Overexpression of prolylcarboxypeptidase enhances plasma prekallikrein activation on Chinese hamster ovary cells

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Shariat-Madar, Z., E. Rahimy, F. Mahdi, and A. H. Schmaier. Overexpression of prolylcarboxypeptidase enhances plasma prekallikrein activation on Chinese hamster ovary cells. Am J Physiol Heart Circ Physiol 289: H2697–H2703, 2005.—Plasma prekallikrein (PK) complexes with its receptor, high-molecular-weight kininogen (HK), on human umbilical vein endothelial cells (HUVEC). When assembled on endothelial cells, PK is activated to plasma kallikrein independent of factor XIIa by the serine protease prolylcarboxypeptidase (PRCP, *K*ₘ = 9 nM). PRCP was shown to be a PK activator when isolated from HUVEC (J Biol Chem 277: 17962–17969, 2002) and produced as a recombinant protein (Blood 103: 4554–4561, 2004). To additionally confirm that human PRCP is a physiological PK activator, PRCP was overexpressed in Chinese hamster ovary (CHO) cells. CHO cells were transfected with full-length PRCP under the control of a cytomegalovirus promoter, and CHO recombinant PRCP was expressed as a fusion protein with COOH-terminal enhanced green fluorescence protein (EGFP). The presence of recombinant PRCP in transfected CHO cells was detected by real-time RT-PCR, immunoblot, and immunoprecipitation. PRCP mRNA and PK activation were two- to threefold higher in transfected than in control CHO cells. The increase in PRCP-induced PK activation in the transfected CHO cells paralleled the increase in PRCP antigen expression, as determined by anti-PRCP and anti-green fluorescence protein antibodies. PK activation of the transfected cells was blocked by small interfering RNA to PRCP. Anti-PRCP antibody and Z-Pro-Pro-aldehyde dimethyl acetate also blocked PK activation (IC₅₀ = 0.01 and 7.0 mM, respectively). Localization of PRCP in intact cells observed via confocal microscopy and flow cytometry also confirmed overexpression of PRCP on the external membrane. These investigations independently confirm that PRCP is expressed on cell membranes and that PRCP expression increases PK activation.

Plasma kallikrein–kinin system; high-molecular-weight kininogen

PROLYLCARBOXYPEPTIDASE (PRCP) was initially described as bradykinin (BK)- and angiotensin II-inactivating enzyme. Recently, it was recognized to be a plasma prekallikrein (PK) activator in endothelial cells [human umbilical vein endothelial cells (HUVEC)] and cell matrix (8, 10, 13, 14). When the high-molecular-weight kininogen (HK)-PK complex binds to HUVEC membranes, PK is rapidly converted to plasma kallikrein by PRCP (9, 14). Formed plasma kallikrein then liberates BK from HK, which leads to vasodilation and subsequent nitric oxide and PGI₂ formation and tissue plasminogen activator liberation by propagation of the signal through the BK type 2 receptor (19). This pathway of activation is important for wound healing, angiogenesis, high blood pressure regulation, thrombosis risk, and, perhaps, metabolic syndrome (7, 12, 15, 17). Therefore, an understanding of the regulation and activation of plasma PK has a broad biological interest.

PRCP was first described as being associated with lysosomes and membranes (3, 17, 18). Recently, it has been characterized in more detail (14). Colocalization studies show that the protein is present on the cell surface and in lysosomes (14). It colocalizes with gC1qR, urokinase plasminogen activator receptor (uPAR), and cytokeratin 1 (14). However, PRCP does not completely colocalize with lysosomal-associated protein 1 (3). Because very little is known about PRCP and its expression on cell surfaces, we characterized the function of PRCP by overexpressing the protein in Chinese hamster ovary (CHO) cells. Our results show that overexpressed PRCP in CHO cells is located to the cell surface. Overexpressed PRCP in CHO cells also enhances plasma PK activation.

MATERIALS AND METHODS

Materials. A biotinylation kit and ImmunoPure streptavidin-horse-radish peroxidase dihydrochloride [turbo-3,3′,5′,5′-tetrabromobenzene dihydrochloride (Turbo-TMB)] were purchased from Pierce Chemical (Rockford, IL); prestained and low-molecular-weight standards, nitrocellulose and polyacrylamide, from Bio-Rad (Richmond, CA); HUVEC, endothelial cell growth medium, and trypsin-neutralizing buffer from Clonetics (San Diego, CA); trypsin-EDTA and enhanced green fluorescence protein (EGFP) vector from GIBCO Laboratories (Grand Island, NY); HD-Pro-Phe-Arg-p-nitroanilide (S2302) from DiaPharma (Franklin, OH); human HK (18 U/mg) and PK (21 U/mg) from Research Enzyme Laboratory (South Bend, IN); EcoRI and T4 DNA ligase from New England Biolabs (Beverly, MA); DNA polymerase from Stratagene (La Jolla, CA); and DMEM-Ham’s F-12, fetal bovine serum (FBS), CHO-S-SEMM II (serum-free medium), and OPTI-MEM I from American Type Culture Collection (Manassas, VA); and Lipofectamine 2000 from Invitrogen (Carlsbad, CA). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Proteins, peptides, and antibodies. The peptide acetyl-[KTFN-QRYLVADKYWK]-amide (KTIF16), corresponding to the indicated amino acid sequences of PRCP, was prepared and used to immunize goats for the production of antisera (anti-KTIF16 antibody) at Quality Controlled Biochemicals (Hopkinton, MA). This peptide is named using the one-letter code of the first three amino acid residues followed by the number of amino acids in the peptide unless otherwise stated. Antisera produced by this peptide were affinity purified on a column that immobilized its respective peptide. Anti-KTIF16 antibody was biotinylated according to the procedure of Pierce Chemical (Rockford, IL). Briefly, 1 mg of anti-KTIF16 was dialyzed against 0.01 M sodium phosphate and 0.15 M NaCl, pH 7.4. Sulfo-NHS-LC-Biotin
was added to anti-KTF16 antibody to give a fourfold molar excess of Sulfo-NHS- LC-Biotin over anti-KTF16 antibody. After incubation for 30 min at room temperature, the sample was loaded onto a 10-ml Econo-Pac 10 DG column (Bio-Rad). Biotinylated anti-KTF16 antibody was monitored by absorbance at 280 nm using an extinction coefficient of 14 for antibody to determine its protein concentration.

Plasmid. For construction of the vector containing human PRCP (GenBank Assesorion No. 431320) for CHO cell transfection, the coding region of human PRCP was amplified by PCR using pMT/Bip/V5-His-CPR as a template. The forward and reverse primers were 5′-CGGAATTCTCAGTGCTGCTTTCCCGCACT-3′ and 5′-CGGAATTTCAGTGGCTTTCCCCGACT-3′, respectively (the underlined nucleotides of the primers denote the two EcoR I sites; Table 1). The PCR product was digested with EcoR I and then ligated to a previously digested pEGFP-C1 (Invitrogen). Next, PRCP cDNA was transferred to six-well plates containing 3 ml of serum-free DMEM and to lower risk of contamination. Melting profiles showed generation of a specific product. The PCR mixture (50 µl) consisted of each primer at 0.2 µM, 0.02 µM probe, and 1 µg of RNA and TaqMan Universal Master Mix (2×) or platinum Taq. Reaction conditions were as follows: 50°C for 30 min and then 95°C for 5 min followed by 35 cycles at 95°C for 1 min and 38 cycles at 60°C for 1 min. Real-time PCR data were expressed as a relative quantity based on the ratio of fluorescent change. Negative controls (samples without polymerase) were performed in parallel during different determinations to assess melting curve and ensure equivalent assay conditions. cDNA products were analyzed for purity by gel electrophoresis and sequencing. All assays were performed in triplicate and are reported as means.

Real-time quantitative RT-PCR. Real-time quantitative (Tagman) PCR analysis was carried out using SuperScript one-step RT-PCR with platinum Taq according to the manufacturer’s instructions (Invitrogen), with minor modifications. Briefly, measurements were performed using the iCycler iQ real-time PCR detection system (Bio-Rad). The primers (Invitrogen) and probes (Integrated DNA Technologies, Coralville, IA) directed to a unique site of γ-actin (control) and the catalytic region of PRCP were designed. Probes were 5′-labeled with 6-carboxyfluorescein amide and a downstream 3′ black hole quencher dye (BHQ-1, Integrated DNA Technologies; Table 1). Specific primers for the real-time PCR were used for the RT-PCR assay examining expression of PRCP RNA in CHO cells. A one-step real-time RT-PCR was used to reduce handling of the sample and to lower risk of contamination. Melting profiles showed generation of a specific product. The PCR mixture (50 µl) consisted of each primer at 0.2 µM, 0.02 µM probe, and 1 µg of RNA and TaqMan Universal Master Mix (2×) or platinum Taq. Reaction conditions were as follows: 50°C for 30 min and then 95°C for 5 min followed by 35 cycles at 95°C for 1 min and 38 cycles at 60°C for 1 min. Real-time PCR data were expressed as a relative quantity based on the ratio of fluorescent change. Negative controls (samples without polymerase) were performed in parallel during different determinations to assess melting curve and ensure equivalent assay conditions. cDNA products were analyzed for purity by gel electrophoresis and sequencing. All assays were performed in triplicate and are reported as means.

Small interfering RNA. The GenBank database was searched for unique sequences within the PRCP that had no identity with known cellular genes. Four sites in the PRCP transcript were chosen as targets for small interfering RNA (siRNA), and siRNA were synthesized at Integrated DNA Technologies. A double-stranded siRNA targeted to the translation initiation site on PRCP was more effective at a lower concentration than that targeted to the other three selected sites. The 19-nt siRNA duplex designed to target this site in the PRCP transcript was 5′-GACUCCUCUGGUGUAUATT-3′. The integrity and viability of the CHO cells after transfection with siRNA were

Table 1. Primer and probe sequences used to quantify gene expression by RT-PCR and real-time PCR

<table>
<thead>
<tr>
<th>Genes Sequences</th>
<th>Product Size, bp</th>
<th>GeneBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-PRCP</td>
<td>75</td>
<td>431320</td>
</tr>
<tr>
<td>Probe: 5′-56-FAM-CTTGTTGTTGCTGAACATCG3′/BHQQ-1/3′</td>
<td>87</td>
<td>18256836</td>
</tr>
<tr>
<td>Primer 1: 5′-GGTGGCTGAACCTGAAATG-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer 2: 5′-GTCAACAGGAGGAGGAC-3′</td>
<td></td>
<td></td>
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<tr>
<td>γ-Actin</td>
<td>1,365</td>
<td>431320</td>
</tr>
<tr>
<td>Probe: 5′-HEX-TACCCACCTAGTACCCCGCATGCTGA3′/BHQQ-1</td>
<td>2,238</td>
<td>55763</td>
</tr>
<tr>
<td>Primer 1: 5′-GGTGGCTGAATCGTCTCTCTG-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer 2: 5′-CAGTGGCTGTTCTCTGCT-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFPR-PRCP</td>
<td>1,365</td>
<td>431320</td>
</tr>
<tr>
<td>Primer 1: 5′-GGTGGCTGAACCTGAAATG-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer 2: 5′-GTCAACAGGAGGAGGAC-3′</td>
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H-PRCP, human prolylcarboxypeptidase; EGFPR-PRCP, enhanced green fluorescence protein-PRCP fusion protein; 5′-56-FAM, 5′-labeled with 6-carboxyfluorescein amide; 3′-BHQ 1, 3′-labeled with black hole quencher dye 1; HEX, hexachlorofluorescein.
verified by trypan blue. Transfection of siRNA into CHO cells was carried out in a six-well plate using Lipofectamine 2000 according to the manufacturer’s instructions with slight modifications. Two microliters of Lipofectamine 2000 were diluted in 50 μl of Opti-MEM I, and the mixture was incubated for 5 min at room temperature. During this incubation period, 0.5–3 μl of siRNA (20 μM) were mixed with 50 μl of Opti-MEM I and incubated for 25 min at room temperature for complex formation. Then 100 μl of the siRNA-Lipofectamine mixture were added to each well containing 5 × 10⁴ CHO cells in suspension.

**Immunofluorescence analysis.** EGFP-PRCP-transfected CHO cells were plated on glass coverslips and incubated for 2 h. The cells were washed once at room temperature with 0.01 M sodium phosphate and 0.15 M NaCl, pH 7.4 (PBS), fixed in 2% paraformaldehyde (diluted in PBS) for 20 min, and then washed three times with PBS. Next, the cells were incubated with 50 mM NH₄Cl for 10 min and washed in PBS. The cells were permeabilized using 0.1% Triton X-100 in PBS (5 min) and then washed three times. To reduce nonspecific reactivity, the cells were incubated in blocking solution (5% FCS in PBS) for 30 min. Then the cells were stained using a 1:50 dilution of polyclonal anti-PRCP antibody for 1 h at 4 °C, washed, and stained with Alexa Fluor 594-conjugated anti-goat secondary antibody for 1 h at 4 °C (Molecular Probes, Eugene, OR). After the slides were washed, they were covered with antiadhesive mounting medium (Molecular Probes) and visualized on the laser scanning confocal microscope as previously described (4, 5). The cells were imaged at ×60 magnification.

**Flow cytometry.** EGFP-PRCP protein expression was measured in PRCP-transfected cells via flow cytometry as described elsewhere (4). Briefly, the cells were harvested and washed with cold PBS, fixed with an equal volume of 4% paraformaldehyde in PBS (10 min at room temperature), washed, and permeabilized with 0.1% saponin in Hanks’ balanced salt solution. For detection of PRCP antigen, the cells were stained with anti-PRCP protein antibodies at a final concentration of 30 μg/ml for 1 h at 4°C. Subsequently, the cells were washed three times in Hanks’ balanced salt solution containing saponin and incubated with Alexa 647-conjugated sheep anti-goat IgG antibody (1:500 dilution; Molecular Probes) for 60 min at 4°C. The presence of EGFP-PRCP antigen was measured directly as well. After they were washed again, the cells were analyzed for PRCP protein expression with a flow cytometer (Epics-C, Coulter Electronics, Hialeah, FL) as previously reported (4).

**Confocal microscopy.** Confocal microscopy for PRCP distribution in the CHO cells was performed as previously described (14). CHO and PRCP-transfected CHO cells were grown on tissue culture slides and exposed to vehicle (PBS) or antibody. Endothelial cell growth medium was removed, rabbit IgG (1:100), rabbit serum (1:100), or anti-PRCP (1:100) antibody was added, and the cells were incubated for 1 h at 37°C. The cells were washed and fixed in 2% paraformaldehyde for 15 min and washed with 50 mM glycine in PBS for 5 min at room temperature. PRCP was identified using 300 nM FITC goat anti-rabbit IgG (Calbiochem, La Jolla, CA). The antibody-treated cells were then washed, covered with antiadhesive Prolong mounting medium (Molecular Probes), mounted, and analyzed as previously described (14).

**Gel electrophoresis and immunoblot analysis.** Proteins were separated on a 12% SDS polyacrylamide gels and then transferred to nitrocellulose membranes at 8 mA overnight. The electroblots were incubated in blocking buffer [5% (wt/vol) dry milk with 0.1% (wt/vol) BSA, 0.05% Tween 20, 0.15 M NaCl, and 20 mM Tris-HCl, pH 7.4] for 1 h (4). Then the membrane was incubated with antibody against PRCP at 37°C for 2 h. After the nitrocellulose membrane was washed, it was incubated with horseradish peroxidase-conjugated anti-goat antibody (1:2,000 dilution). The specific reactivity of antibody with electrophoretically separated PRCP was detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL). All steps were carried out at room temperature.

**Endothelial cell culture.** HUVEC were obtained and cultured according to the recommendations of Clonetics. Cells between passages 1 and 5 were subcultured onto fibronectin-treated, 96-well plates 24 h before the start of the experiment as previously reported. Cell viability was determined using trypan blue exclusion. Cell numbers was determined by counting on a hemocytometer.

**Statistical analysis.** Values are means ± SD of n different experiments. PRCP activity in wild-type and PRCP-transfected CHO cells was determined by measuring substrate hydrolysis (S2302 or Ala-Pro-p-nitroanilide) and compared between the two groups. Differences were considered significant at P < 0.05.

**RESULTS**

**Generation of increased PRCP mRNA in transfected CHO cells.** To investigate the influence of PRCP expression on PK activation on cell surfaces, we created CHO cells that overexpress PRCP. We used a vector that attached the GFP (EGFP) to PRCP (Fig. 1A). The resulting EGFP-PRCP fusion protein allowed generation of transfected CHO cells that were labeled with the fluorescent reporter protein. Investigations were performed to examine EGFP-PRCP and PRCP mRNA expression in transfected CHO cells. Total RNA was isolated from PRCP-transfected CHO cells, and RT-PCR was performed (Fig. 1B). As predicted, a 2,181-bp band for transfected EGFP-PRCP was detected in the cDNA from the transfected cells with the use of primers to GFP. In the same RNA preparation, the 1,365-bp cDNA for human PRCP was isolated from PRCP-transfected CHO cells with use of primers for PRCP (Fig. 1B). The identity of EGFP-PRCP in the RT-PCR product was confirmed by restriction enzyme analysis and sequencing (data not shown).

**Fig. 1. Generation of enhanced green fluorescence protein (EGFP)-prolylcarboxypeptidase (PRCP) in Chinese hamster ovary (CHO) cells.** A: schematic representation of the PRCP construct used to generate transfected cells under control of cytomegalovirus (CMV) promoter. pA, polyadenylation. B: total RNA was isolated from PRCP-transfected CHO cells and then amplified by RT-PCR. Amplified DNA was resolved on a 1% agarose gel. With use of primers for green fluorescence protein (GFP) and human PRCP, the expected 2,293- and 1,365-bp bands for recombinant EGFP-PRCP (EGFP-PRCP) and human PRCP (PRCP), respectively, in transfected CHO cells are shown in lanes 1 and 2. C: detection of PRCP in wild-type CHO cells, PRCP-transfected CHO cells, and human umbilical vein endothelial cells (HUVEC) by real-time RT-PCR. Values (means from 3 independent experiments) are expressed in arbitrary units.
Further studies using primers and a probe directed to the catalytic region of PRCP determined the relative amount of PRCP mRNA in RT-PCR (Table 1, Fig. 1C). The relative expression of PRCP in CHO cells was determined using γ-actin as external control. The lower-threshold thermal cycle in PRCP-transfected than in wild-type CHO cells (CT = 26 vs. 31) showed that overexpression of PRCP in transfected CHO cells resulted in increased mRNA expression of PRCP.

**PRCP functional expression on CHO cells.** Investigations next proceeded to determine whether expression of PRCP activity is increased in CHO cells. PK was activated in CHO cells. In the absence of added HK or PK, there was no hydrolysis of S2302 (data not shown). After HK and PK were assembled on the cells, wild-type CHO cells formed some plasma kallikrein, which hydrolyzed S2302 (Fig. 2A). PK-activating activity in PRCP-transfected CHO cells was increased twofold over that in untransfected CHO cells (Fig. 2A). In comparison, PK-activating ability was similar in HUVEC and transfected CHO cells (Fig. 2A). Addition of 100 μM antipain blocked all PK activation in the cells, consistent with previous studies (8, 13, 14) (Fig. 2A).

Additional investigations confirmed that the CHO cell PK activator was PRCP. Wild-type and PRCP-transfected CHO cells were treated with siRNA for 24 h before measurement of PK activation (Fig. 2B). PK-activating ability declined 50% in wild-type CHO cells and 75% in PRCP-transfected CHO cells (Fig. 2B). These data indicated that the specific PK-activating enzyme in wild-type and transfected CHO cells was mostly PRCP.

Next, investigations were performed to determine whether PK-activating activity was increased in lysates of transfected CHO cells. PK-activating ability in lysates of CHO cells...
transfected with PRCP was three to four times that in lysates of wild-type CHO cells (Fig. 2C).

Additional studies determined whether human PRCP antigen was increased in transfected CHO cells. An immunoblot of an anti-PRCP immunoprecipitate showed a predominantly ~88-kDa band (Fig. 2D), which represents a fusion protein of PRCP and GFP. The identity of the smaller (~114-kDa) band is not known. Because the molecular mass of EGFP-PRCP was higher than that of PRCP (13, 14), further studies were performed to determine whether antibody to EGFP would recognize the fusion protein of the same size. Immunoprecipitated CHO EGFP-PRCP immunoblotted with anti-EGFP at ~88 kDa (Fig. 2E). Alternatively, EGFP, when directly added to the SDS-PAGE on immunoblot, was ~28 kDa (Fig. 2E). This information indicated that PRCP-transfected CHO cells produced a PRCP of ~60 kDa, which is consistent with the band identified in endothelial cells (13).

Inhibition of PRCP on transfected CHO cells. Investigations next determined whether the PRCP on transfected CHO cells is subject to the same inhibitors as PRCP on endothelial cells. Increasing concentrations of antibody to PRCP inhibited the kallikrein-forming activity of PRCP-transfected CHO cells (IC50 = 10 μM antibody; Fig. 3A). Furthermore, the PRCP inhibitor Z-Pro-Prolinal inhibited the kallikrein-forming activity of PRCP on wild-type and transfected CHO cells (IC50 = 7 mM; Fig. 3B). In addition to PK-activating activity of CHO cells, the active enzyme in transfected cells could hydrolyze the chromogenic substrate Ala-Pro-p-nitroanilide (Km = 0.4 mM; data not shown). Furthermore, increasing concentrations of Z-Pro-Prolinal also inhibited Ala-Pro-p-nitroanilide-hydrolyzing activity in PRCP-transfected and wild-type CHO cells (IC50 = 1.7 mM; Fig. 3C).

Fluorescent microscopy and flow cytometry studies of PRCP-transfected CHO cells. To determine the cellular localization of the product of pEGFP-PRCP, stable CHO cell transfections were performed. At 24 h after transfection of pEGFP-PRCP, GFP expression was examined by laser scanning confocal microscopy. The immunofluorescent pattern of transfected CHO cells labeled with GFP and polyclonal antibody to human PRCP is shown in Fig. 4A. A typical cell staining pattern of colocalization was observed with GFP and anti-PRCP antibody when the double-labeled images were merged. Flow cytometry studies revealed that ~90% of the cells were GFP positive (data not shown). Untransfected CHO cells expressed some PRCP antigen on their cell membrane (Fig. 4B). When transfected, CHO cells showed an increased rightward shift of PRCP expression on the flow cytogram.

DISCUSSION

The present investigations were initiated to determine by an independent means that PRCP is expressed on cell membranes and that its expression results in increased PK activation. Previous investigations from our laboratory indicated that PRCP on endothelial cell lysates activates PK only when bound to HK linked to microtiter plates (13). Further investigations show that a recombinant PRCP produced in insect cells activates PK only when bound to HK with a Km equal to that seen in isolated endothelial cell PRCP (Km = 7–17 nM) (14). On laser scanning confocal microscopy, endothelial cell PRCP colocalizes with the kininogen multiprotein receptor complex

Fig. 3. Characterization of PRCP-transfected CHO cells. A: effect of anti-PRCP antibodies (1–60 μM) or goat IgG on PK activation in PRCP-transfected CHO cells. After incubation of 20 nM HK and PK in the absence or presence of anti-PRCP or IgG for 1 h, plasma kallikrein activity was determined by hydrolysis of 0.8 mM S2302. B: influence of 0.01–10 mM Z-Pro-Pro aldehyde dimethyl acetate (Z-Pro-Prolinal) on PK activation on wild-type and PRCP-transfected CHO cells. CHO cells were incubated with HK and PK in the absence or presence of increasing concentrations of Z-Pro-Prolinal, and 0.8 mM HD-Pro-Phc-Arp-p-nitroanilide was added. Hydrolysis of substrate was observed for 1 h in the absence or presence of the inhibitor. C: ability of Z-Pro-Prolinal to block Ala-Pro-p-nitroanilide (A-P-pNA) hydrolysis. Ala-Pro-p-nitroanilide (1 mM) was incubated with PRCP-transfected and wild-type CHO cells, and hydrolysis of the substrate was measured for 1 h in the absence or presence of increasing concentrations of Z-Pro-Prolinal. Values are means of 3 replicates from 3 independent experiments. Data were normalized to uninhibited enzyme activity on the cell surface.
of gC1qR, uPAR, and cytokeratin 1 on nonpermeabilized cultured endothelial cells (4, 5, 14). Because PRCP is known to be enriched in cellular lysosomes, we sought an independent means to determine whether it is also expressed on the membrane (3, 17). The present investigation indicates that transfected CHO cells produce increased PRCP mRNA and antigen, which are constitutively expressed on the cell membranes, resulting in increased plasma PK activation.

Initial studies characterized the overexpression of PRCP mRNA in transfected CHO cells. The fusion protein of GFP-PRCP helped identify the cells in which PRCP expression was increased after transfection. We were surprised to observe that, on real-time RT-PCR, the primers to human PRCP also were able to amplify and detect CHO cell mRNA. Although the CT of expression of the RT-PCR from the mRNA from the wild-type cells lagged behind that from transfected CHO cells, it was present. The fusion protein GFP-PRCP was detected by antibodies to GFP and PRCP. Once the size of GFP (~28 kDa) is subtracted from the total fusion protein, the predicted size of the transfected PRCP is ~60 kDa, which is similar to the band that was purified from endothelial cells and produced in insect cells. The identity of the minor 114-kDa band detected by the anti-PRCP antibody is unknown (Fig. 2D). It is possible that it could be previously activated enzyme in a covalent complex with one of its natural inhibitors.

Our investigations also examined the cellular expression of PRCP. Via flow cytometry and laser scanning confocal microscopy, constitutive and overexpressed human PRCP is observed on the external membrane of nonpermeabilized cells. Flow cytometry shows some wild-type CHO cell PRCP on the cell membrane. This finding indicates that our antibody to PRCP detects CHO cell PRCP antigen. When CHO cells are transfected with human PRCP, membrane expression is increased. Similarly, the GFP label of the GFP-PRCP protein and PRCP antigen colocalize on the membrane of nonpermeabilized CHO cells. These latter findings indicate that, when transfected into cells, a large pool of the expressed protein becomes externalized on the membrane. Because PRCP does not specifically colocalize with lysosomal-associated protein 1, a lysosomal marker, but does colocalize with uPAR, a membrane receptor, it appears to cycle through the endosomal pathway (14).

In summary, this study demonstrates that stable overexpression of human PRCP in CHO cells significantly raises the membrane expression of the PK-activating ability of these cells. This information is consistent with our finding that PRCP is constitutively present on cultured endothelial cells, allowing
for PK activation. Furthermore, in gene-trapping experiments that targeted membrane proteins exported to the cell membrane, PRCP was trapped (16). These previous and present studies indicate that PRCP is a PK-activating serine protease (8, 13, 14). However, with the use of antisense oligonucleotides and siRNA, no reduction of cell PK-activating activity was complete, suggesting that other enzymes on cell membranes might also activate PK. The complete physiological activity of PRCP is unclear. We speculate that it contributes to basal plasma PK activation in the intravascular compartment for BK liberation. The reasons for this assessment are twofold: 1) PK probably saturates all the plasma HK bound to endothelial cell membranes, and 2) C1 inhibitor-knockout mice have constitutive angioedema as a result of BK liberation (2, 6). This activity should be important for control of blood pressure (4, 10). It is tempting to speculate that membrane or cell PRCP activity should be important for control of blood pressure (4, 10). This work was supported by National Heart, Lung, and Blood Institute Grant HL-52779 (to A. H. Schmaier) and American Heart Association Grant SDG-030193N (to Z. Shariat-Madar).

**GRANTS**

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