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Collagen Type I Selectively Activates Ectodomain Shedding of the Discoidin Domain Receptor 1: Involvement of Src Tyrosine Kinase

Barbara E. Slack,* Marina S. Siniaia, and Jan K. Blusztajn

Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, Massachusetts 02118

Abstract The discoidin domain receptor 1 (DDR1) is a receptor tyrosine kinase that is highly expressed in breast carcinoma cells. Upon binding to collagen, DDR1 undergoes autophosphorylation followed by limited proteolysis to generate a tyrosine phosphorylated C-terminal fragment (CTF). Although it was postulated that this fragment is formed as a result of shedding of the N-terminal ectodomain, collagen-dependent release of the DDR1 extracellular domain has not been demonstrated. We now report that, in conjunction with CTF formation, collagen type I stimulates concentration-dependent, saturable shedding of the DDR1 ectodomain from two carcinoma cell lines, and from transfected cells. In contrast, collagen did not promote cleavage of other transmembrane proteins including the amyloid precursor protein (APP), ErbB2, and E-cadherin. Collagen-dependent tyrosine phosphorylation and proteolysis of DDR1 in carcinoma cells were reduced by a pharmacologic Src inhibitor. Moreover, expression of a dominant negative Src mutant protein in human embryonic kidney cells inhibited collagen-dependent phosphorylation and shedding of co-transfected DDR1. The hydroxamate-based metalloproteinase inhibitor TAPI-1 (tumor necrosis factor-α protease inhibitor-1), and tissue inhibitor of metalloproteinase (TIMP)-3, also blocked collagen-evoked DDR1 shedding, but did not reduce levels of the phosphorylated CTF. Neither shedding nor CTF formation were affected by the γ-secretase inhibitor, L-685,458. The results demonstrate that collagen-evoked ectodomain cleavage of DDR1 is mediated in part by Src-dependent activation or recruitment of a matrix- or disintegrin metalloproteinase, and that CTF formation can occur independently of ectodomain shedding. Delayed shedding of the DDR1 ectodomain may represent a mechanism that limits DDR1-dependent cell adhesion and migration on collagen matrices. J. Cell. Biochem. 98: 672–684, 2006.

Key words: zinc-dependent metalloproteinase; amyloid precursor protein; tyrosine phosphorylation; TIMP (tissue inhibitor of metalloproteinase)

The discoidin domain receptor 1 (DDR1) is a receptor tyrosine kinase-like phosphoprotein that is highly expressed in breast carcinoma, in epithelial cells of the lung, kidney, and colon, and in brain [Sanchez et al., 1994; Alves et al., 1995; Vogel, 1999]. It has a discoidin-like domain in its N-terminal extracellular domain, a predicted transmembrane region, and a C-terminal tyrosine kinase domain [Vogel, 1999]. Of the five splicing variants of DDR1 that have been described to date [Alves et al., 1995, 2001; Perez et al., 1996], DDR1a is the shortest, encoding a protein of 876 amino acids. DDR1b contains an additional 37 amino acids in the juxtamembrane domain, including the motif LLXNPXY, which, when phosphorylated on the tyrosine residue, provides a docking site for the Shc adapter protein [Vogel et al., 1997]. A second member of this family, DDR2, is expressed in many tissues, including brain,

Abbreviations used: ADAM, a disintegrin and metalloproteinase; APP, amyloid precursor protein; CTF, C-terminal fragment; DDR1, discoidin domain receptor 1; L-685,458, [(2R,4R,5S)-2-Benzyl-5-(Boc-amino)-4-hydroxy-6-phenylhexanoyl]-Leu-Phe-NH2; MMP, matrix metalloproteinase; PKC, protein kinase C; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; sAPPα, APP soluble ectodomain; TACE, tumor necrosis factor-α converting enzyme; TAPI-1, tumor necrosis factor-α protease inhibitor-1; TIMP, tissue inhibitor of metalloproteinase.

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*Correspondence to: Dr. Barbara E. Slack, Department of Pathology and Laboratory Medicine, Boston University School of Medicine, 715 Albany Street, Rm. L808, Boston, MA 02118. E-mail: bslack@bu.edu

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muscle, and heart, and is found in stromal cells surrounding ovarian and lung tumors [Vogel, 1999].

In 1997, two groups reported that DDR1 and DDR2 are collagen receptors [Shrivastava et al., 1997; Vogel et al., 1997]. The addition of collagen to the medium of cells expressing either DDR1 or DDR2 resulted in tyrosine phosphorylation of these receptors with a time-course that was delayed in onset and lasted for up to 18 h. Whereas DDR1 responded to both fibril-forming collagens (e.g., collagen types I, II, III, and V) and the network-forming collagen type IV, DDR2 phosphorylation was strongly stimulated only by collagen types I and III [Vogel et al., 1997].

The known functions of DDR1 include promotion of cell adhesion and migration on collagen matrices, and modulation of cell proliferation [Kamohara et al., 2001; Vogel et al., 2001; Franco et al., 2002; Dejmek et al., 2003]. DDR1 is important for axon growth of cerebellar granule neurons [Bhatt et al., 2000], and for normal development of the mammary gland [Vogel et al., 2001]. DDR1 expression and activation are triggered by apoptotic stimuli, and promote cell survival by a mechanism dependent on activation of MAPK and induction of p53 [Ongusaha et al., 2003].

The recent demonstration that collagen type I caused the delayed appearance of a tyrosine phosphorylated C-terminal fragment (CTF) of DDR1 in a breast carcinoma cell line suggested that DDR1 phosphorylation might promote its proteolysis [Vogel, 2002]. Generation of the CTF was blocked by a zinc metalloproteinase inhibitor, indicating that DDR1 might constitute a novel target for limited proteolysis by a family of enzymes known as disintegrin metalloproteinases, or secretases [Hooper et al., 1997; Arribas and Borroto, 2002; Vogel, 2002].

Secretases cleave transmembrane proteins near the external face of the membrane, resulting in shedding of their ectodomains [Hooper et al., 1997; Arribas and Borroto, 2002], and many are members of the ADAM (a disintegrin and metalloproteinase) family. ADAM17, also known as tumor necrosis factor-α converting enzyme (TACE), catalyzes shedding of the majority of transmembrane proteins known to undergo secretory processing, although ADAM9, ADAM10, and ADAM12 also act on selected substrates in some cell lines [Schlöndorff and Blobel, 1999; Arribas and Borroto, 2002]. Ectodomain shedding may occur constitutively, and it may be up-regulated by receptor ligands, and by direct activators of protein kinase C (PKC). However, the mechanisms linking signaling pathways to proteolysis are not well understood [Arribas and Borroto, 2002]. Selective proteolysis of some transmembrane proteins may also be catalyzed by ligand binding, as in the case of Notch, which is cleaved by ADAM17 after binding to its ligand Delta [Fortini, 2002]. Shedding allows paracrine signaling by membrane-tethered growth factors, and, in some cases, it is a prerequisite for an additional cleavage event, usually within the transmembrane domain, that generates a signaling-competent CTF [Ebinu and Yankner, 2002].

The results of the present investigation demonstrate that collagen causes a specific, delayed stimulation of DDR1 ectodomain shedding, in the absence of an effect on other transmembrane proteins that are subject to cleavage by more general stimuli. Inhibition of Src tyrosine kinase activity by two different methods reduced collagen-dependent tyrosine phosphorylation of full-length DDR1, and shedding of its ectodomain. Two inhibitors of zinc-dependent metalloproteinases, TAPI-1 and TIMP-3, caused dose-dependent reductions in collagen-evoked shedding of the DDR1 ectodomain, but did not block generation, or phosphorylation, of the CTFs, suggesting that liberation of DDR1 CTFs may be mediated by a distinct, collagen-regulated protease. Ectodomain shedding may constitute part of an inhibitory feedback loop that serves as a constraint on the ability of DDR1 to promote adhesion and migration of cells in the presence of collagen [Franco et al., 2002; Dejmek et al., 2003].

MATERIALS AND METHODS

Cell Culture, Transfection, and Treatments

A431 cells (a human epidermoid carcinoma cell line) and human embryonic kidney (HEK) 293 cells were were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen). T-47D breast carcinoma cells obtained from the American Type Culture Collection (ATCC, Manassas VA) were grown in modified RPMI 1640 medium (ATCC)

supplemented with 10% fetal bovine serum, 0.2 IU/ml bovine insulin (Invitrogen), and penicillin/streptomycin (Invitrogen). Cells were maintained at 37°C in 95% air, 5% CO_2. Cells were serum-deprived prior to an experiment by incubating them overnight in serum-free DMEM. Rat-tail collagen type I solubilized in 0.02N acetic acid (BD Biosciences, Bedford, MA) was diluted in cold DMEM before being added to cell cultures. Controls received equivalent amounts of acetic acid in DMEM. The Src inhibitor 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1) (Alexis Biochemicals, San Diego, CA) was dissolved in dimethylsulfoxide (DMSO) to make a stock solution that was diluted in DMEM for cell treatments. Controls received equivalent concentrations of DMSO. The hydroxamic acid-based zinc metalloproteinase inhibitor TAPI-1 (Peptides International, Louisville, KY), and the γ-secretase inhibitor L-685,458 (Bachem, King of Prussia, PA) were dissolved in DMSO to make stock solutions that were diluted in DMEM. Controls received equal amounts of DMSO. Final concentrations of DMSO did not exceed 0.5%. The cDNA encoding DDR1b under the control of a CMV promoter was a generous gift of Dr. Teizo Yoshimura [Matsuyama et al., 2003]. Plasmids encoding tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, and TIMP-3, and the corresponding empty pBLAST vector, were purchased from InvivoGen (San Diego, CA). The cDNA constructs encoding wild-type Src (Srcwt) and dominant negative Src (K296R/Y528F) (SrcRF), and the corresponding empty pUSE vector, were purchased from Upstate (Lake Placid NY). Transient transfections were carried out using Lipofectamine Plus reagent (Invitrogen). Cells were plated onto 10 cm dishes and allowed to grow overnight. The next day they were transfected with vectors encoding DDR1b (3 μg/dish), together with plasmids encoding either TIMP-1, -2, or -3, Srcwt or SrcRF, or the corresponding empty vector. The plasmids were mixed with the Plus and Lipofectamine reagents in serum-free DMEM according to the manufacturer's instructions, and added to cell cultures. After 3 h, the transfection solutions were replaced with growth medium. Approximately 24 h later, the medium was replaced with serum-free DMEM containing collagen or vehicle, and the cells were incubated for the indicated time periods.

Preparation of Cell and Media Extracts and Immunoblot Analysis

Conditioned medium and cells were collected and processed for immunoblotting as previously described [Slack et al., 2001]. Samples were size-fractionated by SDS–PAGE and transferred to polyvinylidene difluoride membranes. Antibodies to the N- and C-terminus of DDR1 and to E-cadherin, and anti-phosphotyrosine antibodies (clone PY99) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to the amyloid precursor protein (APP) ectodomain (clone 6E10) were from Signet Laboratories (Dedham, MA), and antibodies raised against the C-terminus of APP were purchased from Zymed laboratories (San Francisco, CA). ErbB2 antibodies were from BD-Biosciences, TIMP antibodies from Chemicon International (Temecula, CA), and antibodies to Src (clone 327) were obtained from Calbiochem (San Diego, CA). Protein bands were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences, Inc., Boston MA, or Pierce, Rockford, IL) and quantitated with a Kodak Image Station and 1D Image Analysis software.

Statistical Analysis

Results were analyzed by 1- or 2-way analysis of variance (ANOVA), followed by Fisher's post-hoc least significant difference test. Results are expressed as mean ± SEM. Differences were considered to be significant at $P < 0.05$.

RESULTS

Collagen Type I Selectively Stimulates Shedding of the DDR1 Ectodomain in a Time- and Concentration-Dependent Fashion

Serum-deprived A431 cells were incubated in serum-free DMEM containing 10 μg/ml type I collagen for varying periods of time. The conditioned medium was collected and assayed by immunoblotting with antibodies raised against the N-terminus of DDR1. A protein with an estimated molecular mass of ~60 kDa was detected in the medium of collagen-treated cultures at 6 and 24 h, but not at earlier time-points (Fig. 1A). Full-length DDR1b has an estimated molecular mass of ~100 kDa, and an apparent mass of approximately 120 kDa on SDS gels [Vogel et al., 1997]. A protein of ~120 kDa was detected in A431 cell lysates

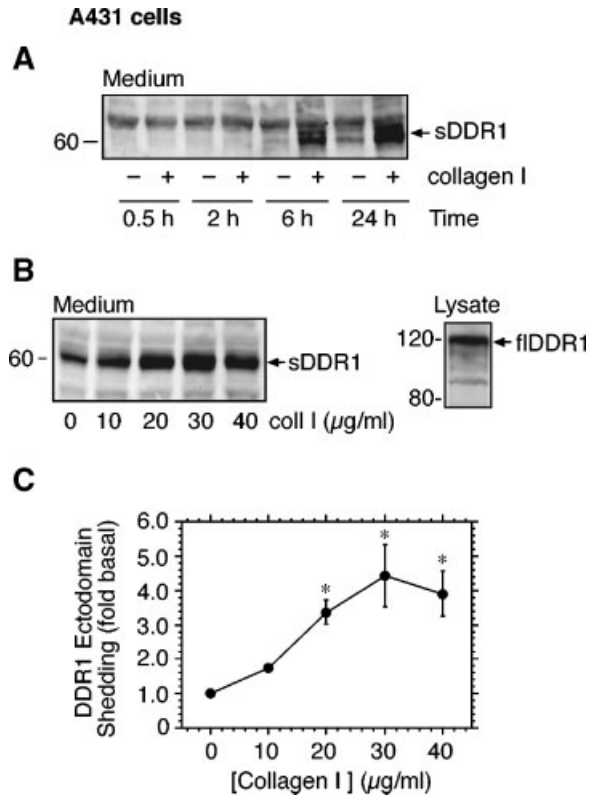

Fig. 1. Collagen type I activates shedding of the DDR1 ectodomain in A431 cells. **A**: Serum-deprived A431 cells were treated with collagen type I (10 μg/ml) or a vehicle control in DMEM. Conditioned media were analyzed for the presence of the soluble DDR1 ectodomain (sDDR1) by immunoblotting with antibodies to the DDR1 N-terminus. **B**: Serum-deprived A431 cells were treated for 24 h with a range of concentrations of collagen type I in DMEM. **Left panel**: conditioned medium sDDR1 content. **Right panel**: full-length DDR1 in cell lysates. **C**: Results from 3 to 6 experiments were normalized to basal release and expressed as mean ± SEM. *Significantly different from control values.

using an antibody against the DDR1 C-terminus (Fig. 1B, right panel). The results suggest that, in the presence of collagen type I, and after a delay of several hours, an N-terminal fragment of DDR1b is shed from A431 cells.

The release of the soluble DDR1 ectodomain (sDDR1) evoked by collagen type I was concentration-dependent, and saturable, with a maximal increase observed at 30 μg/ml (Fig. 1B, left panel, and C). To assess the specificity of collagen's effect on DDR1, ectodomain shedding of three additional transmembrane proteins, APP, the epidermal growth factor receptor family member ErbB2, and E-cadherin, was examined in A431 cells exposed to collagen type I (Fig. 2). The secreted ectodomain of APP, termed sAPPα, accumulated over time in the medium of A431 cells (Fig. 2A, upper panel). However, in contrast to DDR1, APP ectodomain shedding was not affected by the presence of collagen type I in the medium, even at concentrations that caused maximal release of the DDR1 ectodomain (cf. Fig. 2B, upper panel, and Fig. 1). Shedding of ErbB2 and E-cadherin was similarly unaffected by collagen (Fig. 2A,B, middle and lower panels).

Shedding of the DDR1 Ectodomain Coincides With the Appearance of Tyrosine Phosphorylated CTFs

To determine if collagen-evoked DDR1 shedding occurs in other cell lines, experiments were next carried out using the breast carcinoma cell line T-47D. The addition of collagen type I (10–40 μg/ml) to the medium caused a concentration-dependent increase in sDDR1 release that approached maximum levels at a concentration of 40 μg/ml (Fig. 3A). Collagen did not affect the release from these cells of either sAPPα or the E-cadherin ectodomain (not shown). In agreement with an earlier report [Vogel, 2002], collagen also induced the appearance in cell lysates of a ~60 kDa fragment that was detected by antibodies to the DDR1 C-terminal (Fig. 3B). This band often appeared as a doublet. Full-length DDR1 and the CTFs were tyrosine phosphorylated 24 h after the addition of type I collagen to the medium (Fig. 3B). Levels of tyrosine phosphorylated CTFs (Fig. 3B, right panel) increased in a concentration-dependent fashion in the presence of collagen type I, as did total CTFs (not shown). Whereas full-length DDR1 became tyrosine phosphorylated within 1 h following exposure to collagen type I, the appearance of phosphorylated CTFs in DDR1 immunoprecipitates was significantly delayed, and coincided with the appearance of the ectodomain fragment in the medium (Fig. 3C). To confirm the identity of the phosphorylated bands, anti-phosphotyrosine immunoprecipitates were prepared from control and collagen-treated cultures, and immunoblotted with antibodies to the DDR1 C-terminus (anti-DDR1-CT). DDR1-immunoreactive bands corresponding in size to full-length DDR1 and a ~60 kDa CTF were detected in anti-phosphotyrosine immunoprecipitates derived from collagen-treated cultures (Fig. 3D). Thus, exposure to collagen type I results in the delayed formation of tyrosine phosphorylated DDR1 CTFs coincident with

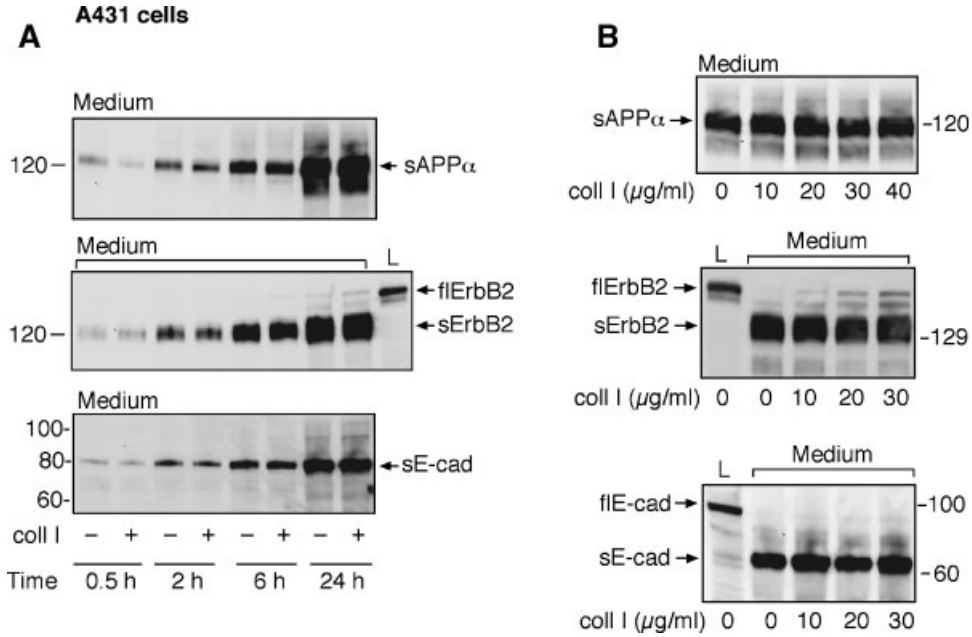

Fig. 2. Collagen type I does not affect constitutive ectodomain shedding of other transmembrane proteins. A431 cells were treated for various time periods with 10 μg/ml collagen type I (**A**), or for 24 h with a range of concentrations of collagen type I (**B**). Conditioned media were analyzed by immunoblotting with antibodies to the soluble ectodomains of APP (sAPPα, **upper panels**), ErbB2 (sErbB2, **middle panels**), or E-cadherin (sE-cad, **lower panels**). Full-length ErbB2 (flErbB2) and full-length E-cadherin (flE-cad) in cell lysates (L) are also indicated. Numbers to the left or right of the blots indicate approximate positions of molecular mass markers in kDa.

shedding of the ectodomain, and over the same concentration range.

Induction of Tyrosine Phosphorylation and Ectodomain Shedding of DDR1 by Collagen Type I Is Partly Dependent on Src

Others have shown that collagen-evoked DDR1 phosphorylation can be blocked either by abolishing the autocatalytic activity of DDR1 via mutation of the kinase domain [Vogel et al., 2000], or by treating cells with a pharmacological Src inhibitor [Dejmek et al., 2003]. T-47D cells were treated with DMEM containing collagen type I or vehicle for 24 h in the presence of PP1 (10 μM) or DMSO. PP1 reduced collagen-evoked tyrosine phosphorylation of full-length DDR1 and its CTFs, and shedding of the ectodomain, by approximately 80% (Fig. 4A,B). The abundance of full-length DDR1 (total) was slightly reduced, to $80 \pm 15\%$ of control in the presence of collagen, and was further reduced to $51 \pm 11\%$ of control in the combined presence of collagen I and PP1 (mean ± SEM, $n = 4$)(Fig. 4C, left panel). Generation of the major DDR1 CTF in the presence of collagen was slightly depressed by PP1, although the effect was not statistically significant (Fig. 4C, both panels). Similar results were obtained when these experiments were repeated in A431 cells (not shown).

To further test the role of Src in DDR1 activation, HEK 293 cells, which do not express endogenous DDR1, were transiently co-transfected with DDR1b and a cDNA construct encoding either wild-type Src (Srcwt), dominant negative Src (SrcRF), or the corresponding empty vector. The release of sDDR1b by collagen type I was reduced in cells co-expressing SrcRF, but not in cells co-expressing Srcwt (Fig. 5A, upper panel). This effect of SrcRF was statistically significant, and averaged approximately 50% in three experiments (Fig. 5B). Levels of full-length DDR1 in the absence of collagen were similar in all groups (Fig. 5A, middle panel). The two Src constructs were expressed at comparable levels in transfected cells (Fig. 5A, lower panel). Overexpression of Srcwt increased tyrosine phosphorylation of full-length DDR1, and of a major DDR1 CTF, even in the absence of collagen (Fig. 5C). In cells co-expressing SrcRF, tyrosine phosphorylation of full-length DDR1 by collagen type I was

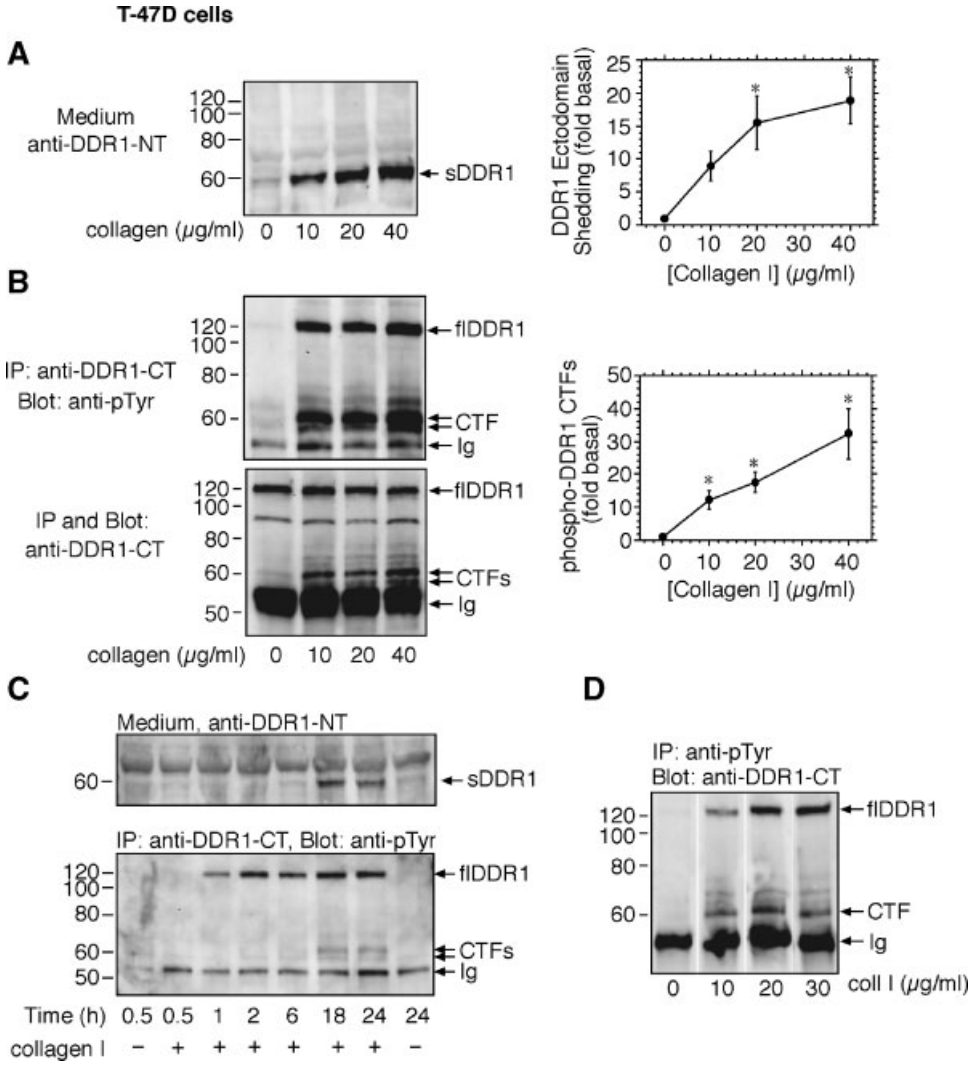

Fig. 3. DDR1 shedding evoked by collagen type I coincides with the formation of tyrosine phosphorylated CTFs. **A**: Serum-deprived T-47D cells were incubated for 24 h with different concentrations of collagen type I in DMEM. **Left panel**: Conditioned medium was analyzed for sDDR1 content using antibodies to the DDR1 N-terminal (anti-DDR1-NT). **Right panel**: Normalized values were expressed as mean ± SEM from five experiments. *Significantly different from control values. **B**: **Left panel**: Immunoprecipitates were prepared using antibodies to the DDR1 C-terminal (anti-DDR1-CT), and analyzed by immunoblotting with anti-phosphotyrosine antibodies (anti-pTyr) (**upper panel**), or with anti-DDR1-CT (**lower panel**). **Right panel**: Levels of phosphorylated CTFs were normalized, and expressed as mean ± SEM from five experiments. *Significantly different from control values. **C**: Cells were treated for various time periods with 20 μg/ml collagen type I, and analyzed for sDDR1 in the medium (**upper panel**), or for tyrosine phosphorylation in anti-DDR1-CT immunoprecipitates (**lower panel**). **D**: Cells were treated with various concentrations of collage type I for 24 h. Immunoprecipitates were prepared with anti-pTyr, and immunoblotted with anti-DDR1-CT.

significantly reduced to 62 ± 3% (mean ± SEM, n = 3) of that observed in vector-transfected cells (Fig. 5C).

Induction of DDR1 Shedding by Collagen Type I Is Blocked by TAPI-1 and TIMP-3

Ectodomain shedding of many transmembrane proteins is mediated by zinc-dependent metalloproteinases of the ADAM family [Arribas and Borroto, 2002]. Accordingly, T-47D cells were next treated with collagen type I in the presence of varying concentrations of the zinc metalloproteinase inhibitor TAPI-1. Controls received DMSO, adjusted so that all groups received the same final concentration. TAPI-1 caused a concentration-dependent inhibition of collagen evoked sDDR1 release (Fig. 6A). Curve-fit analysis yielded an estimated IC_{50} for TAPI-1 of 3.4 μM (Fig. 6A, lower panel). TAPI-1 did not significantly reduce tyrosine

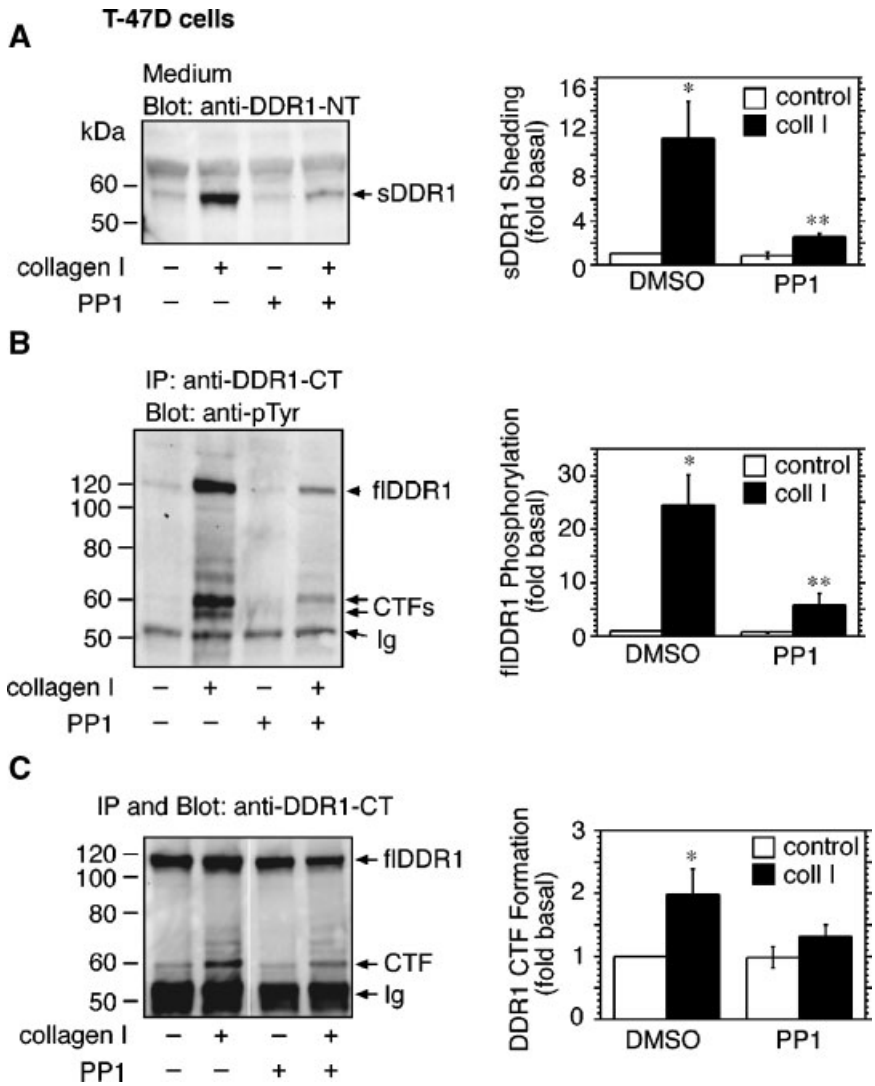

Fig. 4. The Src inhibitor PP1 reduces collagen-dependent DDR1 tyrosine phosphorylation and ectodomain shedding. Serum-deprived T-47D cells were incubated for 18 h with DMEM containing 20 μg/ml type I collagen or vehicle in the presence of PP1 (10 μM) or DMSO. **A**: Conditioned medium was analyzed for sDDR1 content. **B**: Immunoprecipitates were prepared using anti-DDR1-CT, and analyzed by immunoblotting with anti-pTyr. **C**: Cell lysates were subjected to successive immunoprecipitation and immunoblot analysis using anti-DDR1-CT. Results were quantitated, normalized to basal release and expressed as mean ± SEM from four experiments (**right panels**). *Significantly different from corresponding control group; **Significantly different from collagen-treated cultures.

phosphorylation of full-length DDR1, or of DDR1 CTFs (Fig. 6B, upper panel). Notably, total levels of DDR1 CTFs were also unaffected by the inhibitor (Fig. 6B, lower panel), even at a concentration (30 μM) that reduced DDR1 ectodomain shedding by 80% (cf. Fig. 6A).

We next examined the sensitivity of DDR1b shedding to tissue inhibitors of metalloproteinases (TIMPs), HEK cells were transiently transfected with a cDNA vector encoding DDR1b, together with a construct encoding either TIMP-1, TIMP-2, TIMP-3, or the corresponding empty vector. The addition of collagen type I to the medium caused the release of a large N-terminal fragment of DDR1b into the medium (Fig. 7A). Shedding was significantly inhibited by co-expression of TIMP-3, but was largely unaffected by expression of TIMP-1 or TIMP-2 (Fig. 7A, upper panel, and B). Levels of full-length DDR1 were not changed by co-expression of any of the TIMPs (Fig. 7A, lower panel). Despite causing a reduction in shedding, TIMP-3 did not alter tyrosine phosphorylation of full-length DDR1, or the DDR1 CTF, in the presence of collagen (Fig. 7C). TIMPs are secreted proteins [Baker et al.,

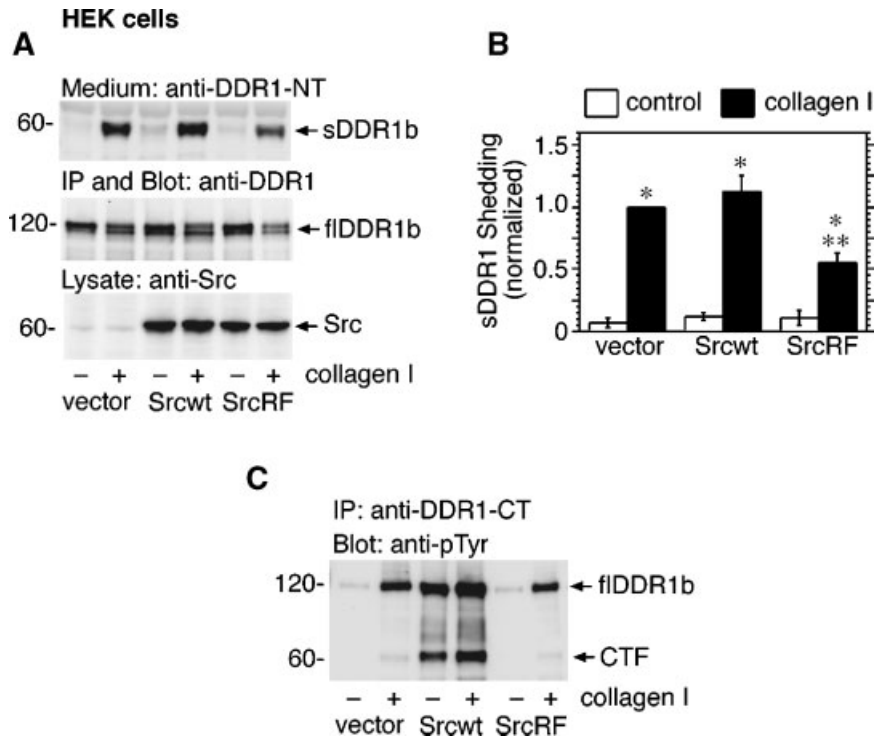

Fig. 5. A dominant negative Src mutant inhibits DDR1 ectodomain shedding. HEK cells were transiently transfected with DDR1b together with wild type Src (Srcwt), dominant negative Src (SrcRF) or an empty vector. The cells were incubated for 18 h in DMEM with collagen type I (40 μg/ml) or vehicle. **A**: Conditioned medium was collected and analyzed for sDDR1 content (**upper panel**). Immunoprecipitates were prepared using anti-DDR1-CT, and analyzed by immunoblotting with anti-DDR1-CT (**middle panel**). Lysates were analyzed for expression of Src (**lower panel**). **B**: sDDR1 release was quantitated and results from three experiments were normalized and expressed as mean ± SEM. *Significantly different from control cultures. **Significantly different from collagen-treated vector transfectants. **C**: Immunoprecipitates were prepared using anti-DDR1-CT antibodies, and analyzed by immunoblotting with anti-phosphotyrosine antibodies.

2002], and, as expected, all of the TIMPs expressed in HEK cells were released into the medium of the corresponding transfected cultures (Fig. 7D).

Inhibition of γ-Secretase Activity Did Not Inhibit DDR1 Ectodomain Shedding or CTF Generation Induced by Collagen

A number of transmembrane proteins that undergo ectodomain shedding are also substrates for γ-secretase [Ebinu and Yankner, 2002]. In order to determine if the DDR1 CTF is cleaved by γ-secretase, or alternatively, if it is generated by γ-secretase-mediated cleavage of a larger precursor, T-47D cells were treated with collagen type I or vehicle in the presence of DMSO or L-685,458, a specific γ-secretase inhibitor [Shearman et al., 2000]. The inhibitor did not affect DDR1 ectodomain shedding, or CTF generation, in cells treated with collagen type I (Fig. 8A). In contrast, levels of the CTF derived from constitutive α-secretory cleavage of APP, a known substrate of γ-secretase [Ebinu and Yankner, 2002], were consistently increased by at least four-fold in the presence of L-685,458 (Fig. 8B), indicating that γ-secretase activity in T-47D cells was effectively inhibited by this compound.

DISCUSSION

Our results show that collagen type I promotes cleavage of DDR1, and release of its ectodomain, via a mechanism that is dependent on zinc-dependant metalloproteinases and Src. The effect of collagen is specific, in that shedding of other transmembrane proteins, including APP, ErbB2, and E-cadherin, was not affected by the addition of collagen to the medium. An early study reported that DDR1 (also known as mammary carcinoma kinase-10, or MCK-10) is constitutively cleaved within its extracellular domain to form a membrane-anchored kinase domain and a soluble ectodomain fragment

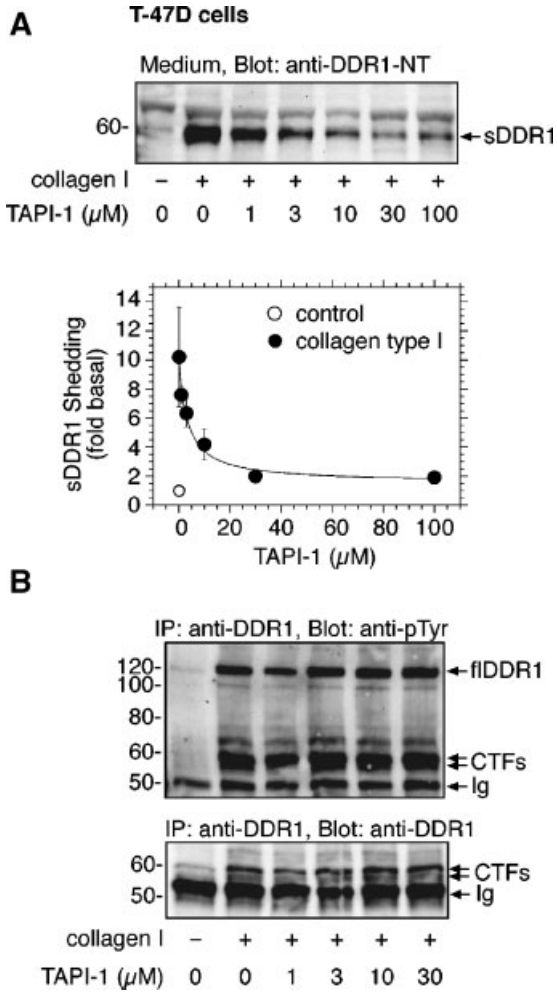

Fig. 6. TAPI-1 inhibits collagen-dependent ectodomain shedding of DDR1, but not generation of CTFs. Serum-deprived T-47D cells were incubated for 18 h in DMEM containing vehicle or collagen type I (20 μg/ml) in the presence of increasing concentrations of TAPI-1. Controls received DMSO. **A**: Conditioned medium was collected and analyzed for sDDR1 content (**upper panel**). Results from three experiments were normalized to basal release and expressed as mean ± SEM (**lower panel**). Open symbol, control; closed symbols, collagen type I. **B**: Immunoprecipitates were prepared using anti-DDR1-CT, and analyzed by immunoblotting with anti-pTyr (**upper panel**) or anti-DDR1-CT (**lower panel**).

[Alves et al., 1995]. Subsequently, it was discovered that exposure to collagen type I results in the generation of a 62 kDa cell-associated CTF of DDR1. The size of the fragment suggested that the cleavage site was close to the plasma membrane, within the extracellular domain [Vogel, 2002], a prediction borne out by the present study, which documents the appearance of an N-terminal fragment of DDR1 in the medium of collagen-treated cells.

Collagen-evoked tyrosine phosphorylation of full-length DDR1 was evident within 60 min in T-47D cells (Fig. 3C), and lasted for at least 24 h, consistent with earlier studies [Shrivastava et al., 1997; Vogel et al., 1997]. Levels of tyrosine phosphorylated DDR1 CTFs, and ectodomain release, both increased in a concentration-dependent fashion following prolonged exposure to collagen type I, although the DDR1 fragments were not detected until hours after phosphorylation of full-length DDR1 was first apparent (Fig. 3C). The delay in DDR1 proteolysis suggests that synthesis of a proteinase might be necessary for DDR1 cleavage to occur, and indeed, the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 is increased by DDR1 [Hou et al., 2002]. However, given the range of substrates recognized by the metalloproteinases implicated in shedding [Arribas and Borroto, 2002], merely increasing expression of such a proteinase would be expected to cause a global increase in shedding of multiple proteins, which was not the case here. Alternatively, tyrosine phosphorylation of DDR1, or binding to its ligand collagen, or both, might increase its suitability as a protease substrate, perhaps by inducing a conformation change that exposes the cleavage site, but this would not explain the observed delay in proteolysis.

DDR1 autophosphorylates in response to collagen binding [Vogel et al., 2000]. The Src inhibitor PP1 nearly abolished tyrosine phosphorylation of DDR1 evoked by collagen [Dejmek et al., 2003]; an observation confirmed by the present study. Moreover, PP1 almost completely blocked collagen-induced ectodomain shedding in both cell lines tested (Fig. 4 and not shown). Overexpression of a dominant negative Src mutant protein in HEK cells caused a reduction of approximately 50% in shedding of co-transfected DDR1b (Fig. 5A), and reduced collagen-evoked tyrosine phosphorylation of full-length DDR1 to 62 ± 3% (mean ± SEM, $n = 3$) of that observed in vector-transfected control cells (Fig. 5C). The relatively larger inhibitory effects of PP1 compared to SrcRF might reflect direct inhibition of the intrinsic kinase activity of DDR1, since PP1 has been shown to affect a number of kinases in addition to Src [Bain et al., 2003; Tatton et al., 2003]. Nevertheless, when considered in conjunction with the effect of dominant negative Src, the results suggest that autophosphorylation of DDR1 and activation of a Src-family

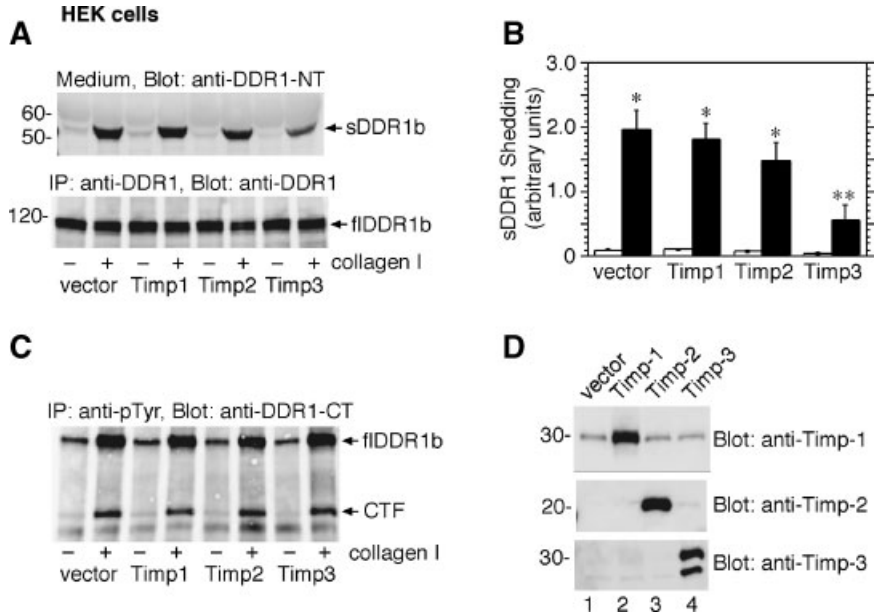

Fig. 7. TIMP-3 inhibits collagen-dependent ectodomain shedding of DDR1b. HEK cells were transiently transfected with DDR1b together with TIMP-1, TIMP-2, TIMP-3, or an empty vector. The cells were incubated for 18 h in DMEM with collagen type I (40 μg/ml) or vehicle. **A**: Conditioned medium was collected and analyzed for sDDR1 content (**upper panel**). Immunoprecipitates were prepared using anti-DDR1-CT, and analyzed by immunoblotting with anti-DDR1-CT (**lower panel**). **B**: Results from three experiments are expressed as mean ± SEM. *Significantly different from control cultures. **Significantly different from collagen-treated vector transfectants. **C**: Immunoprecipitates were prepared using anti-phosphotyrosine antibodies, and analyzed by immunoblotting with anti-DDR1-CT. **D**: Conditioned medium was analyzed for TIMP levels by immunoblotting.

kinase are both required for maximal phosphorylation and cleavage of DDR1 in response to collagen. Since collagen binds directly to DDR1, it seems reasonable to propose that Src is recruited to autophosphorylated DDR1 following activation of the latter by collagen, and phosphorylates additional sites. The cytoplasmic domain of DDR1b contains a number of proline-rich regions exhibiting the core sequence required for binding to SH3 domains (PXXP) [Mayer, 2001], and one binding site for the Src SH3 domain (RXXPXXP) [Feng et al., 1994]. Indeed, in HEK cells co-transfected with DDR1b and Srcwt, Src was found to co-immunoprecipitate with DDR1b even in the absence of collagen (not shown), although we were unable to demonstrate co-precipitation of endogenous DDR1 and Src from T-47D cell lysates. Overexpression of Srcwt also resulted in tyrosine phosphorylation of DDR1b even in the absence of collagen, suggesting that DDR1b is a substrate for Src kinase (Fig. 5C). However, Srcwt overexpression alone did not increase DDR1 shedding (Fig. 5A). This might indicate that autophosphorylation of DDR1 in response to collagen precedes Src activation, and is necessary for shedding of the ectodomain.

The generation of a DDR1 CTF induced by collagen was reported to be reduced by the furin inhibitor decanoyl-RVKR-chloromethylketone, and completely blocked by batimastat, a hydroxamate-based inhibitor of zinc-dependent metalloproteinases [Vogel, 2002]. TAPI-1, another inhibitor of zinc-dependent metalloproteinases, caused a dose-dependent inhibition of DDR1 ectodomain shedding with an apparent IC_{50} of 3.4 μM (Fig. 6). This was similar to the concentration of TAPI-1 previously found to inhibit receptor-coupled release of the APP ectodomain [Slack et al., 2001]. DDR1b shedding was also blocked by TIMP-3, but not TIMP-1 or TIMP-2 (Fig. 7). Both the matrix metalloproteinases (MMPs), and ADAM proteinases, are sensitive to hydroxamate-based inhibitors [Black and White, 1998], and to TIMPs [Baker et al., 2002], and both may be activated by removal of an inhibitory prodomain by furin-like proprotein convertases [Milla et al., 1999; Kang et al., 2002]. However, the majority of proteins that undergo regulated

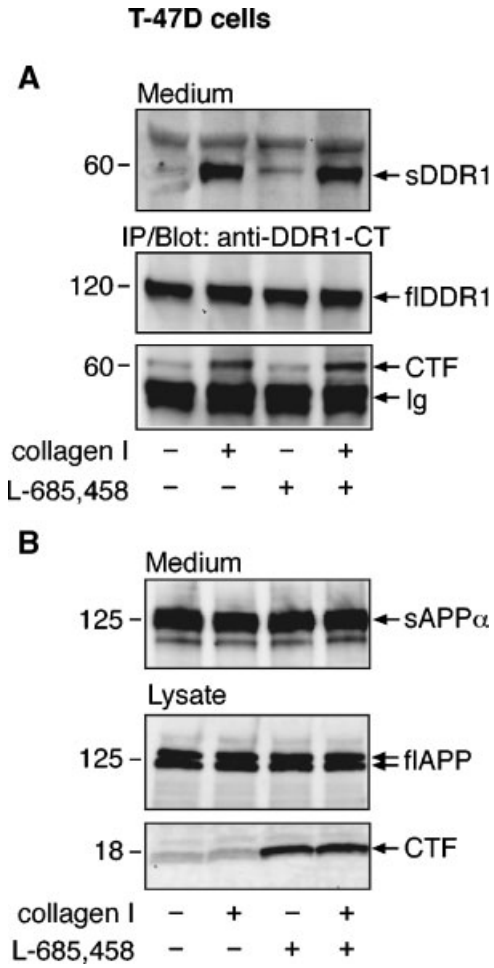

Fig. 8. Collagen-dependent generation of DDR1 CTFs is not mediated by γ-secretase. Serum-deprived T-47D cells were incubated for 24 h in DMEM containing vehicle or collagen type I (20 μg/ml) in the presence of DMSO or L-685,458 (10 μM). **A**: Conditioned medium was analyzed for sDDR1 content (**upper panel**). Immunoprecipitates were prepared using anti-DDR1-CT, and analyzed by immunoblotting with anti-DDR1-CT (**middle** and **lower panels**). **B**: Conditioned medium was analyzed for sAPPα content (**upper panel**). Lysates were probed with antibodies to the APP C-terminal. Arrows indicate full-length (fl) APP (**middle panel**) and its ~9 kDa CTF (**lower panel**). Approximate positions of molecular mass markers (in kDa) are indicated on the left.

shedding appear to be cleaved by TACE [Arribas and Borroto, 2002]. TIMP-3 inhibits both ADAM10 and TACE, whereas TIMP-1 inhibits ADAM10 but not TACE [Amour et al., 1998, 2000]. Both these ADAMs are expressed in HEK cells [Slack et al., 2001], and our results could suggest that collagen-dependent DDR1 shedding is mediated either by TACE alone, which would account for the resistance of the response to TIMP-1, or by both TACE and ADAM10. The results, however, do not rule out the possible involvement of other metalloproteinases in the response, including, for example, ADAM12, or ADAM19, both of which may act as sheddases [Arribas and Borroto, 2002] and are inhibited by TIMP-3 [Baker et al., 2002].

Strikingly, although TAPI-1 effectively inhibited release of the DDR1 ectodomain, it had no effect on the generation of DDR1 CTFs by collagen in T-47D cells (Fig. 6). Similarly, TIMP-3 reduced DDR1 shedding, but not levels of the phosphorylated CTF, in collagen-treated DDR1 transfectants (Fig. 7). Neither formation, nor degradation, of DDR1 CTFs appeared to be dependent on γ-secretase activity, as indicated by the failure of the inhibitor L-685,458 to alter CTF levels (Fig. 8). That DDR1 CTF formation was reportedly blocked by the broad-spectrum metalloproteinase inhibitor batimastat [Vogel, 2002], but not by TAPI-1, could indicate that the generation of CTFs in response to collagen type I may be mediated by a metalloproteinase activity that is distinct from that involved in shedding.

DDR1 promotes cell adhesion and migration on collagen, and modulates cell proliferation [Kamohara et al., 2001; Vogel et al., 2001; Franco et al., 2002; Dejmek et al., 2003]. However, the physiological significance of collagen-evoked DDR1 ectodomain shedding is unknown. Release of the ectodomain could abrogate DDR1-dependent effects of collagen, or it might subserve a paracrine or autocrine function, as is the case for membrane-anchored growth factors [Arribas and Borroto, 2002; Blobel, 2005]. Ectodomain shedding of some transmembrane proteins is followed by additional cleavages within the remaining CTF by a presenilin-containing complex known as γ-secretase. Successive cleavage of Notch, APP, and ErbB4 by ADAM proteases and γ-secretases results in the liberation of a CTF that translocates to the nucleus and activates transcription [Ebinu and Yankner, 2002]. However, the γ-secretase inhibitor L-685,458 did not affect collagen-dependent formation of DDR1 CTFs, indicating that DDR1 is not processed in the same way as these other transmembrane proteins.

The results suggest that ectodomain shedding of DDR1 may regulate cellular responses to collagen. Further work is needed to determine if the resultant cleavage products play a role in signaling.

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