Recombinant Human Extrinsic Pathway Inhibitor

PRODUCTION, ISOLATION, AND CHARACTERIZATION OF ITS INHIBITORY ACTIVITY ON TISSUE FACTOR-
INITIATED COAGULATION REACTIONS*

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Previous studies have shown that extrinsic pathway inhibitor (EPI) is an effective inhibitor of factor Xa alone or factor VIIa-tissue factor complex in the presence of factor Xa. Since tissue factor exposure is implicated in thrombogenesis, we hypothesized that EPI may be valuable in the treatment of some thromboembolic episodes. Furthermore, EPI may be an important factor in bleeding complications in hemophiliacs. In this study, human EPI was expressed in baby hamster kidney cells using a mammalian expression vector. Transfected cells expressed 1-2 μg/mL of recombinant EPI (rEPI) which was purified to homogeneity by heparin-Sepharose chromatography, ion-exchange chromatography, and reverse phase high performance liquid chromatography. Purified rEPI exhibited a specific activity of 30,000 units/mg and migrated as a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an apparent molecular weight of 42,000. In addition, the NH₂-terminal sequence of rEPI was identical to that of HepG2 EPI and HeLa EPI. The ability of rEPI to inhibit factor X activation by a complex of factor VIIa-tissue factor was then examined in the presence and absence of plasma concentrations of human factors VIII and IX. Using relipidated human brain tissue factor apoprotein, rEPI inhibited the factor VIIa-mediated activation of factor X half-maximally at 2.5 and 1 nM in the presence and absence of factors VIII and IX, respectively. Using monolayers of a human bladder carcinoma cell line (J82) as the source of tissue factor, the activation of factor X by cell-bound factor VIIa was inhibited half-maximally by 5 nM rEPI in the presence of factors VIII and IX, and at 0.8 nM EPI in the absence of factors VIII and IX. The proteolytic activity of J82 cell-bound factor Xa toward prothrombin was inhibited half-maximally at ~5 nM EPI, while the amidolytic activity of factor Xa in solution was inhibited by rEPI with a Kᵣ of 130 pm. Recombinant EPI also inhibited the amidolytic activity of factor VIIa half-maximally at 10 nM rEPI in the presence of relipidated tissue factor apoprotein and calcium. These results indicate that, in the presence of plasma concentrations of factors VIII and IX, at least 10 times the plasma concentration of EPI is required to reduce factor VIIa-dependent factor X activation one order of magnitude in vitro. In the absence of functional factor VIII and IX, rEPI at plasma levels was a potent inhibitor of factor VIIa-mediated factor X activation, and this activity presumably accounts for the inability of hemophiliacs to initiate hemostasis via the extrinsic pathway.

The extrinsic pathway of mammalian blood coagulation is initiated when factor VII or factor VIIa binds to its cofactor, tissue factor. The factor VIIa-tissue factor complex then activates either factor IX or factor X by limited proteolysis which eventually leads to thrombin formation and a fibrin clot. In contrast to every other serine protease involved in blood coagulation, factor VIIa-tissue factor does not appear to be regulated to any significant extent in vivo by antithrombin III, the principal regulator of coagulation proteases. Based in part on studies reported by Hjort in 1957 (1), two different laboratories have identified a novel plasma protein that recognizes the factor VIIa-tissue factor complex (2, 3). This protein, designated as extrinsic pathway inhibitor (EPI) by Rapaport’s laboratory (5), has now been purified to homogeneity from human plasma (6) and the conditioned, serum-free medium of the HepG2 hepatoma cell line (7, 8).

Purified preparations of plasma EPI were heterogeneous when examined by SDS-PAGE and exhibited major bands at 40 and 46 kDa and minor bands at 55, 65, 75, 90, and 130 kDa (6). The 46-kDa form of EPI and higher M₅ forms were shown to be associated with apolipoprotein A-II in mixed disulfide linkages (6). All forms of plasma EPI were recognized by antibodies directed against EPIs amino and carboxyl termini (6). The amino acid sequence of the first 20 residues of plasma EPI was identical to that observed for HepG2 EPI with the exception of residue 5 where leucine was identified in plasma EPI and glutamic acid was found in HepG2 EPI (6). The majority of plasma EPI has been shown to circulate in complex with plasma lipoproteins, although the functional significance of this interaction is not fully understood.

The amino acid sequence of human EPI has recently been deduced from the nucleotide sequence of a cDNA coding for EPI (11). The abbreviations used are: EPI, extrinsic pathway inhibitor; rEPI, recombinant extrinsic pathway inhibitor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; Heps, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.

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1 The abbreviations used are: EPI, extrinsic pathway inhibitor; rEPI, recombinant extrinsic pathway inhibitor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; Heps, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.

2 Extrinsic pathway inhibitor is apparently identical to lipoprotein-associated coagulation inhibitor as designated by Broze and coworkers (4).
The EPI cDNA codes for a mature protein consisting of 276 amino acids (M, 32,000) with 18 cysteines and three canonical N-linked glycosylation sites (9). The sequence of EPI revealed a highly positive amino terminus, three tandemly repeated Kunitz-type serine protease inhibitory domains, and a highly positively charged carboxyl terminus (9). Site-directed mutagenesis experiments indicated that the Kunitz domain 1 interacts with the factor VIIa active site while the Kunitz domain 2 binds to the factor Xa active site (10). Mutation of the reactive-site residue of the Kunitz domain 3 had no effect with respect to factor VIIa/Xa inhibition (10).

The mechanism whereby EPI inhibits factor VIIa-tissue factor factor is currently thought to occur in two discrete steps (4, 11). In the first step, EPI binds to factor Xa through the interaction of its Kunitz domain 2 and the reactive site of factor Xa. In the second step, EPI Kunitz domain 1 interacts with the active site of factor VIIa. This mechanism appears to be operative on cell-surface tissue factor as well as soluble tissue factor generated from reconstituted tissue factor apoprotein (5, 12).

In the present study, we have expressed human EPI in stably transfected baby hamster kidney cells and purified the protein from cell culture media. The molecular properties of the recombinant EPI were compared with that observed for EPI synthesized by HepG2 and HeLa cells. Finally, we have investigated the inhibitory activity of EPI toward factor VIIa-tissue factor in the presence of physiological levels of factor VIII and factor IX using soluble and cell-surface expressed tissue factor. Our data indicate that EPI may be responsible, in part, for the severity of bleeding episodes observed in hemophiliacs. Our findings also provide support for the use of recombinant EPI as adjunct therapy in the treatment of some thrombocytopenic diseases, although doses in excess of 10 times the plasma concentration of EPI may be required to achieve a therapeutic effect.

**EXPERIMENTAL PROCEDURES**

**Materials**—Heparin-Sepharose CL-4B, Mono Q, Mono S, Pro RPC 10/10, and SDS low M, standard kit were obtained from Pharmacia LKB Biotechnology Inc. Affi Gel 10 was obtained from Bio Rad. Benzoyl-7-Asp-Glu-Arg-p-nitroanilide (S-2222), H-D-Ile-Pro-Arg-p-nitroanilide (S-2288), and H-D-Phe-Pip-Arg-p-nitroanilide (S-2288) were purchased from Sigma. Corynebacterium diphtheriae diphtheria toxin (100 mg/ml) was obtained from Ny-Nordisk, Bagsvaerd, Denmark. Factor VIII was purified by immunoaffinity chromatography using a von Willebrand Factor polyclonal antibody column (22) and a factor VIII-specific monoclonal antibody column (23).

**Preparation of Anti-EPI NH2-terminal Peptide Antibodies**—Antibodies against a 10-residue peptide (Glu-Glu-Asp-Glu-Glu-Thr-Ile-Ile-Cys) in the amino-terminal end of EPI was prepared in rabbits as follows. Synthetic peptide (50 mg) was coupled to diphtheria toxin (1.1, w/w) with 5% glutaraldehyde in 0.3 M NaCl (pH 7.0). Following coupling, excess glutaraldehyde was removed by dialysis. Rabbits were administered four doses of coupled peptide (equivalent to 100 mg of peptide) in Freund's adjuvant at 28-day intervals. Antiserum was harvested 41, 69, and 97 days after the initial injection. The anti-EPI NH2-terminal peptide IgG did not inhibit EPI activity and was routinely diluted 1:5000 for immunoblot analysis of EPI samples.

**General Methods**—Standard DNA techniques were carried out as described (23). Synthetic oligonucleotides were prepared by solid-phase phosphoramidite chemistry on an automated synthesizer (Applied Biosystems model 380A). Nucleotide sequence determinations were performed by the dye deoxychain-terminiation technique (24). Amino acid sequence analyses were carried out by automated Edman degradation using an Applied Biosystems 470A Gas-liquid Sequencer.

**Peptides**—were synthesized using N'-t-Boc amino acids with suitably protected side chains and methylbenzhydryl resin on an automatic peptide synthesizer (Applied Research model 430). Peptides were cleaved with 95:5 (v:v) with 5% glutaraldehyde in 0.3 M NaCl (pH 7.0). Following coupling, excess glutaraldehyde was removed by dialysis. Rabbits were administered four doses of coupled peptide (equivalent to 100 mg of peptide) in Freund's adjuvant at 28-day intervals. Antiserum was harvested 41, 69, and 97 days after the initial injection. The anti-EPI NH2-terminal peptide IgG did not inhibit EPI activity and was routinely diluted 1:5000 for immunoblot analysis of EPI samples.

**CDNA Cloning**—Approximately 50,000 colonies from a cDNA library (25, 26) prepared from mRNA isolated from the human liver-derived cell line HepG2 (ATCC HB 8065) were screened by filter hybridization with two 32P-labeled oligonucleotide probes, NOR-895 and NOR-896, using stringent hybridization conditions. NOR-895 (CATGATTGCTTTACATGGGCCATC) is complementary to position 310-333 and NOR-896 (GGGAACCTTGGTTGATTGCGGAG) is complementary to position 727-750 in the known EPI cDNA sequence (9). Five colonies hybridized to both probes, and one of these colonies was selected for further work. The presence of the EPI gene was further confirmed by sequencing. An XbaI site immediately after the stop codon was introduced into the gene by replacing the cDNA sequence downstream of the stop codon with a synthetic XbaI-XhoI fragment: 5' ATCGGATCCACGCGCGCCCTCGCTGGGAAGATGAGTGAACGCTGGTGTGCTGGGTCTGGTG-3'.

**Amino acid sequence analyses were carried out by automated Edman degradation using an Applied Biosystems 470A Gas-phase Sequencer.**

**Peptides**—were synthesized using N'-t-Boc amino acids with suitably protected side chains and methylbenzhydryl resin on an automatic peptide synthesizer (Applied Research model 430). Peptides were cleaved with 95:5 (v:v) with 5% glutaraldehyde in 0.3 M NaCl (pH 7.0). Following coupling, excess glutaraldehyde was removed by dialysis. Rabbits were administered four doses of coupled peptide (equivalent to 100 mg of peptide) in Freund's adjuvant at 28-day intervals. Antiserum was harvested 41, 69, and 97 days after the initial injection. The anti-EPI NH2-terminal peptide IgG did not inhibit EPI activity and was routinely diluted 1:5000 for immunoblot analysis of EPI samples.
Similarly, HindIII, BamHI, and EcoRI sites immediately followed by the sequence CCACCATG, corresponding to a consensus eukaryotic transcription initiation site (27), were introduced by replacing the DNA sequence upstream from the Ral site in position 154 in the EPI gene. The synthetic 28-base pair HindIII-Ral site fragment used for the construction had the following sequence: 

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M K K V
HindIII BamHI EcoRI
Rsal
S' 5' - AGCTTTGATCCTAGGACAGTAAAGAGT
T' 3' - CCACCATGTAAGGTGGTACTTCTTTCA
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Expression Vector—From the DNA constructed as described above, the EPI gene was excised as a 936-base pair BamHI-XhoI fragment and was inserted into a mammalian expression vector designated zEM219b (28), replacing the tTA gene of that vector. The zEM219b vector, modified by Dr. Elena Mudrich, ZymoGenetics, Inc., Seattle, WA, also carries an expression unit for the dihydrofolate reductase gene.

Cell Culture, Transfection, and Screening—Baby hamster kidney cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Subconfluent cells were transfected with the EPI expression plasmid by the calcium phosphate-mediated transfection procedure (29) with subsequent glutamine selection (30). Two days posttransfection, cells were treated with trypsin and diluted into selective medium containing 0.4–1.0 μM methotrexate. After 12–14 days, individual colonies were isolated and tested for EPI expression by the EPI assay. One clone (NW 11688–8) was selected for further expression level studies in a nonselective medium without methotrexate in a bicorctor. Cell culture supernatants were collected and employed for the isolation of recombinant EPI.

Purification of Recombinant EPI—Recombinant wild-type EPI was purified from conditioned baby hamster kidney medium by sequential application of heparin-Sepharose, Mono Q, Mono S, and reverse-phase HPLC chromatography essentially as described for the isolation of rEpo EPI.3 Briefly, 100 column volumes of baby hamster kidney culture medium was applied to a heparin-Sepharose column previously equilibrated at 4 °C with 20 mM Tris-HCl (pH 7.5), 10% glycerol (buffer b). Following sample application, the column was washed with buffer b containing 0.2 M NaCl. EPI was eluted from the heparin-Sepharose column with 0.7 M NaCl dissolved in buffer b. Heparin-Sepharose fractions containing EPI activity were then diluted 10-fold in buffer b and applied to a Mono Q HR 5/5 column equilibrated with buffer b. Following a wash with buffer b containing 25 mM NaCl, EPI was eluted from the Mono Q column using a linear NaCl gradient (25–500 mM NaCl) in buffer b. EPI fractions from the Mono Q fractions were pooled, diluted 5-fold in 20 mM sodium citrate (pH 5.0), 10% glycerol (buffer c), and applied to a Mono S HR 5/5 column at a flow rate of 0.4 ml/min. After a brief wash with buffer c, EPI was eluted in a gradient composed of buffer c and 50 mM imidazole-HCl (pH 7.4), 0.6 M NaCl, 10% glycerol. Fractions from the Mono S column containing EPI activity were subjected to a reverse-phase HPLC employing a Pro RPC 10/10 column equilibrated with 0.1 M H3PO4, 0.1 M NaH2PO4, 30% ethanol. The Pro RPC column was eluted with a gradient formed by equilibrating buffer and equilibrating buffer containing 30% ethanol. Recombinant EPI eluted from the Pro RPC column was desalted in a column of Sephadex G-25 equilibrated at 25 °C with 10 mM glycylglycine (pH 7.0), 100 mM NaCl, 165 mM mannitol, and freeze-dried.

Cell Electrophoresis—SDS-polyacrylamide slab gel electrophoresis was performed according to Laemmli (31) using 10% polyacrylamide separating gels. Following electrophoresis, the proteins were visualized by staining with Coomassie Brilliant Blue or subjected to immunoblotting (32). Rabbit antibody against a synthetic peptide found in the amino-terminal end of EPI was used to detect EPI following electrotransfer.

EPI Activity Assay—EPI activity was measured by a slight modification of the method described by Sandset et al. (33). The assay was performed in microtitration plates in a 37 °C water bath. Duplicate aliquots (50 μl) of test sample or standard were pipetted into wells and 100 μl of factor Xa/substrate/tissue factor/calciumpool combination reagent was added to each well. After 10-min incubation, 25 μl of factor X (40 μM) was added to each well. After a further 10-min incubation, 25 μl of S-2222 (2 mg/ml) was added. The reaction was stopped 10 min after the addition of S-2222 with 50 μl of 1 M citric acid (pH 3.0) and the plate read at 405 nm on a microplate reader.

Heat-treated (56 °C) normal pooled plasma was used as the standard EPI sample assuming 1 unit of EPI/ml. Standards and test samples were diluted in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 μg/ml PPACK, 0.2% BSA, 0.5% NaN3, and 0.5% methotrexate to a final concentration of 30 μl factor Xa, dilute human thromboplastin (1,000 units) factor Xa, and 18 mM CaCl2 dissolved in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.02% NaN3.

Inhibition of Factor Xa Amidolytic Activity by Recombinant EPI—Human factor Xa (300 pm) was mixed with various concentrations of factor VIIa, factor IXa, and factor Xa in a linear range of concentrations of each factor. The absorbance at 405 nm was continuously recorded using a Beckman DU-65 spectrophotometer. The velocity (ΔA/min) was plotted versus rEPI concentration, and Kapp, determined for each substrate concentration according to the graphical method of Dixon (34). The Ki, for factor Xa hydrolysis of FXa-1 was determined in the absence of rEPI from a Lineweaver-Burk plot. This Ki, value, together with the three [S] Kapp values, were used to determine K, as the y intercept of a Kapp versus FXa-1 concentration plot.

Analysis of Factor X Activation by Cell-bound Factor VIIa in the Presence of Recombinant EPI—Recombinant tissue factor apoprotein (10 nM effective concentration) and factor VIIa (10 nM) were coinubated in a polystyrene cuvette containing 0.1 M NaH2PO4, 50 mM NaCl, 5 mM CaCl2, 0.5% BSA (buffer a). Following a 10-min incubation at 25 °C, 200 μl of S-2288 (0.4 mM) was added along with various concentrations of rEPI. The absorbance at 405 nm was recorded continuously and initial velocity (ΔA/min) plotted versus rEPI concentration.

Analysis of Factor IX Activation by Cell-bound Factor VIIa in the Presence of Recombinant EPI—Twelve-well plates were initially seeded with 1.3 × 105 382 cells/cm2 and grown to confluence. At confluency, there were 2.0 × 105 cells/cm2. Cells were washed once with buffer B (10 mM Hepes (pH 7.45), 137 mM NaCl, 4 mM KC1, 11 mM glucose) supplemented with 10 mM EDTA and subsequently washed three times with buffer B. Duplicate wells were incubated with factor VIIa (10 nM), factor IX (50 nM), and various concentrations of recombinant EPI in 0.5 ml of buffer A. After incubation for 15 min at 37 °C, 100 μl of (where TBS is 50 mM Tris-HCl (pH 7.5), 100 mM NaCl), 50 mM EDTA was added to stop the reaction. Aliquots (120 μl) of the supernatant were removed, and factor IX coagulant activity generated in the reaction mixtures was determined in a Becton-Dickinson fibrometer using nonactivated factor IX-deficient plasma essentially as described (35). Factor IXa concentrations were calculated from a standard curve of AA/min versus factor Xa concentration. In experiments designed to measure the effect of factor VIII on factor IX activation, a mixture of factor VIII (500 ng/ml) and various concentrations of rEPI (0-10 nM) were each incubated for 15 min at 25 °C. In separate experiments, factor VIIa was replaced by zymogen factor VII (10 PM). The rationale for preincubating factor VII/VIIa with tissue factor, and EPI with factor Xa was 1) to ensure complex formation between factor VII/VIIa with tissue factor, and 2) to realize potential for a factor Xa-mediated mechanism. These two incubation mixtures were then mixed with S-2222 (0.1 mM final concentration) in a polystyrene cuvette in a final volume of 1 ml of buffer A. The absorbance at 405 nm was continuously recorded for 5 min, and factor Xa activity was integrated from a standard curve of AA/min versus factor IX concentrations that was linear in the range 1–100 ng of factor IXa/ml.

Analysis of Factor X Activation by Factor VIIa-Tissue Factor Complex in the Presence of Recombinant EPI—In these experiments, a mixture of factor VIIa (10 PM) and relipidated tissue factor apoprotein (10 nM effective concentration) and factor XI (50 nM) were each incubated for 15 min at 37 °C, 100 μl of (where TBS is 50 mM Tris-HCl (pH 7.5), 100 mM NaCl), 50 mM EDTA was added to stop the reaction. Aliquots (120 μl) of the supernatant were removed, and factor X coagulant activity generated in the reaction mixtures was determined in a Becton-Dickinson fibrometer using nonactivated factor IX-deficient plasma essentially as described (35). Factor Xa concentrations were calculated from a standard curve of AA/min versus factor Xa concentration. In experiments designed to measure the effect of factor VIII and IX on this reaction, a mixture of these proteins was added simultaneously with the chromogenic substrate. The final concentration of factor VIII and factor IX in these systems were 1 unit and 50 nM, respectively.

Analysis of Factor X Activity by Cell-bound Factor VIIa in the Presence of Recombinant EPI—Initially, confluent monolayers of 382 cells were washed as described above. Duplicate wells were then offered factor VIIa (10 nM), factor X (100 nM), and various concentrations of rEPI on 0.5 ml of buffer A. After a 10-min incubation at 37 °C, 100 μl of TBS, 50 mM EDTA was added to each well to stop the reaction. The aliquot (120 μl) was removed and transferred to a
polystyrene cuvette containing 780 μl of TBS, 20 mM EDTA (pH 8.5). Finally, 190 μl of S 2222 (1 mM) was added and the absorbance at 405 nm continuously recorded for 3 min. Residual factor Xa activity was interpolated from a standard curve of ΔAmin/min versus factor Xa concentration. In reaction mixtures containing cofactor VIII and factor IX, concentrations of 1 unit/ml and 50 nM were employed, respectively.

Analysis of Prothrombin Activation by Cell-bound Factor Xa in the Presence of Recombinant EPI—In an earlier report from this laboratory (38), we demonstrated that J82 cells readily bind factor Xa, and cell-bound factor Xa exhibits proteolytic activity toward prothrombin. In these experiments, confluent monolayers of J82 cells in 12-well plates were washed as described above. Wells were subsequently incubated for 30 min at 25 °C with factor Xa (10 nM) in 0.5 ml of buffer A. Wells were then washed three times with buffer A, and duplicate wells were incubated with various concentrations of rEPI for 30 min at 25 °C in 0.5 ml buffer A. Prothrombin (2 μM final concentration) was added to each well, and the incubation carried out an additional 30 min at 25 °C. At this time, an aliquot (10 μl) of the incubation mixture was removed and added to a cuvette containing 890 μl of 50 mM Tris-HCl (pH 8.3), 100 mM NaCl, and 100 μl S-2238 (1 mM). Thrombin concentration was interpolated from a standard curve of ΔAmin/min versus thrombin concentration.

**RESULTS**

**Purification and Structural Characterization of Recombinant EPI**—Recombinant EPI was purified to homogeneity from 33 liters of spent media of stably transfected baby hamster kidney cells with an overall recovery of 32% using a combination of heparin-Sepharose chromatography, ion-exchange fast protein liquid chromatography, and reverse-phase HPLC. A similar purification scheme has been used previously for the purification of trace quantities of EPI present in HeLa cell culture media. The specific activity of recombinant EPI was estimated to be 30,000 units/mg protein. The results of the purification of recombinant EPI produced in baby hamster kidney cells are summarized in Table I. In this purification procedure, heparin-Sepharose chromatography was selected as the initial step since earlier studies demonstrated that HeLa cell EPI possessed a relatively high affinity for heparin-Sepharose. For recombinant EPI, the heparin-Sepharose step results in more than 100-fold purification from culture media. The recovery of EPI activity in each step was 55–86% with the exception of the reverse-phase HPLC where virtually a quantitative recovery was observed. Recombinant EPI eluted from the Pro RPC reverse-phase column as three closely spaced peaks (data not shown). As EPI-specific activity in each peak was identical, all three peaks were pooled for further characterization. The structural differences between the three recombinant EPI isoforms eluting from the Pro RPC column are unknown, although it is probable that the observed heterogeneity reflects different degrees of glycosylation. A similar heterogeneity has been observed by HPLC separation of HeLa cell-derived EPI.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of recombinant EPI from each step in the purification indicated that recombinant EPI was essentially pure after the Mono S-FPLC step. As shown in Fig. 1, purified recombinant EPI migrated as a single band in SDS gels with an apparent molecular weight of 40,000 in the absence of reducing agent and 42,000 in the presence of 10% 2-mercaptoethanol. SDS-PAGE immunoblot analysis of recombinant EPI indicated a single band that migrated with essentially the same molecular weight as HeLa cell and HepG2 cell-derived EPI (Fig. 2).

Although not proved in this study, small differences in the apparent M, values of these proteins are probably due to differences in glycosylation. Amino-terminal amino acid sequence analysis of recombinant EPI indicated a single sequence of Asp-Ser-Glu-Glu-Asp-Glu-Glu-His-Thr-Ile-Ile-Thr-Asp. This sequence is identical to that found for HeLa cell EPI, and HepG2 EPI (9).

**Functional Characterization of Recombinant EPI**—In the initial experiments with purified recombinant EPI, we confirmed the observation by Broze and co-workers (4) that EPI is a slow-acting, tight-binding competitive inhibitor of human factor Xa. In order to determine the inhibitor constant (Kii) for EPI toward factor Xa, we first obtained apparent Kii (Kii,app) values at three different concentrations of a factor Xa-specific chromogenic substrate, and a K, value for this substrate in the absence of EPI from a Lineweaver-Burk plot. The Kii,app values were obtained by the graphical method of Dixon (34). K, was obtained as the y intercept of a plot of Kii,app versus chromogenic substrate concentration (Fig. 3). According to this method, the K, value for the inhibition of factor Xa amidolytic activity by recombinant EPI was 130 pm EPI.

We next determined the effect of recombinant EPI on the amidolytic activity of factor VIIa. In preliminary studies, we observed that recombinant factor VIIa possessed weak amidolytic activity toward Ile-Pro-Arg-p-nitroanilide (S-2288). Relipidated tissue factor apoprotein greatly augmented the amidolytic activity of factor VIIa toward this substrate (Fig. 4) that was maximal at equimolar concentrations of factor VIIa and tissue factor apoprotein (data not shown). Recombinant EPI inhibited the amidolytic activity of an equimolar complex of factor VIIa-tissue factor in a dose-dependent manner with half-maximal inhibition at 10 nm EPI (Fig. 5). Recombinant EPI had little, if any, effect on the amidolytic activity of factor VIIa in the absence of tissue factor, suggesting that EPI recognized a conformation in factor VIIa induced through its association with tissue factor.

As factor VIIa-tissue factor activates both factors IX and X, and EPI inhibited the amidolytic activity of factor VIIa-tissue factor, we next examined whether or not recombinant EPI inhibited factor VIIa-tissue factor proteolytic activity toward factor IX using factor VIIa bound to J82 cell surface-expressed tissue factor. Inconsistent with its effect on factor VIIa-tissue factor amidolytic activity, but consistent with earlier reports, the inhibition of factor IX activation by J82 cell-bound factor VIIa was observed in the presence of sevenfold concentrations of recombinant EPI and in the absence of exogenous factor Xa. Precisely how EPI can inhibit the active site of factor VIIa toward a small chromogenic substrate and

**TABLE I**

| Step                  | Volume | Protein | EPI activity | Specific activity | Recovery |
|-----------------------|--------|---------|--------------|------------------|----------|
|                       | ml     | mg      | units        | unit/mg          | %        |
| Culture medium        | 35,000 | 55,000  | 990,000      | 18               | 100      |
| Heparin-Sepharose     | 570    | 260     | 680,000      | 2,600            | 69       |
| Mono-Q                | 134    | 28      | 389,000      | 12,400           | 38       |
| Mono-S                | 20     | 12      | 320,000      | 26,000           | 32       |
| HPLC                  | 66     | 11      | 320,000      | 30,000           | 32       |

a Protein was measured at 280 nm assuming an E280 of 10.0.

b EPI activity was measured using a chromogenic assay as described under "Experimental Procedures."
FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of recombinant EPI. Reduced and unreduced samples were electrophoresed in a 10% polyacrylamide slab gel according to Laemmli (31). Lane 1, 10 ng of unreduced EPI; lane 2, 10 ng of reduced EPI; lane 3, mixture of reduced standard proteins including phosphorylase b (M, 94,000), bovine serum albumin (M, 67,000), ovalbumin (M, 43,000), carbonic anhydrase (M, 30,000), soybean trypsin inhibitor (M, 20,000), and α-lactalbumin (M, 14,000).

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis of recombinant EPI (lane 1), HepG2 EPI (lane 2), and HeLa EPI (lane 3). Approximately 1 ng of protein was loaded in each well. Following electrophoresis, proteins were detected with rabbit IgG against the amino terminus of EPI.

have no effect on a large protein substrate is unknown and merits further investigation. With the exception of its ability to inhibit factor VIIa-tissue factor amidolytic activity, recombinant EPI appears to exhibit the same molecular properties and inhibitory specificity as that reported for EPI isolated from HepG2 media (7, 8).

Only a limited amount of information is currently available concerning the activation of factor X by factor VIIa-tissue factor factor in the presence of EPI, factor VIII, and factor IX (37). Thrombin-stimulated human umbilical vein endothelial cells express cell-surface tissue factor that is functionally equivalent to purified relipidated tissue factor apoprotein (12). Rapaport and co-workers (12) have demonstrated that endothelial cell-surface tissue factor activity is inhibited by dilute human plasma in a reaction that is blocked by polyclonal antibodies directed against plasma EPI. In addition, EPI isolated from HepG2-conditioned media has recently been shown to inhibit factor VIIa bound to tissue factor expressed on subcultured fibroblasts in a reaction that depended upon exogenous factor Xa (38). In order to gain insight into the inhibitory efficacy of EPI during normal hemostasis, we investigated the effect of various concentrations of recombinant EPI on factor X activation by factor VIIa-tissue factor in the presence and absence of factors VIII and IX. In these studies, relipidated human brain tissue factor apoprotein was used as the tissue factor. Preliminary studies performed in the absence of EPI indicated that more factor Xa was formed in the presence of factors VIII and IX than in their absence, suggesting that trace amounts of factor Xa generated early in the reaction activated factor VIII (39). Factor IXa generated by factor VIIa-tissue factor, in complex with factor VIIIa, presumably activates additional factor X. When increasing amounts of recombinant EPI were added to the above reaction mixtures, an inhibition of factor X activation was observed (Fig. 6). The inhibition of factor X activation by recombinant EPI was considerably more effective in the absence of factors VIII and IX, and half-maximal inhibition was observed at 1 nM recombinant EPI (Fig. 6). In the presence of physiological levels of factors VIII and IX, 2.5 nM recombinant EPI was required to effect half-maximal inhibition. In all of these experiments, factor Xa activity was measured using a chro-
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FIG. 5. Inhibition of factor VIIa-tissue factor amidolytic activity by recombinant EPI. Relipidated, recombinant human tissue factor apoprotein (10 nM effective concentration) and 10 nM factor VIIa were incubated for 15 min with various concentrations of recombinant EPI (0–25 nM). The amidolytic activity of factor VIIa was then determined using S-2288.

FIG. 6. The effect of recombinant EPI on the activation of factor X by factor VIIa-tissue factor in the presence (○) and absence (△) of factor VIII (1 unit/ml) and factor IX (50 nM). Mixtures of factor VIIa-tissue factor (each 10 pm) and factor X (100 nM) and various concentrations of recombinant EPI (0–10 nM) were incubated separately for 15 min at 25 °C. Both mixtures were then combined in a polystyrene cuvette containing S-2222 (0.1 mM) with or without a mixture of factor VIII-IX. The amidolytic activity of factor Xa was determined as described in “Experimental Procedures."

FIG. 7. The effect of recombinant EPI on the activation of factor X by J82 cell-bound factor VIIa in the presence (○) and absence (△) of factor VIII (1 unit/ml) and factor IX (50 nM). Factor Xa activity was determined as described under “Experimental Procedures."

FIG. 8. Inhibition of J82 cell-bound factor Xa by recombinant EPI. Monolayers of J82 cells in 12-well plates were saturated with factor Xa, washed, and subsequently incubated 30 min at 25 °C with various concentrations of recombinant EPI (0–25 nM). Following incubation with EPI, prothrombin (2 pm) was added and incubation continued for an additional 30 min. At this time, an aliquot was removed and thrombin formation determined using S-2238.

We next investigated the effect of recombinant EPI on the activation of factor X by factor VIIa in complex with tissue factor constitutively expressed on the cell surface of a human bladder carcinoma cell line, J82. Initial experiments performed in the absence of EPI demonstrated a linear time course for the generation of factor Xa activity even when the activity was assessed in the initial phase of the reaction. The results indicate that EPI, considered to be a slow-acting inhibitor, has a profound effect on the generation of factor Xa activity even when the activity is assessed in the initial phase of the reaction. The above results suggested that factor Xa in the cell experiments was still capable of activating factor VIII in the absence of these clotting factors. Recombinant EPI completely blocked factor X activation by cell-bound factor VIIa in the absence of factors VIII and IX at a concentration of ~10 nM, or roughly 4 units/ml of EPI assuming a plasma concentration of 2.5 nM EPI (7). In sharp contrast, the rate of factor X activation was reduced by ~70% at 5 units/ml EPI in the presence of factors VIII and IX (Fig. 7).

The above results suggested that factor Xa in the cell experiments was still capable of activating factor VIII in the presence of fairly high concentrations of recombinant EPI. Recent studies performed in our laboratory indicated that J82 cells possess ~28,000-binding sites for human factor Xa (Kd = 1.6 nM factor Xa), and that cell-bound factor Xa readily activates prothrombin (36). Accordingly, we investigated the ability of recombinant EPI to inhibit the activation of prothrombin by cell-bound factor Xa. Fig. 8 illustrates the effect of increasing EPI concentrations in this system. Inhibition of prothrombin activation by EPI was identical to that observed for factor X activation in the presence of these clotting factors. Recombinant EPI completely blocked factor X activation by cell-bound factor VIIa in the absence of factors VIII and IX at a concentration of ~10 nM, or roughly 4 units/ml of EPI assuming a plasma concentration of 2.5 nM EPI (7). In sharp contrast, the rate of factor X activation was reduced by ~70% at 5 units/ml EPI in the presence of factors VIII and IX (Fig. 7).
VIII was mediated by cell-bound factor Xa even at very high concentrations of EPI.

**DISCUSSION**

In this paper, we describe the expression, purification, and biochemical characterization of recombinant extrinsic pathway inhibitor (EPI). A complementary DNA for EPI was isolated from a human hepatoma cell line (HepG2) cDNA library, and plasmids containing this cDNA were constructed and transfected into BHK cells. A producer cell line was established that produced 1–2 mg of EPI/liter of cell culture medium. Recombinant EPI was purified to homogeneity from the BHK cell medium at 32% yield and migrated as a single band in reduced SDS-PAGE with an apparent M, of 42,000. Recombinant EPI exhibited an amino-terminal sequence identical to that reported for HepG2 cell and HeLa cell-derived EPI, although small differences in M, were observed for these three proteins in immunoblotting experiments. These small differences in M, may be due to differences in glycosylation of EPI by HepG2, HeLa, and BHK cells.

Purified recombinant EPI preparations exhibited a specific activity of 30,000 units/mg in a tissue factor inhibition assay. This value is approximately 2-fold higher than that reported for purified plasma EPI and EPI isolated from HepG2 conditioned media (6). HeLa cell-derived EPI exhibited a specific activity of 20,000 units/mg by the same method used to assay recombinant EPI. The reason(s) for the significant differences in specific activity values between recombinant EPI and that reported for plasma and HepG2 EPI are unknown, but may reflect subtle differences in the assays and reagents employed.

According to earlier reports, EPI does not inhibit the proteolytic activity of factor VIIa-tissue factor in the absence of factor Xa (2, 3). This finding was confirmed in the present study using factor IX as the substrate for factor VIIa-tissue factor. However, in separate studies, we observed that tissue factor augmented the amidolytic activity of factor VIIa toward human factor X by factor VIIa-tissue factor in the presence of factors VIII and IX. Our findings do not shed light on the mechanisms associated with the observed therapeutic effectiveness of infused plasma-derived or recombinant factor VIIa in arresting bleeding episodes in hemophiliacs with circulating antibodies against factor VIII (49–50). Presumably, these individuals have normal circulating levels of EPI which, as noted in a single patient, do not appear to change following infusion of recombinant factor VIIa (46). One explanation for the beneficial effect of recombinant factor VIIa in the treatment of inhibitor patients may be that factor VIIa directly activates factor X in the absence of tissue factor but in the presence of calcium and an appropriate phospholipid membrane as proposed by several groups (47–49). EPI appears to recognize only that conformation in factor VIIa generated by its association with tissue factor. The rate of factor X activation by factor VIIa-phospholipid would appear to be insignificant given the relatively low affinity of factor VII/VIIa for acidic phospholipids (50, 51). On the other hand, factor X has a relatively high affinity for mixed phospholipid vesicles (52), and conceivably mechanisms are operative that would allow high affinity binding of factor VIIa to the phospholipid membrane through its affinity for factor X. This possibility is currently under investigation in our laboratory.

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