activities (Kandasamy *et al.*, 2010). Calpains are believed to be activated by an initial insult via a rise of intracellular Ca^{2+} from the ER, the mitochondria or an influx of extracellular Ca^{2+}, as caused for example by oxidative stress (Harwood *et al.*, 2005). Since MMPs are also Ca^{2+}-dependent enzymes (Tallant *et al.*, 2010) and may be activated by oxidative stress (cf. §1.2), they are indeed very likely to perform claimed calpain functions. Likewise, because caspase activity requires a free sulfhydryl group in the catalytic site, many caspase inhibitors target this part of the enzyme. MMPs possess an essential cysteine with a sulfhydryl group in the latent proform that is also a key regulator of enzyme activity. This suggests that caspase inhibitors may activate MMPs by interacting with the latency-conferring Cys in the propeptide (Van Wart and Birkedal-Hansen, 1990).

In addition to the cleavage of common substrates in the apoptotic pathway, granzyme B directly activates pro-caspases, whereas granzyme A induces caspase-independent cell death (Vandenabeele *et al.*, 2005). The parallelisms between MMPs and granzymes are striking, as they both are extracellular proteases that can be transported into the cell, for example by endocytic mechanisms (cf. §3.2.1). Conversely, whereas granzymes are mostly known for their intracellular substrates and their extracellular functions only start to gain appreciation (Boivin *et al.*, 2009), the reverse is true for MMPs. Calpains may activate or inactivate pro-caspases, whereas caspase 3 cleaves the general calpain inhibitor calpastatin. Thus caspase, calpain and granzyme cascades are tightly interrelated (Harwood *et al.*, 2005; Demon *et al.*, 2009). Interestingly, calpain 2 and calpastatin were identified as MMP candidate substrates, as well as various cathepsins (cf. Table 3), which translocate from the lysosomal lumen in response to apoptotic stimuli and trigger apoptosis (Turk and Turk, 2009).

Besides the apparent overlap in (pro-)apoptotic substrate cleavage (cf. Figure 7), and inhibitor profiles, other elements hint for a participation of MMPs in the apoptotic cascade and the cross-talk with (pro-)apoptotic proteases. Nitrosative stress, which promotes apoptosis by activation of mitochondrial apoptotic pathways, inhibits caspases by S-nitrosylation (Kim *et al.*, 2002), whereas MMPs were shown to be activated by S-nitrosylation and consequently induced apoptosis (cf. §1.2) (Gu *et al.*, 2002). Hence, under specific conditions, such as oxidative and nitrosative stress, MMPs may indeed exert some caspase functions. In support of this, early activation of MMPs in response to oxidative stress and cleavage of the apoptotic caspase-3 substrate PARP-1 preceded caspase-3 activation in various studies (Ruta *et al.*, 2009; Yang *et al.*, 2010). As already mentioned, in stressed dopaminergic neurons, intracellular activated MMP-3 was shown to be a pro-apoptotic signaling molecule upstream of caspase-3 (but not by direct cleavage of MMP-3) (Choi *et al.*, 2008), whereas MMP-2 was found to be an apoptotic effector molecule downstream from caspase-3 in spontaneous apoptosis of endothelial cells (Shapiro *et al.*, 2010; Yarbrough *et al.*, 2010). Conversely, mitochondrial MMP-1 was shown to confer resistance to apoptosis (Limb *et al.*, 2005).

Additional data enhance the links between MMPs and apoptotic substrate conversions. Like calpastatins inhibit calpains, the tissue inhibitors of metalloproteinase (TIMPs) are natural MMP inhibitors (Brew and Nagase, 2010). TIMPs have been shown to inhibit (TIMP-1 and TIMP-2) or stimulate (TIMP-2 and TIMP-3) apoptosis and were also detected intracellularly (cf. Table 4). These findings suggest that the intracellular functions of TIMPs may contribute to the regulation of apoptosis by inhibiting MMP function or by other independent mechanisms. As a consequence, the extracellular effects of MMPs, for instance the cleavage of cytokines (e.g. pro-TNF-α) and cytokine receptors (Cauwe *et al.*, 2007), may work synergistically with intracellular substrate cleavages in the regulation of apoptosis.

Common (pro-)apoptotic substrates and inhibitor profiles, MMP activation and action in early apoptotic phases, and cross-talk with pro-apoptotic proteases all point to MMPs as executioners of a novel form of caspase-independent cell death. Caspases traditionally held the predominant role as prime mediators of apoptotic execution. However, recent evidence has accumulated that non-caspases, including calpains, cathepsins, granzymes and the proteasome have roles in mediating and promoting cell death (Vandenabeele *et al.*, 2005). Since modulation of apoptosis seems to be a leitmotiv in the intracellular functions of MMPs, they may soon be considered as the next addition to the growing list of apoptotic proteases. Further research into the influences of intracellular MMPs on both caspase-dependent and caspase-independent apoptosis may unveil new modes to prevent pathological cell death, or conversely, to induce apoptosis in cancer cells and pathological autoreactive cells.

4. Intracellular MMP substrates degraded outside cells

It is increasingly recognized that a subset of proteins owns both intracellular and extracellular functions

Table 7. Extracellular localization mechanisms of intracellular MMP substrates.

Non-classical secretion mechanisms		Cellular exit during cell death	
DIRECT TRANSMEMBRANE TRANSLOCATION		**APOPTOTIC BLEBS/BODIES**	
Galectin-1	(Schafer *et al.*, 2004)	Annexin I	(Arur *et al.*, 2003)
Galectin-3	(Lukyanov *et al.*, 2005)	La	(Casciola-Rosen *et al.*, 1994)
LYSOSOMAL SECRETION		Calreticulin	(Rosen and Casciola-Rosen, 1999)
Cathepsin D	(Mambula *et al.*, 2007)	Histone H2A	(Schiller *et al.*, 2008)
HMGB1	(Gardella *et al.*, 2002)	Histone H2B	(Schiller *et al.*, 2008)
HSP70	(Mambula *et al.*, 2007)	Histone H4	(Schiller *et al.*, 2008)
EXOSOME-MEDIATED SECRETION		Jo-1	(Rosen and Casciola-Rosen, 1999)
Actin-β/γ	(Thery *et al.*, 2001)	PARP	(Rosen and Casciola-Rosen, 1999)
α-Actinin-4	(Simpson *et al.*, 2008)	Vimentin	(Boilard *et al.*, 2003)
Alix	(Thery *et al.*, 2001)	**ALARMINS**	
Annexin I	(Thery *et al.*, 2001)	α-defensins	(Yang *et al.*, 2009)
Carbonic anhydrase II	(Simpson *et al.*, 2008)	Annexin I	(Bianchi, 2007)
Cofilin-1	(Thery *et al.*, 2001)	Galectin-1	(Cambi and Figdor, 2009)
Cyclophilin A	(Yu *et al.*, 2006)	Galectin-3	(Cambi and Figdor, 2009)
EF 1-α1	(Yu *et al.*, 2006; Thery *et al.*, 2001)	HDGF	(Bianchi, 2007)
EF2	(Thery *et al.*, 2009)	HMGB1	(Bianchi and Manfredi, 2007)
Enolase-α	(Yu *et al.*, 2006)	HSP70	(Bianchi, 2007; Kono and Rock, 2008)
Ezrin	(Thery *et al.*, 2009; Simpson *et al.*, 2008)	HSP90	(Bianchi, 2007; Kono and Rock, 2008)
Ferritin light chain	(Thery *et al.*, 2001)	Nucleolin	(Bianchi, 2007)
GAPDH	(Thery *et al.*, 2009; Simpson *et al.*, 2008)	**NETOSIS**	
Galectin-3	(Thery *et al.*, 2001)	Actin-β/γ	(Brinkmann *et al.*, 2004; Urban *et al.*, 2009)
Histone H2A	(Thery *et al.*, 2001)	α-Actinin-1	(Urban *et al.*, 2009)
Histone H2B	(Thery *et al.*, 2001)	α-Actinin-4	(Urban *et al.*, 2009)
Histone H4	(Thery *et al.*, 2001)	α-defensins	(Urban *et al.*, 2009)
HSC70	(Thery *et al.*, 2009; Simpson *et al.*, 2008)	Annexin I	(Brinkmann *et al.*, 2004)
HSP70	(Thery *et al.*, 2009)	Cytochrome c	(Brinkmann *et al.*, 2004)
HSP90	(Thery *et al.*, 2001; Yu *et al.*, 2006)	Enolase-α	(Urban *et al.*, 2009)
Malate dehydrogenase	(Simpson *et al.*, 2008)	Histone H2A	(Brinkmann *et al.*, 2004)
Moesin	(Thery *et al.*, 2009; Simpson *et al.*, 2008)	Histone H2B	(Brinkmann *et al.*, 2004)
Myosin	(Thery *et al.*, 2009; Simpson *et al.*, 2008)	Histone H4	(Brinkmann *et al.*, 2004)
Peroxiredoxin-1	(Thery *et al.*, 2001)	Myosin	(Urban *et al.*, 2009)
PGK1	(Yu *et al.*, 2006)	Tubulin-α	(Brinkmann *et al.*, 2004)
PGM1	(Simpson *et al.*, 2008)		
Profilin-1	(Thery *et al.*, 2001)		
Rab GDI-β	(Simpson *et al.*, 2008)		
Rho GDI-α/β	(Thery *et al.*, 2009; Simpson *et al.*, 2008)		
Tubulin-α/β	(Thery *et al.*, 2001)		
14-3-3 proteins	(Thery *et al.*, 2001; Simpson *et al.*, 2008)		
SECRETION BY VESICLE SHEDDING			
Annexin I	(Simpson *et al.*, 2008)		
Galectin-1	(Cooper and Barondes, 1990; Harrison and Wilson, 1992)		
Galectin-3	(Simpson *et al.*, 2008; Mehul and Hughes, 1997)		
Histones	(Simpson *et al.*, 2008)		
HSP27	(Simpson *et al.*, 2008)		
HSP70	(Simpson *et al.*, 2008)		
HSP90	(Simpson *et al.*, 2008)		

The meaning of the acronyms can be found in Table 3 and 5.

(Butler and Overall, 2009a). This phenomenon is denominated "protein multifunctionality" and a classical example is the identity of the intracellular phosphomannose receptor with the extracellular receptor of insulin-like growth factor II (Morgan *et al.*, 1987). These functions may be very diverse and even affect physiopathology in opposite ways. For example, the upregulation of intracellular heat shock protein 70 (HSP70) is mostly cytoprotective and induces anti-apoptotic mechanisms in cells, whereas increased levels of extracellular HSP70

are generally immunostimulatory and augment the synthesis of co-stimulatory molecules and pro-inflammatory cytokine and chemokines (Asea, 2007). These multifunctional proteins may travel between intracellular and extracellular compartments in non-conventional ways. Alternatively, the intracellular protein pool may leak from the cell after necrotic loss of membrane integrity. Here, we discuss the specific circumstances in which intracellular proteins may be accessed and modulated by extracellular MMPs. Indeed, many of the intracellular (candidate) substrates in Tables 3 and 5 have also been found extracellularly. Hence, quite opposite to the previous chapter, in which it is described how MMPs enter cells and specific subcellular compartments to cleave intracellular proteins, this chapter deals with intracellular proteins that exit the cell and are proteolysed by MMPs in the extracellular milieu.

4.1. Extracellular localization mechanisms of intracellular MMP substrates

4.1.1. Non-classical secretion of intracellular MMP substrates

The classical secretion of soluble proteins requires transportation into and through the endoplasmic reticulum (ER) and the Golgi apparatus. Signal peptides target proteins for translocation into the ER lumen. However, most intracellular proteins do not possess a signal sequence for secretion but leave the cell by ER–Golgi independent secretion pathways. Although the details of the unconventional secretory mechanisms remain elusive for most of these proteins, four main pathways have emerged as potential means of non-classical cell exit: direct membrane translocation, secretory lysosomes, multivesicular body (MVB)-derived exosomes and membrane vesicle secretion (Nickel and Seedorf, 2008; Prudovsky *et al.*, 2008; Nickel and Rabouille, 2009). Examples of intracellular MMP (candidate) substrates secreted by one of these four mechanisms are given in Table 7.

A first mechanism consists of *direct translocation across the plasma membrane*, for example by direct interaction of membrane lipids and spontaneous penetration of the lipid bilayer, as proposed for galectin-3 (Lukyanov *et al.*, 2005). Alternatively, translocation may require the cooperation of a molecular apparatus of integral and peripheral membrane proteins (e.g. galectin-1) (Schafer *et al.*, 2004; Delacour *et al.*, 2009). The *endolysosomal pathway* is a second pathway for unconventional secretion and involves the sequestration of soluble, cytoplasmic proteins into endolysosomes. These secretory lysosomes have characteristics of both lysosomes and secretory granules. The mechanisms by which cytoplasmic proteins may enter the lumen of the secretory lysosomes have not been fully elucidated yet, but may involve the highly conserved ABC cassette transport proteins, which can pump large and small molecules across membranes. A second trigger, extracellular ATP, is proposed to promote the fusion of secretory lysosomes with the cell membrane, which releases their content into the extracellular milieu (Mambula *et al.*, 2007; Nickel and Rabouille, 2009). Interestingly, pro- and activated MMP-9 forms were identified in secretory lysosomes of reactive astrocytes (cf. §3.2.2) (Sbai *et al.*, 2010), suggesting that proteolysis may already occur before or during secretion.

A third pathway is the *secretion by exosomes derived from multivesicular bodies* (MVBs). Exosomes are 40–100 nm vesicles, which originate from the inward budding of MVBs to form intraluminal vesicles with concomitant engulfment of cytosolic components. Although MVBs primarily deliver cytoplasmic proteins for degradation by fusion with lysosomes, they can also release internal vesicles or exosomes into the extracellular space following their fusion with the plasma membrane (Simpson *et al.*, 2008; Thery *et al.*, 2009). As can be deduced from Table 7, many MMP (candidate) substrates have been found in exosomes. Interestingly, both pro- and functionally active MT1-MMP forms are present in exosomes and are ideally situated to cleave intracellular proteins upon secretion (Hakulinen *et al.*, 2008). Finally, a fourth mechanism for non-classical release of proteins is the *secretion of membrane vesicles*. In contrast with the endosomal origin of exosomes, these "shedding vesicles" are generated by direct budding from the plasma membrane. According to their characteristics (size, density, appearance, sedimentation, lipid composition, protein markers) and cell type of origin, they have been termed ectosomes, shedding vesicles, shedding bodies, microparticles, membrane particles, exovesicles and microvesicles, without much consensus in terminology throughout the literature (Cocucci *et al.*, 2009; Nickel and Rabouille, 2009). Here, we will not differentiate between various subtypes and use the general term "membrane vesicles". Upon release, both exosomes and membrane vesicles circulate in the extracellular space adjacent to the site of discharge, where they can rupture and release the enclosed proteins. However, some of these vesicles travel considerable distances by diffusion within tissues and appear in biological fluids such as blood and even urine. In addition, recent data have emerged showing that they can fuse with other cells as a means of direct intercellular communication, which may play important roles in infections and immune functions, coagulation and tumor progression (Thery *et al.*, 2009; Cocucci *et al.*, 2009).

4.1.2. Exposure of intracellular substrates to extracellular MMPs by various forms of cell death

Apoptosis is characterized by a cascade of structural remodeling steps, which contribute to the progression

towards cell death, but also prepare the cell for removal by phagocytes, preventing unwanted immune responses. Apoptotic cells initially become rounded and retract from neighboring cells, accompanied by a period of dynamic plasma membrane blebbing and culminating in the shedding of apoptotic bodies and small apoptotic blebs (Taylor *et al.*, 2008). Interestingly, intracellular proteins undergo a striking redistribution during apoptosis and become concentrated in and on the surface of apoptotic blebs. Small blebs contain molecules associated with the ER and the membrane skeleton, whereas apopotic bodies are enriched with nuclear proteins (Casciola-Rosen *et al.*, 1994; Rosen and Casciola-Rosen, 1999). Whereas the shedding of apopotic bodies and blebs seems to be a means of safely breaking the cell apart into more manageable pieces for phagocytosis, the meaning of the intracellular protein translocation onto the cell surface is less evident. However, since both calreticulin and annexin I trigger ligation and engulfment by phagocytes (Arur *et al.*, 2003; Gardai *et al.*, 2005), the surface localization of these intracellular proteins may constitute "eat-me signals" for phagocytosis. In this way, various MMP (candidate) substrates are exposed for degradation by extracellular MMPs (cf. Table 7) (Schiller *et al.*, 2008; Casciola-Rosen *et al.*, 1994; Rosen and Casciola-Rosen, 1999) and will be discussed in the connected pathological context in §4.2.

In the absence of phagocytes, or when necrotic cells outnumber the phagocytes, apoptotic cells are not cleared in time and progress to secondary necrosis. Necrotic cell death results in the discharge of the intracellular content into the extracellular milieu, and will allow for the full intracellular protein pool to come in contact with extracellular MMPs. In principle, this may be caused by any condition that destroys the cellular integrity, by physical stresses, such as heat, cold, positive or negative pressure and irradiation at various wavelengths. Chemical insults such as pH and toxins, biochemical activation of various classes of enzymes (e.g. lipases), porines (e.g. complement) and membrane channels, and finally infections with cytopathogenic viruses and other micro-organisms, all contribute to the live scenario of MMPs acting on intracellular substrates.

Furthermore, some intracellular proteins are released from necrotic cells before the loss of membrane integrity. These specific damage-associated molecular patterns (DAMPs) or "alarmins" are released from primary and secondary necrotic cells, but not from apoptotic cells. Hence, they function as endogenous adjuvants and activate the innate and adaptive immune systems, signaling the "danger" of immunogenic cell death and tissue damage (Bianchi, 2007; Kono and Rock, 2008). Various proteins have been proposed to function as alarmins, many of which were identified as MMP (candidate) substrates (cf. Table 7) (Bianchi, 2007; Kono and Rock, 2008; Yang *et al.*, 2009). Modulation of these pro-inflammatory molecules may account for some of the multiple immunomodulatory roles of MMPs (Cauwe *et al.*, 2007; Manicone and McGuire, 2008). Indeed, hyperactivation by MMP-mediated cleavage would strongly enhance inflammation, whereas an inactivating proteolytic effect may be required for the termination of the pro-inflammatory effect, much like the thrombin-mediated cleavage and dampening of HMGB1 activity (Ito *et al.*, 2008).

Although apoptosis and necrosis seemed to be the two fates of choice for a dying cell, it has recently become clear that some phagocytic cells also succumb via the formation of extracellular chromatin structures, which were first identified in neutrophils and were termed neutrophil extracellular traps or NETs (Brinkmann *et al.*, 2004). Upon activation by pro-inflammatory stimuli (IL-8, lipopolysaccharide (LPS), bacteria, fungi, activated platelets), neutrophils start a program that leads to the formation of NETs and to their death, termed NETosis. This involves gradual disintegration of the nuclear membranes and loss of granule integrity, with subsequent filling of the cell with nuclear material, mixed with cytoplasmic and granule contents. Hence, these NETs consist of bundled chromatin fibers (DNA and histones), decorated with (antimicrobial) granular and cytoplasmic proteins. NETs capture and kill microbes such as bacteria, fungi and parasites using these antimicrobial proteins as well as bactericidal histones (Brinkmann and Zychlinsky, 2007; Papayannopoulos and Zychlinsky, 2009). Indeed, histones and histone-derived fragments were shown to have a variety of antimicrobial functions, including bacterial cell membrane permeabilization, penetration into the membrane, and binding and neutralization of bacterial LPS toxicity. Interestingly, a portion of these histone fragments have been shown to be produced from precursor histones via specific cleavage by endogenous proteases (Kawasaki and Iwamuro, 2008). Since histones were identified as MMP-2 candidate substrates (cf. Table 3), and endogenous or exogenous ROS are required for NET formation and may activate MMPs (cf. §1.2), MMPs may very well be the activators of histone microbicidal activity, intracellularly before the NETs are released, or extracellularly after NET expulsion. This would indeed be very similar to the respective intracellular and extracellular activation of murine and human α-defensins, as discussed in §3.3.1. MMP-9, which is present in tertiary neutrophil granules, was also found on these NETs (Brinkmann *et al.*, 2004), and is thus ideally positioned to participate in proteolytic defensin and histone activation. A recent proteomic study identified 24 different NET-associated proteins, a surprisingly restricted set, since the chromatin comes in contact with the full cytoplasmic contents during NETosis and the cell membrane ruptures during NET release (Urban *et al.*, 2009). Interestingly, half

Table 8. Overview of the cleavage sites of various MMPs in αB-crystallin.

Cleavage sites*	MMP-9†	MMP-2	MMP-8	MMP-10	MMP-12	MT1-MMP	MT2-MMP	MT3-MMP	MT4-MMP	MT5-MMP	MT6-MMP
His7-Pro8	✓						✓	✓			
Pro16-Phe17	✓										
Arg22-Leu23	✓	✓									✓
Pro46-Phe47	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓
Tyr48-Leu49	✓		✓		✓	✓				✓	
Ser53-Phe54				✓		✓	✓	✓			✓
Phe54-Leu55	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Trp60-Phe61	✓	✓		✓		✓	✓	✓			✓
Glu67-Met68	✓	✓				✓	✓	✓	✓		✓
His83-Phe84											✓
Glu88-Leu89	✓	✓	✓					✓	✓		✓
Asp96-Val97											✓
Phe113-Ile114		✓		✓	✓	✓	✓	✓			✓
Lys121-Tyr122	✓	✓	✓		✓			✓			✓
Pro130-Leu131	✓						✓	✓			✓
Tyr132-Ile133	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓
Lys150-Gln151	✓	✓	✓	✓			✓	✓	✓	✓	✓

*The cleavage sites are compiled from Shiryaev *et al.* (2009) and Starckx *et al.* (2003).
†MMP-9 also partially processes the following peptide bonds: Met1-Asp2; Asp2-Ile3; Ala4-Ile5; Asp25-Gln26; Glu30-His31; Leu32-Leu33; Ser35-Asp36; Thr40-Ser41; Thr42-Ser43; Phe47-Tyr48; Arg69-Leu70; Asn78-Leu79; Glu99-Val100; Val100-His101; Gly102-Lys103; Gly112-Phe113; Tyr122-Arg123; Ser136-Leu137; Pro160-Ile161;Val169-Tyr170 (Starckx *et al.*, 2003).

of these NET proteins are MMP (candidate) substrates (cf. Table 7), again pointing to the potential modification of NET activities by MMPs. NETosis is restricted to neutrophils, but the formation of different kinds of extracellular traps (ETs) was also observed in other granulocyte cell types, such as mast cells and eosinophils (von Kockritz-Blickwede and Nizet, 2009).

In conclusion, whereas MMPs may cleave various substrates within cells, it is clear that they can also access many intracellular proteins in the extracellular milieu. Since many of the proteins that leave cells by unconventional ways are molecules with multiple functions, that differ according to their location in the extracellular versus intracellular milieu, the modulation of these substrates by MMPs adds an additional layer of complexity to MMP inhibition in pathology. Indeed, inhibition of potential detrimental effects of MMPs on these bimodal substrates will require milieu-specific inhibitors that do not enter the compartment where substrate cleavage is beneficial.

4.2. Extracellular proteolysis of intracellular substrates in physiology and pathology

4.2.1. Extracellular proteolysis of intracellular autoantigens in organ-specific autoimmune diseases: multiple sclerosis

Multiple sclerosis (MS) is a chronic neurological disorder of the CNS, characterized by the breakdown of the BBB, perivascular infiltration of inflammatory cells, leading to the formation of the so-called "vascular cuffs", and multiple regions of focal myelin and neuronal loss (lesions or plaques). MMPs may contribute to these pathogenic events by increasing the permeability of the BBB, enhancing demyelination by the degradation of myelin (glyco) proteins, e.g. myelin basic protein (MBP), by the generation of antigenic peptides, and by the facilitation of infiltration and migration of immune cells through the ECM and the basal membrane (Opdenakker and Van Damme, 1994; Cuzner and Opdenakker, 1999; Opdenakker *et al.*, 2003; Agrawal *et al.*, 2006; Rosenberg, 2009). While attempting to identify the components of the myelin sheath that provoke the autoimmune reaction in MS, van Noort *et al.* (1995) isolated αB-crystallin (αB-crys) as a prominent target in myelin from MS brains, but not in healthy brain myelin. In addition, αB-crys is the most abundant gene transcript present in early active MS lesions, whereas such transcripts are absent in normal brain tissue (Chabas *et al.*, 2001). MMP-9 cleaves αB-crys at multiple sites *in vitro* (cf. Table 8) and released various immunodominant and cryptic epitopes of αB-crys. Whereas both intact αB-crys and the immunodominant peptide 1-16 stimulated T-cell proliferation *in vitro*, neither intracerebral injection of the MMP-9-generated fragments nor injection of intact αB-crys triggered immediate neuroinflammation in an unprimed host *in vivo* (Starckx *et al.*, 2003). Besides being a major MS autoantigen, αB-crys was discovered to possess neuroprotective and anti-apoptotic functions in the brain. αB-crys knockout mice are more susceptible to development of experimental autoimmune encephalomyelitis (EAE), a murine MS model, and administration of recombinant αB-crys ameliorated EAE symptoms (Ousman *et al.*, 2007). Hence, degradation of αB-crys by MMP-9 may be pathogenic by the ablation of these protective functions

(Starckx *et al.*, 2003). This is supported by the finding that young gelatinase B-deficient mice are resistant to EAE development (Dubois *et al.*, 1999). The loss of protection at a more advanced age may be explained by the fact that various MMPs proteolyse αB-crys at multiple scissile bonds (cf. Table 8) (Shiryaev *et al.*, 2009) and may compensate for the absence of MMP-9-mediated αB-crys destruction.

4.2.2. Extracellular proteolysis of intracellular autoantigens in systemic autoimmune diseases: SLE

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease characterized by the production of high titers of autoantibodies directed against ubiquitous intracellular, and mostly nuclear, autoantigens. The diverse symptoms of SLE range from rash and arthritis through anemia and thrombocytopenia to serositis, nephritis, seizures, and psychosis. Pathogenic autoantibodies and circulating immune complexes are the primary cause of tissue damage in patients with SLE (Rahman and Isenberg, 2008). More than 100 different autoantibodies have been found in SLE patients, and these arise from the dysregulation of all of the key components of the immune system (Sherer *et al.*, 2004). Apoptotic cells have been increasingly accepted to have initiating and propagating effects in SLE (Munoz *et al.*, 2005). Indeed, lymphocytes of SLE patients show accelerated apoptosis rates (Emlen *et al.*, 1994; Denny *et al.*, 2006). In addition, in SLE, phagocytes have decreased phagocytic activities, which results in a defective clearance of apoptotic cells (Ren *et al.*, 2003; Gaipl *et al.*, 2005). Hence, the overload of apoptotic cells progresses to secondary necrosis and intracellular proteins become accessible to post-translational modifications (Dieker and Muller, 2009), for example by proteases (cf. §4.1.2). Altered intracellular autoantigens and danger signals released from necrotic cells create a pro-inflammatory environment in which dendritic cells engulfing apoptotic cells may become activated instead of tolerogenic (Savill *et al.*, 2002; Viorritto *et al.*, 2007). These activated "immunogenic" dendritic cells may in turn activate autoreactive T and B cells, leading to the production of autoantibodies and formation of pathogenic immune complexes. Infection or environmental exposures may raise the apoptotic burden to initiate autoimmune disease and cause relapses. Furthermore, apoptotic cells cluster intracellular and nuclear proteins on the surface of apoptotic blebs and apoptotic bodies (cf. §4.1.2), many of which are major SLE autoantigens.

Hence, the question was raised whether MMPs may modify these systemic autoantigens exposed on the apoptotic cell surface, as well as the overload of SLE autoantigens released during (secondary) necrosis. To investigate this, a system of dying monocytic leukemia cells was used with MMP-9 as a model protease. This "one-dimensional degradomics system" identified adenylyl cyclase-associated protein-1 or CAP1 as novel and highly efficient substrate of MMP-9 during cellular necrosis (Cauwe *et al.*, 2008). Indeed, cleavage of CAP1 was more efficient than the cleavage of gelatin, the physiological MMP-9 substrate. In addition, CAP1 was also degraded by other MMPs (cf. Table 5), but at physiological concentrations, MMP-9 was the only (tested) MMP that caused its degradation. CAP1 is a cytoskeletal protein involved in the promotion of actin filament turnover (Moriyama and Yahara, 2002; Bertling *et al.*, 2004) and it was identified as an autoantigen in SLE (Frampton *et al.*, 2000) and rheumatoid arthritis (Kinloch *et al.*, 2005). Moreover, intact CAP1 was identified in the urines of patients with systemic autoimmune diseases such as SLE, vasculitis and Sjögren's syndrome, as well as in healthy control urines. Whereas healthy control urines did not contain activated MMP-9, urine samples of patients with clinical parameters suggesting renal failure showed increased levels of pro-MMP-2 and pro-MMP-9, and the appearance of activated forms of both gelatinases, as evidenced by substrate zymography. Interestingly, in some patient urines, an inverse relation was observed between the levels of intact CAP1 and activated forms of MMP-2 and MMP-9, and one urine sample of an SLE patient contained CAP1 fragments. These findings, together with the high turnover rate of CAP1 by MMP-9, suggest that this cleavage may occur *in vivo* (Cauwe *et al.*, 2008). In a recent study, a pro-apoptotic role is described for CAP1. Upon apoptosis induction by various stimuli, CAP1 translocates rapidly to the outer mitochondrial membrane even before caspase activation. CAP1-knockdown cells are resistant to apoptosis inducers, showing that the mitochondrial translocation of CAP1 is proapoptotic, possibly by shuttling apoptosis-inducing actin to the mitochondria (Wang *et al.*, 2008). MMP-9 was found in mitochondria (cf. §3.2.5 and 3.3.3) and its efficient cleavage of CAP1 may result in defective apoptosis, pushing the cell death program towards immunogenic necrosis. Alternatively, CAP1 may be cleaved by MMP-1, which was found to confer resistance to apoptosis when associated with mitochondrial membranes (cf. §3.2.5 and 3.3.6 and Figure 7) (Limb *et al.*, 2005).

By developing and applying a two-dimensional degradomics (2DD) approach to THP-1 cytosol (cf. §2), the intracellular degradome of MMP-9 was further expanded and this demonstrated that about two thirds of the identified (candidate) substrates are autoantigens described in one or multiple autoimmune conditions, and in cancer (e.g. annexin I, nucleolin, citrate synthase, cyclophilin A, HMGB1/2, α-enolase, histidyl-tRNA synthetase, HSP27, HSP90, phosphoglycerate kinase 1) (Cauwe *et al.*, 2009). From Tables 3 and 5, it is clear that cleavage of systemic autoantigens may be a general MMP function, and that

cleavage by MMPs is predictive of autoantigen status, as was described for the caspases and granzyme B (Rosen and Casciola-Rosen, 1999). Indeed, on top of the above-mentioned autoantigens targeted by MMP-9, MMPs (may) cleave many nucleic acid-associated autoantigens such as histones, hnRNPs, La, PARP-1, SmD3, nucleophosmin and cytoplasmic autoantigens including various elongation factors, cytoskeletal proteins, calpastatin, and cathepsins G (Sherer *et al.*, 2004; Hoffmann *et al.*, 2009; Cauwe *et al.*, 2009).

Proteolysis of systemic autoantigens by caspases and granzymes was proposed to be immunogenic by the release of normally "hidden" cryptic epitopes (Rosen and Casciola-Rosen, 1999). However, granzyme B may both release and destroy immunodominant epitopes (Darrah and Rosen, 2010). In addition, a study with granzyme B-deficient mice showed that granzyme B is not required for the development of pristane-induced SLE and may even have a protective effect, as the granzyme B-deficient mice showed increased mortality after pristane treatment (Graham *et al.*, 2005). This suggests that autoantigen cleavage does not necessarily lead to the stimulation of autoimmunity. As cleavage by MMPs is also predictive for autoantigen status, a similar question remains. Will proteolysis by MMPs lead to the release of tolerance-breaking neo-epitopes or will degradation by MMPs disrupt immunodominant epitopes and contribute to the silent removal of (abundant) intracellular proteins after necrosis and tissue injury?

In addition, the complex consequences of systemic autoantigen cleavage by MMPs may not be restricted to the alteration of the substrate's immunogenicity. Indeed, by cleavage of calreticulin and annexin I at the surface of apopotic cells, MMPs may abolish recognition by phagocytes in SLE (cf. §4.1.2), contribute to the clearance deficiency and further tip the balance to immunogenic secondary necrosis. Alternatively, ingestion of apoptotic cells has been suggested to be tolerogenic in the absence, and immunogenic in the presence, of danger signals (Savill *et al.*, 2002; Viorritto *et al.*, 2007). Hence, if MMPs degrade and inactivate danger signals, as discussed for the above-mentioned alarmins (cf. §4.1.2), they may promote tolerance and dampening of inflammation.

In conclusion, further examination of the effects of proteolysis by MMPs on the immunogenicity and function of systemic autoantigens may yield interesting insights into the etiology and relapsing-remitting mechanisms of complex systemic autoimmune diseases.

4.2.3. Extracellular proteolysis of intracellular autoantigens in acute necrotic conditions

Fulminant hepatic necrosis, septic shock, ischemic conditions, acute respiratory distress syndrome (ARDS), tumor lysis syndrome, pre-eclampsia during pregnancy, hemolysis due to malaria, and severe traumatic and burn injuries, are all acute conditions accompanied by massive cellular necrosis and tissue injury. These conditions depend on a rapid "cleaning" system to remove the overload of toxic and immunogenic proteins released from the cells, in order to prevent inflammation and secondary injury. Indeed, when cells die, monomeric and filamentous actins are released into the extracellular space and reach the systemic circulation. In the plasma, where the ionic strength, pH and temperature promote polymerization, actin monomers can form long filaments together with coagulation factor Va, which triggers disseminated intravascular coagulation, if not rapidly resolved. Functional organ decompensation leads to a condition resembling multiple organ dysfunction syndrome (MODS). In addition, the high viscosity of actin filaments, the inhibitory effect of actin on fibrinolysis, and the fact that actin binds adenine nucleotides that activate purinergic receptors are all mechanisms that may lead to secondary tissue injury (Bucki *et al.*, 2008). However, to avoid these toxic effects of extracellular actin, a complex actin-scavenging system exists in the vascular compartment. This system involves two proteins: plasma gelsolin that releases monomers from the toxic filaments, and Gc-globulin, which complexes the freed monomers. Both actin-gelsolin and actin-Gc-globulin complexes are subsequently cleared by the liver phagocytes much more efficiently than the free proteins (half-lives of 30 min versus 1–2 days, respectively). Nevertheless, excessive release of cellular actins or decreased activity of the actin-scavenger system causes severe pathological conditions such as MODS, hepatic necrosis, ARDS, septic shock, and complications of pregnancy (Haddad *et al.*, 1990; Lee and Galbraith, 1992; Dahl, 2005; Meier *et al.*, 2006).

Gelsolin exists in an intracellular and a secreted isoform. Both isoforms are derived from a single gene by alternative transcriptional initiation sites and mRNA processing, which removes the signal sequence and NH_2-terminus to generate cytoplasmic gelsolin. The term "gelsolin" refers to its ability to convert filamentous actin (F-actin) from a "gel" to a "solvent" state by rapid shortening of the filaments (Kwiatkowski *et al.*, 1988). Plasma gelsolin not only binds actin, but also scavenges a variety of potentially inflammatory moieties such as platelet-activating factor, lysophosphatidic acid, sphingosine-1-phosphate and bacterial cell wall constituents. In this way, gelsolin can modulate the exuberance of the host response to sepsis, malaria, burns, trauma, and other acute clinical conditions. The lower the levels of plasma gelsolin, the less favorable the prognosis of acute illness becomes (Bucki *et al.*, 2008; DiNubile, 2008). Plasma gelsolin was identified by 2D-PAGE as a MT1-MMP substrate in plasma (Hwang *et al.*, 2004). In addition to MT1-MMP, gelsolin is cut

into several fragments by various MMPs. MMP-3 cleaves gelsolin most efficiently, followed by MMP-2, MMP-1, MT1-MMP and MMP-9. MMP-3 cleaves gelsolin at Asn416-Val417, Ser51-Met52, and Ala435-Gln436, which results in considerable loss of its depolymerizing activity (Hwang *et al.*, 2004; Park *et al.*, 2006). This suggests that MMPs may weaken the extracellular actin-scavenging system by cleaving gelsolin and enhancing primary and secondary injury in pathological conditions induced by extracellular actin.

However, MMPs may also have a beneficial effect after massive necrosis. Indeed, by cleaving actin, they diminish primary and secondary effects of actin toxicity. By degradation of many essential actin/tubulin-binding proteins such as ezrin, moesin, CAP1, Arp2/3 complex subunits, IQGAP1, profilin, stathmin, and tubulin (cf. Tables 3 and 5), they may inhibit polymerization and branching of the cytoskeleton in the extracellular space and prevent the previously mentioned consequences of massive necrosis (Cauwe *et al.*, 2009).

Hence, cleavage of cytoskeleton-associated proteins after necrosis may have both positive and negative outcomes. The extracellular clearance may be part of a physiological process that clears proteins released by occasional necrosis in the tissues. In steady-state conditions, MMP activity in the circulation is kept in check by the general inhibitor α_2-macroglobulin. Hence, gelsolin will not be cleaved and may exert its actin-scavenging role. However, in acute inflammation, activated MMPs are found in the circulation, where they may inactivate gelsolin with the ensuing pathological consequences. In addition, the outcome may depend on the MMP in charge. Since MMP-9 is less efficient at gelsolin cleavage, it may have mostly beneficial effects by clearing abundant cytoskeletal proteins and by the very efficient degradation of CAP1 (*vide supra*). However, MMP-3 may be mostly pathogenic by weakening the gelsolin scavenging capacity. Since MMP inhibitors were proposed as an interesting line of therapy in acute inflammatory conditions (Hu *et al.*, 2007), determining the balance of pathogenic and beneficial effects of MMPs after massive necrosis may be of critical importance. Indeed, an ideal time-window and duration of inhibition will limit or even prevent toxic side effects (Hu *et al.*, 2007).

4.2.4. Extracellular proteolysis of intracellular autoantigens in amyloid diseases

Protein conformational diseases are diverse disorders characterized by abnormal unfolding, followed by aggregation and progressive accumulation of a disease-associated (glyco)protein. Another common feature of these protein conformational diseases is that they are mostly late-onset illnesses (Surguchev and Surguchov, 2010). The pro-aggregating roles of MMPs in two protein conformational diseases with mostly intracellular accumulation of protein aggregates, namely Parkinson's disease (cf. §3.3.4) and cataract (cf. §3.3.5), were already discussed. Here, we discuss the aggregation of gelsolin, which leads to familial amyloidosis of Finnish type (FAF). FAF is a late-onset autosomal dominant disease, which leads to progressive peripheral neuropathy that involves the cranial nerves and especially the facial nerve. These symptoms are caused by deposition of amyloid in the perineurium, vascular walls, cornea and skin (Luttmann *et al.*, 2010). FAF patients possess mutated plasma gelsolin (D187Y/N), which is aberrantly processed by at least two successive proteolytic events to generate amyloidogenic peptides. D187Y/N mutations abolish Ca^{2+} binding in domain 2, destabilizing and rendering the domain accessible to aberrant proteolysis by an α-gelsolinase, that was identified as furin (Chen, CD *et al.*, 2001). Cleavage of mutated gelsolin by furin at Arg172-Ala173 occurs as it transits through the Golgi apparatus and yields a secreted 68 kDa COOH-terminal fragment (C68) that contains the amyloidogenic region at its NH_2-terminus. C68 is the substrate for a second protease, β-gelsolinase, whose activity yields major 8 kDa and minor 5 kDa amyloidogenic fragments. Incubation of C68 with lysates of the human fibrosarcoma-derived cell line HT1080 (but not with HT1080 culture medium) resulted in cleavage of C68 into the amyloidogenic 8 kDa and 5 kDa peptides, and this was inhibited by the general MMP inhibitor GM6001 and TIMP-2, but not by TIMP-1. These results suggest that the β-gelsolinase is an MT-MMP. Indeed MT1-MMP cleaves C68 *in vitro* at Ala242-Met243, generating the 8 kDa fragment, which is converted into the 5 kDa upon additional incubation with MT1-MMP (Page *et al.*, 2005). Other MMPs also cleave C68 *in vitro* (cf. §4.2.3), i.e. MMP-3, -7, -9, but not MMP-2, indicating that multiple MMPs may contribute to amyloidogenesis. ECM components, such as glycosaminoglycans, accelerate amyloidogenesis at neutral pH, suggesting that gelsolin is cleaved and forms fibrils in the neutral environment of the ECM (Annabi *et al.*, 2001). However, since amyloidogenesis is optimal at low pH (Ratnaswamy *et al.*, 1999) and MMPs may be activated intracellularly, intracellular β-gelsolinase activity by MMPs cannot be excluded.

MMPs were also shown to be involved in fibril formation (and degradation) in AA amyloidosis, caused by aggregation of the acute-phase protein serum amyloid A (SAA) (Stix *et al.*, 2001) and in the most prevalent amyloid disease, Alzheimer's disease, caused by amyloid β (Aβ) deposition in the brain. Interestingly, gelsolin inhibits the fibril formation of Aβ and destabilizes preformed Aβ fibrils (Ray *et al.*, 2000). In addition, cytoplasmic gelsolin reduces Aβ burden in a mouse model of Alzheimer's disease and prevents Aβ-induced apoptotic mitochondrial changes (Antequera *et al.*, 2009). Hence, cleavage of the anti-amyloidogenic gelsolin may have pathogenic effects

in both FAF, Alzheimer's disease, and in acute necrotic conditions (cf. §4.2.3).

In conclusion, the clarification of the roles played by MMPs in these and other amyloid diseases and protein conformational disorders may result in novel therapeutic strategies.

Conclusion

Matrix metalloproteinase substrate identification has evolved from extracellular matrix molecules to secreted and membrane-bound molecules. All these substrates imply the extracellular action of MMPs. Here, we discuss that intracellular localization of MMPs may not be an artifact by mislocalizations. Activated MMPs are found in many subcellular compartments where they cleave intracellular proteins or even exhibit non-proteolytic functions acting as transcription factor (Eguchi *et al.*, 2008) or antimicrobial agent (Houghton *et al.*, 2009). Indeed, most MMPs are modular entities that often share protein domains with members of sister enzyme families, including the "a disintegrin and metalloproteinase" (ADAM) family and the "a disintegrin and metalloproteinase with thrombospondin-like motif" (ADAMTS) enzymes. Therefore, it is not surprising that biological functions of specific MMPs depend on protein domains different from the catalytic site. A recent prominent example of such MMP multifunctionality is the anti-apoptotic activity of the hemopexin domain, discovered for human MMP-2 and MMP-9 (Redondo-Munoz *et al.*, 2010) and the non-catalytical unwinding of collagen by collagenases and aggrecanases (Nagase and Fushimi, 2008).

Searching for common denominators in the intracellular actions of MMPs, we came across various interrelated pathophysiological settings, such as cancer, cellular stress, apoptosis, degenerative and protein conformational diseases. Indeed, under cellular stress, MMPs may be activated by ROS and RNS, and exert pathological actions such as the cleavage of heat shock proteins and other chaperones that are upregulated to avoid stress-induced protein precipitation and that rescue a stressed cell from apoptosis. Hence, cleavage of chaperones is pro-apoptotic and will lead to protein aggregation and cell death, as observed in protein conformational diseases, such as cataract, Parkinson's disease and amyloid disorders. Proteolysis by MMPs even causes direct aggregation and precipitation of amyloidogenic proteins, suggesting that MMP inhibition in such protein conformational diseases may have double benefits. Extended cell death leads to degeneration, for example after ischemia/reperfusion injury in heart and brain. Many observed intracellular cleavages have turned out to have pro-apoptotic effects. However, dual roles in the apoptotic cascade are to be expected. Understanding the roles that MMPs may play in the decision making process as to whether a cell should live or die, may shed new light on the targeting of cancer cells and autoreactive cells in autoimmunity, and the prevention of cell death in degenerative diseases. As a result, an additional reason why MMPs are produced as inactive zymogens seems to be to avoid intracellular damage in healthy cells, whereas stressed cells have various ways to activate the MMPs, such as proteolysis by caspases and oxidative stress. However, as MMP activity is regulated by subtle concentration changes of ROS and RNS, as well as by phosphorylation, it cannot be excluded that MMPs also function as intracellular signaling molecules in cellular homeostasis, by proteolytic or non-proteolytic actions.

It will presumably take a while before we understand the complexities of subcellular enzyme substrate ecosystems, e.g. in inflammation, at the tumor cell invasion front or in a developing organism. Along similar lines of complexities, by degradomics and reverse-degradomics approaches, attempts are made to understand the complex protease web (Overall and Kleifeld, 2006; Kruger *et al.*, 2010). Similar to the Human Genome (HUGO) Project, it is technically possible to elucidate degradomes, to establish the protease web and to remodel exactly and completely any protein as a substrate of proteases and peptidases. Whether the full insights will lead to new treatments remains an unanswered question. However, the definition of a critical activation cleavage within a cascade or a network will remain a valuable approach for the development of novel therapies.

Nevertheless, therapeutic MMP inhibition is still far from home. The well-acknowledged complexities are the extended redundancy within the MMP family for a specific proteolytic action, with another MMP compensating for the inhibited one. The extensive lists of substrates discovered for every single MMP imply that pathogenic substrates will accumulate when inhibiting cleavage of physiological ones. In addition, the same MMP may have a pathological effect in an early disease state and a protective function in the final stages. Hence, inhibitors should not only be MMP-specific, but also time- and substrate-specific. With the discovery of intracellular MMP functions, an additional dimension is added to tissue ecosystems, since MMPs inside or outside cells may have different and even opposite effects on a particular disease outcome. Hence and ideally, an effective MMP inhibitor should also be specific for the specific subcellular locations where the particular MMP action is pathogenic.

In conclusion, what we presently know about intracellular MMP functions and cleavages of intracellular substrates may only be the tip of the iceberg. Further

characterization of intracellular substrates in physiology and pathology is amongst the challenges in current MMP research.

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