Promacrophage-stimulating protein (MSP) is an 80-kDa protein that acquires biological activity after cleavage at an Arg-Val bond to a disulfide-linked aβ heterodimer by serine proteases of the intrinsic coagulation cascade. These proteases, which include serum kallikrein, factor XIIa and factor XIa, are members of the trypsin family of serine proteases. We now report that two other members of the family, nerve growth factor-γ (NGF-γ) and epidermal growth factor-binding protein (EGF-BP), cleave and activate pro-MSP to the disulfide-linked aβ heterodimer. Cleavage of 1.5 nM pro-MSP by 1 nM NGF-γ or EGF-BP at 37 °C was almost complete within 30 min. These concentrations of enzyme are about 2 orders of magnitude less than is required for cleavage by serum kallikrein or factor XIIa. Cleavage of pro-MSP to MSP was associated with a conformational change in the protein, because the cleaved product, but not pro-MSP, was detected by a sandwich enzyme-linked immunosay. Cleavage caused the appearance of biological activity, as measured by chemotactic activity of MSP for resident peritoneal macrophages, by MSP-induced macrophage shape change, and by stimulation of macrophage ingestion of C3bi-coated erythrocytes. These findings suggest the possibility of cooperative interactions between NGF-γ or EGF-BP and pro-MSP in inflammation and wound healing.
a possible role for NGF-γ and EGF-BP as pro-MSP convertases in vitro.

EXPERIMENTAL PROCEDURES

Reagents—Human plasma MSP was purified as previously described (3). NGF-γ and EGF-BP were prepared according to the method of Darling and Shooter (13). The concentration of NGF-γ and EGF-BP was active-site-titrated with NPNGB by the method of Chase and Shaw (14). Purified human serum kallikrein was from Enzyme Research Laboratories, Inc. (South Bend, IN). Human plasma C1-INH and PPACK were from Calbiochem. Cystine-free Dulbecco’s modified Eagle’s medium was from Speciality Media, Inc. (Lavellette, NJ). RPMI 1640 medium was from Life Technologies, Inc. C1-INH (600 C1/mm) was from Amerham Corp. Protein G-Sepharose was from Pharmacia LKB Biotechnology Inc. Enlightening was from DuPont NEN. Goat anti-rabbit IgG conjugated with alkaline phosphatase was from Sigma. A monoclonal expression vector pcDNA-3, transfection of CHO cells, and cloning of the antibody which recognizes MSP but not pro-MSP, was used in sandwich ELISA to study the conformational change in pro-MSP after cleavage.

Expression of Pro-MSP in CHO Cells—Ligation of a human MSP cDNA with pcDNA-3, transfection of CHO cells, and cloning of the MSP-producing cell CHO-MSP18 were as described (9). Pro-MSP was prepared from cultures of CHO-MSP18 cells in serum-free medium and cysteine-free Dulbecco’s modified Eagle’s medium with 100 μCi/ml of [35S]cysteine for 56 h at 37°C. Culture fluids were harvested and used as a source of pro-MSP. The concentration of pro-MSP was measured with a sandwich ELISA after treatment with serum kallikrein (9).

Cleavage of Pro-MSP in Vitro—Culture fluid 50-μl aliquots, with a concentration of 150 ng/ml of [35S]cysteine-labeled MSP, were incubated for 30 min at 37°C in a water bath with NGF-γ, EGF-BP, or serum kallikrein in 50 μl of Tris buffer, pH 7.6, containing 0.1% NaCl. To test the effect of C1-INH on cleavage of pro-MSP by serum kallikrein or NGF-γ, C1-INH at different concentrations was mixed with kallikrein or NGF-γ at 37°C for 20 min, after which pro-MSP was added for another 30 min of incubation. After treatment, the radiolabeled products were immunoprecipitated, boiled in sample buffer containing 2-mercaptoethanol, run in SDS-polyacrylamide gel electrophoresis, and autoradiographed as previously described (9).

Assay for Macrophage Shape Change—The assay was performed as previously described (9). Briefly, the bottom wells of a multi-well chemotaxis chamber were filled with purified MSP or samples to be tested and then covered with a 10-μm thick polycarbonate membrane with 5-μm holes. Upper wells were filled with 50 μl of macrophage suspensions. During the incubation period of 3 h at 37°C, macrophages migrated through the membrane and remained attached to the antiserum side of the membrane. After air drying and staining with Diff-Quik, migrated cells were counted with an image analyzer. MSP or samples were assayed in duplicate. The results were expressed as the percentage of macrophages with extended morphology.

Cleavage Chemotaxis Assay—The assay was conducted as previously described (9). Briefly, the bottom wells of a multi-well chemotaxis chamber were filled with purified MSP or samples to be tested and then covered with a 10-μm thick polycarbonate membrane with 5-μm holes. Upper wells were filled with 50 μl of macrophage suspensions. During the incubation period of 3 h at 37°C, macrophages migrated through the membrane and remained attached to the antiserum side of the membrane. After air drying and staining with Diff-Quik, migrated cells were counted with an image analyzer. MSP or samples were assayed in duplicate. The results were expressed as the percentage of input macrophages that migrated.

Assay for Macrophage Phagocytosis of EC3bi—The assay was performed as described previously (3) with slight modifications. Briefly, resident macrophages at a concentration of 8 × 106/ml in Dulbecco’s modified Eagle’s medium were cultured in a 24-well culture plate for 45 min at 37°C. After the wells were washed twice to remove nonadherent cells, 0.5 ml of EC3bi were added to each well. The ratio of erythrocytes to macrophages was 5:1. After incubation for 30 min to allow erythrocyte binding to macrophages, MSP or tested samples were added to wells. After incubation for 15 min, nonadherent erythrocytes were washed away, and 0.5 ml of ammonium chloride lysed buffer was added. The buffer was removed after 2.5 min, and the monolayer was stained with Diff-Quik. The percentage of macrophages with ingested erythrocytes was determined by examination of the monolayer with a 100 x oil immersion objective.

Sandwich ELISA for MSP—The ELISA was previously described (15). It used monoclonal antibody anti-MSP 25 as capture antibody and polyclonal rabbit IgG anti-MSP as detection antibody. Although the polyclonal rabbit anti-MSP detects pro-MSP by immunoprecipitation, the sandwich ELISA does not detect pro-MSP, apparently because the epitope with which monoclonal antibody anti-MSP 25 reacts is not accessible.

RESULTS

Cleavage of Pro-MSP by NGF-γ and EGF-BP—We previously reported that pro-MSP could be cleaved by three enzymes that initiate the intrinsic coagulation cascade, serum kallikrein, factor XIIa, and factor Xla (9). Because NGF-γ and EGF-BP belong to the family of glandular kallikreins (10), we tested the capacity of these proteases to cleave pro-MSP. Fig. 1 shows that NGF-γ and EGF-BP completely cleaved the single-chain form of pro-MSP into the α- and β-chains that are characteristic of mature MSP. Cleavage by 100 μg serum kallikrein is shown for comparison. PPACK, an irreversible inhibitor of thrombin, blocked the effects of all three enzymes on cleavage of pro-MSP. Concentration-dependent cleavage of 1.5 μg pro-MSP by NGF-γ and EGF-BP is shown in Fig. 2. Complete cleavage of pro-MSP within 30 min occurred in 1 ng NGF-γ or EGF-BP. Comparison of results with enzyme concentrations of 0.1 μg shows that NGF-γ was slightly more efficient than EGF-BP. In contrast to these glandular kallikreins, we previously showed that 100 μg serum kallikrein was required for complete cleavage of pro-MSP (9).

Time Course of Conversion of Pro-MSP by NGF-γ—Pro-MSP at a concentration of 1.5 μg was incubated with 2 μg NGF-γ at 37°C for different time intervals. Fig. 3 shows about 12% cleavage of pro-MSP as early as 1 min, 60% at 5 min, and 100% at 60 min. A trace of a 48-kDa digested band was seen at 60 min. Traces amount of this band could also be seen after digestion with high concentrations of serum kallikrein or EGF-BP. The band apparently represents the α-chain after cleavage of a short peptide, possibly near the C terminus at residue 471.

Cleavage-dependent Pro-MSP Conformational Change—For
Conversion of pro-MSP by NGF-γ and EGF-BP

quantifying serum MSP concentrations, we previously developed a sandwich ELISA that used a mouse monoclonal anti-MSP capture antibody and a rabbit polyclonal anti-MSP detection antibody (4). As shown in Table I, pro-MSP was not detected by this ELISA, whereas the protein was detected after proteolytic conversion to the mature αβ heterodimer by 50 nm serum kallikrein. Proteolytic conversion of the same concentration of pro-MSP by NGF-γ or EGF-BP resulted in ELISA values comparable with that of kallikrein-digested pro-MSP. Partial digestion of pro-MSP with 10 nm serum kallikrein (resulting in a mixture of pro-MSP and MSP as monitored by immunoprecipitation) was reflected in lower ELISA values. These results indicate that cleavage of pro-MSP by each of these three enzymes was associated with a conformational change that exposed an epitope recognized by the monoclonal capture antibody.

Effect of C1-INH on NGF-γ-mediated Cleavage of Pro-MSP—It was reported that NGF can substitute for the activated first component of complement (C1), in that it can proteolytically activate C4 and C2; like C1, it is inhibited by C1 inhibitor, a protein that blocks enzymatic activity of C1 and serum kallikrein (16). We determined if pro-MSP cleavage activity of NGF-γ could be regulated by C1-INH. Fig. 4 shows that C1-INH inhibited the capacity of NGF-γ to cleave pro-MSP. However, 1 unit/ml C1-INH only partially counteracted 1 nm NGF-γ, and no inhibition was seen at C1-INH concentrations below 0.2 unit/ml (lane 8). In contrast, 0.02 unit/ml C1-INH totally blocked the enzymatic activity of 50 nm serum kallikrein (lane 4).

Cleavage of Pro-MSP by NGF-γ or EGF-BP Produces Biologically Active MSP—To test whether NGF-γ and EGF-BP cleavage generates biologically active MSP, samples were tested for induction of macrophage shape change and phagocytosis of C3bi-coated erythrocytes. As shown in Table II, pro-MSP from CHO-MSP18 cell cultures was inactive in both assays. After incubation of pro-MSP with NGF-γ or EGF-BP, the product induced macrophage shape change and stimulated phagocyto-

FIG. 2. Concentration-dependent cleavage of pro-MSP by NGF-γ and EGF-BP. NGF-γ or EGF-BP at different concentrations was incubated with 1.5 nm radiolabeled pro-MSP at 37 °C for 30 min, followed by the addition of 20 μM PPACK to stop the reaction. A, pro-MSP treated with NGF-γ; B, pro-MSP treated with EGF-BP. Lane 1, normal rabbit IgG control; lane 2, pro-MSP control.

FIG. 3. Time course of pro-MSP cleavage by NGF-γ. [35S]Pro-MSP at a concentration of 1.5 nm was incubated with 2 nm NGF-γ at 37 °C. The reaction was stopped by the addition of PPACK (20 μM) at different times. Reaction mixtures were analyzed by immunoprecipitation, as shown in panel A. Lane 1 is the normal rabbit IgG control. To determine the percentage conversion of pro-MSP, the autoradiograph was scanned with a densitometer and analyzed with the Scan XL software package (panel B).

| Enzyme treatment of pro-MSP | Detected MSP (pm) |
|----------------------------|------------------|
| None                       | 10 ± 10          |
| Kallikrein, 10 nm           | 160 ± 14         |
| Kallikrein, 50 nm           | 810 ± 52         |
| NGF-γ, 5 nm                 | 840 ± 12         |
| EGF-BP, 5 nm                | 990 ± 10         |

We previously reported that pro-MSP is cleaved by enzymes of the intrinsic coagulation cascade, including serum kal-

sis of EC3bi. Biological activity was comparable with that of plasma MSP and to pro-MSP cleaved by serum kallikrein. Fig. 5 shows that pro-MSP acquired chemotactic activity for macrophages after incubation with either NGF-γ or EGF-BP. The activity was comparable with that of MSP purified from human plasma.

DISCUSSION

We previously reported that pro-MSP is cleaved by enzymes of the intrinsic coagulation cascade, including serum kal-
tions of C1-INH at 37 °C. After 30 min of incubation, 1.5 nsf pro-MSP immunoprecipitation was rabbit anti-MSP, except for normal IgG in lane 1, Lanes 1 and 2, pro-MSP. Pro-MSP with kallikrein (lane 3) or kallikrein plus C1-INH (lane 4). Lanes 5–10, pro-MSP plus NGF-γ preequilibrated with C1-INH concentrations of 1, 0.2, 0.04, 0.008, 0.0016, and 0.0 unit/ml.

NGF-γ at was added, followed by incubation for another 30 min. Pro-MSP treated with serum kallikrein (50 nM) or serum kallikrein plus C1-INH (0.02 unit/ml) was used as a positive control for C1-INH action. IgG for immunoprecipitation was rabbit anti-MSP, except for normal IgG in lane 1, Lanes 1 and 2, pro-MSP. Pro-MSP with kallikrein (lane 3) or kallikrein plus C1-INH (lane 4). Lanes 5–10, pro-MSP plus NGF-γ preequilibrated with C1-INH concentrations of 1, 0.2, 0.04, 0.008, 0.0016, and 0.0 unit/ml.

**TABLE II**

Effects of pro-MSP on resident macrophage shape change and phagocytosis of EC3b1 after treatment with NGF-γ, EGF-BP, or serum kallikrein

| Stimulus                             | Cell spreading | Phagocytosis |
|--------------------------------------|----------------|--------------|
| Medium                               | 13 ± 2         | 2 ± 1        |
| MSP                                  | 65 ± 3         | 12 ± 1       |
| Pro-MSP                              | 19 ± 4         | 2 ± 0        |
| Pro-MSP + NGF-γ                      | 71 ± 5         | 20 ± 2       |
| NGF-γ                                | 18 ± 2         | 1 ± 1        |
| Pro-MSP + EGF-BP                     | 73 ± 6         | ND           |
| EGF-BP                               | 14 ± 2         | ND           |
| Pro-MSP + kallikrein                 | 63 ± 2         | ND           |
| Kallikrein                           | 21 ± 1         | ND           |

Pro-MSP (1 nsf) was incubated for 30 min at 37 °C with NGF-γ (5 nsf), EGF-BP (5 nsf), serum kallikrein (50 nsf), or no enzyme. After incubation, samples were tested for capacity to induce macrophage shape change or ingestion of EC3b1, as described under "Materials and Methods." Purified plasma MSP was the positive control. Enzymes at the concentration used for treatment of pro-MSP were also included in the assays. The results for the shape change assay are expressed as the percentage of macrophages with extended morphology. Phagocytosis assay results are expressed as the percentage of macrophages with at least one ingested erythrocyte.

FIG. 4. Effect of C1 inhibitor on cleavage of pro-MSP by NGF-γ. NGF-γ at a concentration of 1 nsf was mixed with different concentrations of C1-INH at 37 °C. After 30 min of incubation, 1.5 nsf pro-MSP was added, followed by incubation for another 30 min. Pro-MSP treated with serum kallikrein (50 nsf) or serum kallikrein plus C1-INH (0.02 unit/ml) was used as a positive control for C1-INH action. IgG for immunoprecipitation was rabbit anti-MSP, except for normal IgG in lane 1, Lanes 1 and 2, pro-MSP. Pro-MSP with kallikrein (lane 3) or kallikrein plus C1-INH (lane 4). Lanes 5–10, pro-MSP plus NGF-γ preequilibrated with C1-INH concentrations of 1, 0.2, 0.04, 0.008, 0.0016, and 0.0 unit/ml.

FIG. 5. Chemotaxtactant activity of pro-MSP after cleavage by NGF-γ and EGF-BP. 1.2 Pro-MSP (1.5 nsf) was incubated at 37 °C for 30 min with NGF-γ (5 nsf), EGF-BP (5 nsf), or no enzyme. Chemotactic response of resident mouse peritoneal macrophages to the treated samples was determined (see “Experimental Procedures”). Purified plasma MSP was the positive control. Panel A, pro-MSP treated with EGF-BP; panel B, pro-MSP treated with NGF-γ.

and mast cell proteases, should be tested for their capacity to either act as pro-MSP convertases or to degrade and biologically inactivate pro-MSP or MSP.

Our data support the hypothesis that cleavage of pro-MSP by the serine proteases listed in Table III causes a conformational change in the protein, because cleavage is associated with exposure of an epitope recognized by a monoclonal anti-MSP antibody (Table I) as well as the appearance of biological activity (Table II, Fig. 5). Although we have demonstrated binding of MSP to its target cells, we have not yet determined if binding is associated with the conformational change in pro-MSP. Despite the presence of other MSP Arg-Val bonds that could be potential sites of serine protease cleavage, with the exception of plasmin, the serum proteases tested did not cause degradation of MSP to biologically inactive fragments under the conditions described.

NGF-γ and EGF-BP are both examples of proteolytic enzymes that occur in complexes with their respective proprotein substrates (10, 11). At high concentrations of the 116-kDa NGF complex, NGF-γ lacks proteolytic activity. But on dilution to 0.5...
Activation of pro-MSP by NGF-\(\gamma\) and EGF-BP

Based on these findings, we can enumerate possible roles of proteolytically active NGF-\(\gamma\) in wound healing and tissue repair as follows. 1) In the promotion of wound contraction, the mechanism is unknown. 2) Activation of serum complement (16) occurs with the release of the C5a leukocyte chemoattractant. In contrast to the classical complement pathway, which begins with antibody-induced activation of C1, there is no antibody requirement for initiation of the complement activation cascade by NGF-\(\gamma\). 3) There is induction of neutrophil migration to a site of injury. Because this occurs in C5-deficient mice, the NGF-\(\gamma\) substrate is apparently not C5 (28). 4) Cleavage and activation of precursor single-chain u-PA can lead to the formation of plasmin and dissolution of fibrin clot (21). 5) Cleavage and activation of pro-MSP may cause stimulation of tissue macrophage motility, pinocytic, and phagocytic activity including ingestion of C3bi-coated cells via the CR3 receptor (3). In vivo experiments in mouse models of tissue injury will be required to explore this possibility.

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TABLE III

| Proteases                        | Enzymatic activity | Mechanism of action |
|----------------------------------|--------------------|---------------------|
| Pro-MSP                          | NGF-\(\gamma\)      | Affecting cell signaling |
| Pro-MSP                          | EGF-BP             | Activating cell growth |
| Pro-MSP                          | Mouse serum kallikrein | Promoting cell migration |
| Pro-MSP                          | Clotting factor XIa | Stimulating clot formation |
| Pro-MSP                          | Clotting factor XIIa| Enhancing platelet aggregation |
| Pro-MSP                          | NGF-\(\gamma\)      | Inducing nerve growth |
| Pro-MSP                          | EGF-BP             | Enhancing cell proliferation |

\(\gamma\) Concentration that caused almost complete conversion of 1.5 \(\mu M\) pro-MSP to the biologically active \(\alpha IIb\beta3\) heterodimer within 30 min at 37 °C. Effects of plasmin, thrombin, urokinase, and Cl- were reported in Ref. 9. Cleavage by trypsin to inactive fragments was observed in the present work (data not shown).

\(\mu M\) or less, NGF-\(\gamma\) autocatalytically activates (19). Active NGF-\(\gamma\) cleaves arginyl bonds at two sites of pro-NGF-\(\beta\) to NGF-\(\beta\), which is the moiety that induces nerve growth in the in vitro assay by which NGF was first discovered (20). Although neither NGF-\(\gamma\) nor EGF-BP can cleave their noncorresponding substrates (11), our work shows that they can both cleave and activate pro-MSP. In addition, NGF-\(\gamma\) has been shown to proteolytically activate zymogens of two other protein systems, complement and plasminogen. NGF-\(\gamma\) can mimic Cl-, in that it can proteolytically activate C4 and C2; like Cl-, it is inhibited by C1-INH, a protein that blocks enzymatic activity of Cl-.

NGF-\(\gamma\) is also capable of cleaving precursor single-chain urokinase-type plasminogen activator to the proteolytically active two-chain u-PA, which mediates conversion of plasminogen to plasmin (21).