Regulation of Human ADAM 12 Protease by the Prodomain

EVIDENCE FOR A FUNCTIONAL CYSTEINE SWITCH*

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The ADAMs (a disintegrin and metalloprotease) are a family of multidomain proteins that are believed to play key roles in cell-cell and cell-matrix interactions. We have shown recently that human ADAM 12-S (meltrin α) is an active metallopeptase. It is synthesized as a zymogen, with the prodomain maintaining the protease in a latent form. We now provide evidence that the latency mechanism of ADAM 12 can be explained by the cysteine switch model, in which coordination of Zn²⁺ in the active site of the catalytic domain by a cysteine residue in the prodomain is critical for inhibition of the protease. Replacing Cys⁷⁷⁹ with other amino acids results in an ADAM 12 proform that is proteolytically active, but latency can be restored by placing cysteine at other positions in the propeptide. None of the amino acids adjacent to the crucial cysteine residue is essential for blocking activity of the protease domain. In addition to its latency function, the prodomain is required for exit of ADAM 12 protease from the endoplasmic reticulum. Tissue inhibitor of metalloprotease-1, -2, and -3 were not found to block proteolytic activity of ADAM 12, hence a physiological inhibitor of ADAM 12 protease in the extracellular environment remains to be identified.

The ADAMs are a family of integral membrane or secreted glycoproteins comprised of several distinct domains. Together with snake venom metalloproteases, they make up the represolysin family of zinc metalloproteases (1–5). The archetypical ADAM protein has a prodomain, metalloprotease domain, disintegrin-like domain, cysteine-rich region, and in the case of membrane-anchored ADAMs, a transmembrane and cytoplasmic domain. We recently made use of an α₂-macroglobulin (α2M) trapping assay to demonstrate that human ADAM 12-S, the secreted form of ADAM 12, is an active metalloprotease (6).

Members of the metzincin superfamily of metallopeptases, including the represolysins and matrix metalloproteases (MMPs), are synthesized as inactive precursors, in which an NH₂-terminal prodomain is responsible for maintaining latency of the protease (7, 8). MMPs are generally secreted as proenzymes; the latent proform is subsequently converted to the active form by proteolytic cleavage of the prodomain. In contrast, it appears that ADAM proteases are converted from a latent proform to an active enzyme before secretion as a result of cleavage of the prodomain by furin or related proteases in the trans-Golgi (6, 9).

The prodomain of all MMPs contains a highly conserved cysteine residue that is part of the mechanism for the blocking activity of the proenzyme (3). This cysteine residue coordinates the zinc ion located at the active site of the catalytic domain and is the basis for the proposed “cysteine switch” model of repression/activation for MMPs (10, 11). The key cysteine residue of the prodomain is also highly conserved in snake venom metalloproteases as well as in those members of the ADAMs family which have been demonstrated to be active proteases (1, 3, 6, 12, 13). It is therefore likely that a similar cysteine switch is part of the mechanism by which the activity of these proteases is regulated.

It is not clear to what extent the regulatory activity of an ADAM or MMP prodomain should be attributed to cysteine coordination of the active site zinc and how much is the result of interaction of other regions of the prodomain with sites in the metalloprotease domain. Treatment of pro-MMPs with thiol-modifying agents results in activation with ensuing autolysis of the propeptide (10, 14). This supports the view that the cysteine-zinc interaction is the key one. On the other hand, Chen et al. (15) showed that disruption of the zinc-cysteine interaction in MMP-3 by chemical modification of the cysteine was not sufficient to activate the proenzyme, whereas subsequent treatment with 4-aminophenylmercuric acetate did result in activation (15). This suggests that, at least in the case of MMP-3, the prodomain is capable of performing its role of maintaining latency even in the absence of a cysteine switch. Single amino acid substitutions in the cysteine switch region of MMP proenzymes, whether of the cysteine itself or of adjacent amino acids, have been shown to result in spontaneous activation of the proenzyme (16–18). The details of how the cysteine switch functions in MMP proenzymes have therefore not been fully resolved, and the relative importance of the cysteine switch to the latency mechanism of ADAM proteases remains an open question.

We decided to explore whether the cysteine switch model can explain latency of the ADAM 12-S proenzyme. Our approach was to substitute amino acids in the prodomain, based on what has been learned from previous studies on MMPs, and to determine the effect this had on protease activity of the ADAM 12 proenzyme, using α2M as a substrate.
EXPERIMENTAL PROCEDURES

Materials—N-Ethylmaleimide (NEM), phenylmethylsulfonyl fluoride, and Nonidet P-40 were from Sigma. BB-94 and BB-3103 were from British Biotech Pharmaceuticals Ltd. (Oxford, United Kingdom). Restriction endonucleases were from New England Biolabs or Roche Molecular Biochemicals. Recombinant human tissue inhibitors of metalloprotease (TIMP)-1, -2, and -3 were provided by Gillian Murphy (University of East Anglia, Norwich, U. K.); they were expressed in NSO mouse myeloma cells and purified as described previously (19, 20).

Plasmid Constructs—Plasmids for expression of full-length ADAM 12-S (p1151), for expression of the same protein lacking a furin cleavage site at the junction between the prodomain and the metalloprotease domain (p1197), and for expression of ADAM 12-S lacking both the prodomain and the metalloprotease domain (p1905) have been described previously (6, 21). Nucleotide and amino acid numbering is according to the ADAM 12-S sequence deposited in the GenBank data base (accession number AF023477).

Plasmids coding for ADAM 12 proteins with amino acid substitutions in the cysteine switch region were constructed as follows. First, plasmid 1197 was modified to give two unique restriction sites in place of the codons for amino acids 177–183. This was done using the method described previously by performing strand overlap polymerase chain reaction on plasmid 1197 to generate a PmlI DNA fragment (nucleotides 716–2163) containing the desired mutation and using this to replace the wild-type PmlI DNA fragment in plasmid 1151 (6). The resulting plasmid (p1265) contains, in addition to the mutated furin cleavage site sequence, the SphI site within the cysteine switch region was changed to eliminate catalytic activity. Insertion of the appropriate double-stranded oligonucleotide at the SphI site produced plasmid 1376, coding for full-length ADAM 12-S with a mutated furin cleavage site plus the Gln351 → Gln mutation and plasmid 1377, containing these mutations plus a Cys179 → His mutation in the cysteine switch.

A modified form of plasmid 1265 was prepared containing a Glu → Gln mutation to eliminate catalytic activity. Insertion of the appropriate double-stranded oligonucleotide at the SphI site in the plasmid 1376 yielded plasmid 1376, coding for full-length ADAM 12-S with a mutated furin cleavage site plus the Gln351 → Gln mutation, and plasmid 1377, containing these mutations plus a Cys179 → His mutation in the cysteine switch.

Table I provides an overview of these ADAM 12-S expression plasmids. Standard recombinant DNA techniques were used throughout (22). Sequencing to confirm the accuracy of the mutagenesis was performed using the Vistra DNA Sequencer 725 (Amer sham Pharmacia Biotech).

Transfection Assays and Immunoblotting—COS-7 cells were transfected with ADAM 12-S expression plasmids by electroporation as described previously (6). Unless stated otherwise, all experiments were performed in medium containing 10% fetal bovine serum (Life Technologies, Inc.). 48–72 h post-transfection, the medium was harvested, concentrated 10-fold using an Amicon Centricron-10 filter, and processed for SDS-PAGE. To evaluate ADAM 12 polypeptides located intracellularly, a cell extract was prepared by washing the cell layer with phosphate-buffered saline and lysing by incubation in 150 mM NaCl, 20 mM Tris (pH 7.4), 1% Nonidet P-40 on ice for 10 min. After centrifugation at 7090 × g for 5 min at 4 °C, the supernatant was prepared for SDS-PAGE. Samples were denatured and reduced by boiling in SDS sample buffer containing dithiothreitol, subjected to SDS-PAGE on Tris-glycine gels (Novex) and transferred to a polyvinylidene difluoride membrane (22). The membranes were incubated either with the 14E3 monoclonal antibody specific for the ADAM 12 cysteine-rich region or with rabbit antiserum 104 specific for the same region (21). After incubation with the appropriate peroxidase-conjugated secondary antibody, detection was performed using the chemiluminescence SuperSignal kit from Pierce or the ECL-Plus kit from Amersham.

Deglycosylation was performed on denatured protein that had been boiled in SDS sample buffer. Protein was precipitated with 8 volumes of acetone to remove excess SDS. The protein pellet was resuspended by boiling in 100 mM 2-mercaptoethanol, 0.1% SDS for 2 min. One aliquot was diluted with an equal volume of 150 mM NaCl, 20 mM Tris (pH 7.4), 1% Nonidet P-40. EDTA was added to 10 mM, and digestion with N-glycosidase F (Roche Molecular Biochemicals) was performed for 16 h at 30 °C. One aliquot was diluted with an equal volume of 100 mM sodium citrate (pH 5.5), phenylmethylsulfonyl fluoride was added to 5 mM, and digestion with endoglycosidase H (Roche Molecular Biochemicals) was performed for 16 h at 30 °C.

Protease Assays—The α2M complex formation assay was used (23–25). ADAM 12 protein from transfected COS-7 cells was prepared in serum-free medium (UltraDOMA medium from BioWhittaker) and concentrated 10-fold using an Amicon Centricron-10 filter. Assays were carried out at 100 mM NaCl, 20 mM Tris (pH 7.4), 10 mM CaCl2, and 0.02% sodium azide. The α2M substrate was either added in the form of fetal bovine serum at a final concentration of 25% or purified α2M at a concentration of 1 μg/μl. ADAM 12 proforms were alkylated by the addition of 1 mM NEM and incubation at 20 °C for 15 min before the addition of α2M. Reactions were terminated after incubation at 37 °C for 16 h by boiling in SDS sample buffer as described above. After electrophoresis to remove α2M purified from three different sources was tested. Adult human and bovine α2M was purified as described previously (26). Fetal bovine α2M was purified by a slightly modified procedure. Briefly, fetal bovine serum (Life Technologies, Inc.) was precipitated with PEG-6000, and the 4–20% precipitate was redissolved in 10 mM sodium phosphate, 10 mM NaCl, 2 mM EDTA (pH 7.4) and loaded onto a DEAE-Sephacel column. The column was eluted with a linear gradient from 10 to 300 mM sodium chloride. α2M-containing fractions, as determined by a protease protection assay, were pooled and loaded on a Zn2+-imidoacetic acid-Sepharose 4B column. The column was washed with 2 volumes of 20 mM sodium phosphate, 150 mM sodium chloride (pH 7.0) and eluted with 100 mM EDTA (pH 7.0). As a final step, the eluate was fractionated on a Superose 6 column in 100 mM sodium phosphate, pH 8.0, resulting in 95% pure α2M as judged by SDS-PAGE. All column materials were from Amersham Pharmacia Biotech. All preparations contained 3.7–3.9 thiol esters/α2M tetramer, as measured by the appearance of thiols upon treatment with mthylamine.

Because side-by-side assays showed that human α2M was cleaved more efficiently than either fetal or adult bovine α2M in purified form, all assays described in this study were performed either with purified human α2M or fetal bovine serum. We reported previously that an ADAM 12-S proenzyme with a mutated cysteine switch did not react with purified bovine α2M (6). This was apparently the result of the preparation of bovine α2M used in the previous study because bovine α2M, when purified and used as above, demonstrated unequivocal activity both with wild-type ADAM 12-S and mutant ADAM 12-S proen-

### Table I

| Plasmid | Description |
|---------|-------------|
| 1151 | Full-length, wild-type human ADAM 12-S |
| 1197 | Mutated furin cleavage site: two amino acids at the prodomain/catalytic domain junction were changed to eliminate cleavage by furin |
| 1214, 1336 | Mutated furin cleavage site and a mutated cysteine switch (Cys179 → Ala or His) |
| 1376 | Mutated furin cleavage site and Glu351 → Gln mutation to eliminate catalytic activity |
| 1377 | Mutated furin cleavage site, mutated cysteine switch (Cys179 → His), and Glu351 → Gln mutation to eliminate catalytic activity |
| 1229 | ADAM 12-S lacking the prodomain; contains Ig κ-chain signal peptide |
| 1305 | ADAM 12-S lacking both the prodomain and the metalloprotease domain; contains Ig κ-chain signal peptide |

Additional plasmids are listed in Tables II and III and correspond to plasmid 1197 with various amino acid substitutions in the cysteine switch of the prodomain.
When an ADAM 12-S proform containing a Cys179 mutation was expressed in COS cells and assayed for proteolytic activity, the results are summarized in Table II. The polypeptide encoded by plasmid 1197 secreted by cells growing in serum-containing medium that was subsequently concentrated, subjected to SDS-PAGE, and immunoblotted with an antibody specific for ADAM 12 showed that the latent ADAM 12-S proform encoded by plasmid 1197 secreted to serum-containing medium, either in the presence or absence of NEM. Samples were analyzed on a 6% polyacrylamide gel. Panel B, analysis of reaction products of ADAM 12 polypeptides with purified human α2M. Protease assays were carried out on concentrated serum-free medium, and the samples were then subjected to electrophoresis on a 6% polyacrylamide gel.

The prodomain of ADAM 12 contains a conserved cysteine residue at position 179 which is predicted to participate in the cysteine switch for maintaining latency of the protease domain. When an ADAM 12-S proform containing a Cys179→Ala mutation (plasmid 1214) was secreted to serum-containing medium, the presence of α2M reaction products showed that this proform is a constitutively active rather than a latent protease (Fig. 1A, lane 3). Consistent results were obtained when assays were performed by first preparing ADAM 12-S protein in serum-free medium and then adding α2M in the form of fetal bovine serum (Fig. 1A, lanes 4–9). In addition, these assays showed that the latent ADAM 12-S proform encoded by plasmid 1197 could be activated by treatment with NEM, which presumably alkylates the free cysteine in the prodomain, thereby destroying the cysteine switch (Fig. 1A, lane 7). NEM treatment had no significant effect on activity of the 68-kDa form nor on activity of the proform containing the Cys179→Ala mutation. Purified human α2M reacted with ADAM 12-S in a similar fashion. It is a more efficient substrate than the preparation of purified bovine α2M which we used in a previous study; the intensity of the α2M reaction products is comparable to that obtained with fetal bovine serum and clearly shows the proteolytic activity of the proform containing the Cys179→Ala mutation (Fig. 1B, lane 3). The reaction products of α2M with the proenzyme encoded by plasmid 1214 are larger than those generated by the wild-type ADAM-12 protease, as would be expected for complexes containing 92-kDa rather than 68-kDa ADAM 12 polypeptides.

Transfection with ADAM 12-S proform plasmids often yielded an additional band that migrated at 68 kDa in addition to the strong band at 92 kDa (Fig. 1, panel A, lanes 7–9 and panel B, lane 3). It is conceivable that this band represents active ADAM 12-S protease, generated either by inefficient cleavage by furin at an alternate site or by autocleavage of the ADAM 12 proenzyme (see next “Results” section). Some of the α2M reaction products generated by activated ADAM 12 proforms comigrated with the products of wild-type ADAM 12 polypeptide (Fig. 1A, lanes 7–9) and possibly derive from reaction of 68-kDa rather than 92-kDa polypeptides. Therefore, for the purpose of evaluating whether a given ADAM 12-S proform was proteolytically active, we judged it to be active only in the event that it produced α2M reaction products larger than those produced by the wild-type 68-kDa polypeptide.

Which Amino Acids Are Required for a Functional Cysteine Switch?—We proceeded to generate a series of proforms with various amino acid substitutions in the cysteine switch region, centered around Cys179. A proform that did not react with α2M was judged to be a latent protease on the condition that it reacted after treatment with NEM. A proform that reacted with α2M when secreted into serum-containing medium or when prepared in serum-free medium that was subsequently incubated with α2M in the absence of NEM was judged to be a constitutively active protease, in which the cysteine switch of the prodomain had been inactivated by the amino acid substitution.

Mutants of the ADAM 12-S proform were expressed in COS cells and assayed for proteolytic activity. The results are summarized in Table II. The polypeptide encoded by plasmid 1197 has the wild-type ADAM 12 cysteine switch, and it has no activity unless assayed in the presence of NEM. Replacing Cys179 with alanine or histidine yields a constitutively active protease (mutants 1214 and 1336). Cysteine is therefore essential for a functional cysteine switch and cannot be replaced even by an amino acid such as histidine, which is capable of coordinating zinc. The two histidine residues in the vicinity of Cys179 could conceivably be involved in coordination of the active site Zn$^{2+}$, similar to the way in which three histidines of the protease domain bind to Zn$^{2+}$. However, mutant 1313 shows that changing these two amino acids does not affect the ability of the prodomain to maintain latency. Gly180 is highly conserved among members of the metzincin superfamily, but mutant 1358 demonstrates that it is not essential for function of the ADAM 12 cysteine switch. Finally, replacement of the ADAM 12 cysteine switch with the cysteine switch of ADAM 17

**RESULTS**

Evaluating Proteolytic Activity of ADAM 12-S Proforms by the α2M Trapping Assay—We employed the α2M trapping assay that we used previously to demonstrate that human ADAM 12 is an active metalloprotease (6). Fig. 1A shows the results of a typical assay, in which ADAM 12/α2M reaction products were detected by immunoblotting with an antibody specific for ADAM 12. When wild-type ADAM 12-S encoded by plasmid 1151 was secreted to the medium, Plasmid 1197 codes for ADAM 12-S with a mutated furin cleavage site. As we have shown previously, cells transfected with plasmid 1197 secreted the 92-kDa proform, consisting of the ADAM 12 prodomain and metalloprotease domain, and disintegrin-like and cysteine-rich domains (6). This proform is a latent protease and therefore does not react with α2M, as evidenced by the absence of the characteristic high M$_s$ bands (Fig. 1A, lane 2).

The prodomain of ADAM 12 contains a conserved cysteine residue at position 179 which is predicted to participate in the cysteine switch for maintaining latency of the protease domain. When an ADAM 12-S proform containing a Cys179→Ala mutation (plasmid 1214) was secreted to serum-containing medium, the presence of α2M reaction products showed that this proform is a constitutively active rather than a latent protease (Fig. 1A, lane 3). Consistent results were obtained when assays

![Fig. 1. α2M trapping assays on ADAM 12-S proteins expressed in COS cells.](Image 60x578 to 286x729)
Protease activity of ADAM 12-S proforms with amino acid substitutions in the cysteine switch of the prodomain

These ADAM 12 polypeptides all contain the KR<sup>107</sup> → NG mutation to prevent cleavage of the prodomain by furin. Note that for mutants 1312 and 1311, Gly<sup>177</sup> is not part of the consensus sequence.

| Plasmid | Cysteine switch | Amino acid residues 177–183 | Protease activity | Protease activity with NEM |
|---------|----------------|-----------------------------|------------------|---------------------------|
| 1197    | ADAM 12        | G S C G S H H               | No<sup>a</sup>  | Yes                       |
| 1214    | Cys<sup>179</sup> → Ala | G S A G S H H               | Yes<sup>b</sup> | NR<sup>c</sup>             |
| 1336    | Cys<sup>179</sup> → His | G S H G S H H               | Yes<sup>b</sup> | NR<sup>c</sup>             |
| 1313    | HH → QQ        | G S C G S Q Q               | No<sup>d</sup>  | Yes<sup>e</sup>            |
| 1338    | Gly<sup>170</sup> → Ala | G S C A S H H               | No<sup>d</sup>  | Yes<sup>e</sup>            |
| 1312    | ADAM 17        | G V C G Y L K              | No<sup>d</sup>  | Yes<sup>e</sup>            |
| 1311    | SVMP<sup>f</sup> | G M C G Y T G              | No<sup>d</sup>  | Yes<sup>e</sup>            |
| 1314    | MMP consensus  | P R C G V F D              | No<sup>d</sup>  | Yes<sup>e</sup>            |

<sup>a</sup> No, no α2M reaction products were observed either when secreted into serum-containing medium or when prepared in serum-free medium and then incubated with fetal bovine serum.

<sup>b</sup> Yes, α2M reaction products were observed, as described under “Results.”

<sup>c</sup> NR, not relevant.

<sup>d</sup> SVMP, snake venom metalloprotease.

or with a consensus snake venom metalloprotease or MMP cysteine switch, yields a fully functional prodomain. We conclude that cysteine is the crucial amino acid of the ADAM 12 cysteine switch and that none of the adjacent amino acid residues is essential.

Replacing Cys<sup>179</sup> with Ala results in an inactivated cysteine switch (mutant 1214). We next asked whether an active cysteine switch could be re-created in the prodomain by placing a cysteine residue in the vicinity of position 179. A series of ADAM 12 proforms was generated in which this inactive cysteine switch was modified by substituting cysteine for the adjacent amino acid residues one at a time. The results are presented in Table III. When cysteine was present at positions –1, +1, or +2 relative to the wild-type position, the result was a constitutively active protease, i.e. the cysteine switch was nonfunctional. A prodomain containing cysteine at position +3 or +4 was functionally indistinguishable from the wild-type prodomain (mutants 1335 and 1343 compared with 1197). Therefore, not only is cysteine the key amino acid residue, but there is a certain flexibility in its position in the prodomain. Together, these data demonstrate that the latency mechanism of the ADAM 12 prodomain can be explained by the cysteine switch model as proposed in 1990 for MMPs (10, 11).

In the Absence of a Functional Cysteine Switch, ADAM 12 Proenzyme Is Capable of Autocleavage—As mentioned above, ADAM 12 proforms in which the cysteine switch was altered either chemically (by alkylation with NEM) or genetically (by substitution of another amino acid for Cys<sup>179</sup>) often yielded a 68-kDa band in addition to the major 92-kDa product. To test for autocatalysis of the ADAM 12-S proenzyme, the 92-kDa proform polypeptide with a wild-type cysteine switch was prepared in serum-free medium by transfecting cells with plasmid 1197. This protein is stable when incubated at 37 °C (Fig. 2, lane 1). Treatment with NEM, in the absence of α2M, resulted in the appearance of a 68-kDa band (Fig. 2, lane 2). The same polypeptide with a Glu<sup>351</sup> → Gln mutation in the catalytic domain to abolish proteolytic activity did not generate a 68-kDa band (Fig. 2, lanes 3 and 4). We therefore concluded that conversion of the 92-kDa polypeptide to a 68-kDa form was caused by autocatalysis. A similar result was obtained for an ADAM 12-S proform where the cysteine switch was inactivated genetically. Cells transfected with plasmid 1336, encoding the proenzyme with a mutated furin cleavage site and with a Cys<sup>179</sup> → His mutation in the cysteine switch, secreted predominantly the 92-kDa form, with a lesser amount of 68-kDa polypeptide (Fig. 2, lane 5). The processing was a result of autocleavage rather than cleavage by furin because the same proform with the Glu<sup>351</sup> → Gln mutation yielded a 92-kDa band only (Fig. 2, lane 6).

The Prodomain Is Required for Secretion of ADAM 12 Protease—When wild-type ADAM 12-S is expressed in COS-7 cells, a steady-state level of the 92-kDa proenzyme can be detected in cell extracts by Western blotting; these are presumably polypeptides that have not yet reached the trans-Golgi, where a furin-like protease removes the prodomain (Fig. 3A, lane 1). If an ADAM 12-S protein lacking the prodomain is synthesized (encoded by plasmid 1229), it accumulates in the cell and is not secreted (Fig. 3A, lanes 2 and 5). Retention of this polypeptide is related to the metalloprotease domain because an ADAM 12-S polypeptide lacking both the prodomain and the metalloprotease domain is secreted efficiently (Fig. 3A, lanes 3 and 6). To determine in which subcellular compartment the polypeptide encoded by construct 1229 was located, we analyzed the glycosylation pattern of intracellular and secreted ADAM 12 proteins. ADAM 12-S has five predicted N-glycosylation sites, two of which are in the prodomain (21). Processed and secreted 68-kDa ADAM 12-S protease is glycosylated, as evidenced by increased mobility on SDS-PAGE after treatment with N-glycosidase F (Fig. 3B, lanes 1 and 2). These carbohydrates are resistant to removal by endoglycosidase H, as expected for a protein that has traversed the Golgi (Fig. 3B, lane 3). ADAM 12-S protein synthesized without the prodomain (plasmid 1229) is endoglycosidase H-sensitive and therefore has not been processed by enzymes in the medial Golgi (Fig. 3B, lanes 4–6). We conclude that the prodomain is required for translocation from the ER to the Golgi apparatus, possibly by assisting the metalloprotease domain in folding to a secretion-competent conformation.

Testing Potential Inhibitors of ADAM 12 Protease—Regulation of ADAM 12 protease activity intracellularly appears to be carried out by the cysteine switch of the prodomain, but cleavage of the prodomain by furin means that ADAM 12 is secreted as a constitutively active protease. Are there specific inhibitors in the extracellular environment which take over the function of regulating ADAM 12 after removal of the prodomain? It is well established that MMPs are regulated by a group of physiological inhibitors, the TIMPs (7, 27). It had long been assumed that the specificity of TIMPs was restricted to inhibition of MMPs, but recently TIMP-3 has been shown to inhibit ADAM 17 protease (28). We tested the ability of recombinant TIMPs -1, -2, and -3 to inhibit ADAM 12-S protease. 68-kDa active protease was prepared by transfecting cells with plasmid 1151 and growing them in serum-free medium. After incubation with inhibitor for 15 min, purified human α2M was added, and the reaction was allowed to proceed for 16 h in the presence of the inhibitor. There was no inhibition by any of the TIMPs at
concentrated, and incubated for 16 h at 37 °C in 100 mM NaCl, 50 mM proenzyme. ADAM 12-S protein was prepared in serum-free medium, where proteolytic activity has been ablated by a Glu351 type at a concentration of 1 mM was included for the samples in a mutated furin cleavage site and a Cys 179 by a Glu351 12-S with a mutated furin cleavage site. Plasmid 1376 codes for the monoclonal antibody 14E3. Plasmid 1197 codes for full-length ADAM 4 concentrations up to 500 nM (Table IV). Longer preincubation Tris (pH 7.4), 10 mM CaCl2, 1 mM ZnCl2, and 0.02% sodium azide. NEM metallocarboxypeptidase domain (1095). prodomain (1229), or ADAM 12-S lacking both the prodomain and the COS cells were transfected with plasmids coding for wt full-length wild-type (1151), ADAM 12-S (1151), ADAM 12-S lacking the propeptide, and Cys75 interacts directly with the catalytic zinc ion (29). However, elimination of the cysteine switch by alklylation of Cys75 does not activate the proenzyme (15). There have been conflicting reports on the effect of substituting another amino acid residue for Cys75. In some expression systems this leads to activation of MMP-3, presumably by autocatalysis (16, 30), but it has also been reported that the mutant proenzyme remains latent (17). Alteration of amino acid residues in the vicinity of Cys75 can lead to activation of MMP-3, and ADAM 17 protease activity. Hydroxamate inhibitors such as BB-94 and BB-3103 inhibit MMPs in in vitro enzyme assays when present at low nanomolar concentrations, and BB-94 inhibits ADAM 17 protease with an apparent Ki of 0.54 nm (28). However, no effect on ADAM 12 protease activity was observed with these hydroxamate inhibitors unless they were added at markedly higher concentrations (Table IV). This may reflect a difference in substrate specificity between ADAM 12 and ADAM 17.

**DISCUSSION**

The study of the ADAM family of metalloproteases is a relatively young field, but the extensive research that has been carried out on MMPs can serve as a basis for functional analyses of ADAMs. The most intensively studied proenzyme in the metzincin superfamily is proMMP-3 (stromelysin-1) (7, 8). The three-dimensional structure has been determined by x-ray diffraction analysis, and it supports the cysteine switch model of activation in that the active site of the proenzyme is filled by the propeptide, and Cys75 interacts directly with the catalytic zinc ion (29). However, elimination of the cysteine switch by alklylation of Cys75 does not activate the proenzyme (15). There have been conflicting reports on the effect of substituting another amino acid residue for Cys75. In some expression systems this leads to activation of MMP-3, presumably by autocatalysis (16, 30), but it has also been reported that the mutant proenzyme remains latent (17). Alteration of amino acid residues in the vicinity of Cys75 can lead to activation of proMMP-3, and there is evidence that the amino-terminal region of the prodomain is involved in maintenance of latency (16, 17, 30).

The experiments we describe in this paper demonstrate that Cys179 is a key part of the latency mechanism of the ADAM 12 zymogen. Chemical modification of the thiol group or substitution of another amino acid for Cys179 results in proteolytic activity despite the presence of the prodomain. These results are fully consistent with the cysteine switch model proposed in

**TABLE III**

Effect of moving the cysteine residue of the cysteine switch to a different position

| Plasmid | Cysteine switch | Amino acid residues 177–183 | Protease activity | Protease activity with NEM |
|---------|----------------|----------------------------|-----------------|--------------------------|
| 1214    | Cys179 → Ala    | G S A G S H H              | Yes*            | NR                      |
| 1340    | Cys at -1       | G C A G S H H              | Yes             | NR                      |
| 1197    | Cys at wild     | G S C G S H H              | No              | Yes                     |
| 1341    | Cys at +1       | G S A C S H H              | Yes             | NR                      |
| 1342    | Cys at +2       | G S A G C H H              | Yes             | NR                      |
| 1335    | Cys at +3       | G S A G S C H              | No              | Yes                     |
| 1343    | Cys at +4       | G S A G S H C              | No              | Yes                     |

*Yes, NR, and No are as in Table II.

**TABLE IV**

Inhibition of ADAM 12-S protease

| Inhibitor         | Concentration | Inhibition |
|-------------------|---------------|------------|
| 1,10-Phenanthroline | 1 mM          | Yes        |
| TIMP-1            | 500 nM        | No         |
| TIMP-2            | 500 nM        | No         |
| TIMP-3            | 500 nM        | No         |
| BB-94             | 50 nM         | No         |
| BB-3103           | 1,000 nM      | Partial    |
| BB-3103           | 50 nM         | No         |
| BB-3103           | 1,000 nM      | No         |

We tested two synthetic MMP inhibitors for their effect on ADAM 12 protease activity. Hydroxamate inhibitors such as BB-94 and BB-3103 inhibit MMPs in in vitro enzyme assays when present at low nanomolar concentrations, and BB-94 inhibits ADAM 17 protease with an apparent Ki of 0.54 nm (28). However, no effect on ADAM 12 protease activity was observed with these hydroxamate inhibitors unless they were added at markedly higher concentrations (Table IV). This may reflect a difference in substrate specificity between ADAM 12 and ADAM 17.

**FIG. 2. Immunoblot to assay for autocleavage of ADAM 12-S proenzyme. ADAM 12-S protein was prepared in serum-free medium, concentrated, and incubated for 16 h at 37 °C in 100 mM NaCl, 50 mM Tris (pH 7.4), 10 mM CaCl2, 1 mM ZnCl2, and 0.02% sodium azide. NEM at a concentration of 1 mM was included for the samples in lanes 2 and 4. Samples were analyzed on a 6% polyacrylamide gel and detected with monoclonal antibody 14E3. Plasmid 1197 codes for full-length ADAM 12-S with a mutated furin cleavage site. Plasmid 1376 codes for the corresponding polypeptide where proteolytic activity has been ablated by a Glu→Gln mutation.**

**FIG. 3. Immunoblots of intracellular and secreted ADAM 12-S polypeptides. COS cells were transfected with plasmids coding for full-length wild-type (wt) ADAM 12-S (1151), ADAM 12-S lacking the prodomain (1229), or ADAM 12-S lacking both the prodomain and the metalloprotease domain (1095). Panel A, ADAM 12 polypeptides located intracellularly (lanes 1–3) and in the medium (lanes 4–6) were analyzed on an 8% polyacrylamide gel fol...
coordination of the active site Zn\(^{2+}\), and the protease is “on” when the cysteine is dissociated (10, 11). In view of the fact that none of the amino acids in the vicinity of Cys\(^{179}\) is essential for maintenance of latency, we suggest that a functional cysteine switch can be regarded as a prodomain scaffolding that positions the sulfur atom of a cysteine side chain in the immediate vicinity of the zinc ion. This model is supported by the observation that a mutant ADAM 12 prodomain that fails to repress protease activity can be rescued by substituting a cysteine residue for another amino acid in the propeptide (Table III).

The cysteine switch mechanism appears to be utilized only within the metzincin protease family. Latency in other protease families generally results from the propeptide rendering the active site sterically inaccessible to substrates (8, 31). The latency mechanism of ADAM 12, as well as of most other metzincin proteases, can be viewed as having two components: steric obstruction of the active site and coordination of the catalytic Zn\(^{2+}\) by a prodomain cysteine residue. The relative contribution of the two components to latency may vary from protease to protease and might for example explain why proMMP-3 can remain latent even after dissociation of cysteine from Zn\(^{2+}\). In the case of ADAM 12, steric obstruction by the prodomain is apparently not sufficient to inhibit the protease completely in the absence of a cysteine switch. It should be stressed, however, that the assays used in this study were carried out using α2M as the substrate, and thus there may be a partial inhibitory effect that would be revealed by repeating these assays once the natural substrate has been identified.

Chemical or genetic inactivation of the ADAM 12 cysteine switch yields a proteolytically active proform that is not only capable of cleaving an exogenous substrate, in this case α2M, but becomes autocatalytic, in which case the prodomain itself becomes a substrate. We do not know whether autolysis plays any role in the mechanism for activation of wild-type ADAM 12 proenzyme. Removal of the prodomain does not require autolysis because as we have reported previously, the 92-kDa proenzyme is processed to the 68-kDa form, even in a mutant ADAM 12 protein whose catalytic activity has been eliminated by replacing Glu\(^{351}\) with Gln (6). However, it remains to be seen whether ADAM 12 and other ADAM proteases carry out subsequent trimming of the NH\(_2\) terminus, either by autocatalysis or by intermolecular reaction. It is known that the NH\(_2\) terminal amino acids influence both activity and specificity of some members of the MMP family of proteases, so the precise cleavage position of the propeptide can be a critical determinant of protease function (7).

The cysteine switch may have evolved as a way of supplementing a latency mechanism that relied solely on tight binding of the prodomain to the protease domain. For proteases intended to be secreted in an active form, reducing the affinity of the propeptide/catalytic domain interaction would have a potential advantage in that it could allow for more efficient dissociation of the propeptide after cleavage by furin. By including a cysteine switch component in the latency mechanism, this could be achieved without sacrificing efficiency of inhibition. In this model, after cleavage of the polypeptide chain at the propeptide/catalytic domain junction, the propeptide would not have sufficient affinity for the catalytic domain to act as a competitive inhibitor of the active enzyme. But when tethered to the protease domain, before cleavage by furin, it would have sufficient affinity to position the crucial cysteine residue for coordination of the active site Zn\(^{2+}\). This cysteine switch would ensure that protease activity was fully suppressed in the endoplasmic reticulum and Golgi apparatus before the appropriate activation by furin in the trans-Golgi immediately prior to secretion.

In addition to its function of repressing activity of the metalloprotease domain, the ADAM 12 prodomain is required for secretion of the protease. Retention of ADAM 12-S protein lacking the prodomain in the lumen of the endoplasmic reticulum is not related directly to its protease activity because when COS cells synthesize the same protein with a Glu\(^{351}\) → Gln mutation to eliminate activity, this polypeptide is also retained (2). The most likely explanation for retention is that the prodomain is required for proper folding of newly synthesized ADAM 12 into an active protease. In the absence of the prodomain, the misfolded ADAM 12 polypeptide would be retained in the endoplasmic reticulum and ultimately degraded. We performed α2M trapping assays on intracellular ADAM 12 lacking the prodomain (coded for by plasmid 1229) and were unable to detect protease activity, even though the assay is sensitive enough to detect activity of intracellular ADAM 12-S proforms with a mutated cysteine switch (2). This result is not unexpected because studies on proteases from diverse families have consistently shown that the prodomain is required for folding of a polypeptide into an active protease (32–35).

An area that remains to be investigated is regulation of ADAM protease activity in the extracellular environment. The presence of specific physiological inhibitors would seem particularly important for regulation of proteases that are secreted in an active form rather than as zymogens. Based on structural homology, ADAM proteases fall into two groups. One is comprised of ADAMs 10 and 17, and the other is comprised of ADAM 12 along with nine other ADAMs whose activity has not yet been demonstrated experimentally (5, 6, 36). TIMP-3 is a candidate for regulation of ADAMs 10 and 17 in vitro because it inhibits ADAM 17 efficiently in in vitro assays (28). The failure of TIMPs-1, -2, and -3 to inhibit ADAM 12 protease in vitro raises the possibility that there is an unidentified protease-inhibitory role whose role is to regulate activity of the second group of ADAM proteases, analogous to the role of TIMPs in regulating MMPs. An alternative hypothesis is that cleavage of physiological substrates is initiated by their binding to recognition sites in the ADAM 12 disintegrin or cysteine-rich domains and that inhibition by TIMPs occurs by blocking this substrate-selection step rather than by direct inhibition of the catalytic domain. Of relevance here is the fate of ADAM prodomains after cleavage by furin. Is the role of the prodomain restricted to folding of the protease and maintenance of latency prior to cleavage by furin in the trans-Golgi? Or does the propeptide acquire a new function after secretion into the extracellular space, where it could potentially act as a competitive inhibitor of one or more ADAM proteases?

ADAM prodomains are substantially larger than MMP prodomains (179 amino acids for ADAM 12 versus 82 amino acids for MMP-3). This could be an indication that the prodomain of an ADAM protein has functions in addition to its role in folding and inhibition of the metalloprotease domain. One could envision, for example, that an ADAM prodomain is responsible for regulating the activity of the disintegrin-like and cysteine-rich domains by blocking their binding sites for potential ligands until the protein is secreted. Further studies will be needed to test this possibility.

In conclusion, we have shown that the prodomain of human ADAM 12 modulates the activity of the metalloprotease domain by means of a cysteine switch. The results of this study will be of use in future investigations of latency in both ADAMs and MMPs, ultimately leading to a better understanding of the biological relevance of such molecular mechanisms.

\(^2\) F. Loechel and U. M. Wewer, unpublished observations.
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