Ligand-induced shedding of discoidin domain receptor 1

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Abstract Tyrosine kinases belonging to the discoidin domain receptor (DDR) family are activated upon stimulation with various types of collagen. In response to collagen treatment, immunoprecipitation of DDR1 with an antibody specific to the juxtamembrane region results in co-purification of a previously unrecognized tyrosine phosphorylated protein of 62 kDa molecular weight. Here, this protein is identified as C-terminal cleavage product of the full-length DDR1 receptor and a DDR1-specific shedding enzyme postulated. Shedding of DDR1 can be partially blocked by the furin inhibitor decanoyl-RVKR-chloromethylketone and completely inhibited by the hydroxamate-based inhibitor batimastat. The characteristic of the DDR1 sheddase to be blocked by batimastat suggests that it belongs to the membrane-bound matrix metalloproteinase or disintegrin and metalloproteinase family of proteases.

Key words: Tyrosine kinase; Shedding; Discoidin domain; Collagen; Processing

1. Introduction

Communication between cells amongst each other or between cells and the surrounding extracellular matrix is a highly regulated process. A great diversity of cell surface receptors perceive signals from the outside, transmit them through the plasma membrane and initiate downstream signaling cascades. Many cytokines and growth factors are synthesized as membrane-bound precursors and are released and activated upon proteolysis. In turn, ligand binding and signaling of many cell surface receptors is also regulated by proteolysis. A large number of enzymes have been shown to function as cell surface proteases, collectively termed sheddases, metzincins, secretases or convertases [1–3]. Sheddases are transmembrane metalloproteases with a Zn^{2+} ion mediating catalysis. They usually belong either to the family of membrane-type matrix metalloproteases (MT-MMP) or to the ADAM (a disintegrin and metalloprotease) family.

The diversity of sheddase activity is paralleled by the diversity of their substrates. Well characterized substrates for sheddases are cell adhesion receptors, such as VCAM-1, L-selectin, and syndecan, G protein-coupled receptors, such as the V_2 vasopressin receptor, or membrane-anchored precursors of receptor ligands, such as transforming growth factor-α (TGF-α), tumor necrosis factor-α (TNF-α), Kit ligand and Fas ligand. Much attention has recently also been given to the inappropriate shedding of the β-amyloid precursor protein thereby causing Alzheimer's disease [4]. Clearly, sheddases have a rather wide substrate specificity, which is exemplified by TACE (ADAM17) [5]. Originally, TACE was identified as TNF-α processing enzyme, but it has now been shown to also cleave colony-stimulating factor receptor, AAP, HER4, neuregulin, L-selectin, fractalkine and Notch [6–12]. The TACE-related protease ADAM10 has been implicated in shedding of the L1 adhesion molecule [13]. The shedding of cell surface receptors can be either constitutive or induced by antibodies and other stimuli [14]. Ectodomain shedding of receptor tyrosine kinases (RTK) is reported for the epidermal growth factor (EGF) receptor, HER2 receptor, platelet-derived growth factor receptor, the basic fibroblast growth factor receptor, the nerve growth factor receptor, insulin-like growth factor receptor and the hepatocyte growth factor receptor (c-Met) [15–17]. Several stimuli trigger the shedding of RTK, such as the activation of protein kinase C by phorbol esters. Ligand-induced shedding has been reported for the RTK TrkA upon stimulation with nerve growth factor [18]. Furthermore, the EGF receptor uses the Erk and p38 mitogen-activated protein kinase pathway to trigger ectodomain shedding of TGF-α, TNF-α and L-selectin [19]. Some of the sheddases are initially generated as precursors and are activated by intracellular serine endoproteases of the furin family [10]. Various compounds effectively inhibit sheddases, including Zn-chelating agents like EDTA. Most of the more specific sheddase inhibitors are hydroxamate derivatives and have been shown to block matrix metalloproteinases as well [20]. Several of these inhibitors have entered clinical trials due to potent anti-metastatic or anti-angiogenic properties.

Discoidin domain receptors 1 and 2 (DDR1 and DDR2) are a subfamily of the RTK class showing distinct structural and functional homologies. In their extracellular region both receptors have an N-terminal domain homologous to the *Dictyostelium discoideum* protein discoidin I [21]. A functional homology between DDR1 and DDR2 is given by the fact that both receptors are activated by collagen. Whereas DDR1 activation is achieved by type I to type VI and type VIII collagens, DDR2 is only activated by fibrillar collagens [22,23]. The 160 amino acid long discoidin domain is followed by an approx. 200 amino acid long stalk region. Whereas the

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Abbreviations: ADAM, a disintegrin and metalloproteinase domain-containing protein; DDR, discoidin domain receptors; EGF, epidermal growth factor; MT-MMP, membrane-bound matrix metalloproteinase; RTK, receptor tyrosine kinases; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor-α

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discoidin domain is essential for collagen binding of DDR1, both domains are necessary for receptor signaling [24]. Maximal DDR1 phosphorylation is reached after several hours in adherent cells, but much faster in suspension [23,25].

The cDNA encoding human DDR1 has been cloned from several tissues or carcinoma cells [26,27]. Expression of DDR1 is predominantly seen in epithelial cells, particularly from kidney, lung, gastrointestinal tract, and brain, but also in corneal and dermal fibroblasts [26,28–30]. Up-regulated DDR1 expression has been reported from breast, ovarian, esophagus and brain tumors [31–35].

Thus far, five isoforms of DDR1 (indicated with the affixes a–e) have been cloned as a result of alternative splicing [36]. Compared to the longest c-isoform, the b-isoform lacks six amino acids inserted in the kinase domain between exons 13 and 14 [26]. The a-, d- and e-isoforms arise through alternative splicing in the juxtamembrane region. The deletion of exon 11 encoding 37 amino acids gives rise to DDR1a, the deletion of exons 11 and 12 results in DDR1d. In DDR1e, the first half of exon 10 and all of exons 11 and 12 are missing [36]. Whereas DDR1a retains the reading frame and is therefore an active kinase, the coding sequence of DDR1d and DDR1e goes out of frame and renders both isoforms kinase-dead. Deletion of DDR1 in the mouse germ line resulted in viable animals, which are significantly smaller than their littermates [37]. Female DDR1-null mice show defects in blastocyst implantation and mammary gland development. Furthermore, in primary vascular smooth muscle cells cultivated from DDR1-null mice decreased proliferation, collagen attachment and migration have been observed [38].

Here, we provide evidence that activation of DDR1 led to enhanced activity of a cell surface protease, which cleaves DDR1 in a region proximal to the membrane. Due to the sensitivity towards batimastat, we propose that an ADAM- or MT-MMP-related metalloproteinase is responsible for DDR1 shedding.

2. Materials and methods

2.1. Cell lines, antibodies and other reagents

Human mammary carcinoma T-47D cells were obtained from American Type Tissue Culture Collection and cultivated under recommended conditions. The generation of mouse fibroblast NIH3T3 cells stably expressing DDR1b has been described earlier [26]. Rat type I collagen was purchased from Collaborative Biomedical Products (Bedford, MA, USA). Decanoyl-RVKR-chloromethylketone was from Bachem (Heidelberg, Germany) and batimastat from British Biotech. Polyclonal antibodies to DDR1 were raised against a peptide corresponding to amino acids 505–523 of DDR1b [23]. An antibody against the C-terminus of DDR1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA, amino acids 894–913). Monoclonal anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology (Lake Placid, NY, USA).

2.2. Immunoprecipitation and Western blot analysis

Semi-confluent T-47D cells were stimulated with 10 μg/ml collagen and lysed with 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl (pH 7.5), 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml aprotinin. The cellular lysates were centrifuged at 4°C and 13 000 rpm for 10 min. Equal amounts of protein were subjected to anti-DDR1 immunoprecipitation. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell) and immunoblotted with antibodies diluted 1:1000 (4G10) and 1:500 (DDR1) in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% gelatin overnight. Western blots were incubated with mouse or rabbit peroxidase-coupled secondary antibodies (Bio-Rad) and developed with enhanced chemiluminescence (Amersham). Before reprobing, the membrane was stripped in 70 mM Tris–HCl (pH 6.8), 2% SDS, 0.1% β-mercaptoethanol at 50°C for 15 min.

2.3. Scanning densitometry

Western blot results were quantified by scanning densitometry using the Quantity-One software (Bio-Rad, Munich, Germany).

3. Results

3.1. Copurification of a 62 kDa protein from DDR1 immunoprecipitation

In our previous work, we identified endogenous expression of DDR1 in many carcinoma cell lines, including the human mammary carcinoma cell line T-47D [26]. Overnight stimulation of T-47D cells with 10 μg/ml type I collagen resulted in sustained tyrosine phosphorylation of DDR1 [23]. In this study, we performed a time course experiment and stimulated T-47D cells with collagen for 20 min, 2 h and 18 h. Cell lysates were subjected to immunoprecipitation using an antibody directed against the juxtamembrane region of DDR1b, which is the isoform predominantly expressed in T-47D cells. Western blot analysis with an anti-phosphotyrosine antibody detected an increase in DDR1 phosphorylation concomitant with the duration of collagen stimulation (Fig. 1A). Additionally, three other tyrosine-phosphorylated bands of approximately 120, 105 and 62 kDa coprecipitated with DDR1. Whereas phosphorylation of the 120 and 105 kDa protein was already detected in untreated cells and did not chance upon collagen treatment, phosphorylation of the 62 kDa increased during the time period studied. The 62 kDa band was clearly distinct from the immunoglobulin heavy chain running at 50–58 kDa. Quantification of the Western blot by scanning

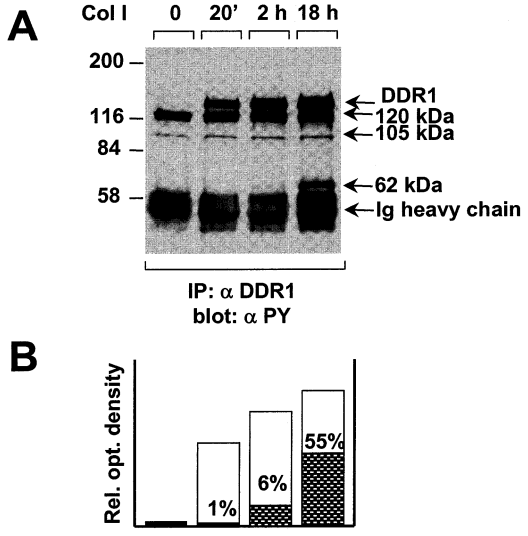

Fig. 1. Collagen-induced processing of DDR1 depends on tyrosine kinase autophosphorylation. A: Human mammary carcinoma T-47D cells were stimulated with collagen for various periods of time. Cell lysates were analyzed by anti-DDR1 immunoprecipitation followed by anti-phosphotyrosine Western blotting. A tyrosine-phosphorylated 62 kDa protein is detected after 2 and 18 h stimulation with collagen. Molecular weight markers are indicated. B: The intensity of DDR1 (open bars) and 62 kDa (checked bars) tyrosine phosphorylation is measured by densitometry. The relative amounts of phosphorylated 62 kDa protein compared to DDR1 are given in percent.

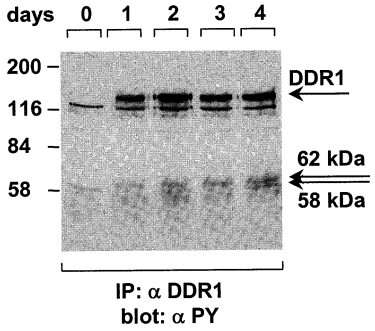

Fig. 2. Sustained presence of DDR1 β-subunit after collagen activation. T-47D cells were stimulated with collagen for up to 4 days. Cell lysates were analyzed by anti-DDR1 immunoprecipitation followed by anti-phosphotyrosine Western blotting. A continuous increase in phosphorylation of the 62 kDa protein is detected through the time period studied. Additionally, a 58 kDa protein is detected after 4 days of stimulation.

densitometry revealed that phosphorylation of the 62 kDa protein was significantly delayed compared to the phosphorylation of DDR1 (Fig. 1B).

3.2. Sustained phosphorylation of 62 kDa protein

Next, we extended the time course of DDR1 activation for several days. T-47D cells were stimulated with collagen and left without further treatment for up to 4 days. Immunoprecipitation and Western blot analysis indicated that DDR1 remains tyrosine-phosphorylated for up to 4 days upon collagen activation (Fig. 2). Again, two proteins with molecular weights of 105 and 120 kDa coprecipitated with unstimulated DDR1 and remained unchanged upon collagen activation. In contrast, phosphorylation of the 62 kDa protein was not seen in unstimulated cells and only appeared upon collagen treatment. At day 4, an additional band of 58 kDa molecular weight was visible. Upon longer exposure of this Western blot, the intensities of phosphorylation for DDR1b and the 62 kDa protein were equally strong compared to the signals seen in Fig. 1 (data not shown).

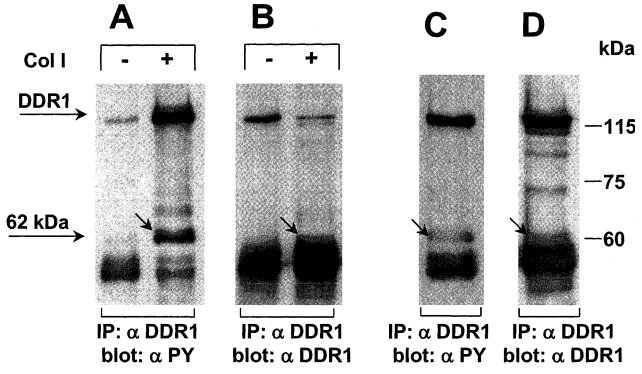

Fig. 3. Processing of DDR1 in response to collagen stimulation. T-47D cells (A,B) and NIH3T3 DDR1b cells (C,D) were stimulated with collagen overnight. Cells lysates were subjected to immunoprecipitation with a DDR1-specific antibody. Bound material was analyzed by Western blotting using an antibody directed against phosphotyrosine (A,C) and reprobed with anti-DDR1 antibody (B,D). The 62 kDa protein (indicated by arrows) is identified as a cleavage product of DDR1 (β-subunit).

3.3. The 62 kDa protein is a C-terminal fragment of DDR1

In order to identify the 62 kDa protein, we reasoned that it could be either a substrate of DDR1 that is tyrosine-phosphorylated upon receptor activation and coprecipitates with the receptor, or it could be a cleavage product of the receptor itself. To test the latter possibility, we treated T-47D cells with collagen overnight. The lysates were subjected to immunoprecipitation with a DDR1-specific antibody as before. Now, however, we not only analyzed the immunoprecipitates by anti-phosphotyrosine Western blotting, but used an antibody specific for the C-terminus of DDR1 (Fig. 3A,B). Indeed, the tyrosine-phosphorylated 62 kDa protein was recognized by the anti-DDR1 antibody, indicating that DDR1 is proteolytically processed in response to ligand activation. Because the antisera for immunoprecipitation and Western blotting were both from the same species in this experiment, the 62 kDa band is detected by the anti-DDR1 antibody with somewhat weaker intensity compared to the anti-phosphotyrosine Western blot. However, the 62 kDa protein was always clearly separated from the immunoglobulin heavy chain (arrow in Fig. 3B).

To demonstrate that DDR1 shedding is not restricted to T-47D cells, we used mouse fibroblast NIH3T3 cells, which had been previously transfected with the full-length DDR1b cDNA [26]. Cells were stimulated with collagen and lysates subjected to anti-DDR1 immunoprecipitation followed by anti-phosphotyrosine Western blotting (Fig. 3C). We detected the 125 kDa DDR1 precursor protein together with the 62 kDa protein also in NIH3T3 cells. Upon reprobing the blot with the anti-DDR1 antibody, we confirmed the identity of the 62 kDa protein as a C-terminal cleavage fragment of DDR1 (Fig. 3D).

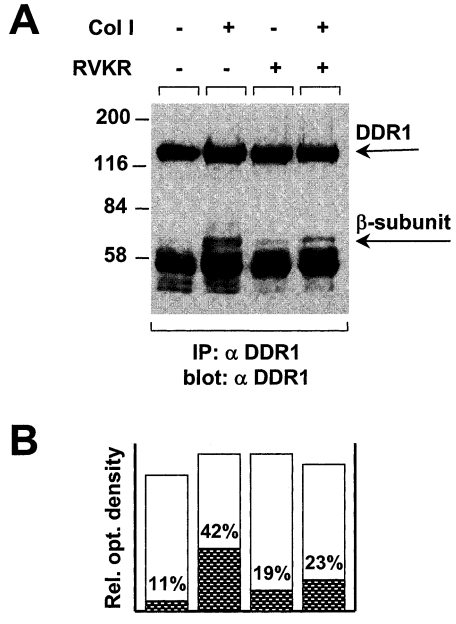

Fig. 4. Furin inhibitor reduces shedding of DDR1. A: T-47D cells were stimulated overnight with collagen in the presence or absence of decanoyl-RVKR-chloromethylketone. Cell lysates were analyzed by anti-DDR1 immunoprecipitation followed by anti-DDR1 Western blotting. A reduction in ligand-induced DDR1 shedding is observed in the presence of the furin inhibitor. B: The intensities of the DDR1 (open bars) and β-subunit (checked bars) signal are measured by densitometry. The percentage of β-subunit compared to unprocessed DDR1 is given.

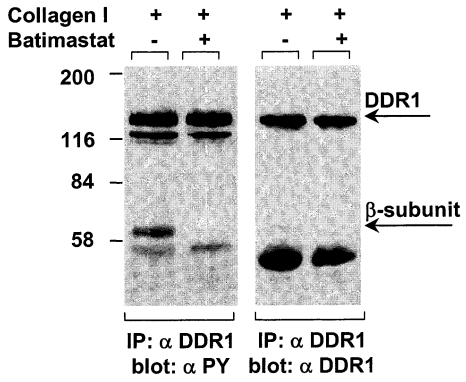

Fig. 5. Batimastat completely blocks DDR1 shedding. T-47D cells were stimulated with collagen in the presence or absence of batimastat. Cell lysates were subjected to anti-DDR1 immunoprecipitation and analyzed by anti-phosphotyrosine Western blotting followed by anti-DDR1 Western blotting. Note the complete absence of DDR1 shedding in the presence of batimastat.

3.4. DDR1 is shed by a furin-like activity

Shedding of a DDR-related RTK, the c-Met receptor, was shown to be achieved by furin cleavage [39]. We therefore wanted to test if DDR1 is also processed by furins. T-47D cells were treated with collagen overnight in the presence or absence of the furin inhibitor decanoyl-RVKR-chloromethylketone at 100 μM concentration. Immunoprecipitation of DDR1 showed that the sheddase activity is partially blocked by the inhibitor (Fig. 4). However, only about 50% reduction of ligand-induced shedding was observed, whereas the inhibitor enhanced the constitutive level of processing seen in unstimulated cells.

3.5. Complete inhibition of DDR1 shedding by batimastat

Since DDR1 processing is only partially inhibited by decanoyl-RVKR-chloromethylketone, we tested batimastat, a hydroxamate-derived inhibitor specific for metzincins. T-47D cells were stimulated with collagen overnight in the absence or presence of 10 μg/ml batimastat. DDR1 phosphorylation was detected by Western blot analysis and expression of DDR1 by reprobing with a receptor-specific antibody (Fig. 5). In the presence of batimastat, DDR1 processing was completely abolished which strongly indicates that ligand-induced DDR1 processing is mediated by a sheddase. Again, due to the conditions used for Western blotting the detection of the 62 kDa protein with the antibody against phosphotyrosine resulted in much stronger signals than with the anti-DDR1 antibody.

4. Discussion

Ectodomain shedding provides crucial mechanisms regulating the signaling capacity of cell surface receptors. On one hand, ligands can be synthesized as transmembrane proforms, inactively sequestered in the tissue and converted to an active form only by shedding. On the other hand, cleaving the ligand binding domain can terminate receptor signaling. Here we found that DDR1 is expressed in mammary carcinoma cells as a 125 kDa full-length receptor and, upon collagen ligand engagement, is tyrosine-phosphorylated and proteolytically processed. We showed that the kinetic of DDR1 cleavage was even more protracted than the process of activation. Whereas collagen stimulation for 2 h resulted in more than 80% maximal DDR1 tyrosine phosphorylation, just 6% of the full-length receptor underwent shedding. Only after 18 h of collagen stimulation was 55% of the receptor processed into a 62 kDa membrane-anchored β-subunit (Fig. 1B). Collagen treatment for 4 days resulted in a further increase in processing. A 58 kDa protein was detected together with the 62 kDa β-subunit suggesting that DDR1 shedding was a stepwise process and sequentially happened at two distinct sites. Alternatively, the 62 kDa protein was converted into the 58 kDa protein by intracellular modification. Taking the molecular size of the β-subunit into account, we calculated that processing of DDR1 took place proximal to the membrane. Membrane-proximal shedding involving residues 10–15 amino acids distal from the transmembrane region has been reported for TNF-α, L-selectin, interleukin-6 receptor and fractalkine [1,9]. However, swapping experiments between different receptors indicated that sheddases have a relaxed specificity for their cleavage sites [7]. As DDR1 cleavage is inhibited by batimastat, an enzyme of the family of MT-MMP or ADAM is most likely involved in DDR1 shedding. Experiments are under way addressing the nature of the shedding enzyme by using cells from knockout mice or inhibitors selective for individual sheddases.

Interestingly, human DDR1 displays the sequence RFRR (amino acids 304–307) in the stalk region, a sequence complying with the consensus site for furin endoproteases. However, the data presented here suggest that this site is not involved in ligand-induced DDR1 shedding. First, furin-like endopeptidases are localized in the *trans*-Golgi network and act during the maturation of membrane-anchored proteins, whereas DDR1 processing takes place in the extracellular space after contacting collagen. Secondly, point mutations switching one or several of the arginines in the recognition sequence to alanine did not influence DDR1 shedding (data not shown). Lastly, while DDR1 sequences from other species have a highly related overall sequence, the RFRR motif is not conserved. Between the DDR1 sequences from human and *Caenorhabditis elegans*, only phenylalanine is conserved in the recognition motif [40]. Although the furin inhibitor decanoyl-RVKR-chloromethylketone partially blocks DDR1 shedding, it may not act on the DDR1 sheddase directly but inhibits the maturation of the ADAM or MT-MMP family member that acts as a DDR1 sheddase. This hypothesis is supported by the observation that the activation of MT5-MMP and ADAM-TS12 takes places by furin-mediated prodomain cleavage and is blocked by decanoyl-RVKR-chloromethylketone [41,42]. So far, we are unable to give a satisfactory explanation for the result showing that DDR1 shedding is also induced by decanoyl-RVKR-chloromethylketone in the absence of ligand.

Several possible mechanisms for the ligand-induced shedding of DDR1 can be proposed, suggesting either a direct or an indirect mode of action (Fig. 6). Three potential mechanisms are proposed here. (i) Collagen binding to the discoidin domain of DDR1 and receptor dimerization may induce changes in the conformation of the stalk region particular of the sequence close to the plasma membrane. These conformational changes could open up a sheddase recognition site. The sheddase would already be present at the membrane in close vicinity to the receptor. (ii) Ligand-induced tyrosine phos-

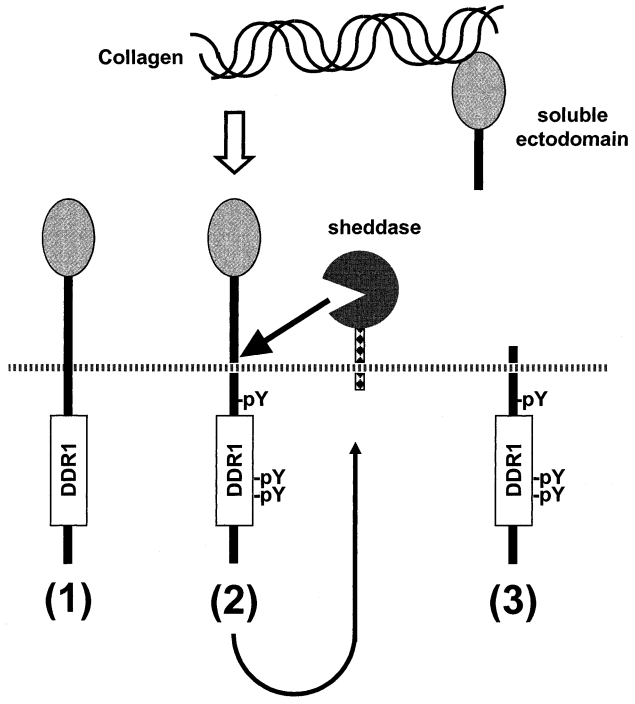

Fig. 6. A model for ligand-induced DDR1 shedding. (1) DDR1 is predominantly present as 125 kDa full-length protein in the absence of collagen. (2) Collagen stimulation induces DDR1 autophosphorylation. Subsequently, the DDR1 sheddase is activated. (3) Membrane-anchored truncated DDR1 remains as tyrosine-phosphorylated 62 kDa protein after ectodomain shedding.

phorylation of DDR1 may induce clustering of a variety of scaffolding and signaling molecules. Amongst others, protein–protein interactions would take place involving phosphorylated tyrosines in the cytoplasmic domain of DDR1 and SH2 or PTB domain containing proteins. These interacting proteins could simultaneously recruit a sheddase molecule and bring it proximal to the receptor. DDR1 is recognized as substrate and cleaved by the sheddase. (iii) Prolonged activation of DDR2 has been shown to result in up-regulation of MMP1 [23]. Therefore, activation of DDR1 may also result in transcriptional up-regulation of proteases. This up-regulation could include a sheddase that cleaves the receptor itself. Additionally more complex mechanisms of protease activation, such as pro-enzyme cleavage or alleviation of inhibitors, may also be considered.

Why do mammary carcinoma cells shed DDR1 in a ligand-dependent manner? We recently showed that cells derived from DDR1-null mice have severely impaired adhesion to collagen and migration on collagen [38]. In a growing mammary tumor, DDR1 may be essential for the initial attachment of invasive cells to the basement membrane collagen. Following DDR1 activation, the cell/matrix contact is terminated by ectodomain shedding, allowing further migration of the cell. Since the β-subunit of DDR1 is still tyrosine-phosphorylated following processing, the proliferative pathways initially triggered by the full-length receptor remain active.

Currently, we can only speculate on the functional role of the ectodomain of DDR1. The ectodomain potentially remains attached to the collagen and blocks further interaction of collagen with full-length signalling-competent receptors. Alternatively, DDR1 ectodomain is transported through body fluids and functions as signalling molecule by binding to molecules other than collagen acting on cells distant from DDR1-expressing cells. More work will be necessary to gain further insight into these mechanisms.

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