Proteolytic Processing of Mullerian Inhibiting Substance Produces a Transforming Growth Factor-β-like Fragment*

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Mullerian inhibiting substance (MIS) is a differentiation factor that causes the Mullerian duct to regress during the development of the male reproductive tract. The active form is a disulfide-linked dimer consisting of two identical 70-kDa subunits. Recently, the amino acid sequence for MIS was deduced from its gene sequence and revealed that the carboxyl-terminal region shares homology with transforming growth factor (TGF)-β. Since TGF-β is produced as a large latent precursor that requires proteolytic activation for activity, we sought to determine if MIS might undergo a similar processing event. Here we demonstrate that typically 5 to 20% of the protein in MIS preparations is cleaved at a site 109 amino acids from the carboxyl terminus. Concurrent cleavages from both chains of the MIS dimer produces a 25-kDa TGF-β-like fragment and a high molecular mass complex derived from the amino terminus of the protein. Although the two fragments are noncovalently linked, they remain tightly associated after cleavage, and thus are structurally organized like TGF-β within its precursor. The same cleavage products also can be generated by limited proteolysis with plasmin, which provides a simple method for converting the entire preparation into the cleaved form. The plasmin-digested MIS is fully active in the organ culture assay.

During the development of the male and female sex organs, the Mullerian duct gives rise to the uterus, vagina, and fallopian tubes, and the Wolffian duct gives rise to the epididymis, vas deferens, and seminal vesicles. Both ducts develop in early embryonic stages with the regression of one or the other duct being a key aspect in sexual development. Wolffian duct regression occurs passively due to lack of testosterone in the female, while regression of the Mullerian duct is an active process controlled by a testicular factor termed MIS. While most TGF-β-like proteins also require proteolytic activation (15), MIS was considered to be an exception because the purified protein was full length and functional in the organ culture assay. During isolation of the recombinant protein, we observed that a fraction of the preparation was cleaved. When processing occurred, both arms of the MIS dimer were cleaved generating a 110-kDa fragment and a 25-kDa fragment. To better understand this event, we have studied the processing in vitro and characterized the fragments by protein sequencing. Cleavage occurs at a mono-basic site which is located at roughly the same position as the cleavage site in TGF-β. Like TGF-β, the MIS cleavage products are tightly associated in a noncovalent complex, but can be separated with boiling or by acidification. While the mechanism through which MIS causes regression is unclear, the similarity in biochemical properties of MIS and the TGF-β precursor suggests that MIS may require proteolytic processing for activity.

MATERIALS AND METHODS

Purification of MIS—CHO cells transfected with the human MIS gene were grown at 37°C in a minimum essential medium without ribonucleosides and deoxyribonucleosides, which was supplemented with 10% fetal bovine serum. The conditioned medium was clarified by filtration and concentrated 15-fold by ultrafiltration. MIS was purified from the concentrate by affinity chromatography using a monoclonal antibody that was raised against the recombinant protein. MIS was eluted from the affinity matrix with 2 M NaSCN, 150 mM
NaCl, 15 mM sodium phosphate, pH 6.3, essentially as described previously (16). The chaotrope was removed and replaced with MIS storage buffer (10% glucose, 300 mM NaCl, 10 mM HEPES, pH 7.5) in a P-6DG desalting column (Bio-Rad). The final preparation was aliquoted and stored at -70°C. MIS was metabolically labeled with [35S]cysteine by pulse-labeling the transfected CHO cells for 30 min with [35S]cysteine (approximately 600 Ci/mmol) in cysteine-free minimum essential medium containing 500 μCi/ml of the radioactive amino acid and then chasing the label for 20 h with complete growth medium. The recombinant protein was purified from the labeling medium by immune precipitation using the monoclonal antibody and eluted from the immune complex with nonreducing electrophoresis sample buffer. After releasing the MIS with nonreducing buffer, a portion of the sample was reduced with 2% 2-mercaptoethanol.

Limited Digestion of MIS with Plasmin—Purified MIS was diluted 1:1 with water and incubated at room temperature with plasmin (Sigma) at a constant MIS to plasmin ratio of 25:1 (w/w). Test samples were incubated for varying lengths of time as noted in the individual figure legends. Preparative samples routinely were digested for 1 h. For gel analysis, the proteolysis was stopped by diluting samples with electrophoresis sample buffer and heating the preparations at 65°C for 10 min. Cleavage products were analyzed by SDS-PAGE on 12% polyacrylamide gels using the Laemmli system (17) and stained with Coomassie Brilliant Blue. Reactions for organ culture assays were quenched by adding fetal bovine serum to 10%; and for gel filtration, by adding acetic acid to 1 M. The organ culture assay was run as previously described (2).

Protein Sequence Analysis—Samples containing either 0.5 nmol of intact protein or 0.25 nmol of the plasmid-derived fragments were subjected to automated Edman degradation in an Applied Biosystems ABI 120A phenylthiohydantoin analyzer. The amino acid compositions of parallel aliquots were determined in a Beckman System 6300 amino acid analyzer.

RESULTS AND DISCUSSION

**Plasmin Digestion of MIS**—Recombinant human MIS was purified by immunoaffinity chromatography from conditioned medium of CHO cells that were transfected with the human gene. On SDS-polyacrylamide gels, the affinity-purified protein migrated under reducing conditions with an apparent mass of 70 kDa (see Fig. 1, lane d). Under nonreducing conditions, the protein migrated with an apparent mass of 140 kDa (see Fig. 1, lane g); however, in addition to dimeric MIS, other high molecular weight disulfide-linked aggregates routinely were observed. These larger forms also were obtained with radioactive MIS which was pulse-labeled with [35S]cysteine and analyzed directly by SDS-PAGE and with preparations that were disrupted with SDS in the presence of 50 mM iodoacetic acid, indicating that the oligomers were not generated by sample preparation. Other groups observed similar high molecular weight forms in natural MIS preparations as well (16, 20). While the organization of disulfide bonds in MIS has not been determined, the presence of the oligomers suggests that one or more cysteines have free sulfhydryl groups. The amino-terminal sequence of the purified protein was Leu-Arg-Ala-Glu-Glu-Pro-Ala-Val-Gly-Thr, which is consistent with the amino terminus of mature MIS.

In addition to the 70-kDa MIS, a fraction of the purified protein exists as a 57-kDa form. The amount of the 57-kDa MIS ranges from 5 to 20% of the total protein. While this form is barely visible in the affinity-purified MIS shown in Fig. 1, lane d, by using metabolically labeled MIS and slightly overexposing the fluorograph, the 57-kDa form is a prominent band on the gel (see Fig. 1, lane a). In addition to the 57-kDa band, a small radioactive band with an apparent mass of 12 kDa was observed, suggesting that the 57- and 12-kDa products may be generated by proteolysis. The 57-kDa form also is present in natural MIS preparations (16, 20), but because it was such a minor component of the preparation, the potential significance of the smaller band was not recognized. Under nonreducing conditions (lane b), the 12-kDa fragment migrates on SDS gels as a dimer of 25 kDa and the 57-kDa band as an oligomer that is obscured by the intact MIS complex. Amino-terminal sequence analysis of the 25-kDa fragment revealed that it was from the carboxyl terminus of MIS. The small fragment is particularly prominent in the cysteine-labeled MIS since this region contains 7 of the 12 cysteines in MIS.

When purified MIS was incubated with plasmin for 1 h at room temperature, the protein was cleaved into a 57-kDa fragment.
fragment and a 12-kDa fragment. The sizes of the fragments and subsequent sequencing of the cleavage products revealed that they were the same fragments we observed as minor components in the purified MIS preparations. Lanes e and h of Fig. 1 show Coomassie Blue-stained gel profiles of plasmin-cleaved MIS under reducing and nonreducing conditions. In the absence of reducing agent, the 12-kDa fragment migrates as a 25-kDa homodimer and the 57-kDa, as a 180-kDa trimer. Both full length and plasmin-digested MIS caused regression of the Mullerian duct in the organ culture assay. The specific activity of the MIS was unaffected by the cleavage. Fig. 2 shows a time course of the cleavage. Within the 2-h incubation period, only the 57- and 12-kDa products were generated.

With longer incubation times or with higher plasmin concentrations, an additional cleavage within the 57-kDa fragment was observed, producing a 33- and a 24-kDa piece. MIS also could be selectively cleaved with trypsin to generate the 57- and 12-kDa fragments, but the reaction was less controlled (data not shown). With trypsin, both fragments were susceptible to further digestion.

Purification and Properties of the Carboxyl-terminal Fragment—While digestion by plasmin resulted in quantitative cleavage of the MIS, the fragments remained associated as a noncovalent complex. Attempts to release the fragments with chaotropes such as 2 M NaSCN or with nonionic detergents failed (data not shown). However, the fragments could be separated by acidifying the sample with acetic acid or after boiling, which are conditions used to separate TGF-β from its latent complex. In 1 M acetic acid, both MIS fragments were soluble and could be separated by gel filtration chromatography in acetic acid. Fig. 3 shows results from gel filtration of plasmin-cleaved MIS after acidification. The two fragments were completely resolved. After removal of the acetic acid by lyophilization, we found that the fragments were soluble in 4 mM HCl as well as in phosphate-buffered saline at neutral pH. After boiling plasmin-cleaved MIS, the small fragment remained soluble, while most of the large fragment precipitated and could be removed by centrifugation. Although TGF-β activity is stable to acid treatment or boiling, these conditions destroyed MIS activity as assessed by the organ culture assay. The two fragments can be dissociated with 1% sodium deoxycholate without altering activity in the organ culture assay, which suggests that the other treatments simply are too harsh for MIS.

The 57- and 12-kDa fragments both were subjected to protein sequencing. The sequence of the large fragment was Leu-Arg-Ala-Glu-Glu-Pro-Ala-Val-Gly-Thr, indicating that
it was derived from the amino terminus of MIS, while the sequence of the smaller fragment was Ser-Ala-Gly-Ala-Thr-Ala-Ala-Asp-Gly-Pro, indicating that it was derived from near the carboxyl terminus. Although the sequence surrounding the plasmin cleavage site differs from the TGF-β activation site, both proteins have basic residues upstream of the cleavage site, which presumably are important for signaling the processing. Other TGF-β-like proteins also share this structural feature (13). The pattern of amino acids surrounding the MIS site, in particular the presence of arginines at positions -1 and -4, conforms with the monobasic sequence which has been proposed as a signal for processing of certain prohormones (19). The presence of alanine at position 2 is also consistent with the model. The 57- and 12-kDa fragments from a preparation of affinity-purified MIS were isolated by preparative SDS-PAGE and subjected to amino-terminal sequencing. The observed sequences were identical with those generated from plasmin-treated MIS. Although the source of the protease is conditioned medium responsible for the processing is unknown, the sequencing results indicate that it and plasmin cleave MIS at the same site.

While the size of the carboxyl-terminal fragment suggested that no further processing had occurred, as a further measure of its integrity we subjected the fragment to amino acid analysis. Table I shows theoretical and observed amino acid compositions for the fragment. The two compositions show a good correlation. In particular, the presence of 9 arginines (since arginine is the last amino acid in the fragment) supports that no other processing has occurred. For comparison, the theoretical and observed compositions for intact MIS are shown, which also show a good correlation.

Fig. 4 shows a schematic summary of the events used to relese the MIS cleavage products. As with TGF-β, the dissociation is a two-step process involving first proteolysis of the two arms of the MIS dimer and subsequent separation of the fragments. While proteolysis of the 70-kDa form of MIS alone does not alter its activity in the organ culture assay, attempts to dissociate the fragments with acid or by boiling, which routinely are used for TGF-β, destroyed MIS activity. Whether this loss of activity reflects that MIS requires both fragments for activity and thus is quite distinct from other TGF-β-like proteins or whether the dissociation treatments are simply too harsh for MIS requires further investigation. The striking structural similarity between MIS and the TGF-β precursor both in the conservation of the processing site and conservation of the amino- and carboxyl-terminal regions as separate domains identify additional features of MIS which are likely to be relevant for its activity.

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REFERENCES