Isolation and characterization of a novel 68000- M_r Ca^{2+} -binding protein of lymphocyte plasma membrane

Raymond J. OWENS and Michael J. CRUMPTON

Imperial Cancer Research Fund Laboratories, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, U.K.

(Received 17 November 1983/Accepted 14 December 1983)

A 68000- M_r protein is a major component of a Nonidet P-40-insoluble fraction of lymphocyte plasma membrane prepared from human B lymphoblastoid cells (BRI 8) and pig mesenteric lymph nodes. The association of the protein with the detergent-insoluble complex depends on free Ca^{2+} concentrations of greater than $10\text{ }\mu\text{M}$. The human and pig 68000- M_r proteins were purified and appear to be homologous on the basis of amino acid composition and peptide mapping. The protein is monomeric, has pI 5.8 and a single high-affinity Ca^{2+} -binding site (K_D $1.2\text{ }\mu\text{M}$). The results are discussed in terms of the possible role of the 68000- M_r protein as an intracellular Ca^{2+} receptor in lymphocytes.

Changes in the cytoplasmic concentration of Ca^{2+} have been implicated in the regulation of many cellular processes, including motility, mitogenesis and intermediary metabolism. The effects of Ca^{2+} appear to be mediated by its interaction with specific Ca^{2+} -binding proteins (Kretsinger, 1979). There is considerable evidence that Ca^{2+} -dependent regulators are involved in the control of the cytoskeleton in non-muscle cells (Dedman *et al.*, 1979). A number of proteins have been characterized that interact with actin in a Ca^{2+} -dependent manner, e.g. HeLa-cell and platelet α -actinins, which cause the bundling of microfilaments (Burridge & Feramisco, 1981; Rosenberg *et al.*, 1981), and gelsolin from macrophages, which causes microfilament shortening (Yin & Stossel, 1981). Another class of actin-binding proteins, represented by villin from intestinal epithelial cells, appears to either fragment or cross-link actin microfilaments depending on the free Ca^{2+} concentration (Bretscher & Weber, 1980a). Polymerization studies on microtubules have demonstrated that Ca^{2+} inhibits tubulin assembly in unfractionated extracts *in vitro* (Weisenberg, 1972; Haga *et al.*, 1974) and *in vivo* (Schliwa, 1976), although tubulin does not bind Ca^{2+} itself.

Ca^{2+} appears to regulate many cellular functions via the ubiquitous Ca^{2+} -binding protein calmodulin (Klee *et al.*, 1980). Calmodulin-dependent

myosin light-chain kinases, which regulate the activity of the actomyosin ATPase in smooth muscle, have been identified in non-muscle cells (Klee *et al.*, 1980). Also, the presence of calmodulin in the highly organized cytoskeletal complex of brush-border microvilli has been demonstrated (Howe *et al.*, 1980). In addition, calmodulin-binding proteins have been identified in this system (Glenney & Weber, 1980; Glenney *et al.*, 1982), and a role for Ca^{2+} in maintaining the structural integrity of the brush-border cytoskeleton has been proposed (Glenney *et al.*, 1980). The interaction of erythrocyte spectrin with actin via polypeptide 4.1 *in vitro* appears to be Ca^{2+} -dependent (Fowler & Taylor, 1980). In addition, the α -subunit of spectrin and its analogues in non-erythroid cells also appears to bind calmodulin (Glenney *et al.*, 1982). Ca^{2+} may thus also be involved in the organization of the membrane-cytoskeleton system of erythrocytes.

In the preceding paper, a detergent-insoluble complex of polypeptides associated with purified preparations of lymphocyte plasma membrane was described (Davies *et al.*, 1984). The detergent-insoluble complex prepared from human lymphocyte plasma membrane comprised three major polypeptides, with M_r 120000, 68000 and 45000, together with several more minor polypeptides, including a pair with M_r 33000 and 28000. A qualitatively similar complex was obtained from pig lymphocyte plasma membrane, although the 120000- M_r polypeptide was less well represented. With the exception of the 45000- M_r polypeptide,

Abbreviations used: SDS, sodium dodecyl sulphate; Dulbecco's PBS, Dulbecco's phosphate-buffered saline medium.

the major polypeptides of the complex occurred exclusively in the detergent-insoluble fraction of the plasma membrane. It was suggested that this detergent-insoluble complex represented a submembranous cytoskeleton in lymphocytes analogous to that found in erythrocytes (Branton *et al.*, 1981). In view of the apparent role of Ca^{2+} in regulating the organization of cytoskeletal structures, we have investigated the effect of Ca^{2+} concentration on the lymphocyte membrane-cytoskeleton system. In the present paper we show that the association of the 68000- M_r polypeptide with the lymphocyte membrane-cytoskeletal complex is Ca^{2+} -dependent and provide evidence that the isolated protein binds Ca^{2+} . A preliminary report of some of this work has been published (Owens & Crumpton, 1983).

Materials and methods

Cells

The human B lymphoblastoid cell line BRI 8 was cultured in RPMI 1640 medium containing penicillin (100 units/ml) and streptomycin (50 $\mu\text{g}/\text{ml}$) and supplemented with 10% (v/v) foetal-calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.). Pig mesenteric lymph nodes were obtained from a local commercial abattoir (British Beef, Watford, Herts., U.K.).

Preparation and Nonidet P-40 extraction of plasma membrane

Plasma membrane was prepared from BRI 8-cell suspensions and from pig mesenteric lymph node as described previously by Crumpton & Snary (1974) and Snary *et al.* (1976). Cells were homogenized in either Dulbecco's PBS (comprising 10 mM-sodium phosphate buffer, pH 7.2, 0.17 M-NaCl, 3 mM-KCl, 1 mM- CaCl_2 and 1 mM- MgCl_2) or Dulbecco's PBS without added CaCl_2 and MgCl_2 . Dulbecco's PBS was routinely used for plasma-membrane preparation. Proteolysis was inhibited by the addition of phenylmethanesulphonyl fluoride and iodoacetamide to the cell suspensions and tissue homogenates, to final concentrations of 1 mM and 10 mM respectively.

The detergent-insoluble fraction of lymphocyte plasma membrane, referred to below as the '20k pellet', was prepared as described in the preceding paper (Davies *et al.*, 1984); a final concentration of 1% (v/v) Nonidet P-40 was used to extract the purified plasma-membrane preparations.

SDS/polyacrylamide-gel electrophoresis

Samples were analysed on 10% (w/v) polyacrylamide slab gels in 0.1% SDS in 25 mM-Tris/192 mM-glycine buffer, pH 8.3, as described by the method of Laemmli (1970). Two-dimensional isoelectric-

focusing-SDS/polyacrylamide-gel electrophoresis was carried out as described by O'Farrell (1975). The pH gradient for the first dimension was generated by using equal volumes of Ampholines (LKB, Bromma, Sweden) pH 3.5–10 and pH 5–7. A Pharmacia flat-bed apparatus (FBE 3000) was used for isoelectric focusing. Samples were run for 3000 V·h in 5% (w/v) polyacrylamide gels containing Ampholine pH 3.5–10 and 6 M-urea. pH gradients after isoelectric focusing were determined by cutting blank gels, run in parallel, into 0.5 cm segments. These were each then soaked in 1.5 ml of distilled water and the pH of each eluate was measured with a pH-meter (Beckman Instruments).

Proteins in gels were detected either by Coomassie Blue staining (Owen *et al.*, 1980) or by silver staining (Morrissey, 1981). Polypeptides were quantified by scanning densitometry with a Joyce-Loebl Chromoscan 3 instrument. The following proteins were used as M_r standards: myosin heavy chain (200 000), β -galactosidase (130 000), phosphorylase *b* (95 000), bovine serum albumin (68 000), ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (34 000), immunoglobulin light chain (25 000) and cytochrome *c* (12 500).

Peptide mapping

Peptide mapping by limited proteolysis was carried out by the procedure of Cleveland *et al.* (1977). Polypeptide bands containing 5–10 μg of protein were excised from SDS/polyacrylamide gels and digested in the gel slices with *Staphylococcus griseus* proteinase (Sigma Chemicals, Poole, Dorset, U.K.) (10–100 $\mu\text{g}/\text{ml}$) for 30 min at room temperature. Peptides were resolved in SDS/15% (w/v)-polyacrylamide gels containing 1 mM-EDTA and detected by silver staining.

Amino acid analysis

Protein (10–15 μg) was hydrolysed in constant-boiling HCl at 110°C for 24 h. The amino acids were subjected to pre-column formation of the *o*-phthalaldehyde derivatives (Jones *et al.*, 1981), which were then separated by reverse-phase high-pressure liquid chromatography by using the Waters system.

Calcium buffers

Known concentrations of ionic Ca^{2+} were obtained by using calcium/EDTA buffers, the compositions of which were calculated from known dissociation constants of EDTA- Ca^{2+} and EDTA- Mg^{2+} complexes (Kim & Padilla, 1978). Buffers for different theoretical free Ca^{2+} concentrations contained 5 mM-EDTA, 5 mM- MgCl_2 and the following concentrations of CaCl_2 : for 0.1 mM- Ca^{2+} , 3.5 mM- CaCl_2 ; for 10 μM - Ca^{2+} , 1.65 mM-

CaCl₂; for 1.0 μ M-Ca²⁺, 0.6 mM-CaCl₂; for 0.1 μ M-Ca²⁺, 0.15 mM-CaCl₂. Calcium buffers were made up in either 10 mM-sodium phosphate buffer, pH 7.2, 0.17 M-NaCl or 10 mM-Tris/HCl buffer, pH 7.4.

Ca²⁺ binding

Ca²⁺-binding activity was measured by the equilibrium gel-filtration method of Hummel and Dryer (Ackers, 1973) with ⁴⁵CaCl₂ (0.55 Ci/mmol; Amersham International, Amersham, Bucks., U.K.). Purified 68000-*M_r* protein was dialysed extensively against 10 mM-Tris/HCl buffer, pH 7.4, containing 50 mM-KCl, at 4°C. Samples (0.5 ml) containing 30 μ g of protein were equilibrated for 60 min at 4°C with known amounts of ⁴⁵Ca²⁺, which were calculated from the specific radioactivity of the ⁴⁵CaCl₂, adjusted for radioactivity decay when necessary. Each sample was then gel-filtered on a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column (10 ml) pre-saturated at the same ⁴⁵Ca²⁺ concentration as the sample. Fractions (0.5 ml) were collected and assayed for radioactivity by liquid-scintillation counting. The amount of protein-bound ⁴⁵Ca²⁺ was determined by measuring the area of the peak of radioactivity eluted from the column above the saturating baseline.

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Purified plasma-membrane fractions were prepared from two sources of lymphocytes, namely human B lymphoblastoid cells (BRI 8) and pig mesenteric lymph nodes (comprising predominantly T and/or null cells on the basis that only about 10% of the cells expressed surface immunoglobulin). The lymphoblastoid cells had been transformed with Epstein-Barr virus and grew continuously in culture, whereas the pig lymphocytes were unstimulated (i.e. quiescent).

Plasma-membrane fractions were isolated by a combination of differential rate and sucrose-density centrifugation after homogenization of the cells in either Dulbecco's PBS or Dulbecco's PBS without added MgCl₂ and CaCl₂. The polypeptide compositions of plasma membranes prepared in the presence of bivalent cations were identical with those of plasma membranes prepared in their absence.

Nonidet P-40 extraction of plasma membrane in the presence of Ca²⁺ chelators

To investigate the effect of Ca²⁺ on the polypeptide composition of the detergent-insoluble

Fig. 1. Effect of Ca²⁺ chelators on the Nonidet P-40-insoluble fraction of lymphocyte plasma membrane

Plasma membranes prepared from human lymphoblastoid cells (BRI 8) and pig lymph-node lymphocytes were extracted with 1% Nonidet P-40 in Dulbecco's PBS containing either 1 mM-CaCl₂ + 1 mM-MgCl₂ or 1 mM-EGTA + 1 mM-MgCl₂ but not CaCl₂. The extracts were centrifuged at 10⁵ g-min (20000 rev./min for 30 min in a Beckman 50.2 Ti rotor) and the resulting detergent-insoluble residues (20k pellets) were analysed on 10% (w/v) polyacrylamide gels in the presence of SDS. Gels were run under reducing conditions and were stained with Coomassie Blue. Human and pig 20k pellets prepared in either the presence (tracks 1 and 3) or in the absence (tracks 2 and 4) of Ca²⁺. The arrows indicate the polypeptides associated with 20k pellet in the presence of 1 mM-Ca²⁺. The *M_r* values of protein standards run in parallel are given on the left of the gels.

fraction, purified plasma membrane (1 mg of protein/ml) was extracted with 1% Nonidet P-40 in Dulbecco's PBS containing either 1 mM-CaCl₂ + 1 mM-MgCl₂ or 1 mM-EGTA + 1 mM-MgCl₂ with no CaCl₂ for 30 min at 4°C. The polypeptide compositions of the resulting detergent-insoluble pellets prepared in the presence and in the absence of Ca²⁺ are shown in Fig. 1. Similar results were obtained with human and pig plasma membranes and they indicated that extraction of the plasma membrane in the presence of the Ca²⁺ chelator (i.e. a free [Ca²⁺] of 0.1 μ M-Ca²⁺) gave pellets that lacked the 68000-*M_r* polypeptide together with the bands of *M_r* 55000, 33000 and 28000. Identical results were obtained with 1 mM-EDTA in place of EGTA + MgCl₂. The concentration of Ca²⁺ required to maintain the association of the 68000-*M_r* polypeptide with the detergent-insoluble fraction of the plasma mem-

Fig. 2. Proportion of the 68000- M_r polypeptide present in 20k pellets prepared at different Ca^{2+} concentrations. Samples (1 mg of protein) of plasma membrane prepared from BRI 8 cells were extracted with 1% (v/v) Nonidet P-40 solutions containing 0.1 μ M–1.0 mM- Ca^{2+} . The polypeptide compositions of the resulting 20k pellets were analysed by electrophoresis on 10% polyacrylamide gels in the presence of SDS under reducing conditions. The amount of the 68000- M_r polypeptide present in each sample was estimated by densitometric scanning of Coomassie Blue-stained gels. The results are represented as the percentages of the amount of 68000- M_r polypeptide in 20k pellets prepared in the presence of 1.0 mM- Ca^{2+} .

brane was then estimated. The 20k pellets were prepared by extracting plasma membrane (1 mg/ml) with Nonidet P-40 solutions containing various concentrations of Ca^{2+} (0.1 μ M–1.0 mM). The polypeptide compositions of the pellets were then quantified by densitometric scanning of Coomassie Blue-stained gels. As shown in Fig. 2, the amount of the 68000- M_r polypeptide in the 20k pellet was progressively diminished by extracting the plasma membrane at decreasing concentrations of Ca^{2+} below 1.0 mM. Complete solubilization of the polypeptide occurred at Ca^{2+} concentrations of less than 10 μ M; the residual percentage of protein represents minor polypeptides in the 20k pellet that co-migrate with the 68000- M_r polypeptide (see Fig. 1).

Nature of the EGTA extract

The 68000- M_r polypeptide was separated from the 20k pellet by making use of its Ca^{2+} -dependent solubility. The 20k pellets were prepared from BRI

8 cells and pig lymphocytes in the presence of 1 mM- Ca^{2+} . The washed pellets were then re-extracted (1 mg of protein/ml) with Dulbecco's PBS, containing 1% Nonidet P-40, 1 mM-EGTA and 1 mM-MgCl₂ but no CaCl₂ for 30 min at 0°C and centrifuged at 100000 g_{av} for 30 min at 4°C. The supernatant was collected and concentrated by ethanol precipitation (95%, v/v, for 16 h at –20°C). The EGTA extract of both human (Fig. 5, track 3) and pig (Fig. 3, tracks 1 and 2) 20k pellets consisted mainly of the 68000- M_r polypeptide (about 60% of the total protein, as judged by densitometric scanning), together with smaller amounts of the 55000- M_r , 45000- M_r , 33000- M_r , and 28000- M_r polypeptides.

Complete release of the 68000- M_r polypeptide from the 20k pellet depended on the presence of Nonidet P-40. Re-extraction of 20k pellets with Dulbecco's PBS containing EGTA and MgCl₂ but no CaCl₂ resulted in the solubilization of only 65–70% of the 68000- M_r polypeptide. A second re-extraction of the pellets with Dulbecco's PBS containing EGTA and MgCl₂ but no CaCl₂ did not yield any further amounts of the polypeptide. All the remaining 68000- M_r polypeptide could be extracted if the insoluble residue was then treated with Dulbecco's PBS containing EGTA, MgCl₂ and 1% Nonidet P-40 but no CaCl₂. These results indicated that most of the 68000- M_r polypeptide behaves as a peripheral membrane protein; a proportion, however, appears to be more tightly bound to the lipid bilayer.

In view of the co-solubilization of the 68000- M_r polypeptide with those of M_r 55000, 33000 and 28000, an important question was whether these polypeptides were associated in any way. As an approach to this question, samples of the EGTA extract were analysed by electrophoresis on SDS/polyacrylamide gels in the presence and in the absence of 1 mM-dithiothreitol. No difference in the polypeptide compositions or mobilities of the extracts were observed under reducing compared with non-reducing conditions (Fig. 3, tracks 1 and 2). Thus none of the polypeptides of the EGTA extract appeared to be linked by disulphide bonds. This result also indicated that the 68000- M_r polypeptide was not serum albumin, which shows a characteristic downward shift in M_r value (68000 to 55000) when run on SDS/polyacrylamide gels under non-reducing conditions (Fig. 3, tracks 3 and 4). Albumin has been shown to be a component of pig but not human lymphocyte plasma membrane (Owen *et al.*, 1980).

Purification of the 68000- M_r protein

The 68000- M_r polypeptide was purified to apparent homogeneity, as judged by one-dimensional and two-dimensional SDS/polyacrylamide-

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of the EGTA extract under reducing and non-reducing conditions*
The 20k pellet, prepared from pig lymph-node lymphocyte plasma membrane, was re-extracted with EGTA as described in the legend to Fig. 2. Samples were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions (1 mM-dithiothreitol) (track 1) and non-reducing conditions (track 2). A sample of pig serum albumin was run in parallel under non-reducing (track 3) and reducing conditions (track 4). The gel was stained with Coomassie Blue. The M_r values of protein standards are given on the left of the gels.

Fig. 5. *SDS/polyacrylamide-gel electrophoresis of stages during the purification of the 68000- M_r protein*
The 20k pellet (track 2) was prepared from BRI 8-cell plasma membrane (track 1) as described in the Materials and methods section. The 20k pellet was washed and re-extracted with Dulbecco's PBS containing 1 mM-EGTA and 1 mM-MgCl₂ but no CaCl₂. The 68000- M_r protein was separated from the other polypeptides of the EGTA extract (track 3) by gel filtration on a Sephacryl S-200 column. Fractions that contained the 68000- M_r protein (Fig. 4, peak I, track 4) and the 33000/28000- M_r polypeptides (peak II, track 5) were combined. All the samples were analysed by electrophoresis on 10% polyacrylamide gels in the presence of SDS under reducing conditions and stained with Coomassie Blue. The M_r values of protein standards are given on the left of the gels.

Fig. 4. *Gel filtration of the EGTA extract*

The EGTA extract prepared from BRI 8-cell 20k pellet was applied to a Sephacryl S-200 (Pharmacia) column (160ml) equilibrated in 20mM-Tris/HCl buffer, pH 7.4. The column was eluted with the same buffer and the eluate monitored for absorption at 280nm. Fractions 70–80 and 100–110, corresponding to the two major A_{280} peaks (I and II), were combined and concentrated by using an Amicon ultrafiltration cell with a PM30 membrane. The column was calibrated with the following standards: bovine serum albumin (M_r 68000), ovalbumin (M_r 45000) and cytochrome *c* (M_r 12500).

gel electrophoresis (Fig. 5, track 4, and Fig. 6), by gel filtration of the EGTA extract. The 20k pellet was prepared from BRI 8-cell plasma membrane and washed by centrifugation to remove Nonidet P-40. The pellet was then re-extracted in the presence of EGTA as described above but without the addition of Nonidet P-40, and the soluble extract was dialysed extensively against distilled water at 4°C. The dialysed extract was freeze-dried and dissolved in 20mM-Tris/HCl buffer, pH 7.4. The 68000- M_r polypeptide was separated from the other components of the EGTA extract by gel filtration on Sephacryl S-200. Two major peaks of protein were eluted from the column (I and II, Fig. 4). A shoulder on the first peak suggested that it overlapped a third peak, which was not completely resolved. To obtain as homogeneous a preparation as possible, only the peak and leading fractions of peak I were combined. The polypeptide compositions of the two peaks (I and II) are shown in Fig. 5 (tracks 4 and 5). The 68000- M_r polypeptide

Table 1. Comparison of the amino acid compositions of the human and pig 68000- M_r proteins

Duplicate samples (10–15 μ g) of purified human and pig 68000- M_r proteins were hydrolysed in 5.7M-HCl at 110°C for 24h, and the amino acids were subjected to formation of the *o*-phthalaldehyde derivatives and analysed by high-pressure liquid chromatography. Proline and hydroxyproline are not detected with this procedure. The amounts of cysteine and tryptophan were not determined.

Amino acid	Amino acid composition (mol/mol of protein)	
	Human 68000- M_r protein	Pig 68000- M_r protein
Asp	84.4	84.4
Glu	89.1	92.8
Ser	56.9	54.1
His	38.8	33.8
Gly	62.9	66.5
Thr	48.4	48.3
Arg	31.1	33.2
Ala	64.0	64.7
Tyr	26.5	28.5
Met	16.6	17.7
Val	16.9	17.9
Phe	27.2	28.2
Ile	20.7	19.1
Leu	62.9	58.3
Lys	62.4	61.3
No. of residues	708.8	709.0

was eluted from the column at the position expected for a globular protein of M_r 68000. No association of this protein with any of the other polypeptides in the EGTA extract was apparent under the conditions used. The purified protein gave a single spot on two-dimensional isoelectric-focusing-SDS/polyacrylamide-gel electrophoresis at a pH of approx. 5.8. To obtain sufficient amounts of the 68000- M_r protein for chemical analysis, 100mg of plasma-membrane protein was required and typically yielded 200 μ g of purified protein. The 68000- M_r protein was purified from pig lymphocyte plasma membrane by a procedure identical with that described above.

Comparison of human and pig 68000- M_r proteins

The 68000- M_r proteins purified from human BRI 8 cells and pig lymphocytes were compared in terms of isoelectric points, amino acid compositions and partial-proteolysis peptide maps. On isoelectric focusing, both the human and pig 68000- M_r proteins gave a cluster of bands that possessed pI values of 5.7–5.9. This result is consistent with that obtained by two-dimensional gel analysis, although the isoelectric variants were not resolved, probably owing to overloading of the gel. The identical isoelectric points of the human and

Fig. 6. One-dimensional peptide mapping of pig and human 68000- M_r proteins

EGTA extracts were prepared from human and pig 20k pellets, and samples were subjected to electrophoresis on a 10% polyacrylamide gel in the presence of SDS. The gels were lightly stained with Coomassie Blue, and the 68000- M_r polypeptide bands were cut out of the gel. The protein in the gel slices was digested with *Staphylococcus griseus* proteinase (10–100 μ g/ml), and the resultant peptides were analysed by electrophoresis on a 15% polyacrylamide gel in the presence of SDS (Cleveland *et al.*, 1977). The peptides were detected by silver staining. The M_r values of protein standards are given on the left of the gels.

pig proteins were consistent with their highly similar amino acid compositions (Table 1). The two proteins also gave very similar one-dimensional peptide maps after digestion with Pronase (Fig. 6). Taken together, the data indicate that the 68000- M_r protein has been highly conserved between the two species studied. For further characterization of the purified 68000- M_r protein, only the protein from human lymphoblastoid cells was used.

Ca^{2+} binding

The involvement of Ca^{2+} in the association of the 68000- M_r protein with the 20k pellet, although at relatively high concentrations (10 μ M), prompted the examination of the interaction of the isolated protein with Ca^{2+} . Binding studies with $^{45}Ca^{2+}$ showed that the 68000- M_r protein bound Ca^{2+} with a stoichiometry of approx. 1:1 and with a K_D of 1.2 μ M (Fig. 7). This indicates that there is a single high-affinity Ca^{2+} -binding site on each molecule.

Discussion

Insolubility in non-ionic detergents has often been used to define 'cytoskeletal' complexes in either whole cells (Osborn & Weber, 1977;

Fig. 7. Measurement of the Ca²⁺-binding activity of the 68000-M_r protein

Samples (60 μg/ml) of the purified 68000-M_r protein were incubated with various amounts of ⁴⁵CaCl₂ in 10 mM-Tris/HCl buffer, pH 7.4, containing 50 mM-KCl for 60 min at 4°C. The amount of protein-bound ⁴⁵Ca²⁺ was then measured by the equilibrium gel-filtration method of Hummel and Dryer, as described in the Materials and methods section.

Sheterline & Hopkins, 1981) or subcellular fractions (Bretscher & Weber, 1980a,b; Luna *et al.*, 1981; Mescher *et al.*, 1981). With the use of this criterion, a complex of polypeptides has been identified in association with purified preparations of lymphocyte plasma membrane. The functional significance of this complex is unclear, though it seems possible that it represents some form of membrane 'cytoskeleton'. A major component of this detergent-insoluble complex is a 68000-M_r polypeptide. This polypeptide accounts for approx. 17% of the total protein of the complex (or about 2.0% of the plasma-membrane protein) (Davies *et al.*, 1984). In the present paper the purification and partial characterization of this polypeptide from human and pig lymphocyte plasma membranes has been described. It is a monomeric protein, and by a number of biochemical criteria, including amino acid composition and peptide mapping, the human and pig 68000-M_r proteins appear to be homologous. Such a high degree of conservation is characteristic of intracellular proteins, particularly those associated with the cytoskeleton, e.g. actin (Vandekerckhore & Weber, 1978), tubulin (Ponstingl *et al.*, 1981; Valenzuela *et al.*, 1981) and calmodulin (Klee *et al.*, 1980).

The properties of the 68000-M_r protein from lymphocyte plasma membrane indicate that it is distinct from another cytoskeleton-associated protein of the same M_r. A major component of the brush-border cytoskeleton of intestinal epithelial cells is a 68000-M_r protein, named fimbrin. This protein is also present in several cultured cells, e.g. chick-embryo fibroblasts (Bretscher & Weber, 1980b). Fimbrin, however, differs from the lymphocyte 68000-M_r protein in terms of amino acid composition and apparent isoelectric point (Glenney *et al.*, 1981). In addition, although fimbrin also binds Ca²⁺, it is released from the detergent-insoluble fraction of brush-border microvilli by the addition of Ca²⁺ rather than by depleting Ca²⁺, as in the case of the lymphocyte protein.

The association of the 68000-M_r protein with the detergent-insoluble fraction of the lymphocyte plasma membrane depends on concentrations of Ca²⁺ in the extraction buffer of greater than 10 μM. This appears to be rather high for a physiological interaction, since cellular concentrations of free Ca²⁺ are estimated to be approx. 0.1 μM (Kretsinger, 1979). However, it is possible that the Ca²⁺ concentrations in the extraction buffers do not reflect the actual concentrations of Ca²⁺ involved in the interaction of the 68000-M_r protein with the plasma membrane *in vivo*. In support of this is the observation that the 68000-M_r protein is present in purified plasma-membrane fractions prepared from cells that have been homogenized in buffers containing physiological concentrations of Ca²⁺. The basis of this discrepancy is not clear, although Walker (1982) showed that similar high concentrations of Ca²⁺ were involved in the interaction of another putative Ca²⁺-binding protein, calelectrin, with synaptic membranes *in vitro*.

The isolated 68000-M_r protein appears to have a single Ca²⁺-binding site, which becomes occupied at micromolar concentrations of Ca²⁺. This concentration of free Ca²⁺ corresponds to that associated with the onset of many Ca²⁺-mediated processes in cells (Kretsinger, 1979). Thus the Ca²⁺-binding activity of the 68000-M_r protein is compatible with a role for the protein as an intracellular Ca²⁺ receptor. Although calmodulin is considered to be the major Ca²⁺-dependent regulator in cells, there is evidence for the existence of a number of other intracellular Ca²⁺ receptors. These include the S100 group of proteins found in neural tissues and the intestinal vitamin D-dependent Ca²⁺-binding proteins (Van-Eldik *et al.*, 1982). Both these proteins have small M_r values, approx. 10000 and 28000 respectively. Recently, a number of polypeptides have been identified in cellular extracts of various tissues, including brain, smooth muscle and striated

muscle, which co-purified with calmodulin on phenothiazine affinity columns (Moore & Dedman, 1982). These polypeptides had M_r values of 67000, 34000, 30000 and 11000 and were shown to interact directly with antipsychotic drugs in a Ca^{2+} -dependent manner. It was suggested that these polypeptides were functionally analogous to calmodulin in that they formed hydrophobic drug-binding sites as a result of their interaction with Ca^{2+} . The similarity of the M_r values of three of these polypeptides and those associated with the lymphocyte plasma membrane is striking. Although the evidence is highly circumstantial, it is conceivable that the lymphocyte 68000- M_r protein is the same as the 67000- M_r protein identified by Moore & Dedman (1982) and that it is representative of a novel class of Ca^{2+} regulators whose function is, as yet, unknown. The association of the protein with a putative plasma-membrane-cytoskeleton complex, presumably on the cytoplasmic face of the membrane, would make it an attractive candidate for mediating cellular processes for which a regulatory role for Ca^{2+} and the cytoskeleton has been inferred.

We are grateful to Dr. M. J. Geisow for advice on the preparation of calcium buffers, and Mr. G. T. Scrase for assistance with the amino acid analyses.

References

- Ackers, G. K. (1973) *Methods Enzymol.* **27**, 441-455
- Branton, D., Cohen, C. M. & Tyler, J. (1981) *Cell (Cambridge, Mass.)* **24**, 24-32
- Bretscher, A. & Weber, K. (1980a) *Cell (Cambridge, Mass.)* **20**, 839-847
- Bretscher, A. & Weber, K. (1980b) *J. Cell Biol.* **86**, 335-340
- Burridge, K. & Feramisco, R. J. (1981) *Nature (London)* **294**, 565-567
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102-1106
- Crumpton, M. J. & Snary, D. (1974) *Contemp. Top. Mol. Immunol.* **3**, 27-56
- Davies, A. A., Wigglesworth, N. M., Allan, D., Owens, R. J. & Crumpton, M. J. (1984) *Biochem. J.* **219**, 301-308
- Dedman, J. R., Brinkley, B. R. & Means, A. R. (1979) *Adv. Cyclic Nucleotide Res.* **11**, 131-174
- Fowler, V. M. & Taylor, D. L. (1980) *J. Cell Biol.* **85**, 361-376
- Glenney, J. R. & Weber, K. (1980) *J. Biol. Chem.* **255**, 10551-10554
- Glenney, J. R., Bretscher, A. & Weber, K. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6458-6462
- Glenney, J. R., Kaulfus, P., Matsudaira, P. & Weber, K. (1981) *J. Biol. Chem.* **256**, 9283-9288
- Glenney, J. R., Glenney, P. & Weber, K. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4002-4005
- Haga, T., Abe, T. & Kurokawa, M. (1974) *FEBS Lett.* **39**, 291-295
- Howe, C. L., Mooseker, M. S. & Graves, T. G. (1980) *J. Cell Biol.* **85**, 916-923
- Jones, B. N., Paabo, S. & Stein, S. (1981) *J. Liq. Chromatogr.* **4**, 565-573
- Kim, Y. S. & Padilla, G. M. (1978) *Anal. Biochem.* **89**, 521-528
- Klee, C. B., Crouch, T. H. & Richman, P. G. (1980) *Annu. Rev. Biochem.* **49**, 489-515
- Kretsinger, R. H. (1979) *Adv. Cyclic Nucleotide Res.* **11**, 1-26
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Luna, E. J., Fowler, V. M., Swanson, J., Branton, D. & Taylor, D. L. (1981) *J. Cell Biol.* **88**, 396-409
- Mescher, M. F., Jose, M. J. & Balk, S. P. (1981) *Nature (London)* **289**, 139-144
- Moore, P. B. & Dedman, J. R. (1982) *J. Biol. Chem.* **257**, 9663-9667
- Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307-310
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007-4021
- Osborn, M. & Weber, K. (1977) *Exp. Cell Res.* **106**, 339-349
- Owen, M. J., Barber, B. H., Faulkes, R. A. & Crumpton, M. J. (1980) *Biochem. J.* **192**, 49-57
- Owens, R. J. & Crumpton, M. J. (1983) *Biochem. Soc. Trans.* **11**, 156-157
- Ponstingl, H., Krauhs, E., Little, M. & Kempf, T. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2757-2761
- Rosenberg, S., Stracher, A. & Burridge, K. (1981) *J. Biol. Chem.* **256**, 12986-12991
- Schliwa, M. (1976) *J. Cell Biol.* **70**, 527-540
- Sheterline, P. & Hopkins, C. R. (1981) *J. Cell Biol.* **90**, 743-754
- Snary, D., Woods, F. R. & Crumpton, M. J. (1976) *Anal. Biochem.* **74**, 457-465
- Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W. J., Kirschner, M. W. & Cleveland, D. W. (1981) *Nature (London)* **289**, 650-655
- Vandekerckhore, J. & Weber, K. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1106-1110
- Van-Eldik, L. J., Zendejui, J. G., Marshak, D. R. & Watterson, D. M. (1982) *Int. Rev. Cytol.* **77**, 1-61
- Walker, J. H. (1982) *J. Neurochem.* **39**, 815-823
- Weisenberg, R. C. (1972) *Science* **177**, 1104-1105
- Yin, H. L. & Stossel, T. P. (1981) *Nature (London)* **281**, 583-586