Purification, cDNA Cloning, and Characterization of a New Serpin with Megakaryocyte Maturation Activity*

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A new member of the serine protease inhibitor (serpin) superfamily with megakaryocyte maturation activity was purified, and its cDNA was cloned and characterized. The predicted amino acid sequence consisting of 380 residues was unique and was 38% identical to the serpin plasminogen activator inhibitor type 2 (PAI-2). The recombinant factor expressed in Chinese hamster ovary cells showed species-specific activity on the induction of megakaryocyte maturation in vitro. When injected into mice, the factor indeed elicited an increase in the number of platelets in plasma. The sequence alignment indicated that the factor possessed a lysine residue at the P1 position, suggesting that it might function as an inhibitor of Lys-specific proteases. Although we could not show any inhibitory activities toward several known Lys-specific proteases, we detected the activity toward protease activity present in the culture supernatant of COLO 201 cells. These results suggested that the protein might influence the maturation of megakaryocytes via action as a serpin.

The generation of megakaryocytes is a complex process dependent on the interaction of hematopoietic progenitor cells, cytokines, and stromal elements (1, 2). The failure of an organism to maintain adequate megakaryocyte numbers leads to thrombocytopenia and consequent bleeding disorders that can result in death. It has been reported that several humoral factors have influences on megakaryocyte development (3). It is believed that megakaryocyte proliferation is dependent on the essential megakaryocyte colony-stimulating factor (Meg-CSF)1.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D88575.

EXPERIMENTAL PROCEDURES

Cell Culture—Cell lines were propagated in protein-free Ham’s F-12 medium, and the culture medium was collected at confluence as described previously (18, 19). Cellular debris was removed by centrifugation and/or filtration though a glass filter. The culture supernatants were concentrated by ultrafiltration in a Pericon cassette (Millipore Corp., Bedford, MA), and the concentrates were dialyzed against 20 mM Tris-HCl buffer (pH 7.4) extensively. To supply the starting material for the large scale purification, cell culture was performed employing the Opti-cell perfusion-culture system (Cellex, Minneapolis, MN).

Purification of Megakaryocyte Maturation Factor—All procedures were performed at 4 °C. The dialyzed protein concentrate was applied to a Matrex Blue-A (Amicon, Beverly, MA) column (5 × 30 cm) equilibrated in 20 mM Tris-HCl buffer (pH 7.4) extensively. After washing, the bound proteins were eluted with the same buffer containing 0.5 M NaCl.

The following are the abbreviations used are: Meg-CSF, megakaryocyte colony-stimulating factor; Meg-POT, megakaryocyte potentiator; IL, interleukin; serpin, serine protease inhibitor; PBS, phosphate-buffered saline; API, Ackromobacter protease I; PCR, polymerase chain reaction; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; PAI, plasminogen activator inhibitor; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethanesulfonic acid; RACE, rapid amplification of cDNA ends.
linear gradient of 0–0.3 M NaCl.

The active fractions (eluted at around 0.1 M NaCl) from the Q-Sepharose column were pooled, and ammonium sulfate was added to a final concentration of 30% saturation. The sample was then applied to a phenyl-Sepharose 4B (Pharmacia) column (2.5 × 20 cm) equilibrated in 50 mM sodium phosphate (pH 7.0) containing 0.05 M ammonium sulfate. After washing, the factor was eluted with a linear gradient of 0–5% saturation of ammonium sulfate.

The active fractions (eluted at about 20% saturation of ammonium sulfate) from the phenyl-Sepharose column were dialyzed against 20 mM MES-NaCl buffer (pH 5.5) and applied to an S-Sepharose (Pharmacia) column (2.5 × 20 cm) equilibrated in 20 mM sodium phosphate, 20 mM NaCl buffer at pH 7.0. After washing, the factor was eluted with a linear gradient of 0–0.5 M NaCl.

The active fractions (eluted at about 0.15 M NaCl) from the S-Sepharose column were pooled, dialyzed against phosphate-buffered saline (PBS), and concentrated on YM-10 membrane in an Amicon cell. The concentrate was applied on a Sephacryl S-200 (Pharmacia) column (2.5 × 120 cm) equilibrated in PBS and eluted with the same buffer.

Amino Acid Sequence—Sequence analysis of peptides obtained from the Achromobacter protease I (API) digest of the purified protein was performed using an Applied Biosystems 477A gas phase sequencer according to the manufacturer's program. The resulting phenylthiolydantoin-derivatives were identified by reversed-phase HPLC with an Applied Biosystem 120A on-line system.

cDNA cloning—cDNA (21) and maintained in an TP55, which was constructed as described previously using the cloned et al.

dium was added, and the procedure was repeated. The viability of the cells was subsequently washed twice with PBS and incubated for 48 h.

Expression of Megakaryocyte Maturation Factor—Chinese hamster ovary cells were transfected with the expression plasmid, pKCR-dhfr-TP55, which was constructed as described previously using the cloned cDNA (21) and maintained in an α-minimal essential medium lacking ribonucleosides and deoxyribonucleosides, supplemented with 10% dialyzed fetal bovine serum (FBS) in the presence of methotrexate (1 μM). The transfectants were grown to confluency in roller bottles (850 cm², Corning, NY) in Ham’s F12 medium supplemented with 5% FBS. The cells were subsequently washed twice with PBS and incubated for 48 h with two changes of the medium. After changing to a serum-free medium (i.e., Ham’s F12 medium containing 5 mg/liter bovine insulin and 1 mg/liter bovine transferrin), the culture was continued for 3 more days, and the spent medium was collected. The fresh serum-free medium was added in the procedure was repeated. The viability of the cells after final collection was greater than 90% as determined by trypan blue exclusion. The purity of the recombinant factor was obtained following the procedure described for the natural factor.

Bioassays—Liquid marrow cultures employing murine bone marrow cells were performed as described by Ishibashi et al. (7) with some modifications. Marrow was flushed from the femurs of BDF1 mice with Opti-MEM I (Life Technologies) supplemented with 5% FBS. Nucleated nonadherent marrow cells (5 × 10⁶) were set up (22) in 96-well plates in 0.1 ml of Opti-MEM I containing 0.5% FBS in the presence or absence of 25 ng/ml of murine IL-3 (Genzyme, Cambridge, MA). After incubation at 37 °C for 4 days, the appearance of megakaryocytes was assayed by histochemical staining for acetylcholine esterase, the marker of murine megakaryocyte, whose activity was measured by the fluorometric method as described (7). One hundred eighty μl of a solution of 0.2% Triton X-100, 1 ml EDTA, 0.12 μl NaCl, 50 mM Hepes (pH 7.5) was added to each well, followed by the addition of 20 μl of acetylcholine iodide to the final concentration of 5.6 mM. After a 3-h incubation at room temperature, the reaction mixture from each well was transferred to the corresponding well of a 96-well plate (MicroFLUOR, Dynatech). Twenty μl of 0.4 mM 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin was then added followed by 160 μl of diluent buffer (0.2% Triton X-100, 1 ml EDTA, 50 mM sodium acetate (pH 5.0)). Fluorescence emission was determined on a fluoroscence concentration analyzer (Pandex, Mundelein, IL) capable of reading 96-well plates and was expressed in arbitrary units.

Human megakaryocyte colony formation was performed as described previously (22). Cell suspensions of 2 × 10⁶ nonadherent mononuclear cells were cultured in 35-mm Petri dishes in 1 ml of ASF 101 medium (Ajinomoto, Tokyo, Japan) containing 0.3% agar, 20% human plasma (AB type), and various concentrations of factors tested. The cultures were incubated for 14 days at 37 °C in a humidified atmosphere of 5% CO₂ and 5% O₂. For murine megakaryocyte colony formation, 20% fetal bovine serum was used instead of human plasma.

Human megakaryocyte colonies were detected by the alkaline phosphatase anti-alkaline phosphatase technique using the monoclonal antibody to GP IIb/IIIa (P2, Cosmobio, Tokyo, Japan) as described previously (22). Culture plates were dehydrated in the Petri dishes using filter paper and fixed with cold 2.5% formalin-secotion for 1 min. The fixed plates were washed twice with PBS and covered with 2 ml of PBS containing 10 units/ml agarase (Calbiochem) for 7 min at 37 °C. After two washes in PBS, immunostaining was performed using an alkaline phosphatase anti-alkaline phosphatase immunostaining kit (Zymed, San Francisco, CA). The plates were then counterstained with Mayer's hematoxylin for 10 min. Megakaryocyte colonies were defined as groups of three or more P2-positive cells. Murine megakaryocyte colonies were enumerated after histochemical staining for acetylcholine esterase and counterstaining with hematoxylin as described (7).

For the in vivo assay, C57BL/6 mice were treated with 10 daily intraperitoneal injections of either 5 mg/kg (about 100 μg/100 μl buffer/ head) of the recombinant factor or control buffer. On day 10, after 4 h of the last injection, blood was sampled from the orbital plexus of anesthetized mice using siliconized micropipettes. The white blood cell count was taken using an electronic Coulter counter.

Binding Assay—The pure recombinant factor (50 μg/ml, Fig. 2) was coated on 96-well plastic dishes overnight at 4 °C. After blocking with 1% bovine serum albumin, dilutions of mAbs (0.46 mg/ml-1000 ng/ml) were added and allowed to bind to the immobilized recombinant factor. After washing, the bound mAbs were detected with horseradish peroxidase-conjugated goat anti-murine IgG (Bio-Rad). Absorbance readings were taken at 450 and 570 nm.

Protease Assay—Protease activity was determined from the rate of increase in absorbance at 405 nm using H-Val-Leu-L-Leu-4-nitroanilide (S-2251, Dai-ichi Chemical, Tokyo, Japan) as a substrate. In brief, the reaction mixture containing 0.5 mg/ml S-2251 and the desired concentration of enzyme preparation in 0.1 ml of 50 mM Tris-HCl buffer (pH 7.5) was incubated at room temperature for 60 min, and then the absorbance at 405 nm was read.

RESULTS Purification of Megakaryocyte Maturation Factor—In initial experiments, we surveyed about 40 human cell lines for their ability to induce the megakaryocyte from murine bone marrow cells in the presence of murine IL-3 by measuring acetylcholine esterase activity (a specific marker for murine megakaryocyte). All cell lines tested were grown in the protein-free media. The activity in their conditioned media was measured in the presence of neutralizing antibody to IL-6 because many cell lines tested produced this cytokine in large quantities. We chose the conditioned medium of A431 cells as the starting material for the purification of megakaryocyte maturation factor, since this cell line produced a higher activity, which was not inhibited by the antibody.
To purify the megakaryocyte maturation factor in adequate quantities, A431 cells were grown in an Opti-cell perfusion-culture system to near confluency using a serum-containing medium, which was then replaced with the protein-free medium (18, 19). After the medium was changed, cells were cultured for 3 months with the continuous supply of the protein-free medium, and overflowed medium was collected and pooled. In total, 500 liters of the medium were pooled, concentrated, and subjected to the purification.

Initial fractionation was based on the moderate affinity of the factor to Matrex Blue-A column. The bound fraction was collected, dialyzed, and subjected to Q-Sepharose column chromatography followed by phenyl-Sepharose and S-Sepharose column chromatographies. The last two steps were quite effective in removing the contaminant proteins. Active fractions were pooled, concentrated, and further fractionated by gel filtration column chromatography (Fig. 1). Calibration of the column with molecular weight standards indicated that the apparent molecular weight of the active fraction was about 55,000. On SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, this final preparation showed diffuse doublet bands of approximately 55 kDa (Fig. 2). The acetylcholine esterase activity coincided well with the doublet bands. After treatment with endoglycosidase F, both protein bands were shifted to 40 kDa, indicating that the factor was glycosylated.

Eighty mg of the purified protein was obtained after the purification process. However, we could not estimate the recovery of the factor, because substantial megakaryocyte maturation activities were detected in other fractions assayed even in the presence of the antibody to human IL-6.

As shown in Fig. 3, the addition of the purified factor to murine bone marrow cells increased the acetylcholine esterase activity in a dose-dependent manner in the presence of murine IL-3. In the absence of IL-3, the factor had little effect on the enzyme activity. Under the microscope, bone marrow cells cultured with the factor contained more megakaryocytes than those cultured without the factor, suggesting that the factor potentiated the appearance of megakaryocytes (data not shown). Therefore, we decided to clone its cDNA to characterize it further.

cDNA Cloning and Expression in Chinese Hamster Ovary Cells of Megakaryocyte Maturation Factor—Several attempts to sequence directly the purified protein from the N terminus were unsuccessful, suggesting that the N terminus of the protein was blocked. Therefore, it was treated with API, and the peptides were separated by reversed-phase HPLC. The amino acid sequences of several peptides were determined. The oligonucleotides corresponding to the two determined sequences (i.e., VEXYDPTNHLGEDTXXNINK and XYIEVTTEGTEAAXG) were prepared and used in a series of PCRs with cDNAs prepared from A431 cells to obtain specific primers. The amino acid sequence predicted from the amplified product contained the determined sequences of the peptides, confirming that this amplified product was generated from the cDNA of the megakaryocyte maturation factor.

The cDNA and the deduced amino acid sequences are shown.
in Fig. 4. The cDNA sequence contains 1950 nucleotides including the 73-nucleotide 5'-untranslated region and 737-nucleotide 3'-untranslated region excepting the poly(dA) tract. The consensus sequence of the serpin superfamily is underlined. The potential N-glycosylation sites and the putative active center of serpin (Lys***) are shown by asterisks and a boldface letter, respectively. The putative polyadenylation signal (double underline), five cysteine residues (open circles), the peptide fragments digested with API (solid boxes), and the stop codon (**) are indicated.

The megakaryocyte maturation factor shows significant sequence similarity to the serpin superfamily of serine protease inhibitors (Fig. 5). It exhibits 27.1% amino acid identity to human PAI-1 (23) and the highest 38.2% to human PAI-2 (24). The P1 residue of serpin, located immediately upstream of the putative reactive center is a critical determinant of the target specificity, and the sequence alignment identified this megakaryocyte maturation factor as a Lys serpin (25).

Chinese hamster ovary cells were transfected with the expression plasmid for the maturation factor, designated pdKCR-dhfr-TP55 and prepared as described under "Experimental Procedures." A clone resistant to 1 \( \mu \text{M} \) methotrexate was selected and cultured in roller bottles, and the supernatant was collected. After concentration and dialysis, the recombinant protein was purified by serial chromatographies on QA-Sepharose, phenyl-Sepharose, and S-Sepharose columns as described under "Experimental Procedures." The purified protein showed a molecular mass of 55 kDa on SDS-PAGE, which was comparable with the natural one (data not shown). It was digested with API, and the resultant fragments were separated by reversed-phase HPLC and sequenced. We could identify peptides that were expected from the cDNA sequence (data not shown). Among four potential N-glycosylation sites, at least two (i.e. Asn68 and Asn215) were shown to be glycosylated, because Asn residues at these sites could not be detected in the sequences of the corresponding API digests.

Biological Effect—Fig. 6 shows the effect of the recombinant megakaryocyte maturation factor on the induction of megakaryocyte from murine bone marrow cells. In the absence of murine IL-3, no effect was observed in cultures treated with the recombinant protein. In the presence of murine IL-3, however, the addition of the recombinant protein increased the acetylcholine esterase activity in a dose-dependent manner. The activity of the recombinant protein was comparable with that of natural protein purified from A431 cells. Under the microscope, an increase in the number of megakaryocyte was definitely observed. The cell populations other than megakaryocyte in the factor-treated culture were not different.
To rule out the possible presence of contaminating protein having megakaryocyte maturation activity, we have prepared several murine monoclonal antibodies (mAbs) against the recombinant protein and examined their effects on the ability of the factor to increase the acetylcholine esterase activity in murine bone marrow cell culture. We have chosen two mAbs (designated 35 and 336) to test this ability, because these two mAbs were shown to have highly similar binding abilities to the purified recombinant protein in an enzyme-linked immunosorbent assay (Fig. 7A). As expected, an irrelevant mAb called 2F, which was prepared against recombinant human IL-5, had no binding activity. We have also performed Western blot analysis and found that both mAbs 35 and 336 detected the 55-kDa bands, but mAb 2F did not (data not shown). Fig. 7B shows the effects of mAbs on the biological activity of the recombinant protein. While mAb 336 decreased the megakaryocyte maturation activity significantly, the other two mAbs (35 and 2F) had no effect. These results suggested that the factor indeed induced the megakaryocyte maturation in vitro. However, a relatively large amount of the factor was required for the action on the murine cells (human IL-11 showed 1000-fold higher activity than the factor), suggesting its species-specific action (Fig. 6).

To test the species specificity, the effect of the factor on megakaryocyte colony formation from human and murine bone marrow cells was compared (Fig. 8). We employed megakaryocyte colony assay instead of acetylcholine esterase assay because, unlike murine cells, the enzyme is not a marker of human megakaryocytes. It should also be noted that the acetylcholine esterase assay cannot distinguish exactly between an increase in the number of megakaryocytes and an increase in the amount of acetylcholine esterase per megakaryocyte. We employed human IL-11 as a standard for the comparison of the activities.

In the absence of IL-3, the factor had no effect both on human and murine cells. On the other hand, in the presence of IL-3, the factor increased the number of megakaryocyte colonies both in human and murine cultures. While the factor was, on a weight basis, more than 1000-fold less active than IL-11 on murine cells, it was only less than 10-fold less active than IL-11 on human cells, clearly indicating the species-specific action of the factor. The human IL-11 was equally potent on both cells, although the number of colonies attained was less in human than in murine cell cultures. These results also made it unlikely that a contaminating cytokine was responsible for megakaryocyte maturation in our assay system, because on a molar basis the factor showed activity comparable with that of IL-11 on human cells. It was also observed that the factor had no effect on formation of myeloid or erythroid colonies (data not shown).

When mice were injected with 100 μg/head of recombinant megakaryocyte maturation factor daily for 10 days, a 40% increase in platelet counts in plasma in comparison with the control animals receiving control buffer was observed (Table I). Neutrophil and erythrocyte counts were essentially unchanged in these animals. Taken together, these results indicated that the factor had Meg-POT activity in vitro and stimulated the platelet production in vivo.

Serpin Activity—Since our factor with the megakaryocyte maturation activity belongs to the serpin superfamily and shows sequence similarity to PAI-1 and -2, we examined the inhibitory activity of the factor to several serine proteases including plasmin, trypsin, and API. Sequence alignment sug-
gested that the factor may have an inhibitory specificity for proteases that cleave at lysine residues. However, no inhibitory activity to these defined proteases was observed. Then we surveyed several cell lines and detected the protease activity in the culture supernatant of COLO 201 cells, which was inhibited by the factor in a dose-dependent manner (Fig. 9A). When the conditioned medium of COLO 201 cells was applied onto the recombinant protein-conjugated Sepharose column, no protease activity was recovered in flow-through fractions, strongly suggesting that the factor trapped the protease (data not shown). Moreover, when the factor was mixed with the conditioned medium, there appeared a new band having higher molecular weight upon SDS-PAGE analysis (Fig. 9B). Since, in general, inhibitory serpins interact with their target proteinase to form complexes that are not dissociable upon boiling in SDS, these results strongly suggested the complex formation between the factor and the putative protease secreted by COLO 201 cells and suggested that the factor was active as an inhibitory serpin.

**DISCUSSION**

In the studies reported in this paper, we have purified, cloned the cDNA for, and characterized a protein that has a megakaryocyte maturation activity. The purified protein was a glycoprotein with a molecular mass of approximately 55 kDa. Amino acid and cDNA analyses indicated that the protein shared considerable sequence similarity with members of the serpin family, including PAI-1 and PAI-2. Serpins share a similar tertiary structure with a reactive center located on an exposed loop near the carboxyl terminus (25, 26). The inhibition by serpin of proteases was mediated by presentation of a reactive site P1–P9 peptide bond with the appropriate P1 specificity for the target enzyme. Although some serpins such as maspin and pigment epithelium-derived factor seem to lack antiprotease activity (27–29), most retain the inhibitory activities toward proteases. Since the sequence alignment identified the factor as a Lys serpin, i.e. the P1 residue in the factor is lysine, we have examined the inhibitory activity of the factor on several proteases that cleave the COOH-terminal side of the
The serpin activity of recombinant megakaryocyte maturation factor. A, the enzyme activity in the culture supernatant of COLO 201 cells was measured in the presence of various concentrations of the factor as described under “Experimental Procedures.” B, SDS-PAGE (12.5% gel) analysis of purified factor (lane 1), COLO 201 supernatant (lane 2), and the mixture of the factor and COLO 201 supernatant (lane 3). The arrows indicate the positions of molecular weight markers. The asterisk indicates the position of the complex between the factor and the putative protease.

Lys residue. However, we could not observe any inhibitory effect on the proteases tested. Instead we detected a protease activity in the culture supernatant of COLO 201 cells and found that the factor inhibited this protease activity. These results indicated that the factor indeed had a protease-inhibitory activity and suggested that the activity upon megakaryocyte development might be mediated through the inhibition of some proteases that degraded some regulatory peptides. However, the identification of the target protease must await for the future studies. It might also be important to identify the peptide(s) that was degraded by the target protease. It should be noted here, however, that a possibility exists that the serpin-protease complex exerts the megakaryocyte maturation activity by binding to a cellular receptor. The existence of such receptors for the complexes is known, e.g. for α1-antitrypsin-neutrophil elastase (30).

The primary function of members of the serpin family is considered to neutralize overexpressed serine protease activity (26). However, in the recent studies, it is becoming evident that serpins have multiple activities on various cell functions. It has been reported that a new serpin termed maspin showed tumor-suppressing activity by reducing tumor cell invasion (27). Pigment epithelium-derived factor, first identified as a secreted product in conditioned medium from cultured fetal human retinal pigment epithelial cells, was shown to be a serpin with neurotropic activity (29). A tumor-associated protein isolated from squamous cell carcinoma tissue of uterine cervix was also shown to be a serpin and was suggested to play an important role in the malignant behavior of the tumor cells (31). It has also been demonstrated that a serpin from the cowpox virus, referred to as CrmA, can inhibit the IL-1β-converting enzyme, which is a cysteine protease, and thus act as a cytokine response modifier by suppressing IL-1β release in response to inflammation (32). In the current study, we have added a new example having megakaryocyte maturation activity. The serpin showed the activity on murine bone marrow cells only modestly that was about 1000-fold less than that of human IL-11. However, apparent species specificity was observed when the activity was measured using human bone marrow cells; the serpin showed the activity comparable with that of human IL-11. Since the serpin had essentially no megakaryocyte-inducing activity in vitro in the absence of murine IL-3, which exhibits Meg-CSF activity, it should be classified with Meg-POT. Moreover, when injected into mouse, the serpin increased the number of platelets in plasma, indicating that it indeed stimulated the production of platelets in vivo.

Until now, several cytokines were shown to have influences on megakaryocyte development. These include IL-1, IL-3, IL-6, IL-11, leukemia inhibitory factor, granulocyte-macrophage colony-stimulating factor, erythropoietin, stem cell factor, and megakaryocyte-potentiating factor (5–11, 33–36). More recently, thrombopoietin (Mpl ligand) was purified and cloned by several groups, and it was shown that this factor was the primary regulator of megakaryocyte development and platelet production (12–16). However, Gurney et al. (17) have shown by employing the gene knockout mice lacking mpl expression, that megakaryocyte numbers and platelet counts were substantially reduced but not completely eliminated. These results indicate that factors other than thrombopoietin are responsible for the residual thrombopoiesis. Our factor may participate in thrombopoiesis through the inhibition of the degradation of some cytokines mentioned above. Such a possibility should be examined in future studies.
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