Engineering Autoactivating Forms of Matrix Metalloproteinase-9 and Expression of the Active Enzyme in Cultured Cells and Transgenic Mouse Brain

Katherine E. Fisher,*,‡ Qing Fei,‡ Ellen R. Laird,§ Jeffrey L. Stock,§ Melanie R. Allen,§ Barbara G. Sahagan,‡ and Christine A. Strick‡

Departments of CNS Discovery and Exploratory Medicinal Sciences, Global Research and Development, Groton Laboratories, Pfizer, Inc., Groton, Connecticut 06340-5146

Received November 20, 2001; Revised Manuscript Received April 10, 2002

ABSTRACT: Matrix metalloproteinases (MMPs) are hypothesized to play an important role in the pathogenesis of several central nervous system disorders. Increased levels of expression of MMP-9 (gelatinase B) and MMP-2 (gelatinase A) have been observed in Alzheimer's disease, stroke, multiple sclerosis, and amyotrophic lateral sclerosis. This suggests an aberrant regulation of MMPs that could lead to inappropriate expression of MMP activity. To allow us to evaluate the effect of increased levels of active MMP-9 in the central nervous system, mutant forms of the enzyme were designed to autocatalytically remove the pro domain, yielding active enzyme. This was accomplished by modifying residues in the cysteine switch autoinhibitor region of the propeptide. Stable cell lines and transgenic mice that express G100L and D103N autoactive forms of human MMP-9 were developed to study the role of dysregulation of MMP-9 in disease.

Matrix metalloproteinases (MMPs)[1] are a family of zinc-dependent endopeptidases that are collectively capable of degrading all the components of the extracellular matrix. Normal physiological processes, including fetal development, inflammatory cell migration, and wound healing, depend on the highly regulated activity of these enzymes and their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (*1*, *2*). Disruption of the tight regulation of activity of these enzymes is believed to contribute to the pathogenesis of tissue destructive diseases such as arthritis (*3*), multiple sclerosis (*4*, *5*), and cancer (*6*). Increased MMP expression levels have also been observed in several central nervous system disorders, including Alzheimer's disease (*7*), Guillain-Barre syndrome (*8*), amyotrophic lateral sclerosis (*9*), and stroke (*10*).

These recent literature reports describing increased levels of MMP-9 expression in degenerate diseases of the central nervous system, along with quantitative RT-PCR data indicating significantly increased MMP-9 messenger RNA levels in Alzheimer's disease brain (D. Wood and B. G. Sahagan, unpublished results), prompted us to focus on investigating the effects of dysregulation of MMP-9 activity in cells of the central nervous system.

Most of the MMPs, including MMP-9, are expressed and secreted from cells as inactive zymogens comprised of an amino-terminal propeptide, a catalytic domain, and a hemopexin-like carboxy-terminal domain. It is widely accepted that the latency of these MMPs is maintained by a cysteine switch mechanism that works through the formation of an intramolecular complex between the single cysteine residue present in a highly conserved region of the propeptide and the essential zinc atom in the catalytic domain, thereby blocking the active site (*11*). This cysteine is contained within an autoinhibitor region of the propeptide (consensus sequence PRCGVPDL) which is highly conserved in all the members of the MMP family except MMP-23 (Figure 1). It has also been shown, however, that the cysteine switch is not solely responsible for maintaining enzyme latency since additional motifs located within the propeptide are necessary to stabilize the propeptide conformation that blocks the active site (*12*−*15*). In vivo, extracellular activation of these MMPs typically results from a two-step process. There is an initial cleavage by an activator protease in an exposed loop of the propeptide domain that destabilizes propeptide binding interactions and disrupts the zinc coordination. This is followed by the final cleavage, usually by an MMP, that releases the mature enzyme (*16*).

Due to this tight regulation of enzyme activity, it is difficult to mimic the potential dysregulation that might be a part of disease pathology. Construction of cell lines and transgenic mice that overexpress MMP-9 would almost certainly result in an increased level of expression of the latent enzyme but not necessarily in increased activity. However, previous studies with MMP-3 have shown that certain mutations in the conserved autoinhibitor region of the propeptide can destabilize the interactions responsible for maintaining enzyme latency and effectively bypass the initial extracellular activation step, resulting in direct expression of the active

* To whom correspondence should be addressed: Global Research and Development, Groton Laboratories, Pfizer, Inc., Eastern Point Road, MS8220-4093, Groton, CT 06340. Telephone: (860) 441-4730. Fax: (860) 686-0637. E-mail: katherine_e_fisher@groton.pfizer.com.

‡ Department of CNS Discovery.

§ Department of Exploratory Medicinal Sciences.

[1] Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; APMA, 4-aminophenylmercuric acetate; RT-PCR, reverse transcriptase polymerase chain reaction.

10.1021/bi012076t CCC: $22.00
Published on Web 06/06/2002

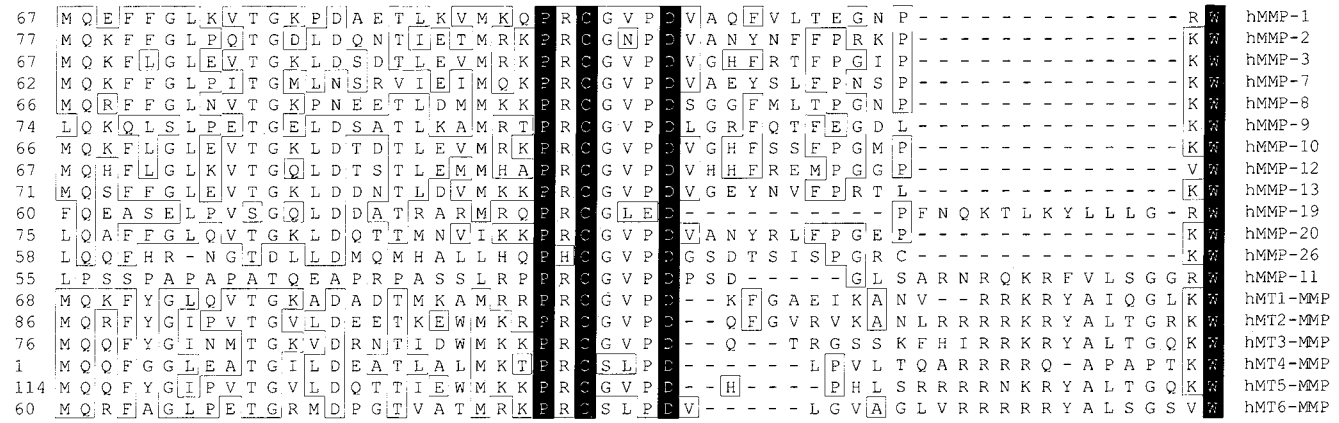

FIGURE 1: Sequence alignment of the autoinhibitor region of known matrix metalloproteinases. The alignment was generated with the program Megalign (DNA Star) using the Clustal method. Amino acid residues conserved in all sequences are boxed and shaded. Unshaded, boxed regions indicate amino acid residues conserved in a majority of the sequences. Accession numbers for sequences from top to bottom are as follows: X54925, M55593, J03209, X07819, J05556, J05070, X07820, L23808, X75308, X92521, Y12779, AF248646, X57766, D26512, Z48482, D85511, AB021225, AF131284, and AF145442.

enzyme (*12*, *14*, *15*, *17*). Because initial attempts to generate an autoactive MMP-9 by constructing a mutant homologous to an autoactive mutant of rat MMP-3 (P93V) yielded only latent enzyme, we set out to generate a series of mutants to identify an autoactivating form of MMP-9.

In this study, we report the identification of several mutations that disrupt the function of the cysteine switch autoinhibitor and generate autoactive forms of human MMP-9. The development and characterization of cell lines overexpressing these mutant forms, as well as the identification and preliminary characterization of transgenic mice expressing detectable levels of active human MMP-9 in their brains, will be described.

EXPERIMENTAL PROCEDURES

MMP-9 Mutant Plasmid Constructions. The cDNA encoding MMP-9 was obtained from G. Goldberg (Washington University, St. Louis, MO). The gene was subcloned into pcDNA3.1 (Invitrogen) as an *Xba*I fragment, and mutations were generated directly in the expression vector using the Stratagene QuikChange site-directed mutagenesis kit according to the manufacturer's instructions. The primers used for mutagenesis are as follows: R98M, 5′GCCATGCGAACACCAATGTGCGGGGTCC3′; R98K, 5′GCCATGCGAACACCAAAGTGCGGGGTCC3′; R98V, 5′GCCATGCGAACACCAGTGTGCGGGGTCC3′; C99S, 5′CGAACCCCACGGAGCGGAGTCCCAGACC3′; G100A, 5′CCCACGGTGCGCTGTCCCAGACCTG3′; G100L, 5′CCCCACGGTGCCTTGTCCCAGACCTG3′; V101A, 5′CCACGGTGCGGTGCTCCAGACCTGGGC3′; P102V, 5′CGGTGCGGGGTCGTGGACCTGGGCAGATTCC3′; and D103S, 5′GTGCGGGGTCCCAAGCCTCGGCAGATTCCAAAC3′.

Note that the nucleotides changed relative to the sequence of wild-type MMP-9 cDNA are underlined. In several cases, an additional silent mutation was added to eliminate problematic secondary structures in the primer. The extension time for the polymerase chain reactions was set at 16 min per cycle for 18 cycles with other parameters set according to the QuikChange site-directed mutagenesis kit specifications. Multiple transformants were selected from each mutagenesis, and DNA was prepared for sequence analysis using the QIAfilter Plasmid Maxi Kit (Qiagen). Full-length sequences for the MMP-9 mutant and wild-type genes were confirmed by automated sequence analysis using an Applied Biosystems sequencer (model 373).

Expression of Mutant MMP-9. Plasmids for each of the mutants were transiently transfected into the human neuroblastoma cell line SH-SY5Y (ATCC, CRL2266) using Lipofectamine Plus reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Approximately 48 h post-transfection, culture supernatants and cell lysates were harvested for zymography gel analysis.

For generation of stable cell lines, MMP-9 cDNA plasmids containing the G100L or D103N mutation were transfected into SH-SY5Y cells. Transformants were selected with 400 μg/mL Geneticin, and individual clones were isolated and expanded. To collect samples for assay, cells were plated at a density of 1×10^5 per well in 96-well tissue culture plates and allowed to adhere overnight. The medium was then replaced with 150 μL of fresh medium, and culture supernatant samples were harvested at 4, 8, 12, and 24 h. Zymography gel analysis was utilized to identify lines expressing high levels of the active enzyme. Supernatants (25 μL samples) from several clones expressing G100L MMP-9 were further analyzed using the BioTrak MMP-9 activity assay (Amersham) according to the manufacturer's instructions to confirm the enzyme's ability to cleave substrate, and to determine the proportions of latent to active enzyme.

Zymography Gel Analysis of Expression in Cell Lines. Novex Zymogram gels [10% tris-glycine polyacrylamide gels with 0.1% gelatin (Invitrogen)] were utilized for this assay according to the manufacturer's protocols. The gels were incubated overnight in the developing buffer for maximum sensitivity. Latent and 4-aminophenylmercuric acetate (APMA)-activated samples of purified MMP-9 were used as standards on all gels.

MMP-9 Activity Assays. For time course analysis of active MMP-9 secretion from G100L/SH-SY5Y cell lines, cells were plated at a density of 2×10^5 cells per well in a 48-well tissue culture plate and allowed to adhere overnight. The following morning medium was removed and replaced with 400 μL of fresh medium. Culture supernatant samples were collected at various time points over the course of 8

days and frozen at −20 °C until they were assayed. Culture supernatants were diluted 1:10 for zymography, and 3 μL was loaded per lane. BioTrak MMP-9 specific substrate cleavage activity assays (Amersham) were performed on 12, 6, or 3 μL undiluted culture supernatant samples. Samples were run in triplicate. Human MMP-9 was used for generation of a standard curve. MMP-9 activity was measured after a 2 h incubation for cell culture samples or after a 1 h incubation for brain protein samples from transgenic mice.

Inhibitor Studies. The ability of the known MMP-9 inhibitor CP-471,474 (*18*) to inhibit the activity of G100L MMP-9 was determined using the BioTrak MMP-9 activity assay. Supernatants were collected from actively growing cultures of the G100L MMP-9 stably transfected SH-SY5Y cells, and 3 μL samples were assayed. CP-471,474 was added to the supernatant-treated wells at half-log dilutions from 1 μM to 0.34 nM for 2 h at 37 °C. The remainder of the assay was carried out according to the standard protocol.

Production of the Mutant MMP9 Transgenic Mice. The transgene was constructed by inserting four copies of the chicken chromatin insulator sequence (*19*) into the multiple cloning region of pHelix 1 (−) (Boehringer Mannheim). An expression cassette fragment consisting of the neuron specific enolase (NSE) promoter (*20*), a random filler sequence, and the SV40 polyadenylation signal sequence was then subcloned into a blunted *Bam*HI site on the pHelix/CI vector such that it was flanked on each side by two copies of the chromatin insulators. Finally, the G100L MMP-9 cDNA was subcloned as an *Xba*I fragment into the *Nhe*I and *Spe*I sites of this vector, replacing part of the random filler sequence. The transgene DNA was purified for microinjection using Elutip columns (Schleicher & Schuell). The production of transgenic founders by pronuclear microinjection was achieved by using well-established procedures (*21*). Pronuclear stage FVB/N embryos were microinjected with the NSE-G100L MMP-9 transgene at a concentration of ∼3.0 ng/μL in 10 mM Tris (pH 7.4) and 0.1 mM EDTA. Microinjected embryos were transferred to pseudopregnant CD-1 females for continued development to term. At weaning, genomic DNA was isolated from the tail tissue by phenol and chloroform extraction. Founder transgenics were identified by PCR using an oligo set specific for the chicken chromatin insulator sequence. Founder mice were bred to FVB/N mates, and transgene positive offspring were used to maintain individual transgenic lines.

Preparation of Samples from Transgenic Mouse Brain. Mice were sacrificed, and the brains were removed, frozen on dry ice, and stored at −80 °C. Extracellular protein extracts were prepared using the method of Rosenberg with some modifications (*22*). Briefly, individual brains were minced in 1% Triton X-100 in PBS (Gibco). The minced brain tissue (400 mg/mL) was incubated at 4 °C for 15 h followed by centrifugation at 14 000 rpm for 10 min at 4 °C. The supernatant was collected and added to 200 μL of pre-equilibrated gelatin beads (Amersham Pharmacia). The mixture of supernatant and gelatin beads was rotated for 2 h at 4 °C. After rotation, the beads were allowed to settle, the supernatant was removed, and the beads were washed with 0.5% Triton X-100 and PBS (PBST) two or three times. MMPs were eluted from the gelatin beads with 10% DMSO and PBST, and the eluent was collected for activity and zymographic analysis. Once prepared, the supernatants were used immediately. For gel analysis by zymography (see above), samples were concentrated using Microcon 50 columns (Amicon). The concentrated sample was mixed (1:1) with Novex tris-glycine SDS sample buffer and incubated for 30 min at room temperature prior to loading.

MMP-9 Immunohistochemistry. Brains were fixed in neutral buffered 10% formalin overnight, embedded in paraffin, and sectioned into 10 μm slices. Sections were mounted on precleaned frosted slides (VWR Scientific). Prior to MMP-9 staining, the sections were deparaffinized by xylene and rehydrated in TBST (0.001% Triton in DAKO TBST). Sections were double blocked for hydrogen peroxidase with HRP blocking reagent (DAKO) and 3% hydrogen peroxide. Then sections were washed with TBST and blocked with DAKO serum free protein blocking reagent. Mouse anti-human MMP-9 antibody (Chemicon) was prebiotinylated and absorbed with horse serum (DAKO animal research kit). The sections were incubated with biotinylated MMP-9 antibody overnight at room temperature. Steptavidin peroxidate (DAKO) was used as a detection enzyme for the substrate DAB (DAKO). Mayer's hematoxylin (Sigma) was used as the counterstain, and the slides were mounted with universal mount (Fisher).

RESULTS

Construction of Mutant Forms of MMP-9. Our goal was to overexpress active MMP-9 for use in cell-based assays and transgenic animals. Since it is unlikely that we could directly express the constitutively active mature enzyme [previous studies indicated that deleting the pro region of rat MMP-3 resulted in very low levels of expression as detected by metabolic labeling (*17*)], we chose to generate an autoactivating form of MMP-9 by introducing mutations that disrupt the function of the cysteine switch autoinhibitor. This technique was used to develop several autoactivating forms of MMP-3 (*12*, *14*, *17*), one of which, rat P93V MMP-3, was successfully overexpressed in transgenic mice (*23*, *24*). On the basis of these data, we generated the homologous mutation, P102V, in human MMP-9. Stable cell lines were generated expressing this P102V mutant form, but unlike rat MMP-3, MMP-9 was secreted only in the latent form. These data are included in Figure 4.

The results from the P102V mutation suggest that not all of the MMP cysteine switch intramolecular complexes involve the same interactions, or at least that the stability and importance of certain interactions may differ for each enzyme. To address this, we generated an extensive set of mutations in the conserved region of the pro domain of MMP-9, using a structure-based approach. Mutations were restricted to this conserved autoinhibitor region so that mature enzyme in its native form could be generated. We used the X-ray structure of human pro-MMP-3 (PDB entry 1SLM) (*25*) and an amino acid alignment of human MMP-3 and MMP-9 to identify potential interactions within the pro domain or between the pro domain and the catalytic domain that could be disrupted upon mutation. Pairs of interacting residues near the cysteine switch were examined for structural evidence of electronic or hydrophobic contributions to fold stability, or for sites where steric crowding could be introduced. The backbone ribbon diagrams in Figure 2 illustrate the selected interactions found in the human pro-

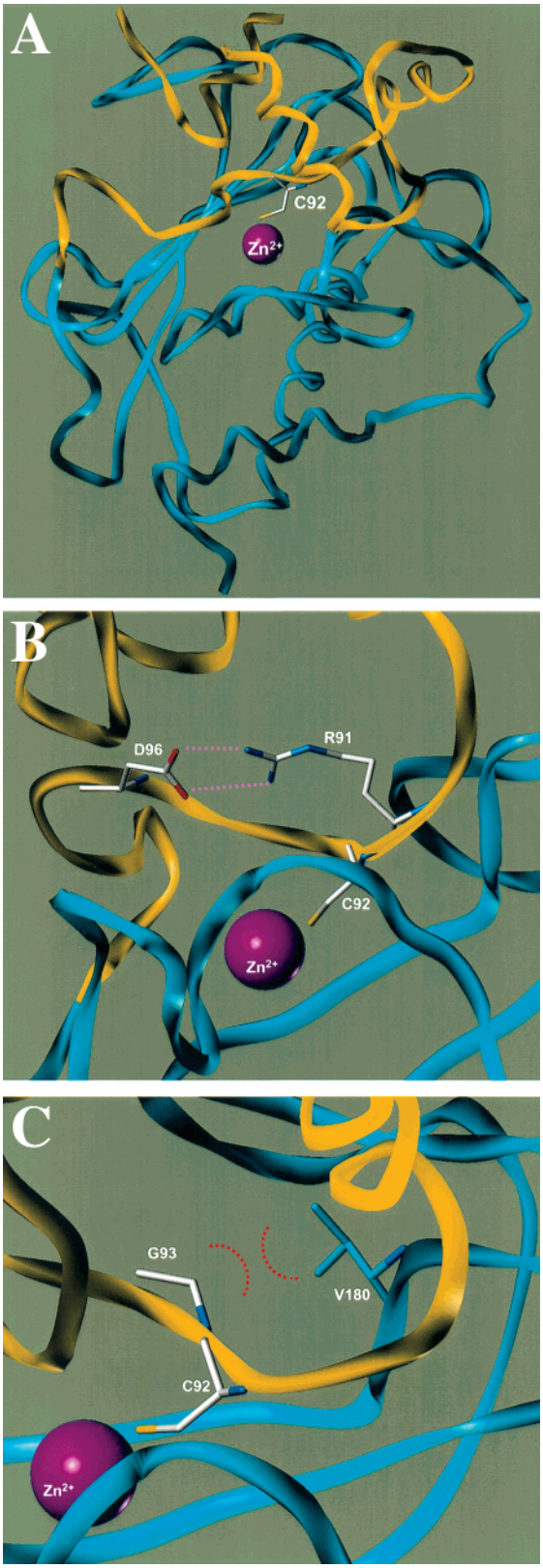

FIGURE 2: Ribbon diagrams showing the intramolecular complexes in human pro-MMP-3 used to design homologous mutations in MMP-9. The yellow corresponds to the pro domain and the blue to the active enzyme. (A) Interaction between the thiol of C92 in the autoinhibitor region and the catalytic zinc ion. (B) Salt bridge between R91 and D96. (C) Steric interaction between G93 and V180. This figure was generated with the program Sybyl (version 6.6, Tripos Inc.).

MMP-3 structure that are expected to be conserved in MMP-9. These include the interaction between the cysteine present

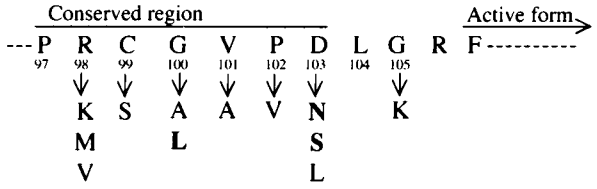

FIGURE 3: Schematic of the mutations generated in the autoinhibitor region of human MMP-9. Bold residues indicate mutations capable of secreting detectable levels of autoactive MMP-9. The numbering corresponds to the full-length MMP-9 amino acid sequence (SWISS-PROT accession number P14780).

in the conserved propeptide and the active site zinc (Figure 2A), the buried salt bridge between R91 and D96 (Figure 2B), hydrophobic interactions between V94 and the catalytic domain, and interactions between G93 and V180 (Figure 2C). Residues homologous to these were identified in MMP-9, and mutations that produced conservative to severe changes were designed (Figure 3). Although the more drastic changes increased the likelihood of achieving the desired effect, the conservative mutations were included to ensure proper protein folding. Some of the mutations that were selected were homologous to those previously tested in MMP-3 (*12*, *14*, *15*, *17*), enabling us to determine whether the cysteine switch mechanism functions similarly in MMP-9 and, in the event that it does, allowing us to assess differences in the requirements for autoactivation of these two enzymes.

Another approach to generating autoactivated MMP-9 involved using the MT-MMPs (membrane-type matrix metalloproteinases) and MMP-11 as models. Although these MMPs have the conserved autoinhibitor region, they can be secreted in the active form due to the presence of a cleavage site for Golgi-associated furin-like endopeptidases immediately upstream of the catalytic domain. These cleavage sites allow for intracellular cleavage which results in direct secretion of the active enzymes (*26*, *27*). On this basis, we designed a mutant form of MMP-9 that incorporated a minimal furin-like cleavage site, KR, immediately upstream of the mature form.

Analysis of Mutant Forms of MMP-9. To analyze the ability of the MMP-9 mutants to produce an active enzyme, plasmid DNA for mutant and wild-type MMP-9 was transiently transfected into the neuroblastoma cell line SH-SY5Y, and culture supernatants and cell lysates were collected for analysis by zymography. The untransfected SH-SY5Y cells express MMP-2 constitutively, but little, if any, MMP-9 is detected by zymography (Figure 4) or activity assay (data not shown). As expected, wild-type MMP-9 constructs expressed the latent enzyme. Each of the mutant forms of MMP-9 expressed detectable levels of enzyme, but in contrast to the wild type, several mutants accumulated significant amounts of active MMP-9 (Figure 4).

Mutation of Cysteine 99. To confirm that the cysteine switch mechanism maintains latency in MMP-9 in a fashion similar to that described for other MMPs, we changed the cysteine at position 99 to a serine. This change converts the sulfur of the thiol to oxygen, thereby preventing chelation to the catalytic zinc ion. The culture supernatants harvested from this C99S mutant contain only very low levels of the latent and active forms of the enzyme (Figure 4A), while the corresponding cell lysates contain a significant amount of the active form (Figure 4B).

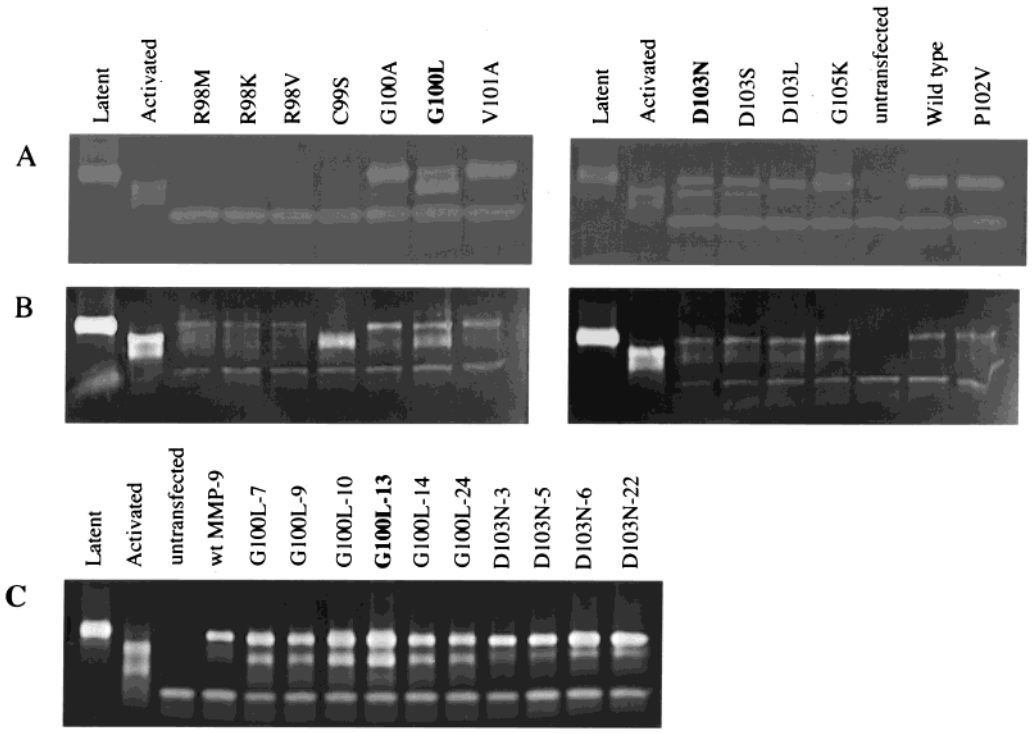

FIGURE 4: Gelatin zymogram of wild-type and mutant forms of MMP-9. Latent lanes contain 0.5 ng of purified latent MMP-9. Activated lanes contain 0.5 ng of purified MMP-9 activated with 1.5 mM APMA for 2 h at 37 °C. Note that the latent form of MMP-9 can be detected by zymography because the SDS present in the gel loading buffer disrupts the inhibition caused by the cysteine switch pro domain, enabling the proenzyme to degrade the gelatin substrate. Untransfected lanes contain proteins from untransfected host cells. The bottom band present across all expression lanes is MMP-2, which is constitutively expressed by SH-SY5Y cells. (A) Culture supernatant (10 μL) collected 48 h after transient transfection with plasmid expressing the specified mutant or wild-type version of MMP-9. (B) Total protein (10 μg) from cell lysates prepared 48 h after transient transfection with plasmid expressing the specified mutant or wild-type version of MMP-9. (C) Culture supernatant (3 μL) collected after 8 h as described in Experimental Procedures from clonal cell lines stably expressing either the G100L or D103N mutation in SH-SY5Y cells.

Mutations at Arginine 98. The structure of human pro-MMP-3 indicated that the conserved arginine in the autoinhibitor region formed a salt bridge with a conserved aspartic acid just downstream (Figure 2B). It has been suggested that this salt bridge may be important in maintaining enzyme latency in human MMP-3 (*15*). Three different substitutions were made for the homologous arginine in MMP-9 (R98K, R98M, and R98V). Culture supernatants from all three mutants produced only very weak bands of activity corresponding to latent and active enzyme on zymography gels as shown in Figure 4A. Analysis of the corresponding cell lysates detected some latent enzyme and a smear at the appropriate size for the active form (Figure 4B).

Mutations at Aspartic Acid 103. Like the R98 mutants, these mutations attempt to diminish or disrupt a possible salt bridge between R98 and D103 (homologous to the R91−D96 interaction shown for human pro-MMP-3 in Figure 2B). The results presented in Figure 4A indicate that all three of the alterations at D103 generate autoactivating MMP-9, although the proportions of latent to active enzyme differ. The D103N and D103S mutations appear to generate a higher proportion of active enzyme than the other mutation at this site.

Mutations at Glycine 100. Mutations were designed at this site to introduce steric encumbrances which might destabilize the chelation of the cysteine at position 99 to the catalytic zinc ion. This was based on the interaction between G93 and V180 in the human pro-MMP-3 structure shown in Figure 2C. Results shown in Figure 4A indicate that the G100A alteration is not sufficient to disrupt the autoinhibitor mechanism, as only latent MMP-9 is detected. Replacing this glycine with the more bulky leucine (G100L), however, results in expression of both the active and latent forms of the enzyme at a ratio of approximately 2:1.

Mutation at Valine 101. The mutation designed at this site, V101A, was intended to diminish hydrophobic contact with the catalytic domain. Results indicated that in MMP-9 this modification was not adequate for disruption of the cysteine switch autoinhibitor because only latent material was detected (Figure 4).

Mutation at Glycine 105. This mutation was generated to insert a sequence (K-R) typical for cleavage by furin-like convertases which might result in secretion of active MMP-9. Data in Figure 4 indicate that the protein was not cleaved at this site during secretion and only latent material was expressed.

Analysis of Cell Lines Expressing G100L or D103N. The G100L and D103N mutations were selected for generation of stable cell lines in SH-SY5Y cells to allow further analysis of the protein product. Zymography assays were used to screen several clonal lines for each mutation as shown in Figure 4C. These data indicate variable levels of expression across the isolated lines, although the G100L series consistently produced a larger proportion of active enzyme. The G100L lines were further analyzed by substrate cleavage assay, and results indicated that 40−70% of the MMP-9 that was produced was active prior to treatment with APMA

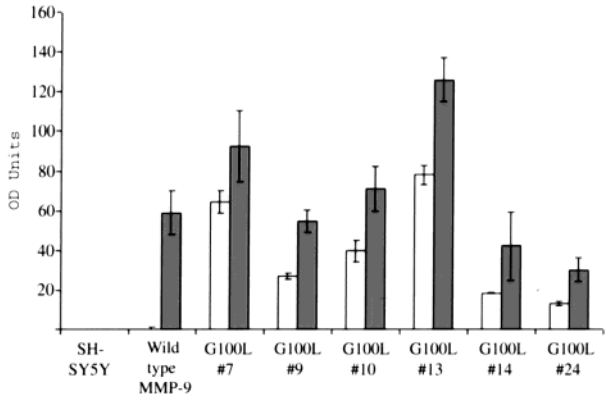

FIGURE 5: Substrate cleavage activity from autoactive and total MMP-9. Twenty-five microliters of the 24 h culture supernatant from six G100L stable cell lines was assayed in the Biotrak MMP-9 activity assay. Empty bars indicate MMP-9 activity present in the unactivated supernatant. The total MMP-9 activity (shaded bars) was determined by activating samples with 1 mM APMA for 2 h at 37 °C. The untransfected SH-SY5Y supernatant was included as a negative control. Supernatants from wild-yype MMP-9 were included for comparison of expression levels.

(Figure 5). The G100L-13 line was selected for further analysis. Expression time course studies indicated that the active enzyme accumulated for more than 24 h and then remained essentially constant for the remainder of an 8 day experiment. Expression culture supernatants collected as described in Experimental Procedures contained active enzyme at concentrations ranging from ~500 to 700 ng/mL as determined by an activity assay.

The active enzyme in undiluted culture supernatants was stable through several freeze−thaw cycles and for up to 3 weeks at 4 °C. However, the majority of the enzyme activity was lost when conditioned medium was filter-sterilized and incubated at 37 °C for 24 h.

Inhibition of Autoactive MMP-9 with a Known Inhibitor. To further characterize the G100L autoactive form of MMP-9, we tested the ability of a known inhibitor, CP-471,474, to inhibit this enzyme. CP-471,474 inhibits the G100L autoactive form of MMP-9 with an IC_{50} of 10.5 nM, a value nearly identical to that reported for the APMA-activated enzyme (*18*).

G100L Transgenic Mice. Human G100L MMP-9 cDNA was expressed in transgenic mice under the control of the neuron specific enolase promoter (*20*). Transgenic mice were viable and fertile with no gross morphological abnormalities observed in their brains. The brains of mice from five transgenic lines were analyzed for MMP-9 expression. In comparison to control, active MMP-9 was detected in all five transgenic lines with line G100L-E showing the highest transgene activity. Gelatin zymography (Figure 6) or enzyme activity assays (data not shown) yielded comparable results. Visualization of the area of lysis on the zymogram suggests that the majority of enzyme detected is in the active form (88 kDa) with small amounts of the latent enzyme (92 kDa) seen. No MMP-9 activity was detected in nontransgenic littermate control mice. As expected, MMP-2 was detected in transgenic and nontransgenic mice in both latent (72 kDa) and active forms (68 kDa).

MMP-9 Immunohistochemistry in G100L Transgenic Mouse Brains. Expression of human MMP-9, but not native murine MMP-9, was detected in all five transgenic lines

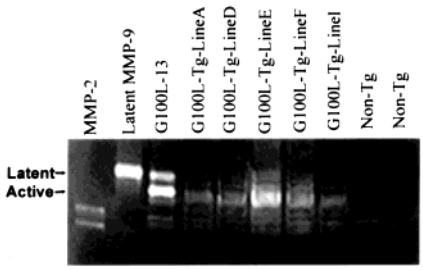

FIGURE 6: Gelatin zymogram of MMP-9 expression in transgenic mice. MMPs were isolated from transgenic (Tg) and nontransgenic littermate control (Non-Tg) mice for zymographic analysis. Recombinant human MMP-9, MMP-2, and the culture supernatant from a cell line expressing active MMP-9 (G100L-13) were used as molecular mass markers. Protein extracts from individual mouse brains were purified by gelatin chromatography and loaded onto a single gel lane. Mouse brains from transgenic lines A, D−F, and I along with nontransgenic littermate control mice were analyzed on a 10% polyacrylamide tris-glycine, 1% gelatin gel. Arrows mark the position of latent (92 kDa) and active MMP-9 (88 kDa).

by Western analysis performed with a panel of MMP-9 antibodies (data not shown). An antibody specific for human MMP-9 was then used to define immunoreactivity in transgenic mouse brain sections. Comparison of MMP-9 expression from the transgenic lines was consistent with the zymographic analysis and enzyme activity assay. No immunoreactivity was seen in brains from nontransgenic littermate controls. The distribution of MMP-9 expression extends from forebrain to cerebellum, including olfactory bulb, cerebral cortex, hippocampus, diencephalon, cerebellum, and brain stem (Figure 7). Higher levels of expression were observed in midbrain and cerebellum, particularly in the cerebral cortex, hippocampus, and diencephalon. MMP-9 immunoreactivity is mainly found in the extracellular space with very little signal seen in the cytoplasm.

DISCUSSION

In this paper, we describe the construction of autoactivating mutant forms of MMP-9. It had been previously shown for MMP-3 that modifying residues in the cysteine switch autoinhibitor region of the propeptide destabilized the interactions responsible for maintaining enzyme latency and effectively bypassed the initial extracellular activation step, resulting in direct expression of the active enzyme (*12*, *14*, *17*).

Our initial attempts to generate an autoactive MMP-9 by constructing a mutant homologous to an autoactive mutant of rat MMP-3 (P93V) yielded only latent enzyme, therefore requiring the generation of the more extensive set of MMP-9 mutants. In contrast, the corresponding mutation in MMP-13 (P99V) was used recently to generate transgenic mice reported to have a significant increase in their level of collagen II cleavage (*28*), suggesting that this mutation is adequate for production of autoactive MMP-13. Although all of the data collected from mutants of the autoinhibitor region of the pro domain of MMP-9 support the cysteine switch mechanism hypothesis, our data also imply that despite significant similarities in the amino acid sequence of the cysteine switch region, there may be distinct differences in the intramolecular interactions or the stability of those interactions for different MMPs.

Like results seen for rat MMP-3 (*12*), mutations at residues 98 and 99 of MMP-9 have a dramatic impact on accumula-

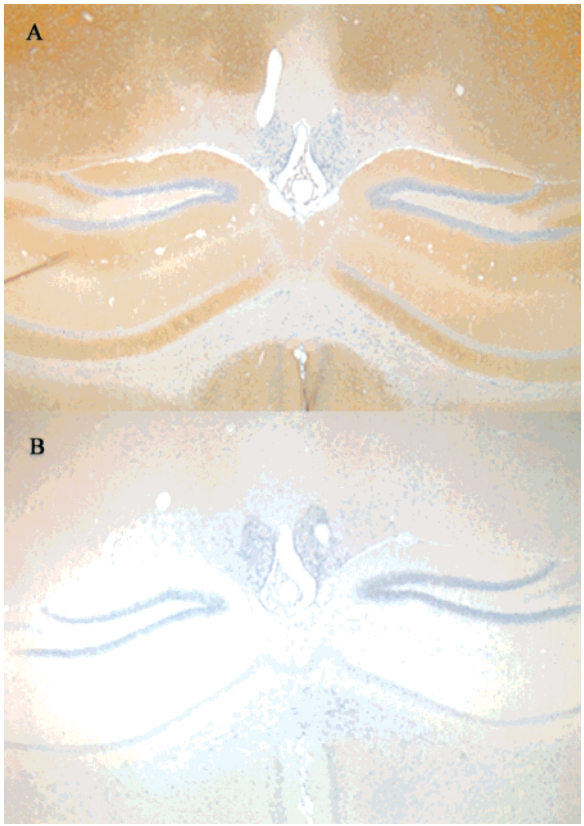

FIGURE 7: Immunohistochemical analysis of MMP-9 transgenic mouse brain. The hippocampal regions of transgenic line GE (A) and nontransgenic littermate control (B) mice are shown. Brain sections were immunostained with mAB 3309 (Chemicon) to detect human MMP-9 expression. Sections were counterstained with hematoxylin to visualize nuclei (magnification, 250×).

tion of enzyme. In the case of the C99S mutation, a significant amount of active but not latent MMP-9 accumulates within the cell. The R98 mutants show a different intracellular profile with both latent and active forms of MMP-9 accumulating at levels not significantly different from those observed in cells producing the wild-type enzyme. However, no enzyme of latent or active molecular mass is detected in the culture supernatant from any of the mutations at R98 or C99. Like Park et al. (*12*), who found that alterations of the cysteine in rat MMP-3 as well as the arginine just upstream resulted in accumulation of only small peptide fragments of MMP-3 in the culture supernatant, we did not detect the active enzyme in the supernatant. They concluded that these mutations caused a complete disruption of the autoinhibitor function, resulting in dramatic autoactivation of the enzyme and its rapid degradation. However, we also monitored intracellular accumulation of the engineered MMP-9s. One possible explanation based on our C99S data is that in mutants with severely disrupted pro domain interactions, the proenzyme is efficiently activated within the cell. And as our results with the MMP-9 C99S mutation and a previous unsuccessful attempt to express significant amounts of mature MMP-3 by deleting the pro domain (*17*) suggest, the active form of MMP may not be efficiently secreted; this would result in the absence of the active enzyme in the culture supernatant. Mutant forms of MMP-9 with less disrupted pro domain interactions (e.g., G100L or D103N) may be only partially activated within the cell. In this case, the latent but "autoactivatable" mutant form is secreted into the culture supernatant, where it undergoes activation, and active enzyme accumulates. However, this explanation suggests that active enzyme would accumulate within the R98 mutant cells, and this is not observed. In this case, the failure of active enzyme to accumulate in the culture supernatant may be due to a low level of expression or secretion efficiency unrelated to MMP activation. Clearly, further experimentation would be necessary to fully understand the impact of these mutations on the activation of MMP-9.

In contrast, several of the mutations which result in secretion of active forms of rat MMP-3 yield only latent enzyme when the homologous mutation is generated in MMP-9. The most interesting of these disparities occurs at the glycine residue just downstream of the cysteine (G100 in human MMP-9). In rat MMP-3, the G93A mutant is reported to express 100% active enzyme (*12*). The homologous mutation in human MMP-9 (G100A) results in secretion of only the latent form, although when we introduced a bulkier leucine (G100L) at this position, we detected secretion of large quantities of the autoactive enzyme. From the pro-MMP-3 structure shown in Figure 2C, it appears that mutations at this site may introduce steric encumbrances that might result in the destabilization of the chelation of the cysteine to the catalytic zinc ion. Our results suggest that this interaction may be more stable in the pro-MMP-9 structure than in rat MMP-3. Other examples that support the idea that different MMPs exhibit differences in the stability of interactions between the cysteine switch region and the active site include alterations at V101 and P102. Several mutations developed at these sites in rat MMP-3 secrete autoactive enzyme (*12*, *17*), but homologous mutations in human MMP-9 (V101A and P102V) produce only latent material.

Many MMPs are activated through a proteolytic cascade involving other MMPs. For example, MMP-3 is an efficient activator of MMP-9 (*29*, *30*). It seems plausible that the stringency of the interactions required for maintaining enzyme latency might increase as one moves through the cascade of activation. This could be one explanation for why more drastic mutations in the cysteine switch region are required to generate autoactive forms of MMP-9 than are required to generate autoactive forms of rat MMP-3. To test this hypothesis, additional mutations would need to be generated to determine if autoactive forms of MMP-9 can be generated by more severe alteration of some of these sites, and similar analyses of additional MMPs involved in activation cascades would need to be performed.

Another approach to generating an autoactive MMP-9 involved insertion of a minimal cleavage site typical for furin-like convertases just upstream of the active form. Although for the MT-MMPs (and MMP-11) cleavage by furin results in direct secretion of the active form of the enzyme, MMP-9 was expressed only in the latent form. This is probably due to the limited cleavage site incorporated in the MMP-9 mutant. The MT-MMPs have up to 11 amino acid residues inserted between the switch loop of the pro chain and the catalytic domain, and the pro chain terminates in an R-X-R/K-R sequence. In the MMP-9 G105K mutant, only the final K-R sequence of the cleavage site was included. This suggests that the minimal cleavage site we chose is not sufficient and/or not accessible without ad-

ditional amino acids between the pro and mature domains. Since our other data indicate that developing autoactivating forms of the various MMPs may be more complex than simply generating mutations that work for other family members, it would be interesting to incorporate larger portions of this cleavage site in the MMP sequence. If these types of insertions were capable of generating autoactive enzyme, this might be a more generally applicable method of producing autoactive forms of a number of MMPs.

Introduction of the modified human MMP-9 transgene into the mouse brain generated mice constitutively producing active MMP-9 in neurons. Further characterization of the autoactive MMP-9 transgenic mice is underway; their reaction to the excitotoxin kainate as well as their response to middle cerebral artery occlusion will be evaluated. We believe that this model system will further our understanding of the impact of dysregulation of MMP-9 in neurodegeneration. A similar approach using autoactive rat MMP-3 successfully expressed in the mammary glands of transgenic mice provided direct evidence that overexpression of activated MMP-3 can effect the basic cellular processes of proliferation and differentiation (*23*, *24*).

Increased levels of expression of MMPs have been measured in chronic and acute neurodegenerative diseases as well as in several animal models of disease. In Alzheimer's disease, MMP-9 has been shown to be present at levels as much as 4-fold higher in the hippocampal region than in age-matched controls (*7*). The enzyme was predominately found in the latent or proenzyme form in the proximity of extracellular amyloid plaques (*31*). In addition, the mRNA level of MMP-9 is increased as much as 6-fold in the superior frontal gyrus and hippocampus in Alzheimer's disease brains (D. Wood and B. G. Sahagan, unpublished data). The level of MMP-9 is also elevated in the cerebrospinal fluid, serum, and leukocytes of patients with multiple sclerosis, and MMP-9 is detected by immunohistochemistry in active and chronic lesions characteristic of the disease (reviewed in ref *32*). Overall, these data demonstrate a significant upregulation of MMP-9 in chronic neurodegenerative disease, suggesting a possible role in disease pathology.

During acute neurodegenerative insults such as stroke, the activated enzyme has also been detected in addition to the significant increase of pro-MMP-9 (*33*). The absence of MMP-9 in MMP-9 null mice (*34*, *35*) or inhibition of enzyme activity by antibodies (*36*) or small molecules (*37*) can ameliorate damage in rodent models of stroke and traumatic brain injury, suggesting a role for the active enzyme in this acute insult. Taken together, these data suggest that an increase in the level of the active enzyme plays a role in acute and perhaps in chronic neurodegenerative conditions. Acute insults may, however, result in a much greater concentration of enzyme at any given time than chronic conditions, thus enabling detection of activity. Under these circumstances, an extreme imbalance of MMPs with their endogenous inhibitors TIMPs (*32*) may exist. For example, data from a gadolinium-enhanced magnetic resonance imaging study suggest that an increase in MMP-9 activity relative to TIMP-1 may signal formation of new lesions in multiple sclerosis (*38*). This same type of imbalance, although not as extreme, may also factor into neuronal loss seen in chronic neurodegenerative conditions.

In this report, we have demonstrated that autoactive forms of MMP-9 can be expressed and secreted at high levels from stably transfected SH-SY5Y cells, that the active enzyme is capable of cleaving the natural and the synthetic substrate, and that the autoactive enzyme can be inhibited by a known MMP inhibitor. Further, we have shown that it is possible to generate transgenic animals with neuronal expression of autoactive human MMP-9. Together, these should provide useful tools in attempts to elucidate the role of active MMP-9 in diseases of the central nervous system.

ACKNOWLEDGMENT

We thank Peter G. Mitchell for intellectual contributions and supply of reagents, Yevette C. Clancy for extensive DNA sequencing support, Douglas E. Wood, Sue A. Yocum, and Jane A. Bennett for reagents and technical advice, Kim P. Kowsz, Mary M. Payette, and Karen L. Steever for trangenic tissue preparation, and Douglas A. Fisher and Dennis E. Danley for critical review of the manuscript.

REFERENCES

1. Borden, P., and Heller, R. A. (1997) *Crit. Rev. Eukaryotic Gene Expression 7*, 159−178.
2. Johnson, L. L., Dyer, R., and Hupe, D. J. (1998) *Curr. Opin. Chem. Biol. 2*, 466−471.
3. Konttinen, Y. T., Ainola, M., Valleala, H., Ma, J., Ida, H., Mandelin, J., Kinne, R. W., Santavirta, S., Sorsa, T., Lopez-Otin, C., and Takagi, M. (1999) *Ann. Rheum. Dis. 58*, 691−697.
4. Waubant, E., Goodkin, D. E., Gee, L., Bacchetti, P., Sloan, R., Stewart, T., Andersson, P. B., Stabler, G., and Miller, K. (1999) *Neurology 53*, 1397−1401.
5. Chandler, S., Miller, K. M., Clements, J. M., Lury, J., Corkill, D., Anthony, D. C., Adams, S. E., and Gearing, A. J. (1997) *J. Neuroimmunol. 72*, 155−161.
6. Kleiner, D. E., and Stetler-Stevenson, W. G. (1999) *Cancer Chemother. Pharmacol. 43*, S42−S51.
7. Backstrom, J. R., Miller, C. A., and Tokes, Z. A. (1992) *J. Neurochem. 58*, 983−992.
8. Creange, A., Sharshar, T., Planchenault, T., Christov, C., Poron, F., Raphael, J. C., and Gherardi, R. K. (1999) *Neurology 53*, 1683−1691.
9. Lim, G. P., Backstrom, J. R., Cullen, M. J., Miller, C. A., Atkinson, R. D., and Tokes, Z. A. (1996) *J. Neurochem. 67*, 251−259.
10. Clark, A. W., Krekoski, C. A., Bou, S. S., Chapman, K. R., and Edwards, D. R. (1997) *Neurosci. Lett. 238*, 53−56.
11. Van Wart, H. E., and Birkedal-Hansen, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5578−5582.
12. Park, A. J., Matrisian, L. M., Kells, A. F., Pearson, R., Yuan, Z. Y., and Navre, M. (1991) *J. Biol. Chem. 266*, 1584−1590.
13. Chen, L. C., Noelken, M. E., and Nagase, H. (1993) *Biochemistry 32*, 10289−10295.
14. Freimark, B. D., Feeser, W. S., and Rosenfeld, S. A. (1994) *J. Biol. Chem. 269*, 26982−26987.
15. Galazka, G., Windsor, L. J., Birkedalhansen, H., and Engler, J. A. (1996) *Biochemistry 35*, 11221−11227.
16. Nagase, H. (1997) *Biol. Chem. Hoppe-Seyler 378*, 151−160.
17. Sanchez-Lopez, R., Nicholson, R., Gesnel, M. C., Matrisian, L. M., and Breathnach, R. (1988) *J. Biol. Chem. 263*, 11892−11899.
18. Rohde, L. E., Ducharme, A., Arroyo, L. H., Aikawa, M., Sukhova, G. H., Lopez-Anaya, A., McClure, K. F., Mitchell, P. G., Libby, P., and Lee, R. T. (1999) *Circulation 99*, 3063−3070.
19. Chung, J. H., Whiteley, M., and Felsenfeld, G. (1993) *Cell 74*, 505−514.
20. Forss-Petter, S., Danielson, P. E., Catsicas, S., Battenberg, E., Price, J., Nerenberg, M., and Sutcliffe, J. G. (1990) *Neuron 5*, 187−197.
21. Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
22. Rosenberg, G. A., Dencoff, J. E., McGuire, P. G., Liotta, L. A., and Stetler-Stevenson, W. G. (1994) *Lab. Invest. 71*, 417−422.
23. Witty, J. P., Wright, J. H., and Matrisian, L. M. (1995) *Mol. Biol. Cell 6*, 1287−1303.

24. Sympson, C. J., Bissell, M. J., and Werb, Z. (1995) *Semin. Cancer Biol. 6*, 159−163.
25. Becker, J. W., Marcy, A. I., Rokosz, L. L., Axel, M. G., Burbaum, J. J., Fitzgerald, P. M., Cameron, P. M., Esser, C. K., Hagmann, W. K., Hermes, J. D., et al. (1995) *Protein Sci. 4*, 1966−1976.
26. Bode, W., Fernandez-Catalan, C., Tschesche, H., Grams, F., Nagase, H., and Maskos, K. (1999) *Cell. Mol. Life Sci. 55*, 639−652.
27. Pei, D., and Weiss, S. J. (1995) *Nature 375*, 244−247.
28. Neuhold, L. A., Killar, L., Zhao, W., Sung, M. L., Warner, L., Kulik, J., Turner, J., Wu, W., Billinghurst, C., Meijers, T., Poole, A. R., Babij, P., and DeGennaro, L. J. (2001) *J. Clin. Invest. 107*, 35−44.
29. Ramos-DeSimone, N., Hahn-Dantona, E., Sipley, J., Nagase, H., French, D. L., and Quigley, J. P. (1999) *J. Biol. Chem. 274*, 13066−13076.
30. Olson, M. W., Bernardo, M. M., Pietila, M., Gervasi, D. C., Toth, M., Kotra, L. P., Massova, I., Mobashery, S., and Fridman, R. (2000) *J. Biol. Chem. 275*, 2661−2668.
31. Backstrom, J. R., Lim, G. P., Cullen, M. J., and Tokes, Z. A. (1996) *J. Neurosci. 16*, 7910−7919.
32. Yong, V. W., Power, C., Forsyth, P., and Edwards, D. R. (2001) *Nat. Rev. Neurosci. 2*, 502−511.
33. Gasche, Y., Fujimura, M., Morita-Fujimura, Y., Copin, J. C., Kawase, M., Massengale, J., and Chan, P. H. (1999) *J. Cereb. Blood Flow Metab. 19*, 1020−1028.
34. Asahi, M., Asahi, K., Jung, J. C., del Zoppo, G. J., Fini, M. E., and Lo, E. H. (2000) *J. Cereb. Blood Flow Metab. 20*, 1681−1689.
35. Wang, X., Jung, J., Asahi, M., Chwang, W., Russo, L., Moskowitz, M. A., Dixon, C. E., Fini, M. E., and Lo, E. H. (2000) *J. Neurosci. 20*, 7037−7042.
36. Romanic, A. M., White, R. F., Arleth, A. J., Ohlstein, E. H., and Barone, F. C. (1998) *Stroke 29*, 1020−1030.
37. Jiang, X., Namura, S., and Nagata, I. (2001) *Neurosci. Lett. 305*, 41−44.
38. Waubant, E., Goodkin, D. E., Gee, L., Bacchetti, P., Sloan, R., Stewart, T., Andersson, P. B., Stabler, G., and Miller, K. (1999) *Neurology 53*, 1397−1401.

BI012076T