Communication

Mutation of Cys\textsuperscript{672} Allows Recombinant Expression of Activatable Macrophage-stimulating Protein*

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We readily produced recombinant pro-macrophage stimulating protein in a mammalian expression system, but it was only weakly active after proteolytic activation. Active macrophage stimulating protein is a disulfide-bonded heterodimer, but in our hands, the subunits of recombinant macrophage stimulating protein were mostly not disulfide bonded. Molecular modeling of the serine proteinase domain of macrophage stimulating protein based on homology to human trypsin suggested that macrophage stimulating protein, but not plasminogen or hepatocyte growth factor, has a Cys residue (672) in close proximity to the Cys residue (578) that forms the intersubunit disulfide link with the other subunit. We hypotho€ized that Cys\textsuperscript{672} might interfere with intersubunit disulfide formation by forming an intrasubunit disulfide with Cys\textsuperscript{578} and therefore mutated Cys\textsuperscript{672} to Ala. After kallikrein activation, the subunits of Cys\textsuperscript{672}→Ala macrophage stimulating protein were fully disulfide linked, and the mutant macrophage stimulating protein had 10–20-fold higher specific activity than the wild type recombinant macrophage stimulating protein.

Macrophage-stimulating protein (MSP)\textsuperscript{1} was originally purified from human serum as a protein that enhances the chemotactic response of murine peritoneal macrophages to the C5a fraction of complement (1). In a screen for proteins that contain kringle domains, Degen and co-workers (2) identified a cDNA that was most closely related to hepatocyte growth factor (HGF) and hence named it HGF-like (HGF-L). MSP and HGF-L were later shown to be the same molecule (3). MSP requires a steady supply of MSP, we began producing recombinant MSP. Our initial attempts to produce recombinant MSP generated preparations with a dramatically reduced specific activity (~50-fold) as compared with material that was purified from a nonrecombinant source. The following studies were undertaken in attempts to determine the underlying mechanism for the reduced activity and to investigate how the activity might be restored to levels observed with naturally produced protein.

MATERIALS AND METHODS

Construction of MSP cDNAs—The human MSP cDNA (GenBank accession number L11924 (11)) was a gift of Professor Sandra Degen, University of Cincinnati. The Cys\textsuperscript{672}→Ala MSP mutant was constructed by polymerase chain reaction from the MSP cDNA with a mutant primer containing a change from TG to GC at nucleotides 2024 and 2025 (5′-CACACCGCTCTGG CCTCCTGGAAAG-3′) and a downstream primer (5′-CTGGCACTAGAAGGCACAGTG-3′) complementary to vector pCDNA3 (Invitrogen, San Diego, CA). All constructs were verified by DNA sequencing.

Expression of MSP—Chinese hamster ovary (CHO) cells deficient in dihydrofolate reductase activity (CHO\textsuperscript{Dhfr}) (Ref. 12) were transfected with calcium phosphate using pBSro2-derived expression vectors (13) containing the wild type human MSP or mutant human MSP cDNAs. Transfected colonies were cloned and analyzed by Western blot using an antibody directed against Escherichia coli derived human MSP. High expressing clones for wild type MSP and mutant MSP were chosen for further analysis. CHO\textsuperscript{Dhfr} cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum, nonessential amino acids, and glutamine/thymidine. Transfected clones were grown in similar medium using 5% dialyzed fetal bovine serum and lacking hypoxanthine/thymidine. Large scale generation of conditioned medium was accomplished by seeding transfected CHO\textsuperscript{Dhfr} clones into roller bottles containing 50% DMEM, 50% Ham's F-12 medium supplemented with 5% dialyzed fetal bovine serum, nonessential amino acids, and glutamine. At 70% confluence, cells were rinsed with phosphate-buffered saline and were used to condition medium, as above, but lacking serum, for 5 days.

Purification, Activation, and Sequencing of MSP—Conditioned medium, with or without concentration by diafiltration and without salt or pH adjustment, was chromatographed by absorption onto heparin-Sepharose (Pharmacia Biotech, Inc.) and eluted with a salt gradient in 20 mM sodium phosphate, pH 7. MSP-containing fractions were identified by Western blotting with rabbit anti-murine proMSP serum. MSP eluted at 0.4 M NaCl. Pooled fractions were dialyzed with 0.02 M Tris, pH 8.5, chromatographed by absorption on Q Sepharose HP (Pharmacia Biotech, Inc.), and eluted with a salt gradient in 0.02 M Tris, pH 8.5. MSP eluted at 0.1 M salt. MSP was activated either by incubation at 37 °C for 1 h with 10 μg/ml human kallikrein (Enzyme Research Laboratories, Inc., South Bend, IN), followed by addition of 1 mM Pefabloc (Boehringer Mannheim), or by passing MSP through a column of kal-

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1 The abbreviations used are: MSP, macrophage-stimulating protein; SPD, serine proteinase domain; HGF, hepatocyte growth factor; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis.

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likrein coupled to cyanogen bromide activated-Sepharose (Pharmacia Biotech, Inc.) at a concentration of 1 mg/ml. Activated samples were dialyzed versus phosphate-buffered saline. N-terminal sequencing was performed on an Immobilon-P® (Millipore, Bedford, MA) blot using a Procise 494 sequencer (PE-ABD, Foster City, CA).

**RESULTS**

We expressed human proMSP in CHO cells and activated the material in vitro with kallikrein. The recombinant material had less than 10% of the specific activity of an MSP sample purified from bovine serum, as tested in a murine colonic crypt attachment bioassay. Furthermore, active MSP from human plasma is reported to be a disulfide-linked heterodimer, but after in vitro kallikrein activation, the 80-kDa tertiary structure does not remain intact. Subunits of our recombinant MSP were mostly not disulfide linked, as judged by SDS-PAGE performed under nonreducing conditions (Fig. 1, lane 4). Incorrect proteolytic processing did not seem to account for this defect, because the existence of the expected N-terminal sequence of the β subunit, VVGGHP, was confirmed by Edman N-terminal sequencing.

The lack of a disulfide bond between the subunits of recombinant MSP suggested that the Cys content of MSP should be examined for a possible cause of this defect and the low activity of our recombinant preparation. The domain and disulfide structures of murine and human plasminogen-related growth factors were first studied with respect to the kringle domains (15). Later the sequences of MSP from chicken (16) and MSP from rat (17) became available. These sequences confirmed that the domain and disulfide structure is highly homologous to that of plasminogen. From the N terminus, the domain structure of plasminogen may be summarized to contain a secretion signal peptide, an N-terminal “hairpin” domain, five kringle domains, and the SPD. The domain structures of MSP and HGF are very similar to that of plasminogen. The main difference is that MSP and HGF have only four kringles. The disulfide structure of plasminogen contains intra- and interdomain disulfide bonds. The intradomain disulfide bonds may be listed as follows: the hairpin domain contains two disulfides, each kringle contains three disulfides, and the SPD contains four disulfides. There is an interdomain disulfide between the second and third kringle, and two disulfides between the α and β subunits because the size-condensing effect of disulfide bonds in nonreduced samples leads to faster migration than reduced samples. Lanes 1 and 5 show single chain proMSP as it is originally purified. Lanes 2 and 6 show the generation of α and β subunits after kallikrein treatment. Lane 4 shows that the disulfide-bonded α/β heterodimer is largely absent in the kallikrein-treated wild type recombinant MSP. In contrast, the subunits of kallikrein-treated Cys672 → Ala mutant MSP are still linked (lane 8).

**FIG. 1. Analysis of MSP by SDS-PAGE.** Wild type human MSP (lanes 1–4, equal amounts of protein were loaded per lane within this group) or Cys672 → Ala mutant human MSP (lanes 5–8, equal amounts of protein were loaded per lane within this group) were analyzed by 10% SDS-PAGE with colloidal Coomassie Blue staining (Novex, San Diego, CA). Dual markers indicate the migration of proMSP, α, or β subunits because the size-condensing effect of disulfide bonds in nonreduced samples leads to faster migration than reduced samples. Lanes 1 and 5 show single chain proMSP as it is originally purified. Lanes 2 and 6 show the generation of α and β subunits after kallikrein treatment. Lane 4 shows that the disulfide-bonded α/β heterodimer is largely absent in the kallikrein-treated wild type recombinant MSP. In contrast, the subunits of kallikrein-treated Cys672 → Ala mutant MSP are still linked (lane 8).

**FIG. 2. Domain and disulfide structure of MSP and plasminogen.** The MSP structure is deduced by homology to plasminogen. Dotted line, cystine; asterisk, activation site.

**FIG. 3. Sequence alignment of the SPDs of human MSP, human plasminogen, and human trypsin.** Numbers above the sequences refer to MSP, and numbering below the sequences refer to trypsin. Cys residues are shaded. Disulfide bonds that are present in plasminogen are drawn as lines.
subunit and the SPD. According to conserved Cys content, every disulfide that is present in plasminogen is also present in HGF and MSP, with one exception: HGF and MSP have only a single disulfide between the α subunit and the SPD (15). HGF from chicken, mouse, and human contain no Cys other than those that are present at positions that are homologous to plasminogen (16). MSP, however contains extra Cys, some of which are conserved in chicken, mouse, rat, and human (16, 17). Thus, murine MSP has a Cys residue in the signal sequence, and murine and human MSP contain an extra Cys in the hairpin domain. MSP from all four species contain three extra conserved Cys in the SPD compared with both plasminogen and HGF (Figs. 2 and 3). Because HGF does not contain extra Cys compared with plasminogen and recombinant HGF of high specific activity is available commercially, we considered whether the extra conserved Cys residues of MSP caused the disulfide bonding defect and low activity that we observed with our recombinant MSP preparation.

Although high resolution structural information is not available for plasminogen, MSP, or HGF, this information is available for other serine proteinases, such as trypsin (18). Fig. 3 shows a sequence alignment of the SPDs of MSP, plasminogen, and mature human trypsin. The trypsin residues that were selected as homologous to the extra Cys of the MSP SPD are numbered3 in Fig. 3 and labeled in Fig. 4. Trypsin has ten Cys residues that form five disulfide bonds, of which four have homologs in MSP (and plasminogen). We used the structure of diisopropylfluorophosphate-inhibited human trypsin described in the Brookhaven file, 1trn, for our modeling. As can be seen in Fig. 4, Lys66 and Arg95 are located on the surface of trypsin on the opposite side of the protein from the Ser127, the homolog of the Cys that forms the intersubunit disulfide. Gln209 and Ser127 are located on the surface in very close proximity. The distance between the α carbons of these residues is 0.61 nm. For comparison, the distances between the α carbons of the disulfide-bonded Cys residues of trypsin range from 0.42 to 0.62 nm. Thus, the inferred close proximity of Cys672 to Cys588 suggests that intrasubunit disulfide formation between these two Cys residues might interfere with intersubunit disulfide formation by Cys588. It should be noted that Cys672 apparently has no Cys residue other than Cys588 with which to interact.

Cys672 of human MSP was mutated to Ala to determine if this would eliminate the proposed intrasubunit disulfide bond. The mutated MSP was expressed, purified, and activated by kallikrein. The Cys672 → Ala mutant MSP has subunits that
are fully disulfide linked (Fig. 1, lane 8) and has significantly more activity than the nonmutant MSP, as judged in a tritiated thymidine uptake assay in NIH 3T3 cells that are expressing transfected murine stk gene (Fig. 5).

DISCUSSION

Wang et al. have reported production of recombinant kalikrein-activated MSP whose activity is comparable with that of serum MSP (10, 19). However, these studies did not address the occurrence of nondisulfide bonded (inactive) material because the recombinant MSP was not analyzed by nonreduced SDS-PAGE.

The Cys residue corresponding to human Cys\textsuperscript{672} is conserved in every species from which MSP has been cloned, and this deserves comment for two reasons. Firstly, the presence of Cys\textsuperscript{672} may result in low specific activity material when MSP from these species is produced recombinantly. Murine recombinant MSP also has the disulfide bonding defect that is described for human MSP in this paper and that is corrected by an analogous Cys to Ala mutation.\textsuperscript{4} Although chicken MSP has been cloned (16), there are no published reports on the recombinant expression of chicken MSP. This may be due, in part, to the complication of low activity described here for recombinant human or murine MSP. Secondly, the conservation of Cys\textsuperscript{672} suggests that the phenomenon described here may have a biological role in the regulation of MSP activity in vivo. In other words, circulating natural proMSP may mimic recombinant MSP by lacking the disulfide bond between the nascent subunits, and activation of proMSP in vivo may require both pro-

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