A novel serine protease predominately expressed in macrophages

Cailin CHEN, Andrew L. DARROW, Jian-shen QI, Michael R. D'ANDREA and Patricia ANDRADE-GORDON[1]
Drug Discovery, Johnson & Johnson Pharmaceutical Research and Development LLC, Welsh and McKean Roads, Spring House, PA 19477-0776, U.S.A.

We have identified a novel serine protease designated EOS by sequence identity searches. The deduced protein contains 284 amino acids with an active form containing 248 amino acids starting from an Ile-Val-Gly-Gly motif. The active form comprises a catalytic triad of conserved amino acids: His^{77}, Asp^{126} and Ser^{231}. It shares 44 % identity with β-tryptase and belongs to the S1 trypsin-like serine-protease family. Interestingly, this gene also maps to human chromosome 16p13.3. The purified protease showed amidolytic activity, cleaving its substrates before arginine residues. Tissue distribution by immunohistochemistry analysis demonstrated that EOS is highly expressed in spleen and moderately expressed in intestine, colon, lung and brain. We confirmed this expression pattern at the mRNA level by performing *in situ* hybridization. The results from both immunohistochemistry and *in situ* hybridization indicate that EOS is associated with macrophages. We corroborated this observation by double immunofluorescence using the anti-EOS antibody and an anti-CD68 antibody, a macrophage specific marker. Furthermore, we have detected a dramatic increase in immune staining of EOS in cultured U937 cells treated with PMA, which represent activated macrophages. This up-regulation is also reflected by elevated EOS mRNA in the PMA-treated U937 cells detected by Northern blotting. Since macrophages have important roles in various pathological conditions, such as wound healing, atherosclerosis and numerous inflammatory diseases, the localization of this novel serine protease to active macrophages may help to further the elucidation of the roles of this gene product in modulating these disorders.

Key words: database sequence identity search, macrophage, serine protease, S1 trypsin/chymotrypsin-like serine protease.

INTRODUCTION

Serine proteases are members of a multigene family of proteins possessing a conserved catalytic site consisting of histidine, aspartic and serine residues. These proteases are involved in the post-translational processing of many polypeptides and have central roles in the regulation of a wide variety of physiological processes, including coagulation, fibrinolysis, fertilization, development, malignancy, neuromuscular patterning and inflammation [1,2]. This trypsin/chymotrypsin-like (S1) serine-protease family, which selectively hydrolyses peptide bonds often C-terminal to a basic amino-acid residue, is gaining recognition due to an increased awareness that these enzymes have pivotal roles in a multitude of diverse physiological processes. In addition to the well-documented involvement in the digestive process, this family of proteins has important roles in the maintenance of homoeostasis. These proteases participate in regulating key amplification cascades through the proteolytic activation of inactive zymogen precursors. Thus, in many instances, the protease substrates within these cascades are themselves the inactive form, or zymogen, of a 'downstream' serine protease. Well-known examples of this serine-protease-mediated regulation include blood coagulation [3], kinin formation [4] and the complement system [5]. Although the regulation of blood coagulation is the key element for maintaining haemostasis, the kinin–kallikrein and complement system are thought to be important components of inflammation and immune reactions [4,5].

Under normal conditions, the body protects itself from the potential damaging effects of proteases with endogenous protease inhibitors (PIs). However, if the balance between activity and protease inhibition is upset due to a decrease in the levels of PIs, the excess protease activity may lead to the development of diseases. One such example is disseminated intravascular coagulation [6], which results in a variety of pathologies, including thrombosis, cerebral infarction, and/or coronary infarction. In other instances, excess cellular proteases are released and activated, overwhelming the steady-state levels of PIs. For example, tryptases have recently been linked to airway diseases. Tryptases are highly expressed in resident mast cells [7], and, currently, at least six distinct cDNAs designated tryptase have been cloned {α, βI, βII, βIII, hTMT (human transmembrane tryptase) and ε-PRSS22 (protease serine S1 family member 22) [7–12]}. The β-tryptase form appears to be the major species purified from mast cells of the lung and skin. Although the exact physiological substrate of this trypsin-like protease is uncertain, *in vitro* studies suggest that its targets include C3 [13], neuropeptides [14], fibrinogen [15], pro-urokinase [16], procollagenase [17], and PAR2 (protease-activated receptor 2) [18–20]. β-Tryptase is the major component of the mast cell secretory granules. Secreted tryptase induces hyper-responsiveness in isolated guinea pig bronchi [21] and increases pulmonary resistance in an *in vivo* sheep airway model [22]. Increased levels of tryptase were also detected in COPD (chronic obstructive pulmonary disease) patients [23]. These results suggest a pathological role for tryptases in asthmatic airway inflammation and obstruction. Thus inhibitors of serine proteases are potentially useful for treatment or prevention of thrombosis, inflammatory diseases, autoimmune diseases and other diseases [24].

Abbreviations used: COPD, chronic obstructive pulmonary disease; DIG, digoxigenin; EK, enterokinase; EST, expressed sequence tag; HA, haemagglutinin; HRP, horseradish peroxidase; hTMT, human transmembrane tryptase; IF, immunofluorescence; IHC, immunohistochemistry; pNA, *p*-nitroanilide; PI, protease inhibitor; PRSS, protease serine S1 family member; RACE, rapid amplification of cDNA ends; SSC, standard saline citrate, Suc, succinoyl.

[1] To whom correspondence should be addressed (e-mail pandrade@prdus.jnj.com).

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Because of the role of proteases in many pathologies, we have engaged in efforts to identify novel serine proteases, which may lead to novel therapeutic approaches for a broad spectrum of diseases. We used a combination of bioinformatic searches of nucleic-acid databases and molecular cloning to isolate and characterize cDNAs encoding new members of this gene family. This resulted in the identification of a trypsin-like serine protease EOS, which is similar to prostasin [25,26] and tryptases [7–12]. The tissue distribution of this gene product indicates that it is predominantly expressed in human macrophages. In addition, we expressed the catalytic region of this serine protease and characterized its amidolytic activity against a panel of chromogenic substrates. The U937 cell-culture system was used to show that the protease EOS gene is differentially responsive to the phorbol ester PMA and is thus up-regulated in a manner similar to a gene that is up-regulated upon macrophage differentiation/activation. These results suggest that this protease may have a role in macrophage-related biological and pathological functions.

EXPERIMENTAL

Materials

General molecular biological methods were in accordance with those previously described in [27]. Oligonucleotides were purchased from Ransom Hill Biosciences (Ransom Hill, CA, U.S.A.) and all restriction endonucleases and other DNA-modifying enzymes were from New England Biolabs (Beverly, MA, U.S.A.), unless otherwise specified. EST (expressed sequence tag) clones were obtained from the IMAGE consortium through Genome Systems (St. Louis, MO, U.S.A.) All construct manipulations were confirmed by dye-terminator cycle sequencing using Allied Biosystem 377 fluorescent sequencers (PerkinElmer, Foster City, CA, U.S.A.).

Identification and cloning of EOS cDNA

A general serine-protease identity search identified a novel protease designated protease EOS within nucleotide sequence databases. A combination of 5′ RACE (rapid amplification of cDNA ends) [28] using human leucocyte marathon ready cDNA (Clontech, Palo Alto, CA, U.S.A.) and a 3′ matching EST clone were used to construct the full-length sequences (GenBank® accession number AF536382).

To construct the zymogen-activation vector, cDNA of a bovine pre-prolactin signal sequence was fused in-frame with the sequence encoding the M2 monoclonal anti-(FLAG epitope) antibody, as described in [29]. For this, five double-stranded oligonucleotides with cohesive ends were annealed. The sequence contained an *Eco*RI or *Not*I site at each end. The prolactin–FLAG sequence was then cloned into a pCDNA3 vector (Invitrogen, San Diego, CA, U.S.A.) to generate prolactinflagpcDNA3 (PFpcDNA3). The cDNA encoding the EK (enterokinase) cleavage site of human trypsinogen I was inserted and fused in-frame downstream of this designed signal sequence (as shown in Figure 8A) to generate the vector PFEKpcDNA3.

The catalytic domain of EOS was generated by PCR from its full-length cDNA using the forward primer 5′-GGGATCTAGAGGACGAGAGTGGCCGTGGC-3′, and the reverse primer 5′-CTCATCTAGAAGCATTAGAAGTGACGCGAGCCTG-3′ containing an *Xba*I restriction-endonuclease site at each end. The PCR product was then digested and inserted into the PFEKpcDNA3 vector. After sequence confirmation, the zymogen cDNA was subsequently transferred into pFastBac1 (Life Technologies, Gaithersberg, MD, U.S.A.) to generate the PFEK-EOS–His_6 expression construct for the production of recombinant baculovirus.

PCR profiling

Human cDNA libraries were purchased from Clontech, except for CHRF-288, a megakaryoblastic cell line and human gel-filtered platelet libraries, which were constructed using the ZAP Express cDNA system (Stratagene, La Jolla, CA, U.S.A.). PCR was performed in 50 μl reaction mixtures containing 1 μl of diluted phage library stock (approx. 10^8–10^{10} plaque-forming units/ml). Reactions were initially denatured at 94 °C for 5 min and subjected to 35 cycles of 94 °C for 20 s (denaturation), 56 °C for 20 s (annealing) and 72 °C for 30 s (extension), followed by a final 10 min extension at 72 °C. The primers used were 5′-GAGAAAGTCAGATTCACAGC-3′ and 5′-CTGCTTAGGGTCTCTTTAGG-3′. A nested primer probe of the sequence 5′-TGAGCGGCCTTTAAGAGTTGAGAGACAGCCGGCAGGGAAT-3′ was radiolabelled using [^{32}P]ATP and T4 polynucleotide kinase. Unincorporated radioactivity was removed using a QIAquick nucleotide removal column (Qiagen, Valencia, CA, U.S.A.). The ^{32}P-end-labelled probe (1×10^5 c.p.m.) was then mixed with 10 μl of each PCR reaction, denatured at 94 °C for 5 min, hybridized at 60 °C for 15 min and cooled to 4 °C. The annealed samples (10 μl) were subjected to electrophoresis in Tris/borate/EDTA non-denaturing 6 % (w/v) polyacrylamide gels (Novex, Invitrogen), dried and exposed by autoradiography. A PCR profile of the cDNA libraries used with β-actin PCR primers and labelled nested primer probe produced a β-actin PCR product in all samples examined as an internal control (results not shown).

Expression and purification of recombinant protease EOS

The recombinant bacmid containing the PFEK-EOS–His_6 construct was prepared from bacterial transformation, selection, growth, purification and PCR confirmation, according to the manufacturer's instructions (Life Technologies). Sf9 insect cells (CRL-1711; ATCC, Manassas, VA, U.S.A.) were transfected with purified bacmid DNA and were incubated at 27 °C. Conditioned medium containing recombinant PFEK-EOS–His_6 baculovirus was collected for viral stock amplification. Sf9 cells were then infected at a multiplicity of infection of 2 at 27 °C for 72 h, and cell pellets were collected. Cells were lysed on ice in buffer A [20 mM Tris/HCl (pH 7.4) containing 500 mM NaCl, 1 % (v/v) Triton X-100, 1 mM EDTA and 1 mM EGTA]. Cell lysates were mixed with anti-FLAG M2-affinity beads (Eastman Kodak Co., New Haven, CT, U.S.A.) and bound at 4 °C for 1 h with gentle rotation. The zymogen-bound resin was transferred to a column and washed with 0.1 M glycine (pH 3.5) into a vial containing a small volume of 1 M Tris/HCl, pH 8.0, and 5 M NaCl. Zymogen cleavage was carried out in a small volume by adding chromatography-purified EK (Sigma, St. Louis, MO, U.S.A.) or recombinant EK (Novagen, Madison, WI, U.S.A.), and incubating overnight at room temperature (19–23 °C) with gentle shaking in a buffer containing 20 mM Tris/HCl (pH 7.4), 250 mM NaCl and 2 mM $CaCl_2$. A 150 μl volume of a 50 % (v/v) slurry of Ni-NTA (nickel-nitrilotriacetic acid; Qiagen) was added to the mixture and was mixed by shaking at 4 °C for 1 h. The nickel beads were washed with washing buffer [20 mM Tris/HCl (pH 7.8), 250 mM NaCl and 15 mM imidazole]. The activated protease EOS was eluted with elution buffer [20 mM Tris/HCl (pH 7.8), 250 mM NaCl and 250 mM imidazole]. Protein concentration was determined by the micro-BCA (bicinchoninic acid) method (Pierce, Rockford, IL, U.S.A.), using BSA as a standard.

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Generation of anti-(protease EOS) antibody and Western blotting

An antigenic peptide with amino-acid sequence VLLPPDY-SEDGAR, encoded by nucleotides 333–372 of protease EOS, was synthesized. The peptide was linked through a terminal cysteine residue to maleimide-activated KLH (keyhole limpet haemocyanin; Pierce), according to the manufacturer's instructions. Unlinked peptide was removed by dialysis against PBS overnight. Rabbit polyclonal antibodies were subsequently generated in two separate animals. The anti-serum was purified on a Protein A–Sepharose™ affinity column (Amersham Biosciences, Piscataway, NJ, U.S.A.).

EOS and other proteases were expressed as in the PFEK–His_6 vector using the Sf9 insect system. Cells infected with recombinant baculoviruses for 72 h were lysed in Laemli buffer. The crude lysates were resolved by SDS/PAGE and transferred on to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, U.S.A.) in Tris/glycine/methanol buffer. Membranes were blocked in PBST [80 mM Na_2HPO_4, 20 mM NaH_2PO_4 (pH 7.5), 100 mM NaCl and 0.05 % (v/v) Tween 20] containing 5 % non-fat dry milk. Membranes were incubated with immune serum, as described above, followed by incubation with donkey anti-rabbit HRP (horseradish peroxidase)-linked secondary antibody (Boehringer Mannheim, Indianapolis, IN, U.S.A.), and detection by ECL® (enhanced chemiluminescence; Amersham Biosciences). The membrane was stripped and probed again with an antibody against the FLAG epitope (Sigma).

U937 cell culture and preparation

The human monoblastic cell line U937 (ATCC) was maintained in RPMI 1640 medium supplemented with 10 % (v/v) heat-inactivated foetal bovine serum (Life Technologies), 2 mM L-glutamine, 10 mM Hepes buffer, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1.0 mM sodium pyruvate and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The cells were incubated in humidified air with 5 % CO_2 at 37 °C and were subcultivated twice each week. The suspension of U937 cells was fixed in 10 % (v/v) neutral-buffered formalin for 15 min at room temperature, washed three times in PBS, then spun on to slides using a Cyto-Tek centrifuge (Miles Scientific, Naperville, IL, U.S.A.). The slides were allowed to air dry at room temperature at least overnight before immunocytochemical staining. The PMA-treated U937 cells became adherent and attached to chamber slides (Nalge Nunc International, Naperville, IL, U.S.A.). The cells were fixed in the same fashion, and were stored at 4 °C until use. The cell staining was carried out in a similar manner to the immunohistochemistry (IHC) described below, except that the H_2O_2 and avidin–biotin blocking steps were omitted.

IHC and immunofluorescence (IF)

Human multiple tissue blocks were purchased from Dako (Carpenteria, CA, U.S.A.). Tissue sections were deparaffinized, hydrated, immersed in Target Retrieval Solution (Dako), and heated twice for 3 min at high power in an 800 W commercial microwave. After the slides had cooled, the endogenous peroxidase was blocked by 3.0 % H_2O_2 for 10 min. Tissue slides were processed through an avidin–biotin blocking system according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, U.S.A.). All reagent incubations and washes were performed at room temperature. To reduce the background, the slides were then incubated with normal blocking serum (Vector Laboratories) for 10 min. Primary antibodies were placed on the slides for 30 min followed by biotinylated secondary antibodies, goat anti-rabbit or horse anti-mouse (Vector Laboratories) for 30 min. After rinsing in automation buffer (Biomeda, Foster City, CA, U.S.A.), the avidin–biotin–HRP complex reagent (Vector Laboratories) was added for 30 min. The slides were then washed and twice treated with the chromogen 3,3′-diaminobenzidine (Biomeda) for 5 min and rinsed in distilled water, counterstained in Mayer's haematoxylin, dehydrated and then mounted on coverslips. Negative controls included replacement of the primary antibody with the antibody diluent buffer (Zymed Laboratories, San Francisco, CA, U.S.A.) and the use of the pre-immunized serum from the same animal or the same species' non-immunized serum. Specificity of the antibody for protease EOS was confirmed by pre-incubating the antibody with its antigen, in approx. 100-fold molar excess to the antibody, overnight at 4 °C. Positive controls were performed using known cellular markers. In the same manner, the double IF staining was performed sequentially on the same section of multiple tissue blocks for EOS and the macrophage marker CD68. The expression of EOS protein was detected with polyclonal EOS antibody and FITC-conjugated sheep anti-rabbit secondary antibody (Sigma). The monoclonal anti-CD68 antibody (Dako) and a Texas-Red-labelled horse anti-mouse secondary antibody (Vector Laboratories) were used for the expression of CD68. The slides were washed following the last secondary antibody and mounted on coverslips with Vectashield mounting medium containing DAPI (4′,6′-diamidino-2-phenylindole) nuclear counter stain (Vector Laboratories).

In situ hybridization

For ribo-probe preparation, a 450 bp fragment from the 3′ untranslated region of EOS cDNA was made by PCR amplification. Oligonucleotides EOS ribo-U, 5′-GCTCTAGACCTGGATTCAGG-CTCGCGTC-3′, and EOS ribo-L, 5′-GCTCTAGACCTGGTGT-CAAATCTCTGTC-3′, were used as primers. The cDNA was ligated into the expression vector pSPT-19 (Boehringer Mannheim). The plasmid was then linearized with *Bam*HI and used as a template to transcribe RNA. The DIG (digoxigenin)-labelled antisense ribo-probe was synthesized *in vitro* with T7 RNA polymerase in the presence of DIG-UTP using the DIG RNA labelling kit (Boehringer Mannheim). The specificity of the ribo-probe was determined by a dot blot against the specific EOS full-length and various non-specific cDNAs (results not shown).

For hybridization, the multiple tissue slides were de-waxed, hydrated and placed in 3 % (v/v) H_2O_2 for 10 min at room temperature. The slides were then digested with pre-diluted pepsin (Research Genetics, Huntsville, AL, U.S.A.) for 10 min at 37 °C. Sections were thoroughly washed and then dehydrated in 100 % (v/v) ethanol and were allowed to air dry for 5 min. The EOS ribo-probe was diluted to 0.1 μg/ml in hybridization buffer (Biomeda). Probe solution (200 μl) was placed on each slide and then heated for 10 min at 98 °C, and incubated at 37 °C for 2 h. Slides were then placed sequentially in a low [2 × SSC (standard saline citrate)] and high (0.1 × SSC) stringency wash at room temperature. The detection was carried out by incubating the slides with anti-(DIG–alkaline phosphatase) Fab fragments (Boehringer Mannheim) for 30 min, followed by alkaline phosphatase substrate NBT (Nitro Blue Tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Enzo Diagnostics, Inc, New York, NY, U.S.A.) reaction. The slides were then counterstained with Nuclear Fast Red (Biomeda), dehydrated and mounted with Permount (Sigma).

Northern blot analysis

Monoblast U937 cells (5×10^5 cells/ml) were treated with the phorbol ester PMA (160 nM) for 0, 2 and 5 days. Total RNA (30 μg), extracted with TRIzol® reagent (Life Technologies),

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was fractionated in 1 % (w/v) agarose–formaldehyde gels and transferred on to Hybond™ N membranes (Amersham Biosciences). Membranes were baked for 2 h at 80 °C under a vacuum and were UV-cross-linked with a Stratalinker (Stratagene). The PCR product amplified using EOS ribo-U and EOS ribo-L as primers was used to generate a radiolabelled probe for EOS. The CD68 cDNA fragment was obtained by reverse transcriptase-PCR from PMA-induced U937 cells using primers CD68-U, 5′-ACTGAGGGGGTTTGGGGTGTG GT-3′, and CD68-L, 5′-CCCTCCTCCCGACTGCATTATCTC-3′, and the actin cDNA fragment (Clontech). Probes were generated by random priming with Klenow DNA polymerase in the presence of ^{32}P-labelled r-dCTP. The hybridization was performed in ExpressHyb™ (Clontech) according to the manufacturer's procedure. Membranes were washed twice in 2 × SSC and 0.1 % (w/v) SDS at room temperature, and twice in 1 × SSC and 0.1 % (w/v) SDS at 50 °C, followed by autoradiography.

Cell fractionation

The full-length protease EOS coding sequence was fused in-frame with a HA (haemagglutinin) and His_6 epitope affinity tag in the mammalian expression vector pCI-neo (Promega, Madison, WI, U.S.A.). This construct was transfected into HEK-293 cells using SuperFect (Qiagen), and stable colonies were selected in the medium supplemented with 1200 μg/ml Geneticin (Invitrogen). The EOS protein expression was confirmed by immunocytochemistry. Cells were collected, washed twice with PBS, and suspended in homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Hepes/NaOH, pH 7.4, and 10 mM dithiothreitol) containing PI [one tablet of Complete™ Mini (Roche Diagnostics, Indianapolis, IN, U.S.A.) per 10 ml]. Cells were homogenized using a tight-fitting Dounce homogenizer (50 strokes). The lysates were centrifuged at 400 ***g*** for 10 min in a microcentrifuge to collect the nuclear fraction and were centrifuged further at 6000 ***g*** for 15 min to pellet the microsomal fraction. The supernatants were then centrifuged at 100 000 ***g*** for 30 min and the resulting pellet (membrane fraction) and supernatant (cytosolic fraction) were collected.

Chromogenic assay

Amidolytic activities of the activated protease EOS–His_6 were monitored by release of pNA (*p*-nitroanilide) from the synthetic substrates. The chromogenic substrates used in these studies were H-D-Pro-HHT-Arg-pNA (where HHT is hexahydrotyrosyl) and H-D-Lys(CBO)-Pro-Arg-pNA (where CBO is carbobenzoxyl) from American Diagnostica (Greenwich, CT, U.S.A.), and H-DL-Val-Leu-Arg-pNA, Suc-Ala-Ala-Pro-Arg-pNA (where Suc is succinoyl) and Suc-Ala-Ala-Pro-Lys-pNA (Bachem, Torrance, CA, U.S.A.). The assay was carried out at 37 °C in 20 mM Tris/HCl (pH 7.8), 250 mM NaCl, and 25 mM imidazole containing 1 mM of each chromogenic substrate. Release of pNA was detected after 60 min incubation on a micro-plate reader (Molecular Devices, Menio Park, CA, U.S.A.) with a 405 nm absorbance filter. The specific activities (nmol of pNA produced/min per μg of protein) are presented.

RESULTS

Identification and characterization of the novel protease EOS gene

Using a nucleic acid database search, we identified a partial cDNA encoding a novel serine protease, designated protease EOS. The full-length sequence was isolated by combining the 5′ RACE product and a 3′ matching EST clone. The nucleotide sequences and deduced amino-acid sequences are shown in Figure 1. This protease comprises a signal peptide of 22 amino acids, a propeptide of 14 amino acids and a mature catalytic domain of 248 amino acids starting from an Ile-Val-Gly-Gly motif, which contains the catalytic triad of serine proteases that has been characterized as the amino-acid residues essential for proteolytic activity. The active-site residues that comprise the catalytic triad are His[77], Asp[126] and Ser[231]. Sequence comparison showed that EOS shares 47.1 % and 44.3 % identity with human proteases prostasin and human β-tryptase respectively. These data clearly indicate that the protease EOS cDNA encodes a member of the S1 (trypsin/chymotrypsin) serine-protease subfamily [30]. An additional BLAST identity search [31] of the GenBank® database was performed for the cDNA. This gene matched 100 % to exons within a genomic clone deposited from the Center for Human Genome Studies at the Los Alamos National Laboratory (GenBank® accession number AC005570). The genomic clone maps to human chromosome 16p13.3, which clusters within the same chromosome locus as β-tryptase [32].

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>Protease EOS:
   1  CCACGCGTCCGACCAGAGTCCAAGCCCTAGGCAGTGCCACCCTTACCCAGCCCAGCCTTG   60
                                                          ><
  61  AAGACAGAATGAGAGGGGTTTCCTGTCTCCAGGTCCTGCTCCTTCTGGTGCTGGGAGCTG  120
                M  R  G  V  S  C  L  Q  V  L  L  L  L  V  L  G  A  A
                                  ><
 121  CTGGGACTCAGGGAAGGAAGTCTGCAGCCTGCGGGCAGCCCCGCATGTCCAGTCGGATCG  180
        G  T  Q  G  R  K  S  A  A  C  G  Q  P  R  M  S  S  R  I  V
                 ⇑
 181  TTGGGGGCCGGGATGGCCGGGACGGAGAGTGGCCGTGGCAGGCGAGCATCCAGCATCCTG  240
        G  G  R  D  G  R  D  G  E  W  P  W  Q  A  S  I  Q  H  P  G

 241  GGGCACACGTGTGCGGGGGGTCGCTCATCGCCCCCCAGTGGGTGCTGACAGCGGCGCACT  300
        A  H  V  C  G  G  S  L  I  A  P  Q  W  V  L  T  A  A  H  C
              ><                                              •
 301  GCTTCCCCAGGAGGGCACTGCCAGCTGAGTACCGCGTGCGCCTGGGGGCGCTGCGTCTGG  360
        F  P  R  R  A  L  P  A  E  Y  R  V  R  L  G  A  L  R  L  G

 361  GCTCCACCTCGCCCCGCACGCTCTCGGTGCCCGTGCGACGGGTGCTGCTGCCCCCGGACT  420
        S  T  S  P  R  T  L  S  V  P  V  R  R  V  L  L  P  P  D  Y

 421  ACTCCGAGGACGGGGCCCGCGGCGACCTGGCACTGCTGCAGCTGCGTCGCCCGGTGCCCC  480
        S  E  D  G  A  R  G  D  L  A  L  L  Q  L  R  R  P  V  P  L
                              •
 481  TGAGCGCTCGCGTCCAACCCGTCTGCCTGCCCGTGCCCGGCGCCCGCCCGCCGCCCGGCA  540
        S  A  R  V  Q  P  V  C  L  P  V  P  G  A  R  P  P  P  G  T
                                                ><
 541  CACCATGCCGGGTCACCGGCTGGGGCAGCCTCCGCCCAGGAGTGCCCCTCCCAGAGTGGC  600
        P  C  R  V  T  G  W  G  S  L  R  P  G  V  P  L  P  E  W  R

 601  GACCGCTACAAGGAGTAAGGGTGCCGCTGCTGGACTCGCGCACCTGCGACGGCCTCTACC  660
        P  L  Q  G  V  R  V  P  L  L  D  S  R  T  C  D  G  L  Y  H

 661  ACGTGGGCGCGGACGTGCCCCAGGCTGAGCGCATTGTGCTGCCTGGGAGTCTGTGTGCCG  720
        V  G  A  D  V  P  Q  A  E  R  I  V  L  P  G  S  L  C  A  G
                                       ><
 721  GCTACCCCCAGGGCCACAAGGACGCCTGCCAGGGTGATTCTGGGGGACCTCTGACCTGCC  780
        Y  P  Q  G  H  K  D  A  C  Q  G  D  S  G  G  P  L  T  C  L
                                             •
 781  TGCAGTCTGGGAGCTGGGTCCTGGTGGGCGTGGTGAGCTGGGGCAAGGGTTGTGCCCTGC  840
        Q  S  G  S  W  V  L  V  G  V  V  S  W  G  K  G  C  A  L  P

 841  CCAACCGTCCAGGGGTCTACACCAGTGTGGCCACATATAGCCCCTGGATTCAGGCTCGCG  900
        N  R  P  G  V  Y  T  S  V  A  T  Y  S  P  W  I  Q  A  R  V

 901  TCACTTCTAATGCTAGCCGGTGAGGCTGACCTGGAGCCAGCTGCTGGGGTCCCTCAGCCT  960
        T  S  N  A  S  R  *

 961  CCTGGTTCATCCAGGCACCTGCCTATACCCCACATCCCTTCTGCCTCGAGGCCAAGATGC  1020
1021  CTAAAAAAGCTAAAGGCCACCCCACCCCCCACCCACCACCTTCTGGCTCCTCTCCTCTTT  1080
1081  GGGGATCACCAGCTCTGACTCCACCAACCCTCATCCAGGAATCTGCCATGAGTCCCAGGG  1140
1141  AGTCACACTCCCCACTCCCTTCCTGGCTTGTATTTACTTTTCTTGGCCCTGGCCAGGGCT  1200
1201  GGGCGCAAGGCACGCAGTGATGGGCAAACCAATTGCTGCCCATCTGGCCTGTGTGCCCAT  1260
1261  CTTTTTCTGGAGAAAGTCAGATTCACAGCATGACAGAGATTTGACACCAGGGAGATCCTC  1320
1321  CATAGCTGGCTTTGAGGACACGGGGACCACAGCCATGAGCGGCCTCTAAGAGCTGAGAGA  1380
1381  CAGCCGGCAGGGAATCGGAACCCTCAGACCCACAGCCGCAAGGCACTGGATTCTGGCAGC  1440
1441  ACCCTGAAGGAGCTGGGAAGTAAGTTCTTCCCCAGCCTCCAGATAAGAGCCCCGCCGGCC  1500
1501  AATCCCTTCATTTCAACCTAAAGAGACCCTAAGCAGAGAACCTAGCTGAGCCACTCCTGA  1560
1561  CCTACAAAGTTGTGACTTAATAAATGTGTGCTTTAAGCTGCC  1602
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Figure 1 cDNA and amino-acid sequences of novel protease EOS

The full-length nucleotide and deduced amino-acid sequences of novel protease EOS (GenBank® accession number AF536382) are presented. The highly conserved amino-acid residues of the catalytic triad, histidine, aspartic acid and serine, are indicated by ●. The first four amino acids of the mature activated protease, just downstream of the presumed zymogen-activation sequence, are underlined. A predicted signal-peptidase-cleavage site is denoted by ⇑. At the 3′ end, the presumed polyadenylation sequence is underlined. Above the nucleotide sequence, the exon/intron junctions are delimited by >< for each position, which were determined by alignments with the genomic clone (GenBank® accession number AC005570).

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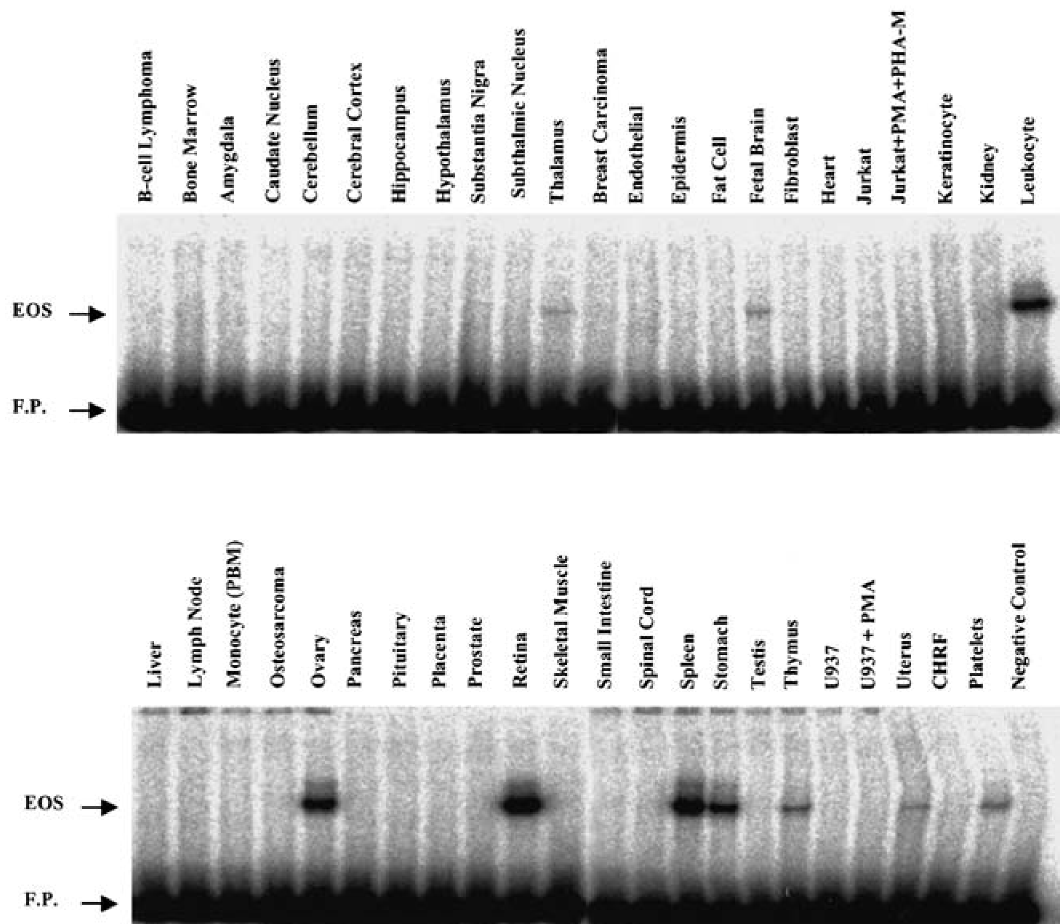

Figure 2 Tissue distribution of EOS

PCR profiling reveals the limited distribution of protease EOS. A series of cDNA libraries of human tissues and cell lines were used as templates in analytical PCR reactions with an amplimer pair of oligonucleotides directed against protease EOS. The amplified products for each reaction were then hybridized to a ^{32}P-labelled nested primer probe and product–probe heteroduplexes were separated from free probe (F.P.) by electrophoresis on non-denaturing gels, which were dried and exposed for autoradiography. The intensity of the indicated product probe correlates with the relative abundance of that particular mRNA in the tissue type analysed. PBM, peripheral blood monocyte; PHA-M, phytohaemagglutinin.

mRNA tissue distribution

As seen in Figure 2, the PCR profile shows that this gene is highly expressed in peripheral leucocytes, ovary, retina, spleen and stomach. Moderate protease EOS mRNA expression is seen in thymus, uterus and platelets, as well as some brain tissues, such as thalamus and foetal brain. Of particular significance is that protease EOS mRNA is not expressed in pancreas, liver or prostate, tissues normally found to express numerous serine-protease genes. Moreover, the gene was not detected in CHRF cells, a megakaryocytic origin leukaemia cell line. It was unexpected that we detected the protease EOS PCR product in cDNA libraries constructed from human gel-filtered platelets. However, since the platelets used to construct the cDNA library contained low levels of contaminating erythrocytes and other blood cells (< 1 per 10^3 platelets), we cannot rule out the possibility of leucocyte contamination, which would generate a false-positive signal in this sensitive PCR assay. Importantly, this novel protease is not expressed in U937 cells treated with PMA for 3 h. These data agree with subsequent experimental results in which the EOS protein was barely detected in U937 cells, but was expressed in U937 cells treated with PMA for several days (see Figures 6 and 7; and results not shown). The latter causes the U937 cells to differentiate to macrophages (from suspension to attached cells) at least 16–24 h after PMA treatment.

Novel protease EOS is expressed in the macrophage

The tissue distribution of protease EOS mRNA by PCR profiling is highly restricted to specific tissues, such as spleen and stomach, rather than ubiquitous expression. In order to understand better the function and biological relevance of this gene, we investigated the cellular expression pattern of this protein. For this purpose, we have generated a polyclonal anti-EOS antibody using an antigenic peptide deduced from the EOS cDNA. The specificity of the antibody was tested by Western blot analysis after affinity-column purification. As can be seen in Figure 3, the antibody specifically recognizes protease EOS, but not other related S1 serine-protease family members, trypsin I, prostasin and protease C-E/tryptase ε-PRSS22. No protease EOS binding was detected using pre-immune serum or the anti-(protease EOS) antibody pre-absorbed with the EOS antigenic peptide (Western blot assay, results not shown). Since all proteases used in this Western analysis contained a FLAG-epitope tag, the same membrane was probed with anti-FLAG antibody to indicate equivalent amounts of recombinant protease expression (Figure 3, lower panel). The parental Sf 9 cell

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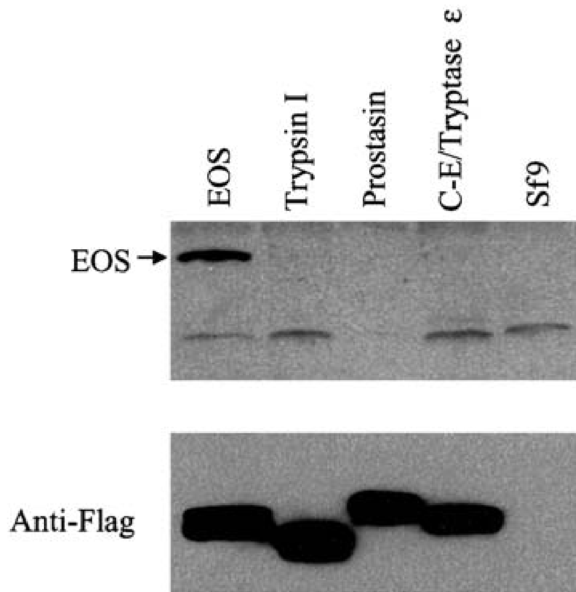

Figure 3 Anti-EOS sera specifically reacts with EOS protein

Crude extracts of Sf9 cells alone or Sf9 cells infected with recombinant baculovirus expressing protease EOS or the related proteases trypsin, prostasin, and tryptase ε, were resolved by SDS/PAGE and analysed by immunoblotting with the antibody raised against the novel protease EOS. Protein–antibody binding was visualized by enhanced chemiluminescence. Since these recombinant proteases were fused to the FLAG epitope tag, the immunoblot was stripped and probed with anti-FLAG M2 monoclonal antibody to confirm equivalent levels of recombinant protease expression.

extracts were prepared in parallel and were used as a negative control (Figure 3).

The cellular localization of EOS protein was performed by IHC in multiple tissue blocks containing 60 different human tissue specimens, including thyroid, spleen, uterus, prostate, testis, ovary, pancreas, lung, liver, kidney, heart, stomach, small intestine, large intestine, brain and adrenal tissues. Immunostained cells were most prominent in the spleen, small and large intestine, lung and brain. The upper panel of Figure 4 shows examples of staining in spleen, lung and colon. In spleen, strong EOS staining was scattered throughout the red pulp parenchymal area, but was nearly absent in white pulp. In the lung, the positive cells either lie on top of the alveolar lining cells or are free in the alveolar space. Some of these cells appear to contain phagocytosed material. The positive cells were seen in the submucosal layer of colon. In contrast, only background staining was observed with the negative controls using normal rabbit serum or pre-immune serum (results not shown). We assessed further the distribution of EOS mRNA by *in situ* hybridization. Using a DIG-labelled protease EOS cRNA riboprobe, we obtained a similar expression pattern as compared with immunoreactivity detected using the anti-(protease EOS) polyclonal antibody. As shown in the lower panel of Figure 4, the positive hybridized cells are in purple.

The unique localization of the protease EOS immunoreactive cells within the tissues mentioned above were examined at high magnifications (results not shown) and revealed that the predominant positive cell type in these tissues appears to be the macrophage. Macrophages display a kidney-bean-shaped nucleus, which tends to look like two separate nuclei. In addition, macrophages have strong phagocytic capability. In the spleen,

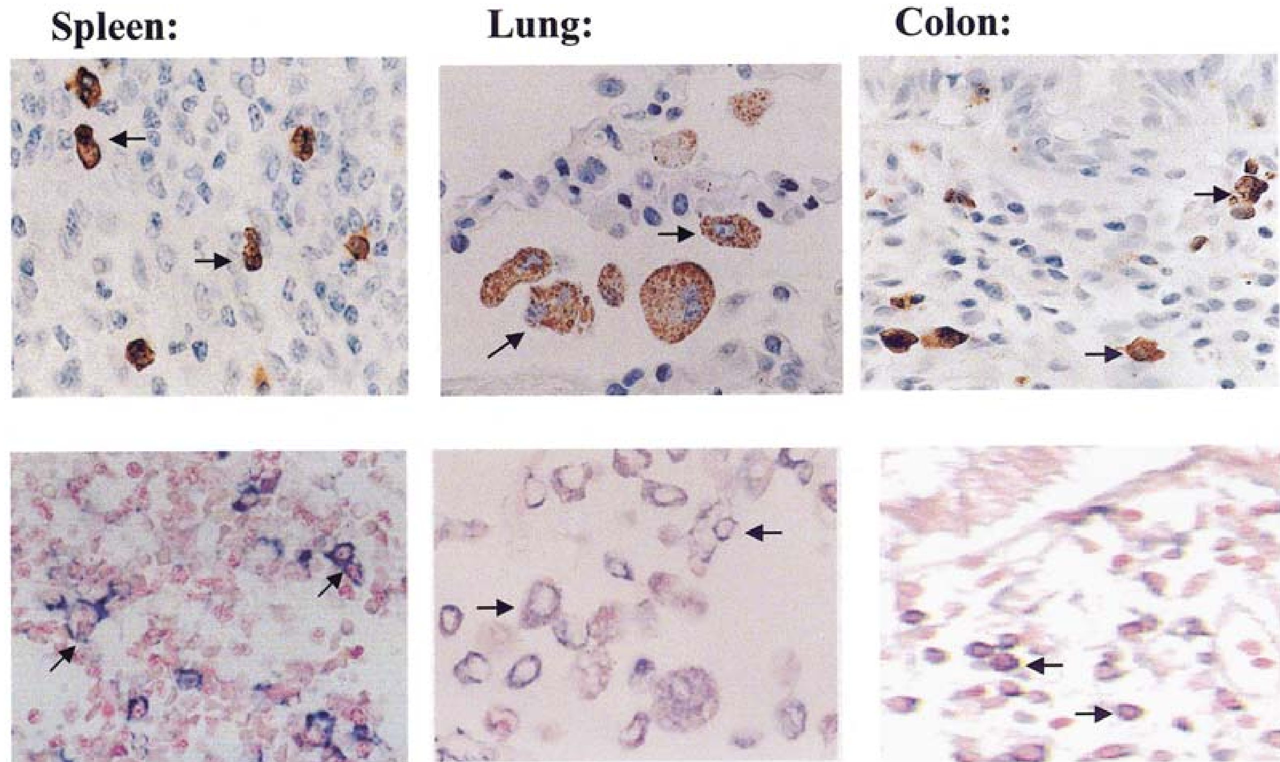

Figure 4 Novel protease EOS is expressed in a subset of macrophage-like cells

Localization of protease EOS protein by IHC (upper panel), and mRNA by *in situ* hybridization (lower panel), in human spleen, lung and colon. Under the conditions used, the positive anti-EOS immunoreacting cells show a brown stain and positive EOS mRNA detection is purple (arrows).

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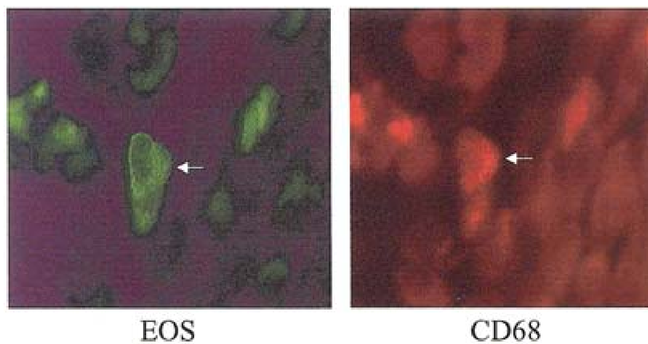

Figure 5 Superimposable staining of EOS and macrophage marker CD68

Double IF was performed using the anti-(protease EOS) antibody and an antibody against CD68, a macrophage-specific marker. The double IF generates a superimposable staining pattern, with EOS shown in green (left panel) and CD68 showing in red (right panel). This substantiates that protease EOS is expressed in macrophages.

phagocytic macrophages would be expected to accumulate red-blood-cell-derived material. In the lung, macrophages are extremely active phagocytes responsible for cleaning debris from the alveolar gas-exchange surface. As mentioned above, we detected phagocytosed material in these positive-staining cells (Figure 4). Within these cells, immunostaining was localized to discrete cytoplasmic granules.

To confirm that the protease EOS immunostaining cells were macrophages, we conducted double-IF staining. Anti-EOS and anti-CD68, a macrophage-specific marker, were sequentially exposed to these multiple tissue blocks. As can be seen in Figure 5, the cells expressing the macrophage marker CD68 were also positive for EOS protein expression. In this particular experiment, a FITC-conjugated secondary antibody recognizing the primary anti-(protease EOS) antibody stained the cells green (left panel), while the Texas-Red-conjugated antibody against the CD68 primary antibody stained the cells red (right panel). This double-IF co-localization confirms that the protease EOS is expressed in macrophages.

EOS is up-regulated by phorbol ester PMA in U937 cells

To investigate further the functional linkage between EOS and macrophages, we monitored the correlation of differentiation of monoblasts to active macrophages and the regulation of the EOS gene. It is well accepted that the monoblasts or peripheral blood monocytes can be differentiated into macrophages upon PMA stimulation [33]. In the present study, we have used the human monoblastic U937 cell line treated with PMA to obtain active macrophages. U937 cells, which normally grow in suspension, display a smooth surface and extended pseudopodia and become adherent to each other and to the surface of the culture dish upon PMA treatment (results not shown). These morphological changes were observed after 16–20 h, and samples were collected after 48 h of PMA treatment. Vimentin, as a control for immunoreactivity, was expressed in both untreated and treated cells (Figures 6A and 6B). The macrophage surface marker CD68 was not expressed in unstimulated cells (Figure 6E), but was expressed in more than 90 % of cells after PMA stimulation (Figure 6F). Likewise, protease EOS was not detected before treatment (Figure 6C), and dramatic increases in immunostained cells (80 %) were obtained in cells treated with PMA (Figure 6D).

EOS up-regulation was also demonstrated at the RNA level by Northern blot analysis. As shown in Figure 7(A), transcripts for both the protease EOS and the macrophage marker CD68 could not be detected at zero time. However, increased levels of both protease EOS and CD68 could be seen after 48 h treatment with PMA. A further increase in both of these mRNA transcripts was observed after 5 days of treatment. In contrast, β-actin, which was used for normalization, showed no increase with PMA treatment. Similar results were observed at the protein level by Western blotting (Figure 7B) using the specific antibody against protease EOS. EOS protein was undetected in unstimulated U937 cells, whereas a dramatic increase can be seen after 2 days of PMA treatment, and actin remained unchanged. Taken together, these data suggest that protease EOS is up-regulated upon macrophage activation.

EOS is mainly expressed in microsomal fraction of mammalian cells

To examine the subcellular localization of EOS, we have created an EOS expressing stable cell line. EOS protein was undetectable in the conditioned medium collected from these cells, as well as PMA-induced U937 cells or even Sf9 cells infected with the recombinant EOS baculovirus (results not shown). In these stably transfected HEK-293 cells, the EOS protein expression was initially examined by immunocytochemistry (results not shown), where the fluorescent EOS staining appeared within the cytoplasmic compartment. Subcellular fractionation was performed to examine the EOS protein localization in further detail. The results indicate that protease EOS is mainly expressed in the microsomal fraction (Figure 8). This subcellular compartmentalization is consistent with our experience of expressing the EOS protein in Sf9 cells, where it requires extraction with 500 mM NaCl and 1.0 % (v/v) Triton X-100, presumably because it is also localized within the microsomal compartment when expressed in insect cells.

Expression and functional amidolytic activity

A major difficulty in studying the members of the serine-protease family is the general requirement of zymogen activation through limited proteolysis. Since these enzymes are synthesized as inactive zymogen precursors, the generation of recombinant serine proteases results in a purified protein possessing little or no detectable activity. To circumvent this dilemma, we developed a zymogen-activation vector that permits the generic activation of expressed heterologous serine-protease catalytic domains, as shown in Figure 9(A). Pre-signal sequence from prolactin was fused to the FLAG epitope, which allows the immunological reaction of the expressed zymogen. Pro-sequence encoding the EK cleavage site is just downstream. At the C-terminus is a His_6 affinity tag to facilitate purification. The *Xba*I site(s) allows for the insertion of appropriately tailored cDNA fragments encoding the catalytic domain. Thus the catalytic domain of a serine-protease cDNA will be positioned in-frame with respect to the EK pro-sequence, such that the expressed zymogen may be generically activated by EK treatment. We have used this vector to activate various trypsin-like serine proteases, such as human trypsin I, prostasin [26] and neuropsin [34] (results not shown). We have applied this methodology to characterize the amidolytic activity of protease EOS. The zymogen-activation vector containing protease EOS is shown schematically in Figure 9(A). The purified recombinant zymogen was treated with EK and aliquots were analysed by SDS/PAGE. The mobility of the EK-processed protease was compared with the untreated zymogen. As shown in Figure 9(B), the mobility of protein was increased following EK treatment, consistent with the pro-sequence being cleaved by the

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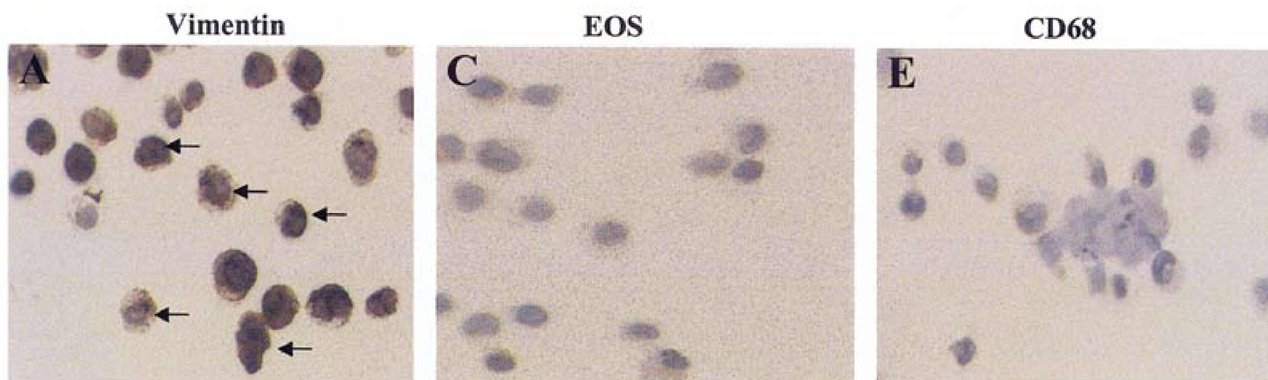

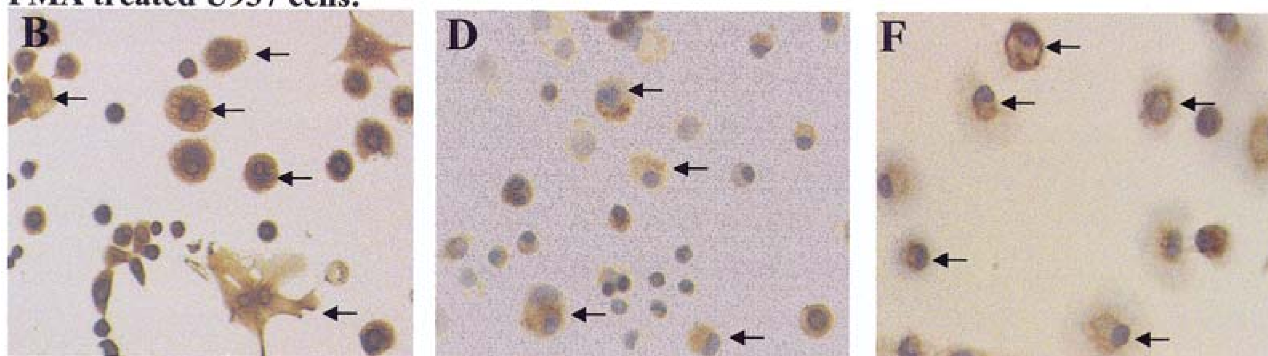

Figure 6 Up-regulation of protease EOS protein by phorbol ester in U937 cells is detected by immunocytochemistry

Cultured monoblast U937 cells that were untreated (upper panel), or treated with phorbol ester PMA (160 nM) for 5 days (lower panel). The cells were set in chamber slides, fixed with 10 % (w/v) formalin/saline solution and analysed by immunocytochemistry. The immunostaining was performed with antibodies against vimentin, protease EOS and CD68, as indicated. The positive immunoreaction is shown as dark brown under the conditions used (arrows).

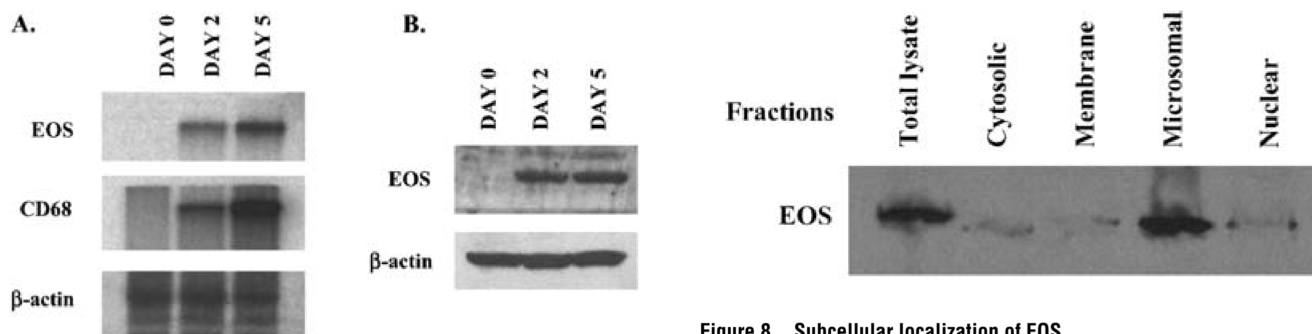

Figure 7 Up-regulation of protease EOS by phorbol ester in U937 cells

Monoblast U937 cells were untreated, or treated with phorbol ester PMA (160 nM) for 0, 2 and 5 days. (**A**) Total RNA (30 μg) was analysed by ^{32}P-labelled probes for EOS, CD68 or β-actin. (**B**) Cell extracts (30 μg) were resolved by SDS/PAGE and analysed by immunonblotting with antibodies specific for protease EOS or actin.

Figure 8 Subcellular localization of EOS

HEK-293 cells stably expressing full-length EOS–HA–His$_6$ were collected and homogenized using a tight-fitting Dounce homogenizer. The subcellular fractions were obtained by differential centrifugation, and the EOS protein within each fraction was detected by Western blotting.

EK. The purified protein was further confirmed by EOS-specific antibody blotting (results not shown). However, the subcellular localization of EOS has rendered this protein difficult to be extracted from infected Sf9 cells. Figure 9(C) shows the specific activities of protease EOS on a panel of amidolytic substrates. Several commercially available pNA substrates with trypsin-like specificities were used to demonstrate activity. H-DL-Val-Leu-Arg-pNA is the best substrate so far identified for EOS, with a K_m of 6.9 mM.

DISCUSSION

As an ongoing effort to identify novel members of the S1 trypsin/chymotrypsin serine-protease family, we have identified and characterized a series of serine-protease homologues. The present paper describes the initial characterization of a novel S1 serine protease, designated protease EOS. The cDNA was identified by BLAST identity searching and the deduced protease

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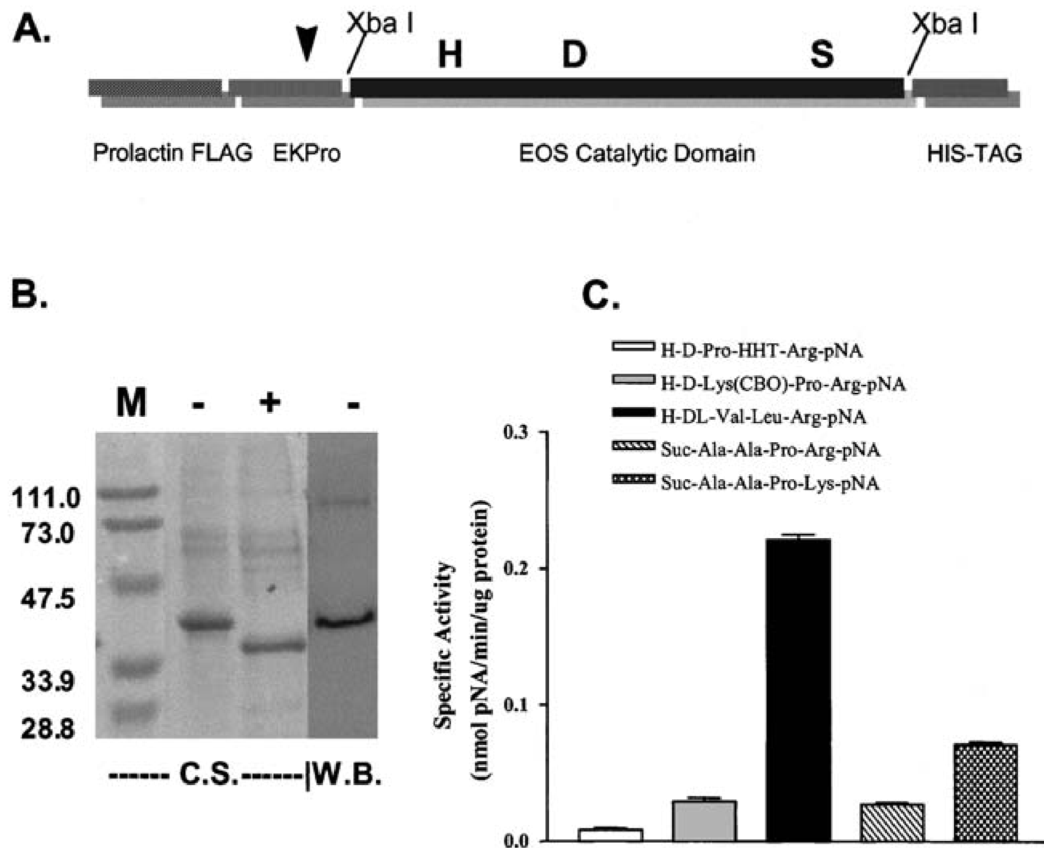

Figure 9 Expression and functional amidolytic activity of EOS

(**A**) Schematic diagram of the zymogen-activation vector. Pre-signal sequence from prolactin was fused to the FLAG epitope. Just downstream is the pro-sequence encoding the EK cleavage site. At the C-terminus is a His_6 affinity tag (HIS). The *Xba*I site(s) allows for the insertion of appropriately tailored cDNA fragments encoding the catalytic domain. (**B**) SDS/PAGE analysis of expressed, purified protease EOS zymogen and activated protease produced using baculovirus-infected Sf9 insect cells. The first two lanes are of Coomassie-Blue-stained (C.S.) polyacrylamide gels containing the purified recombinant EOS, either untreated (−) or digested with EK (+). The Western blot (W.B.) shows the purified protease EOS zymogen (− EK treatment) probed with the anti-FLAG monoclonal antibody M2. Lane M indicates the relative positions of size markers with molecular masses given in kDa to the left of the gel. (**C**) Specific activities of the purified and activated protease EOS assayed using a set of commercially available protease substrates.

EOS amino-acid sequence shares 44 % identity with human β-tryptase. The catalytic domain contains the histidine, aspartic and serine residues that are conserved and necessary for enzymic activity of S1-family serine proteases. Cellular localization demonstrates that protease EOS is predominantly expressed in macrophages. Furthermore, EOS is up-regulated when monoblast U937 cells are stimulated with PMA, a condition that mimics the activation of macrophages.

The localization of the protease EOS gene to human chromosome 16p13.3, which is also the genetic locus of the α- and β-tryptase genes [32], is of interest. More recently, other members of this family, such as hTMT [11,12], esp-1/testisin (PRSS21) [35,36] and tryptase ε-PRSS22 [12], have also been mapped to this locus. This is interesting in light of the genetic data suggesting that, in some populations, chromosome 16 contains determinants of susceptibility to asthma [37]. Tryptase is produced abundantly in mast cells [38] and consequently exists at high levels in lung. Moreover, it is released from granules in response to inflammatory stimuli associated with asthma [23,39,40]. More recently, increased levels of tryptase in COPD patients have been reported [23]. The fact that this novel protease EOS is highly expressed in lung may suggest a role in airway inflammatory diseases. However, our data clearly demonstrate that EOS is located in macrophages. Thus it is likely that the cell-type-specific transcriptional regulatory elements for this gene and those controlling tryptase expression differ significantly. Although the role of protease EOS in airway disease remains unclear, our preliminary results show that this protease, unlike β-tryptase, does not activate PAR2 (results not shown). A selective inhibitor of this protease may help to elucidate its functional role in airway disease as well as in other pathogenic states.

Although we note that full-length EOS contains a classical signal peptide, as predicted by a modified von Heijne method [41], it does not appear to be secreted. EOS protein was undetectable in the conditioned medium collected from EOS stably transfected mammalian cells, PMA-induced U937 cells or even Sf9 cells infected with the recombinant EOS baculovirus. Subcellular fractionation of the full-length EOS, stably transfected into HEK-293 cells, has shown that protease EOS is mainly expressed in the microsomal compartment. This subcellular compartmentalization is consistent with our experience in purifying the EOS protein from Sf9 cells, where it requires extraction with high salt and 1 % (v/v) Triton X-100, even though, in the case of the insect cells, the construct contains a signal sequence. We have used this bovine prolactin-signal-sequence-containing construct to express several other related serine proteases using Sf9 cells, which are secreted following infection. In fact, the localization of EOS is similar to several other members of this gene family, such as chymase [42], tryptase [43] and granzymes [44], which are localized in granules or secretory vesicles. Thus protease EOS may possibly

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be targeted to secretory granules. Whether EOS could be released upon stimuli as these proteases remains to be determined.

Differentiated blood cells express a wide array of proteases, which are likely to have specific roles in various pathological states. Although granzymes from cytotoxic T-cells and natural killer cells [45], elastase and collagenases from neutrophils [46], and chymase and tryptase from mast cells [47–49] have been intensively investigated, the proteases from eosinophils are only currently being identified molecularly and the roles of the eosinophil proteases remain to be elucidated [35,50]. In the present study, data at both the protein and mRNA levels clearly show that protease EOS is up-regulated in active macrophages. Since macrophages are involved in atherosclerosis, wound healing and inflammatory diseases, future studies of protease EOS may contribute to our understanding of the roles of macrophage in these pathological conditions.

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