Divalent cations stabilize the conformation of plasma cell membrane glycoprotein PC-1 (alkaline phosphodiesterase I)

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The plasma cell-membrane glycoprotein PC-1 is an ectoenzyme with alkaline phosphodiesterase I/5′-nucleotide phosphodiesterase (EC 3.1.4.1) and nucleotide pyrophosphatase (EC 3.6.1.9) activities. It contains sequence motifs which closely match the consensus EF-hand (helix–loop–helix) Ca^{2+}-binding regions of parvalbumin, troponin-C and calmodulin, and its enzymic activity is increased in the presence of divalent cations and decreased in the presence of chelating agents. We have undertaken experiments to determine whether divalent cations affect the conformation of the PC-1 protein, as assessed by their effect on thermal stability, resistance to proteolysis and binding of polyclonal antibodies to the whole native protein and monoclonal antibodies to a putative Ca^{2+}-binding region. Divalent cations were found to protect solubilized PC-1 against thermal denaturation and proteolysis. They also stabilized PC-1 on intact cells; this form was much more resistant to proteolysis than Triton X-100 solubilized PC-1. Ca^{2+}, Mg^{2+} and Zn^{2+} ions were equally effective. Monoclonal antibodies to the bacterially expressed C-terminal EF-hand homology region only bound to mammalian PC-1 in the absence of Ca^{2+}. In contrast, the great majority of polyclonal antibodies to native PC-1 bound regardless of whether Ca^{2+} was present or not, but with increased binding when Ca^{2+} was present. These results provide evidence that divalent cations bind to PC-1 and stabilize its conformation.

INTRODUCTION

PC-1 was originally described as a marker of terminally differentiated B cells within the lymphoid system [1]. It was later shown to be expressed on chondrocytes, capillary endothelium, renal tubules, epididymis and salivary gland duct epithelium in the mouse [2]. PC-1 is a type II integral membrane glycoprotein consisting of two identical subunits of ~ 120 kDa held together by disulphide bonds [3–7]. It has been shown to have both 5′-nucleotide phosphodiesterase (5′NPDE) (EC 3.1.4.1) and nucleotide pyrophosphatase (EC 3.6.1.9) activities, and is thus established to be an ectoenzyme [8,9]. It is capable of hydrolysing phosphodiester bonds of nucleotides (e.g. cyclic AMP [10,11]) and nucleic acids (e.g. 3′ end of single-stranded DNA and RNA [10,11]) and pyrophosphate bonds of nucleotides (e.g. ATP and ADP [10,11]) and nucleotide sugars (e.g. UDP-galactose [12]).

The enzyme activities of PC-1 are increased in the presence of divalent cations and decreased in the presence of EDTA [8], suggesting that divalent cations bind to the protein; however, no direct evidence on this point is available. The binding of divalent cations to proteins sometimes results in a conformational change [13], rendering them more resistant to digestion by proteases or to thermal denaturation [14–16]. This observation, together with the detection of sequence similarity between two regions of PC-1 and the EF-hand Ca^{2+}-binding motif [13,17] (see Figure 1), prompted us to examine the effects of divalent cation binding on the stabilization of PC-1 against thermal denaturatation and proteolysis. We have also generated monoclonal antibodies to the bacterially expressed C-terminal EF-hand homology region of PC-1, and have shown that these antibodies only bind to PC-1 when Ca^{2+} is absent, suggesting a conformational change of PC-1 upon the binding of Ca^{2+}.

EXPERIMENTAL

Materials

The following compounds were obtained from the sources identified: G418 (Geneticin; Gibco, U.S.A.), *p*-nitrophenyl phenylphosphonate and glutathione–agarose (Sigma, Australia); $Na^{125}I$ (100 mCi/ml) (Amersham, Australia); Pansorbin (fixed *Staphylococcus aureus*) (Calbiochem, Australia); Protein G–Sepharose 4 Fast Flow (Pharmacia, Sweden); staphylococcal V8 protease (Miles Laboratories, U.K.); Complete Freund's Adjuvant (Commonwealth Serum Laboratories, Australia); fluorescein-conjugated sheep anti-mouse immunoglobulin (Silenus Laboratories, Australia). All other reagents were of analytical grade.

Animals

BALB/c mice were purchased from the Central Animal Facilities, Monash University.

Cell Lines

The mouse myeloma cell lines NS-1 [18] and SP2/0 [19], the mouse-PC-1 cDNA transfected Ltk^- fibroblast cell line *pLs.1* [20] and the pSV2-neo vector alone transfected cell line *NeoL* were grown at 37 °C in Dulbecco's Modified Eagle's Medium containing 10% (v/v) foetal bovine serum, 2 mM glutamine, 100 i.u./ml penicillin and 100 i.u./ml streptomycin, in an atmosphere of air plus 10% CO_2. *pLs.1* and *NeoL* cells were grown in medium supplemented with 0.4 mg/ml G418 (Gibco). The cell lines NS-1 and *pLs.1* express PC-1, whereas *NeoL* cells do not. *SABI* cells are Ltk^- cells transfected with human PC-1 cDNA in

Abbreviations used: DTT, dithiothreitol; 5′NPDE, 5′ nucleotide phosphodiesterase.

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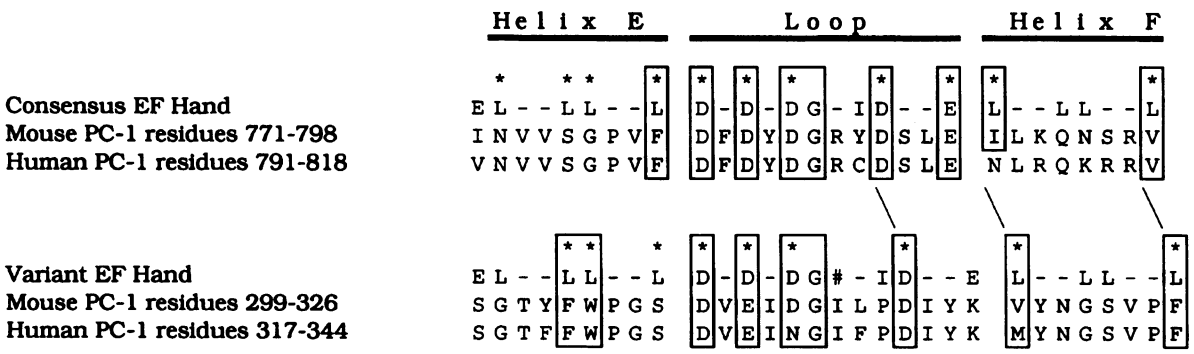

Figure 1 The putative EF-hand Ca^{2+}-binding domains of PC-1

The consensus sequence of the EF-hand Ca^{2+}-binding motif as described for parvalbumin [17] is represented, followed by the putative mouse and human PC-1 EF-hand sequences below. Sequence numbering is based on the assumption that initiation of translation begins at the first methionine [5]. The columns labelled L* in the consensus sequence usually contain hydrophobic residues in the E and F helices (Leu, Ile, Val, Phe, Met, Trp); the columns labelled D* in the consensus sequence usually contain amino acids capable of using an oxygen atom as a Ca^{2+} ligand (Asp, Asn, Glu, Gln, Ser, Thr). Positions where any amino acid may occur are indicated by dashes. The boxed lettering represents sequence similarity among the mouse and human PC-1 sequences and the consensus. Conservative substitutions have been allowed. The upper group of sequences represents the sequence which most closely resembles the EF-hand consensus sequence. In the lower group, a single gap (#) has been introduced in the consensus sequence to maximize sequence identity. A single amino acid insertion occurs in this position in the crystallographically characterized EF hand of bovine intestinal Ca^{2+}-binding protein [30] and in the Ca^{2+}-binding protein S-100 PAPI-b from bovine brain [31].

the expression vector pSVT7, and express enzymically active human PC-1 protein at their surface.

Antibodies

The monoclonal mouse anti-(mouse PC-1) antibody IR-518 has been previously described [2,20–22]. Polyclonal antibodies to native human PC-1 were generated by multiple intraperitoneal injections of *SABI* cells into syngeneic C3H/HeJ mice without adjuvant. Monoclonal antibodies to the C-terminal EF-hand homology region of human PC-1 were generated as follows. The cDNA sequence encoding the C-terminal 140 amino acids of human PC-1 was isolated by the PCR, and subcloned into the bacterial expression vector pGEX-KT [23], in which fusion proteins are formed with glutathione *S*-transferase from *Schistosoma japonicum*. The resulting fusion protein was soluble in water when induced at 25 °C, and was purified by affinity chromatography on glutathione–agarose, emulsified in Complete Freund's Adjuvant, and used to immunize BALB/c mice. Hybridomas were made by fusion of spleen cells with the non-producer cell line Sp2/0, using standard methods [21].

Rabbit antibodies to the mouse transferrin receptor (R190) were made by immunization with the purified receptor in Complete Freund's Adjuvant.

Cell-surface radioiodination

Cells ($\sim 2 \times 10^7$) were washed twice in PBS and labelled with 1 mCi of $Na^{125}I$ by the lactoperoxidase method [21]. Iodinated cells were then divided into equal aliquots before being subjected to immunoprecipitation and proteolysis, as described below.

Immunoprecipitation

Cells were harvested, washed twice (450 *g*, 5 min, 4 °C) in PBS (145 mM NaCl, 7.5 mM Na_2HPO_4, 2.5 mM $NaH_2PO_4.2H_2O$, pH 7.1) and lysed in 0.5 ml of 0.5 % (v/v) Triton X-100 in PBS (lysis buffer) for 30 min on ice. Insoluble debris was removed from cell lysates by centrifugation (2550 *g*, 5 min, 4 °C). Cell lysates were pre-cleared for 30 min at 4 °C on a rotating wheel with 50 μl of 10 % (v/v) Pansorbin, washed twice in wash buffer [50 mM Tris/HCl, pH 8.3, 0.6 M NaCl, 0.5 % (v/v) Triton X-100] and once in lysis buffer. The samples were then centrifuged (10000 *g*, 2 min, 25 °C) to remove the Pansorbin cells. Pansorbin cells were substituted for Protein G–Sepharose beads when the immunoprecipitates were used in enzyme assays. After pre-clearing, supernatants were removed, and 1 μl of anti-(mouse PC-1) monoclonal antibody ascites fluid (IR518 [22]) was added. Previous experiments have shown that the monoclonal antibody IR518 does not inhibit enzyme activity [20]. After 30 min on ice, a precipitating rabbit anti-(mouse immunoglobulin) antibody was added. To immunoprecipitate the transferrin receptor, 20 μl of a polyclonal antibody (R190) were added. After a further 30 min on ice, 100 μl of 10 % Pansorbin cells was added, and the lysates left at 4 °C for another 30 min on a rotating wheel. Immunoprecipitates were pelleted as described earlier, and washed twice in wash buffer before assaying for 5′-nucleotide phosphodiesterase activity or SDS/PAGE.

5′NPDE assay

Immunoprecipitates were washed twice (10000 *g*, 20 s, 25 °C) in assay buffer (0.15 M NaCl, 20 mM Tris/HCl, pH 8.1) containing 1 mM $CaCl_2$or 1 mM EDTA. The assay was carried out in a total volume of 600 μl of assay buffer containing 0.5 mM $CaCl_2$ or 0.5 mM EDTA, and 1 mM *p*-nitrophenyl phenylphosphonate [24]. The immunoprecipitates were left at 37 °C on a rotating wheel until a yellow colour developed, indicative of hydrolysis. They were then centrifuged (10000 *g*, 20 s), the supernatants removed and the reactions stopped with 1 mM dithiothreitol (DTT) before measuring the A_{400}. The absorbance due to the substrate alone in assay buffer with or without Ca^{2+} ions was subtracted from all readings. Conversion rates were calculated using ϵ 18320 $M^{-1} \cdot cm^{-1}$ [24]. Measurements were always taken on the linear portion of the curve.

Thermal stability

PC-1 was immunoprecipitated from *pLs*.1 cells as described earlier. The pellets were washed twice (10000 *g*, 20 s, 25 °C) in 1 mM $CaCl_2$ or 1 mM EDTA in assay buffer. They were then resuspended in 300 μl of assay buffer and incubated at 37, 45, 65 or 85 °C for 10 min. The pellets were then centrifuged (10000 *g*, 20 s, 25 °C) and washed twice in assay buffer containing 1 mM $CaCl_2$. 5′NPDE activity was measured as described earlier.

Proteolysis by V8 protease

Proteolysis of isolated ^{125}I-labelled PC-1 or ^{125}I-labelled transferrin receptor was carried out in a total volume of 100 μl of proteolysis buffer I (10 mM Hepes, pH 7.1, 0.1 M KCl) containing 1 mM $CaCl_2$, 1 mM $ZnCl_2$, 1 mM $MgCl_2$ or 1 mM EDTA and graded amounts of V8 protease. The samples were then left at 37 °C for 30 min. To precipitate the proteins, the samples were left on ice for 30 min with 9 vol. of ice-cold acetone, and then centrifuged at 10000 *g* for 10 min at 4 °C. The pellets were resuspended in SDS-sample buffer [62 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 2.3% (v/v) SDS and 50 mM DTT] and heated to 100 °C for 2 min before being subjected to SDS/PAGE and autoradiography, as described below.

Proteolysis of intact ^{125}I-labelled NS-1 cells was carried out in a total volume of 100 μl of proteolysis buffer II (5 mM Hepes, pH 7, 150 mM NaCl, 4 mM KCl) containing 1 mM $CaCl_2$ or 1 mM EDTA. Graded amounts of V8 protease were added, and the cells left at 37 °C for 30 min. The cells were then centrifuged (250 *g*, 5 min, 4 °C) and washed twice with proteolysis buffer II. The cells were lysed in Triton X-100 and PC-1 was immunoprecipitated and analysed by SDS/PAGE as described below.

Flow cytometry

Mouse Ltk$^-$ cells that had been transfected with human PC-1 (*SABI* cells) or with vector alone (*NeoL* cells) were stained using saturating amounts of monoclonal or polyclonal mouse anti-(human PC-1) antibody followed by saturating amounts of fluorescein-conjugated sheep anti-(mouse immunoglobulin) and analysed by flow cytometry using a Becton–Dickinson FACSCAN.

SDS/PAGE

The Laemmli buffer system was used [25]. Gels were stained, destained and dried before exposure at −70 °C with a Dupont Lightning Plus Intensifying Screen and Fuji RX Medical X-ray film.

RESULTS

Thermal stability of PC-1

The effectiveness of Ca^{2+} ions in stabilizing PC-1 against thermal denaturation was investigated. Immunoprecipitated PC-1 was preincubated at 37, 45, 65 or 85 °C for 10 min in the presence of Ca^{2+} ions or EDTA before the measurement of 5′NPDE activities in the presence of Ca^{2+} ions (Figure 2). Pre-incubation in the presence of EDTA for 10 min at 37 °C decreased enzyme activity to ~ 50% of that observed in the presence of Ca^{2+} ions. At 65 °C, preincubation in the presence of EDTA resulted in the complete loss of activity, but 50% of the activity was retained in the presence of Ca^{2+} ions. At 85 °C, the activity was completely lost even in the presence of Ca^{2+} ions.

Resistance to proteolysis by V8 protease in the presence of Ca^{2+} ions

NS-1 cells were surface radiolabelled with Na^{125}I, and PC-1 was immunoprecipitated from cell lysates using the monoclonal antibody IR518. Immunoprecipitates were treated with V8 protease in the presence or absence of Ca^{2+} ions for 30 min at 37 °C, before analysis of precipitated proteins by SDS/PAGE and autoradiography. V8 protease was chosen in these experiments because its activity is independent of divalent cations [26]. It was found that in the presence of Ca^{2+} ions (Figure 3a, lanes C–E), PC-1 was more resistant to proteolysis by V8 protease

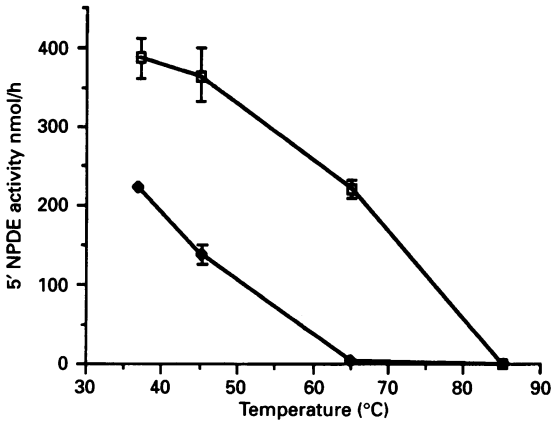

Figure 2 Ca^{2+} ions stabilize PC-1 against thermal denaturation

PC-1 was immunoprecipitated from *pLs.1* cell lysates using the anti-(mouse PC-1) monoclonal antibody IR518. The samples were pre-incubated at 37, 45, 65 or 85 °C for 10 min in the presence of 1 mM Ca^{2+} (□) or 1 mM EDTA (◆), and then assayed for 5′NPDE activity (means ± S.E.M., $n = 3$) in the presence of 0.5 mM Ca^{2+} at 37 °C as described in the Experimental section.

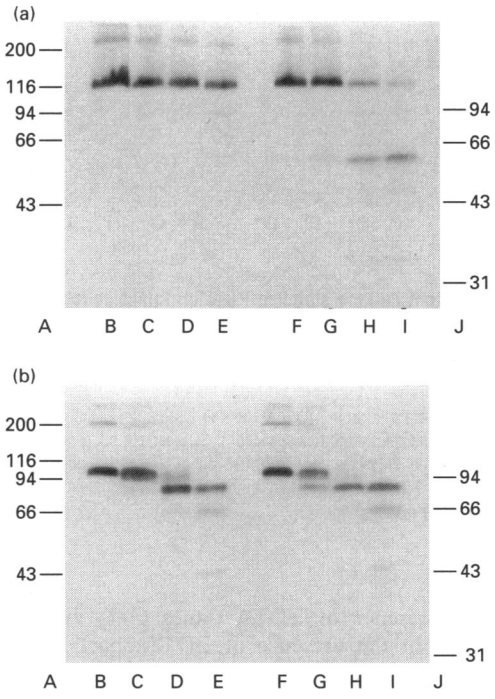

Figure 3 Ca^{2+} ions stabilize PC-1 against proteolysis

Mouse PC-1 (~ 120 kDa) (**a**) and the transferrin receptor (~ 90 kDa) (**b**) were immunoprecipitated from Triton X-100 lysates of surface-radio-iodinated NS-1 cells using the monoclonal anti-PC-1 antibody IR518 or a polyclonal anti-(transferrin receptor) antibody (R190). The immunoprecipitates were incubated in 0 (lanes B and F), 2 (lanes C and G), 10 (lanes D and H) and 50 μg/ml (lanes E and I) V8 protease in the presence of 1 mM Ca^{2+} (lanes B–E) or 1 mM EDTA (lanes F–I). The samples were incubated at 37 °C for 30 min and the proteins then precipitated with acetone. They were then resuspended in SDS-sample buffer and heated at 100 °C for 2 min before being subjected to SDS/PAGE on a 10% (w/v) polyacrylamide gel and autoradiography at −70 °C for 2 days with an intensifying screen. Lanes A and J contain the molecular-mass (kDa) markers.

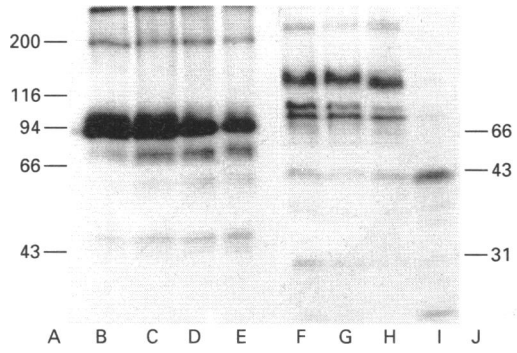

Figure 4 Mg^{2+} and Zn^{2+} ions stabilize PC-1 against proteolysis

The transferrin receptor (~ 90 kDa; lanes B–E) and PC-1 (~ 120 kDa; lanes F–I) were immunoprecipitated from Triton X-100 cell lysates of radio-iodinated NS-1 cells using a polyclonal anti-(transferrin receptor) antibody or the monoclonal anti-PC-1 antibody IR518. The immunoprecipitates were incubated in 50 μg/ml V8 protease in the presence of 1 mM Ca^{2+} (lanes B and F), 1 mM Mg^{2+} (lanes C and G), 1 mM Zn^{2+} (lanes D and H) or 1 mM EDTA (lanes E and I) at 37 °C for 30 min. The proteins were then precipitated with acetone, resuspended in SDS-sample buffer and heated at 100 °C for 2 min before being subjected to SDS/PAGE on a 10% (w/v) polyacrylamide gel and autoradiography at −70 °C for 2 days with an intensifying screen. The band at ~ 200 kDa in lanes B–E represents transferrin receptor dimers. The band at ~ 230 kDa in lanes F–H represents the PC-1 dimer. Lanes A and J contain the molecular-mass (kDa) markers.

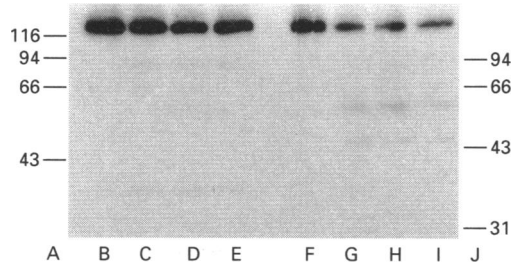

Figure 5 Divalent cations stabilize PC-1 on intact cells

NS-1 cells were surface-radio-iodinated and incubated in 0 (lanes B and F), 50 (lanes C and G), 100 (lanes D and H) and 200 μg/ml (lanes E and I) V8 protease in the presence of 1 mM Ca^{2+} (lanes B–E) or 1 mM EDTA (lanes F–I) at 37 °C for 30 min. The cells were then washed and PC-1 immunoprecipitated from Triton X-100 cell lysates using the anti-PC-1 monoclonal antibody IR518. The samples were resuspended in SDS-sample buffer and heated at 100 °C for 2 min before being subjected to SDS/PAGE on a 10% (w/v) polyacrylamide gel and autoradiography at −70 °C for 4 days with an intensifying screen. Lanes A and J contain the molecular-mass (kDa) markers.

than in the presence of EDTA (lanes G–I). At 2 μg/ml V8 protease, and in the presence of EDTA, partial digestion of PC-1 was observed (lane G). Digestion was almost complete with 50 μg/ml V8 protease (lane I). In the presence of Ca^{2+} ions, at least 10 μg/ml V8 protease (lane D) was required for detectable digestion, and further proteolysis was not apparent even at 50 μg/ml (lane E). Thus the presence of Ca^{2+} ions increased resistance of PC-1 to proteolysis by at least 10-fold.

As a control, the effect of Ca^{2+} ions on the resistance of the transferrin receptor to proteolysis was investigated. The transferrin receptor is an integral membrane glycoprotein, very similar in structure to PC-1 (both are class II transmembrane proteins made up of two identical disulphide-bonded chains and have similar sized intracellular and extracellular domains). The transferrin receptor is not known to bind divalent cations. As seen in Figure 3(b), the extent of proteolysis of the transferrin receptor was the same in the presence (lanes C–E) or absence (lanes G–I) of Ca^{2+} ions.

Resistance to proteolysis by V8 protease in the presence of Mg^{2+} and Zn^{2+} ions

The effects of Mg^{2+} and Zn^{2+} ions on PC-1 were also examined. NS-1 plasmacytoma cells were surface-radio-iodinated, and PC-1-immunoprecipitated using the monoclonal antibody IR518. The immunoprecipitates were treated with V8 protease and processed as described in the Experimental section. It was found that PC-1 (~ 120 kDa) was much more resistant to digestion with 50 μg/ml V8 protease in the presence of Ca^{2+} ions (Figure 4, lane F), Mg^{2+} ions (lane G) and Zn^{2+} ions (lane H) than in the presence of EDTA (lane I). There was no significant difference in the extent of digestion between the three divalent cations. In comparison, the extent of proteolysis of the transferrin receptor (~ 90 kDa) was the same in the presence or absence of Ca^{2+}, Mg^{2+} and Zn^{2+} ions (lanes B–D) or EDTA (lane E).

When the immunoprecipitates were incubated in the presence of the different divalent cations for 10 min, then washed twice in the absence of divalent cations or EDTA and subjected to proteolysis, PC-1 maintained its resistance to proteolysis (results not shown). These results suggest that the affinity of binding to the divalent cations is high and/or that the kinetics of dissociation from PC-1 are slow.

Resistance of PC-1 on intact cells to proteolysis by V8 protease

The previous experiments indicated that detergent-solubilized and immunoprecipitated PC-1 was stabilized by divalent cations against degradation by V8 protease. The stability of the protein on intact cells was also investigated. NS-1 plasmacytoma cells were radiolabelled with $Na^{125}I$ and graded amounts of V8 protease were added. The cells were left at 37 °C for 30 min, washed, and PC-1 was immunoprecipitated from cell lysates using the monoclonal antibody IR518. The samples were then processed as described in the Experimental section, and analysed by SDS/PAGE and autoradiography. It was found that PC-1 on intact cells was more resistant to proteolysis in the presence of Ca^{2+} ions (Figure 5, lanes C–E) than in the presence of EDTA (lanes G–I). Furthermore, it was found that PC-1 on intact cells was generally more resistant to proteolysis by V8 protease than when isolated as an immunoprecipitate (compare Figures 3a and 5, lanes G–I). Even at 200 μg/ml V8 protease and in the presence of EDTA (Figure 5, lane I), PC-1 was not completely cleaved from the cell surface. These findings suggest that on the surface of cells, PC-1 adopts a conformation rendering cleavage sites less accessible to the protease than when the protein is solubilized in Triton X-100.

Monoclonal antibodies to the C-terminal EF Hand region of PC-1

A bacterial fusion protein consisting of the C-terminal 140 amino acids of human PC-1 followed by glutathione *S*-transferase from *S. japonicum* was made in the vector pGEX-KT, and monoclonal antibodies to this protein were generated by standard procedures. The immunogen was incubated with 1 mM Ca^{2+} immediately before injection in Complete Freund's Adjuvant, in the hope that the fusion protein would fold correctly around the metal ions. The resulting antibodies were expected to bind to PC-1 only in the presence of Ca^{2+} ions.

Screening for antibody reactivity on intact mouse L cells expressing human PC-1 (*SABI* cells) in the presence of Ca^{2+} ions

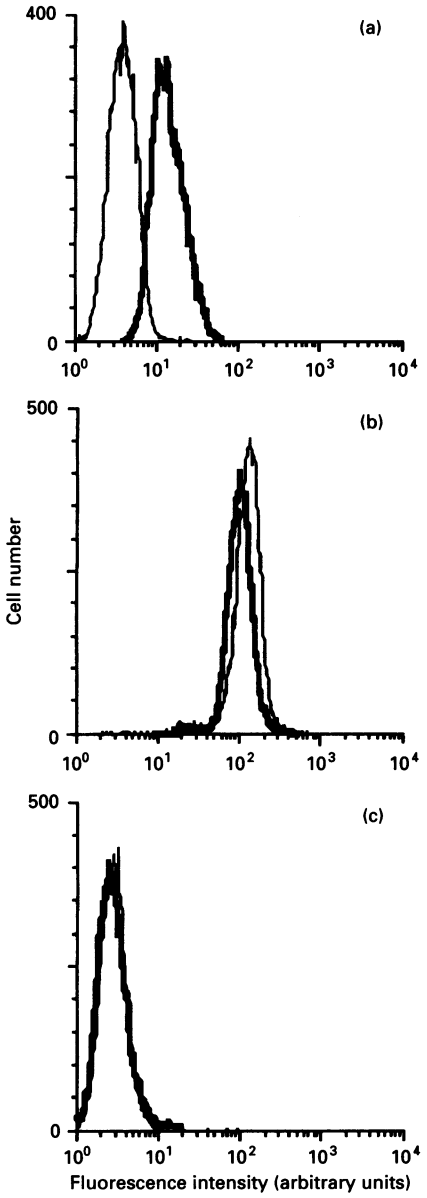

Figure 6 Recognition of PC-1 by monoclonal and polyclonal antibodies to human PC-1 in the presence of Ca^{2+} ions

Antibody reactivity to PC-1 on transfected cells was assessed by immunofluorescence and flow cytometry in the presence or absence of Ca^{2+} ions. (**a**) Binding of the monoclonal mouse anti-(human PC-1 EF-hand) antibody 4H4.89 to *SABI* cells (mouse L cells transfected with human PC-1). (**b**) Binding of polyclonal mouse anti-(*SABI* cells) to *SABI* cells. (**c**) Binding of 4H4.89 antibody to *NeoL* cells (mouse L cells transfected with vector alone). In each panel, the thin line represents antibody binding in the presence of 1 mM Ca^{2+} and the bold line represents antibody binding in the presence of 1 mM EDTA.

revealed no positive clones. However, re-screening on *SABI* cells that had been fixed in methanol allowed the isolation of four clones. All of these reacted with human PC-1 but only after fixation with methanol, suggesting that the monoclonal antibodies only bound to an unfolded form of the antigen. The antibodies did not react with *NeoL* cells that did not express PC-1.

In view of the findings that divalent cations stabilize PC-1 against proteolysis, we hypothesized that the EF-hand regions might unfold at least partially in the absence of Ca^{2+}, as has been shown for other Ca^{2+}-binding proteins [13,17]. Accordingly, we re-examined the binding of these monoclonal antibodies to intact cells in the presence of EDTA. Three of the four monoclonal antibodies bound to PC-1 on intact living *SABI* cells in the presence of EDTA, but not in the presence of Ca^{2+}. Representative data are shown in Figure 6. In the presence of EDTA, strong staining was seen. However, in the presence of Ca^{2+} ions, staining was abolished to background levels (Figure 6a). In marked contrast, polyclonal antibodies to human PC-1 bound to the native protein expressed on mouse L cells, with slightly more staining in the presence of Ca^{2+} than in its absence (Figure 6b). Because the great majority of epitopes recognized by antibodies on native proteins are conformational [27], these results suggest that the C-terminal EF-hand homology region of PC-1 unfolds in the absence of Ca^{2+}, but that the folding of the remainder of the molecule is largely unchanged.

DISCUSSION

Previous work has shown that the enzymic activities that are now ascribed to PC-1 require divalent cations [8]. The most likely explanation would be that divalent cations bind directly to PC-1, although there has been little other evidence for such a proposal. The binding of metals or other small ligands to proteins often stabilizes their conformation, as judged by increased resistance to thermal denaturation or proteolysis (see [13–16]). The current studies were designed to examine this question in the case of PC-1.

Our results show that divalent cations substantially protect the enzyme activity of Triton-X-100-solubilized PC-1 against thermal denaturation. In addition, divalent cations protect PC-1 against proteolytic degradation, both on intact cells and after solubilization in Triton X-100. Accordingly, it seems likely that the decreased stability of PC-1 in the absence of divalent cations reflects local or global unfolding due to removal of their stabilizing effect.

In many Ca^{2+}-binding proteins, Ca^{2+} is bound via paired EF-hand motifs consisting of helix–loop–helix structures first described in parvalbumin, but also found in calmodulin and many other Ca^{2+}-binding proteins [13,17,28–30]. The Ca^{2+} ion is bound in the loop via co-ordination with oxygen groups on the highly conserved aspartic acid residues [13], although variant mechanisms of binding also exist (see below). At the present time, the location of the postulated divalent cation-binding regions of PC-1 are unknown. However, the C-terminal amino acid sequences of both mouse and human PC-1 contain a region which is highly homologous to the EF-hand consensus sequence, and therefore represents a strong candidate for a divalent-cation-binding region (Figure 1). A second candidate region lies in the central portion of the sequence of PC-1. This region is not as close a match to the classic EF-hand sequence as seen in parvalbumin, but closely matches the critical residues of the variant EF hand of bovine intestinal Ca^{2+}-binding protein [30] and the Ca^{2+}-binding protein S-100 PAPI-b from bovine brain [31], both of which have a single amino acid insertion at this point (see Figure 1). EF hands are typically paired, and the fact that PC-1 is a homodimeric protein might suggest that these regions lie at the interface between subunits, or that the internal and C-terminal putative EF-hand regions may be in close proximity in the folded structure.

In contrast to our initial expectations, monoclonal antibodies raised to the bacterially expressed C-terminal EF-hand homology region bound only to intact cells in the absence of Ca^{2+}, suggesting

that the bacterial fusion protein immunogen in Complete Freund's Adjuvant did not fold correctly around the added Ca^{2+} ions, and that the resulting antibodies were directed to an unfolded form of the sequence. Polyclonal antibodies raised to the native form of human PC-1 were made by immunization of mice in the absence of adjuvants with intact mouse L cells expressing human PC-1. Their binding to native human PC-1 was almost independent of Ca^{2+}, with slightly more binding in its presence than in its absence (Figure 6). Because the great majority of epitopes recognized by antibodies on native proteins are conformational [27], it seems reasonable to suggest that in the absence of divalent cations, the C-terminal EF-hand region of PC-1 undergoes localized unfolding. We have attempted to make monoclonal antibodies to this region that bind only in the presence of Ca^{2+}, which would provide additional support for this concept, but so far such antibodies have proven elusive.

It seems reasonable to postulate that the binding of divalent cations to the putative EF-hand regions of PC-1 explains the observed stabilization of the protein, but the possibility remains that these regions do not represent the true metal-binding regions of PC-1. It is interesting to note that similar helix–loop–helix divalent cation-binding regions exist in which some or most of the ligand-coordinating oxygens are provided by the main-chain backbone carbonyl groups (see [13,30–33]). Definitive determination of the metal-binding regions of PC-1 will require the elucidation of its three-dimensional structure.

We have previously noted that PC-1 on intact cells is highly resistant to pepsin, trypsin and cathepsins B and D, both at pH 5 and 7 [20]. The current observations indicate that PC-1 is also much more resistant to proteolysis when on the surface of intact cells than after solubilization in Triton X-100. Immune responses typically occur in the environment of a local inflammatory reaction. Inflammatory cells such as granulocytes, macrophages and mast cells are rich in protease activity. The stability of PC-1 in its physiological setting on the membrane and in the presence of divalent cations may therefore reflect a need to resist proteolytic attack.

This work was supported by the Anti-Cancer Council of Victoria and the National Health and Medical Research Council of Australia. We thank Ms. Joan Curtis and Dr. Emanuela Handman for help in production of monoclonal antibodies.

Received 9 May 1994; accepted 16 June 1994

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