ADAM12 Is a Four-leafed Clover
THE EXCISED PRODOMAIN REMAINS BOUND TO THE MATURE ENZYME

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The ADAMs (a disintegrin and metalloprotease) comprise a family of multidomain proteins with metalloprotease, cell adhesion, and signaling activities. Human ADAM12, which is implicated in diseases such as cancer, is expressed in two splice forms, the transmembrane ADAM12-L and the shorter and soluble ADAM12-S. ADAM12 is synthesized as a zymogen with the prodomain keeping the metalloprotease inactive through a cysteine-switch mechanism. Maturation and activation of the prodomain involves the cleavage of the prodomain in the trans-Golgi or possibly at the cell surface by a furin-peptidase. The aim of the present study was to determine the fate of the prodomain following furin cleavage. Here we demonstrate that, following cleavage of the human ADAM12-S prodomain in the trans-Golgi by a furin-peptidase, the prodomain remains non-covalently associated with the mature molecule. Accordingly, both the 68-kDa mature form of ADAM12-S and the 25-kDa prodomain could be detected using domain-specific antisera in immunoprecipitation and Western blot analyses of human serum ADAM12 and purified recombinant human ADAM12. Using electron microscopy after negative staining we have furthermore obtained the first visualization of a full-length ADAM molecule, human ADAM12-S, and report that it appears to be a compact clover composed of four leaflets, one of which is the prodomain. Finally, our data demonstrate that the presence of the metalloprotease domain appears to be sufficient for the prodomain to remain associated with the mature ADAM12-S. Thus, we conclude that the prodomain of human ADAM12-S is an integral domain of the mature molecule and as such might have specific biological functions in the extracellular space.

The ADAMs (a disintegrin and metalloprotease) are a large family of more than 30 proteins that belong to the metzincin family of zinc-dependent proteases (1) and are multidomain proteins with protease, adhesion, fusion, and signaling activities (for recent ADAM reviews, see Refs. 2–5). From the N terminus, the domain structure of a prototype ADAM consists of a signal peptide, a prodomain, a metalloprotease domain, a disintegrin-like domain, a cysteine-rich domain, an epidermal growth factor (EGF)-like domain, a transmembrane domain, and a cytoplasmic tail. In addition, splice forms exist for several ADAMs; i.e. for ADAM 9, 12, and 28, shorter secreted and soluble forms have been described (6, 7). ADAMs are synthesized as proforms, and in the secretary pathway the prodomain keeps the metalloprotease inactive through a cysteine-switch mechanism, thus preventing intracellular degradation (8, 9). The prodomain also acts as an intramolecular chaperone to facilitate proper folding and efficient secretion of ADAMs (9–13). Milla and co-workers (12) recently demonstrated that the ADAM17 prodomain keeps the catalytic domain in a more open conformation that is inactive. Following cleavage of the prodomain in the trans-Golgi or possibly at the cell surface by a furin-peptidase or by autocatalysis, ADAM molecules are proteolytically active and considered mature (7, 9). The fate and function of the prodomain following cleavage and secretion have remained elusive.

ADAM12, initially called meltrin-α, was first described in 1995 as a transmembrane protein involved in muscle cell fusion (14). In 1998, we identified and cloned human ADAM12 (6). The gene for human ADAM12 resides on chromosome 10q26 and encodes two different forms: a transmembrane form, ADAM12-L, and an alternatively spliced secreted form, ADAM12-S (4, 6). Human ADAM12-S has all the extracellular domains but lacks the transmembrane and cytoplasmic domains. Instead, the EGF-like domain is followed by a stretch of 33 unique amino acids. ADAM12 is expressed in high amounts in tissues characterized by excessive growth, including human placenta (6, 15) and tumors (16–20). ADAM12-S can be detected in body fluids, including serum (15, 21) and urine (22) and appears to be an important biomarker of disease involving tissue growth. Thus we have recently demonstrated that ADAM12 is a promising marker in prenatal diagnostics (21, 23) and that urinary levels of ADAM12 correlate with breast cancer status and stage (22). Little is known about the structure of ADAMs and their domains and, to date, only the crystal structures of the catalytic domains of ADAM17 (24) and ADAM33 (25) and the disintegrin and cysteine-rich domains of ADAM10 (26) have been resolved. The only data that exist on ADAM12 structure comes from homology modeling of the metalloprotease domain and selective inhibitors (27, 28). Here we report on the structure of ADAM12 and show that following cleavage and secretion the prodomain of human ADAM12 is not degraded but remains associated with the rest of the molecule. Importantly, this persistent association of the prodomain is observed both for ADAM12 in serum under physiological conditions as well as for recombinant purified ADAM12. In support of this notion, electron microscopic analysis of full-length ADAM12-S and its fragments demonstrates that the prodomain represents one of the leaves of an apparent four-leafed clover-shape ADAM12. These data represent
the first experimental information on the overall structural organization of ADAM12 and the first visualization of a full-length ADAM molecule.

**EXPERIMENTAL PROCEDURES**

Immunoprecipitation and Western blot of human pregnancy serum, non-pregnancy serum (21), and purified recombinant ADAM12 and its fragments (29) were performed as described using mouse monoclonal antibodies recognizing the ADAM12 disintegrin domain (6E6, 8F8, 6C10) or polyclonal antibodies against the prodomain (rb132), the metalloprotease domain (rb128), or the cysteine-rich domain (rb122) (30, 31).

Recombinant full-length human ADAM12 and its fragments were produced by transfecting human 293-EBNA cells with ADAM12 cDNA and purifying ADAM12 from conditioned, serum-free culture medium by gelatin-Sepharose, cation exchange, and concanavalin A or heparin-Sepharose affinity chromatography essentially as described (29, 32). In some studies, gel filtration chromatography using a Superdex 200 column was performed. Equilibration of the column and elution of ADAM12 was performed using a buffer containing 50 mM sodium phosphate and 150 mM NaCl, pH 7.0. The constructs used were (a) cDNA encoding full-length human ADAM12-S (A12) (GenBank accession number NM_021641), (b) cDNA encoding the prodomain, the metalloprotease domain, and the disintegrin domain (A12-PMD) (amino acids 1–512), (c) cDNA encoding the prodomain and the metalloprotease domain (A12-PM) (amino acids 1–419), and (d) cDNA encoding the disintegrin, the cysteine-rich domain, and the EGF-like domain (A12-DC) (amino acids 417–707). For all fragments the ADAM12 signal sequence was utilized, except for the A12-DC where the Ig κ-chain leader sequence from the pSecTaqB vector (Invitrogen) was used (6). These recombinant proteins are biologically active in cell adhesion and/or protease assays (29, 32).

For mass spectrometry, the relevant bands were excised from the polyacrylamide gel, reduced, alkylated using iodoacetamide, and digested by trypsin. The resulting fragments were extracted, purified using C18 ZipTip (Millipore), and measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometry on an AutoFlex instrument (Bruker, Bremen). For identification, the mass spectral patterns of the fragments were matched against the ADAM12 sequence and used for searching against several databases.

Visualization of the structure of the various forms of ADAM12 was performed by transmission electron microscopy after negative staining with 0.75% uranyl formate (33). For immunolabeling, ADAM12 samples were first incubated with domain-specific antibodies (6E6 to the disintegrin domain (6E6, 8F8, 6C10) and analyzed by Western blotting with polyclonal antibodies to the cysteine-rich domain (rb122). The 68-kDa band representing ADAM12 (arrow) is present in pregnancy serum only; the 50-kDa band in both lanes represents IgG heavy chain. D, pregnancy and non-pregnancy serum was immunoprecipitated (IP) with polyclonal antibodies to the prodomain (rb132) and analyzed by Western blotting with polyclonal antibodies to the cysteine-rich domain (rb122). The 68-kDa band representing ADAM12 (arrow) is present in pregnancy serum only; the 50-kDa band in both lanes represents IgG heavy chain.

**RESULTS AND DISCUSSION**

We have previously demonstrated that ADAM12 is produced in high amounts by placenta and is present in pregnancy serum, but not in non-pregnancy serum (6, 15). More recently, we have presented evidence that maternal serum levels of ADAM12 are reduced in pregnancies with fetal chromosomal abnormalities as well as in preeclampsia, and as such ADAM12 is a promising biomarker in prenatal screening (21, 23). Western blot analysis of pregnancy serum using antibodies to the cysteine-rich domain of ADAM12 revealed a 68-kDa single band (15). To further test whether the prodomain is also present, human pregnancy serum was analyzed by Western blot using a mixture of antibodies to the prodomain and to the cysteine-rich domain. We found the 25-kDa prodomain to be present in pregnancy serum but not in non-pregnancy serum (Fig. 1, A and B). To test the interaction between the 68-kDa and the 25-kDa chains of ADAM12, two complementary co-immunoprecipitation experiments were performed. Pregnancy and non-pregnancy serum ADAM12 was immunoprecipitated by antibodies specific to the prodomain or the disintegrin domain followed by Western blot using antibodies to the cysteine-rich domain or the prodomain, respectively (Fig. 1, C and D). Under these physiological conditions, the prodomain of ADAM12-S remained associated with the rest of the molecule. Based on these results, the apparent molecular mass of mature ADAM12-S in pregnancy serum is estimated to be ~100 kDa.

**FIGURE 1. Association of the ADAM12 prodomain with the rest of the ADAM12 molecule in human serum.** A, Western blot (WB) of pregnancy (P) and non-pregnancy (NP) serum (2 µl of serum/ lane) with a mixture of polyclonal antibodies to the cysteine-rich domain (rb122) and to the prodomain (rb132) under reducing (Red) and non-reducing (Non-Red) conditions. B, Western blot of pregnancy and non-pregnancy serum with a monoclonal antibody to the disintegrin domain of ADAM12 (6E6) under reducing and non-reducing conditions. C, pregnancy and non-pregnancy serum was immunoprecipitated (IP) with polyclonal antibodies to the prodomain (rb132) and analyzed by Western blotting with polyclonal antibodies to the cysteine-rich domain (rb122). The 68-kDa band representing ADAM12 (arrow) is present in pregnancy serum only; the 50-kDa band in both lanes represents IgG heavy chain. D, pregnancy and non-pregnancy serum was immunoprecipitated (IP) with polyclonal antibodies to the prodomain (rb132).
We next analyzed the relationship between the prodomain and the rest of the molecule using purified human recombinant full-length ADAM12 and its fragments (Fig. 2A). ADAM12 proteins were purified from cell supernatants by gelatin-Sepharose, cation-exchange chromatography, and concanavalin A affinity chromatography (Fig. 2B). In some studies, gel filtration was used as an additional purification step after the concanavalin A-Sepharose (the final step in ADAM12 purification). C, gel filtration chromatography elution profile for A12 using a Superdex 200 column. D, SDS-PAGE analysis of purified A12, A12-PMD, and A12-PM; bands visualized by Coomassie Blue staining. E, Western blot (WB) of A12 protein that had been further purified by gel filtration (as in C) with a mixture of polyclonal antibodies to the cysteine-rich domain (rb122) and the prodomain (rb132). F and G, Western blot of A12, A12-PMD, and A12-PM with polyclonal antibodies to the metalloprotease domain (rb128) or to the prodomain (rb132), respectively. H, SDS-PAGE analysis of purified A12 under reducing (Red) and non-reducing (Non-Red) conditions; bands visualized by Coomassie Blue staining.

### Table 1: Tryptic peptides of purified human ADAM12

| Domain                  | Residues | Molecular Mass MH⁺ | Sequence |
|-------------------------|----------|--------------------|----------|
| Pro-domain              |          |                   |          |
| 49–57                   | 1014.56  | 1014.55            | SGDLWIPVK |
| 63–71                   | 1091.60  | 1091.59            | NHPEVLNIR |
| 78–85                   | 999.58   | 999.60             | ELINLNER  |
| 86–110                  | 2724.31  | 2724.27            | NGLATASSPTEHLQDGTDVSLAR |
| 127–142                 | 1659.76  | 1659.76            | GYSDSAVSLSTCSGLR |
| 215–225                 | 1290.71  | 1290.71            | YVELVADNR  |
| 241–250                 | 1151.64  | 1151.64            | LIEIAHVVDK |
| 251–258                 | 1078.62  | 1078.62            | FYPLNR    |
| 259–272                 | 1616.84  | 1616.82            | TVLVAVVAMDKR |
| 273–290                 | 2223.98  | 2223.98            | CFVQDQFFTLHEFLDWR |
| 376–397                 | 2426.04  | 2425.97            | GGMGVAQASGYFPFMVIFSSCSR |
| 407–419                 | 1491.74  | 1491.72            | GMGVCLPILPEVR |

**We next analyzed the relationship between the prodomain and the rest of the molecule using purified human recombinant full-length ADAM12 and its fragments (Fig. 2A). ADAM12 proteins were purified from cell supernatants by gelatin-Sepharose, cation-exchange chromatography, and concanavalin A affinity chromatography (Fig. 2B). In some studies, gel filtration was used as an additional purification step after the concanavalin A-Sepharose (the final step in ADAM12 purification). C, gel filtration chromatography elution profile for A12 using a Superdex 200 column. D, SDS-PAGE analysis of purified A12, A12-PMD, and A12-PM; bands visualized by Coomassie Blue staining. E, Western blot (WB) of A12 protein that had been further purified by gel filtration (as in C) with a mixture of polyclonal antibodies to the cysteine-rich domain (rb122) and the prodomain (rb132). F and G, Western blot of A12, A12-PMD, and A12-PM with polyclonal antibodies to the metalloprotease domain (rb128) or to the prodomain (rb132), respectively. H, SDS-PAGE analysis of purified A12 under reducing (Red) and non-reducing (Non-Red) conditions; bands visualized by Coomassie Blue staining.**
that the prodomain was cleaved and remained associated with the rest of the molecule both in ADAM12 composed of the prodomain, the metalloprotease domain, and the disintegrin domain (A12-PMD) as well as in purified ADAM12 composed of the prodomain and the metalloprotease domain (A12-PM) (Fig. 2, B and D). Because of their similar molecular size and migration pattern under reducing conditions, the two domains of A12-PM cannot be discriminated by SDS-PAGE and Coomassie Blue staining (Fig. 2D). However, Western blot analysis using polyclonal antibodies specific for each of the two domains clearly demonstrated the presence of both domains in A12-PM (Fig. 2, F and G). Mass spectrometry of the purified A12-PM protein validated this conclusion (Table 1). These results strongly suggest that, under the experimental conditions used, the interaction between the prodomain and the metalloprotease domain appears to be sufficient to keep the prodomain associated with the rest of the molecule.

One potential mode of interaction between the prodomain and the metalloprotease domain would be through the formation of a disulfide bond between the unpaired cysteine residues of the two domains following cleavage. However, when SDS-PAGE of purified recombinant ADAM12 was performed under non-reducing conditions, the prodomain migrated separately from the 68-kDa mature form (Fig. 2H). A similar analysis demonstrated that the prodomain of pregnancy serum ADAM12 migrated separately from the 68-kDa form under both reducing and non-reducing conditions (Fig. 1A). These two results suggest that the prodomain is not associated with the rest of the molecule by disulphide bonds, but instead by a non-covalent affinity binding. Notably the 68-kDa band migrated slightly faster under non-reducing conditions (Fig. 1, A and B), demonstrating the existence of intra-disulphide bonds in the mature form of ADAM12-S. We hypothesize that under physiological conditions, inhibitors, substrates, and even downstream ADAM12 domains can affect the affinity of the prodomain for the metalloprotease domain and potentially replace or displace the prodomain. Such interactions might dictate the activity of the metalloprotease activity of ADAM12.

To further determine how the prodomain is structurally related to the remainder of the molecule and to begin to visualize the structure of the entire ADAM12 molecule, we performed negative staining and electron microscopy analyses of full-length recombinant human ADAM12-S (Figs. 3 and 4A), A12-PMD, A12-PM, and A12-DC (Fig. 4, B–D). The predominant structure of the ~100 kDa full-length molecule was found to be that of a compact clover composed of four apparently globular domains (Fig. 3). The size of the whole molecule is ~8 nm, and the leaves measure ~3 nm. Only a few extended molecules were observed. The particles were adsorbed to the carbon film in random orientations and thus exhibited less than four visible domains in some cases, but the maximum number of clover leaves observed was four. Occasionally ADAM12 dimers were observed (Fig. 3, arrowheads), where individual ADAM12 molecules were laterally associated. The different recombinant fragments exhibited the same overall structure as the full-length molecule, but with reduced numbers of globular domains (Fig. 4). The
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A12-PMD fragment has three globular domains (Fig. 4B), and the A12-PM and the A12-DC fragments each have two domains (Fig. 4, C and D, respectively). To identify the individual domains, we used monoclonal and affinity-purified polyclonal domain-specific antibodies labeled with colloidal gold (34). When full-length ADAM12-S was incubated with an affinity-purified polyclonal rabbit IgG directed against the prodomain, the images showed one gold particle bound to one of the globular domains (Fig. 4E). Gold-labeled monoclonal antibody directed against the disintegrin-like domain produced similar images, with a gold particle on one domain (Fig. 4F). These data demonstrate that the prodomain is associated with the mature ADAM12 molecule and importantly represent the first visualization of a full-length ADAM molecule.

We conclude that the mature secreted ADAM12 is a clover-shaped molecule and that the prodomain is an integral domain of mature ADAM12. Fig. 5 represents a schematic of ADAM12-S based on the collective current knowledge about the structure of human ADAM12-S. We propose that the prodomain of human ADAM12 might influence the functional activities of ADAM12 not only inside the cell as previously reported, but also in the extracellular space.

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