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Cellular distribution of p68, a new calcium-binding protein from lymphocytes

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A Ca^{2+}-binding protein of mol. wt. 68 000 (p68) is a major component of a Nonidet P-40 insoluble fraction of human and pig lymphocyte plasma membrane. An affinity-purified rabbit antibody has been produced against p68 and used to study its cellular distribution. The antibody stained fixed and permeabilised human B lymphoblastoid cells, peripheral blood lymphocytes and sections of human tonsil. Whole cells, however, were not stained, indicating that the protein was not represented at the cell surface. This assignment was consistent with the detection of p68 in immunoprecipitates from biosynthetically- but not surface-labelled cells. It is concluded that p68 is located on the cytoplasmic face of the plasma membrane. Subcellular fractionation experiments confirmed that p68 was largely membrane-bound in lymphocytes, although a small soluble fraction (~10% of the total) was detected. Sub-fractionation of lymphocyte membranes revealed that p68 was associated not only with the plasma membrane but also with other endomembrane systems. As judged by immunoprecipitation, p68 was present in a variety of cultured cell lines of both lymphoid and non-lymphoid origin. p68 demonstrated a diffuse distribution in fixed and permeabilised fibroblasts which did not correspond to the distribution of either microfilaments or intermediate filaments. However, in detergent-extracted cells the protein was localised in a lamina-like network. A similar immunofluorescent staining pattern has recently been observed for spectrin-related proteins in the detergent-resistant cytoskeleton of fibroblasts. It is suggested that p68 is part of a sub-membranous cytoskeletal complex not only in lymphocytes but also in other cell types.

Key words: calcium-binding/cytoskeleton/lymphocyte plasma membrane

Introduction

With the exception of erythrocytes, little is known about the components of the inner surface of the plasma membrane of eukaryotic cells. In erythrocytes, a macromolecular complex of proteins underlies the plasma membrane and is defined as the erythrocyte cytoskeleton (Yu *et al.*, 1973). The major protein of this cytoskeleton is spectrin which, as a tetramer, is bound to short actin protofilaments (Pinder and Gratzer, 1983). This results in the formation of a molecular lattice which is attached via ankyrin to band 3, the anion transport protein (Bennett and Stenbuck, 1980; Branton *et al.*, 1981). Recently, a number of studies have revealed spectrin-related molecules in a variety of non-erythroid cells, including fibroblasts (Levine and Willard, 1981; Burridge *et al.*, 1982) and lymphocytes (Levine and Willard, 1983; Nelson *et al.*, 1983). The major spectrin-related protein in non-erythroid cells, fodrin (Glenney and Glenney, 1983), appears to be associated with the plasma membrane (Bennett *et al.*, 1982). This raises the possibility that a sub-membranous complex, similar to the erythrocyte cytoskeleton, may occur in non-erythroid cells. However, to date, functional analogues of ankyrin have not been identified in such cells.

More direct indication of such a complex has recently been obtained from studies on lymphocytes. Using the extraction conditions of Yu *et al.* (1973) a detergent-insoluble complex of proteins has been identified in association with purified plasma membrane prepared from mouse (Mescher *et al.*, 1981), pig and human lymphocytes (Davies *et al.*, 1984). Amongst the major polypeptides of the complex are actin (Mescher *et al.*, 1981 and unpublished observations) and a 68 000 mol. wt. protein. Interestingly, fodrin appears to be only a relatively minor component of the complex (unpublished observations). The 68 000 mol. wt. protein (p68) has been purified from human and pig lymphocytes and partially characterised (Owens and Crumpton, 1984). p68 is a monomeric acidic protein and appears to have been highly conserved between the two species studied. The association of the protein with the detergent-insoluble complex depends upon the calcium concentration and isolated p68 binds calcium.

Here we describe the preparation of an antibody to p68 and its use to study the cellular and subcellular distribution of the protein.

Results

Preparation of anti-(p68)

As the 68 000 mol. wt. calcium-binding protein of lymphocyte plasma membrane (p68) could only be obtained in small amounts (~10 $\mu g/10^{10}$ cells), an indirect method was used to prepare an antibody to the protein. Purified plasma membrane prepared from human B lymphoblastoid cells (BRI 8) was extracted with Nonidet P-40 (NP-40) to yield the detergent-insoluble fraction of the membrane, referred to as the '20-K pellet' (Davies *et al.*, 1984). Antibodies against the 20-K pellet were elicited in a rabbit. As judged by immunoblotting, the antiserum contained antibodies against a 68 000 mol. wt. and 55 000 mol. wt. polypeptide of the 20-K pellet (Figure 1, tracks 1 and 3). The antiserum also detected polypeptides of ~120 000 mol. wt. and 45 000 mol. wt. though the reactivities were very weak.

Since the association of p68 with the 20-K pellet depends upon the presence of Ca^{2+} (Owens and Crumpton, 1984), a 20-K pellet prepared in the presence of a Ca^{2+} chelator, EGTA, lacks p68 (Figure 1, track 2). When a sample of 20-K pellet depleted of p68 was exposed to the anti-(20-K pellet) serum in an immunoblotting experiment, no 68 000 mol. wt. polypeptide was detected (Figure 1, track 4). It was concluded

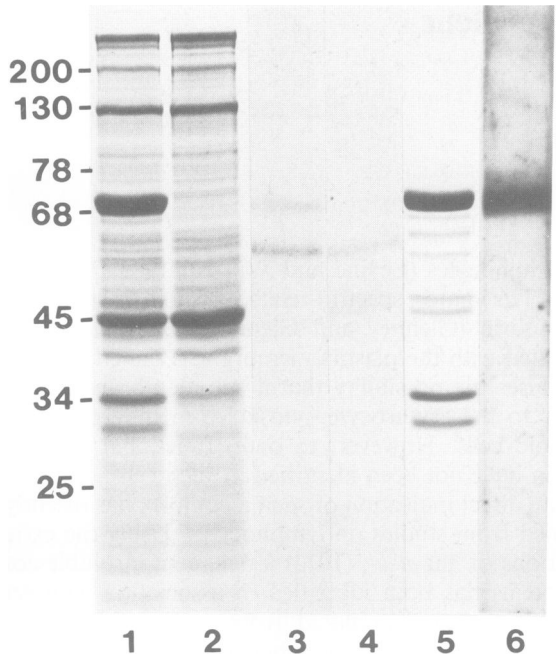

Fig. 1. Specificity of anti-(20-K pellet) serum. 20-K pellet prepared from BRI 8 cells in either the presence **(tracks 1** and **3)** or absence of Ca^{2+} **(tracks 2** and **4)** was separated on 10% polyacrylamide SDS gels and either stained with Coomassie blue **(tracks 1** and **2)** or transferred onto diazotised paper and exposed to the anti-(20-K pellet) serum and [^{125}I]protein A **(tracks 3** and **4)**. In the same way the polypeptides released by EGTA from 20-K pellet prepared in the presence of Ca^{2+} **(track 5)** were subjected to immunoblotting with the affinity-purified antibody against p68 **(track 6)**. The numbers refer to the mol. wt. (x 10^{-3}) of protein standards.

that the anti-(20-K pellet) serum contained antibodies against p68.

An affinity-purified antibody against p68 was produced by absorbing the rabbit anti-(20-K pellet) serum to a column of p68-Sepharose CL-4B. After washing with salt, bound antibody was recovered by elution with 3.5 M KSCN; it represented ~0.5% of the applied immunoglobulin. The specificity of this affinity-purified antibody (anti-p68) was investigated by immunoblotting and immunoprecipitation. A partially purified fraction of p68 (Figure 1, track 5) was electrophoretically transferred to diazotised paper and exposed to the purified antibody (Figure 1, track 6). The purified antibody appeared to react exclusively with p68. Anti-(p68) specifically immunoprecipitated a 68 000 mol. wt. polypeptide from [^{35}S]methionine-labelled lymphocyte plasma membrane which had been solubilised with NP-40 in the presence of EGTA (Figure 3, track 6). As discussed above, p68 is released from the detergent-insoluble complex of the membrane under these conditions. The results of immunoblotting and immunoprecipitation indicate that the purified antibody was specific for p68 and thus could be used to investigate the cellular distribution of the protein.

Immunofluorescent staining of lymphocytes

Anti-(p68) did not give detectable staining of whole, unfixed or *p*-formaldehyde-fixed, human peripheral blood lymphocytes or human B lymphoblastoid cells (BRI 8), indicating that the antigen is most probably not represented at the cell surface. On the other hand, the antibody bound specifically to the cytoplasm of lymphocytes that had been fixed and permeabilised. The staining was concentrated at the cell periphery and in some of the lymphoblastoid cells the staining appeared to be patchy (Figure 2a and b). The antibody also stained lymphocytes in frozen sections of human tonsil with the fluorescence largely restricted to the cytoplasmic rim around each of the cells (Figure 2c). This result was confirmed when sections were stained by an immunoperoxidase method and counter-stained with Meyer's Haemalum (data not shown). Controls in which tissue sections and lymphocyte cell smears were incubated with preimmune rabbit immunoglobulin G showed no significant immunofluorescent staining.

Immunoprecipitation from lymphocytes

The distribution of p68 in lymphocytes was investigated further by immunoprecipitation. In these experiments immunoprecipitation of class I (i.e., HLA-A,B,C) antigens by the monoclonal antibody W6/32 was used as a control. These cell surface antigens comprise a 43 000 mol. wt. transmembrane glycosylated polypeptide which is non-covalently associated with β_2-microglobulin (Barnstable *et al.*, 1978a). The antibody, W6/32, recognises the whole antigen only (Barnstable *et al.*, 1978b).

Human lymphoblastoid cells (BRI 8) were either surface-labelled by lactoperoxidase-catalysed iodination or biosynthetically labelled with [^{35}S]methionine. A purified plasma membrane fraction was prepared from the biosynthetically labelled cells. As a control, a preparation of purified plasma membrane was also labelled with ^{125}I by the lactoperoxidase method. NP-40 lysates were made from each of the labelled preparations and equal aliquots subjected to immunoprecipitation with either anti-(p68) or W6/32. A 68 000 mol. wt. polypeptide was immunoprecipitated with the purified antibody from the [^{35}S]methionine-labelled cells but not from the ^{125}I-labelled cells (Figure 3, tracks 2 and 6). The result was not due to the inability of the lactoperoxidase method to label p68 since a 68 000 mol. wt. polypeptide was immunoprecipitated from the ^{125}I-labelled plasma membrane vesicles (Figure 3, track 4). Since the plasma membrane preparation most probably comprises vesicles of mixed orientation (Walsh *et al.*, 1976), polypeptides located on either the cell surface or cytoplasmic side of the membrane will be labelled. The immunoprecipitation data confirm the results of the immunofluorescence experiments and indicate that p68 is located intracellularly in lymphocytes. It seems likely, therefore, that the protein is associated with the cytoplasmic face of the plasma membrane. In the control experiment, the 43 000 mol. wt. 'heavy' chains of the HLA-A,B antigens were immunoprecipitated from lysates of plasma membrane labelled by all three procedures (Figure 3, tracks 1, 3 and 5). The doublet of polypeptides precipitated in track 1 correspond to the heavy chains of HLA-A and HLA-B antigens respectively (Loube *et al.*, 1983).

Sub-cellular distribution of p68 in lymphocytes

Although the above results are consistent with the localisation of p68 at the inner face of the plasma membrane in lymphocytes, the data do not rule out the possibility that the protein occurs elsewhere in the cell. To investigate this question, homogenised BRI 8 cells, which had been labelled biosynthetically for 20 h with [^{35}S]methionine, were fractionated by differential centrifugation into three fractions, namely, the nuclei/mitochondria, microsomal pellet and cytosol; the nuclei/mitochondria fraction also contained unbroken cells. Each of these fractions was assayed for p68 by quantitative immunoprecipitation; HLA-A,B antigens were used as a con-

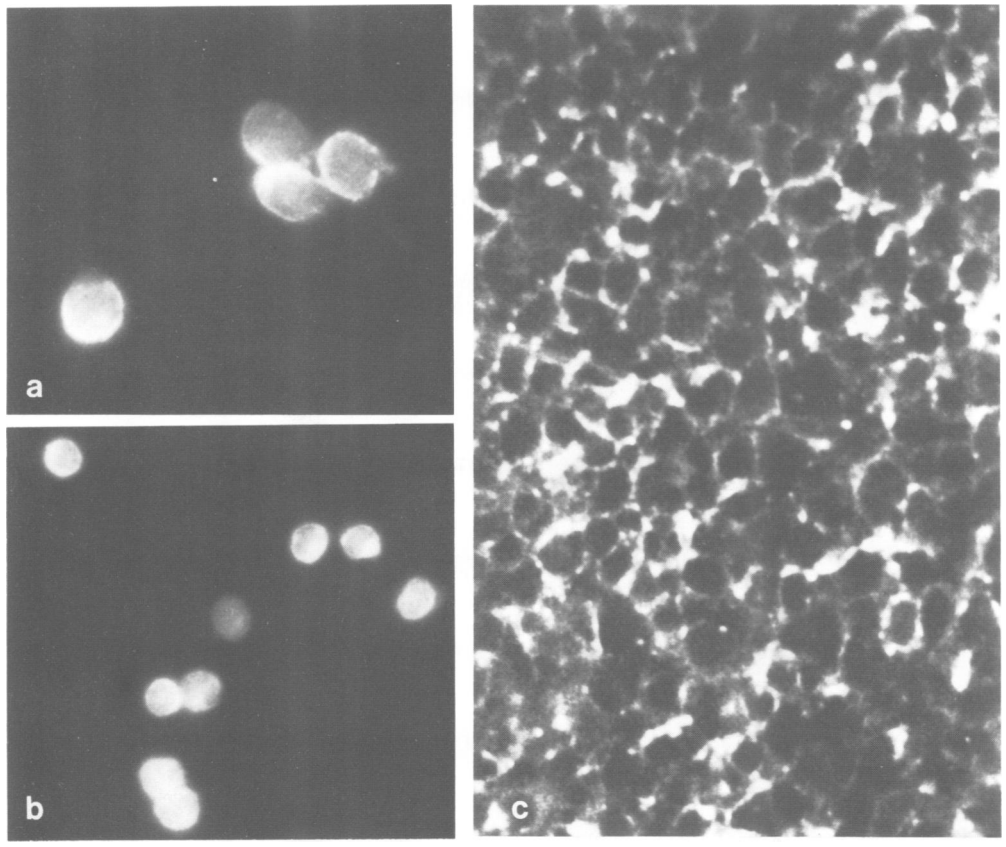

Fig. 2. Indirect immunofluorescent staining of lymphocytes with anti-(p68). Cells and tissue sections were incubated with anti-(p68) and the specifically bound antibody was visualised with either rhodamine or fluorescein-labelled goat anti-(rabbit immunoglobulin G) serum **(a)** human lymphoblastoid B cells (BRI 8); **(b)** human peripheral blood lymphocytes; **(c)** section of human tonsil tissue. Magnifications **a, b,** x 690; **c,** x 860.

trol. The proportion of p68 recovered from the microsomal fraction was similar to that of HLA-A,B indicating that p68 is largely membrane-bound in lymphocytes (Table I). The high proportion of both antigens recovered in the nuclei/mitochondria fraction is due to the presence of unbroken cells and plasma membrane which was trapped around the nuclei (Crumpton and Snary, 1974). This assignment is consistent with the large proportion of 5′-nucleotidase activity in the nuclei/mitochondria pellet; 5′-nucleotidase is a recognised marker of the plasma membrane in animal cells (Evans, 1978). In contrast to the HLA-A,B antigens there was a small but significant amount of p68 in the cytosol fraction, suggesting that there may be a soluble pool of the protein amounting to ~10% of the total cellular p68.

To investigate whether the association of p68 with the microsomal membranes occurs exclusively with the plasma membrane, the following experiment was carried out. BRI 8 cells were incubated with ^{125}I-labelled $F(ab')_2$ fragments of the monoclonal antibody W6/32 as a marker for the cell surface membrane. A microsomal fraction was prepared and the membranes fractionated on a 25–45% (w/w) continuous sucrose density gradient. The position of the plasma membrane in the gradient was indicated by the bound [^{125}I]$F(ab')_2$ W6/32 fragments (Figure 4a). This position agreed with previous data for the sucrose buoyant density of lymphocyte plasma membrane (Crumpton and Snary, 1974; Snary *et al.*, 1976). The five fractions indicated in Figure 4a were separately combined and analysed by immunoprecipitation for the presence of p68 and HLA-A,B antigens. Both antigens were detected in all five membrane fractions (Figure 4b). The membranes of sub-fraction 1 (Figure 4a) from the sucrose gradient are clearly separated from the peak of plasma membrane and on the basis of sucrose density probably comprise endomembranes derived from the endoplasmic reticulum. The HLA-A,B antigens precipitated from sub-fraction 1 (Figure 4b) comprised a polypeptide with mol. wt. ~40 000 in addition to the doublet at a mol. wt. of 43 000. The intensity of the former band was considerably less in sub-fraction 2 and this polypeptide was barely detectable in sub-fractions 3 and 4. The difference between the mol. wt. of the 40 000 and the 43 000 polypeptides coincides with that for the one N-linked glycan unit which is known to be attached to the 43 000 mol. wt. heavy chain of HLA-A,B antigens (Owen *et al.*, 1980). Thus, the 40 000 mol. wt. polypeptide probably represents newly synthesised, that is non-glycosylated, HLA-A,B antigens. Since *de novo* synthesis and glycosylation of membrane proteins occurs in the endoplasmic reticulum, the fact that sub-fraction 1 contains the highest proportion of unglycosylated HLA-A,B antigens supports the view that this fraction is derived from the endoplasmic reticulum. Interestingly, sub-fraction 5, which represents a light membrane fraction (Figure 4a), also contains a high proportion of newly synthesised HLA-A,B antigens. This sub-fraction was not characterised further, but it seems possible that it represents intracellular vesicles derived from the endoplasmic reticulum.

The above results indicate that p68 is associated with the plasma membrane and endomembranes, including the endoplasmic reticulum. Although it is of interest to know whether p68 is concentrated in the plasma membrane fraction, the

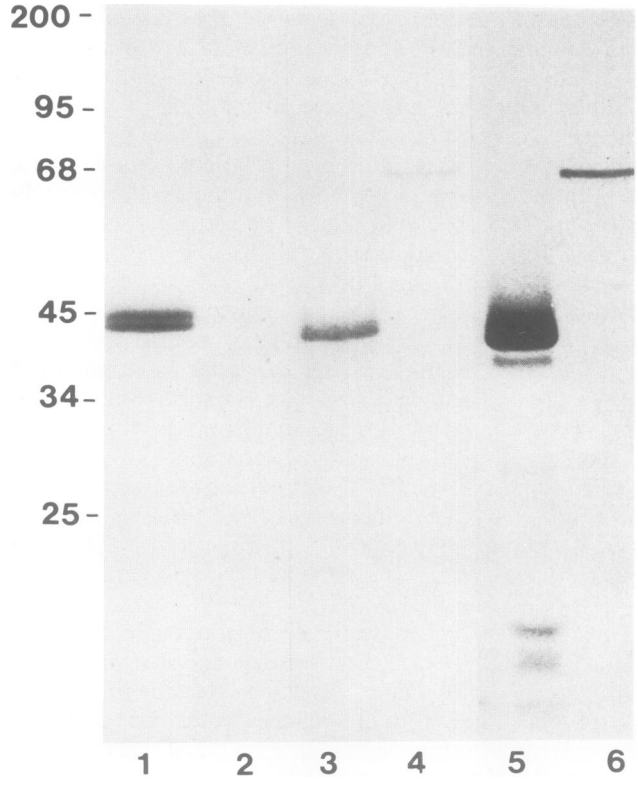

Fig. 3. Immunoprecipitation of p68 and HLA-A,B,C antigens from lymphoblastoid cells. Human lymphoblastoid cells (BRI 8) were labelled either biosynthetically with [^{35}S]methionine or at the surface with [^{125}I]iodine as described in Materials and methods; a purified preparation of BRI 8 cell plasma membrane was also labelled with [^{125}I]iodine. NP-40 lysates prepared from each of the labelled preparations were subjected to immunoprecipitation with either W6/32 (**tracks 1, 3** and **5**) or anti-(p68) (**tracks 2, 4** and **6**). Immunoprecipitates were analysed on 10% polyacrylamide SDS gels under reducing conditions. Autoradiograph of polypeptides immunoprecipitatated from surface-labelled whole cells (**tracks 1** and **2**), surface-labelled plasma membrane vesicles (**tracks 3** and **4**) and biosynthetically-labelled plasma membrane vesicles (**tracks 5** and **6**).

Table I. Sub-cellular distribution of p68

Fraction	% in each fraction			
	Protein	5′-Nucleotidase activity	p68	HLA-A,B antigens
Nuclei/mitochondria/ unbroken cells (5000 *g* pellet)	44.0	68.0	62.0	68.0
Microsomes (20 000 *g* pellet)	9.0	32.0	30.0	30.0
Cytosol (100 000 *g* supernatant)	47.0	0.0	8.0	2.0

The three sub-cellular fractions were prepared from [^{35}S]methionine-labelled (10 μCi/10^6 cells/ml for 20 h) and unlabelled lymphoblastoid B cells (BRI 8). Fractions from unlabelled cells were assayed for protein (Lowry *et al.*, 1951) and 5′-nucleotidase and those from labelled cells were assayed for p68 and HLA-A,B,C antigens by quantitative immunoprecipitation. The amounts in each fraction are expressed as a percentage of the total amount recovered in all three fractions.

present experiment does not provide an answer because the pool size and turnover rate of p68 are unknown. Such information depends upon an assay for p68; this is being developed.

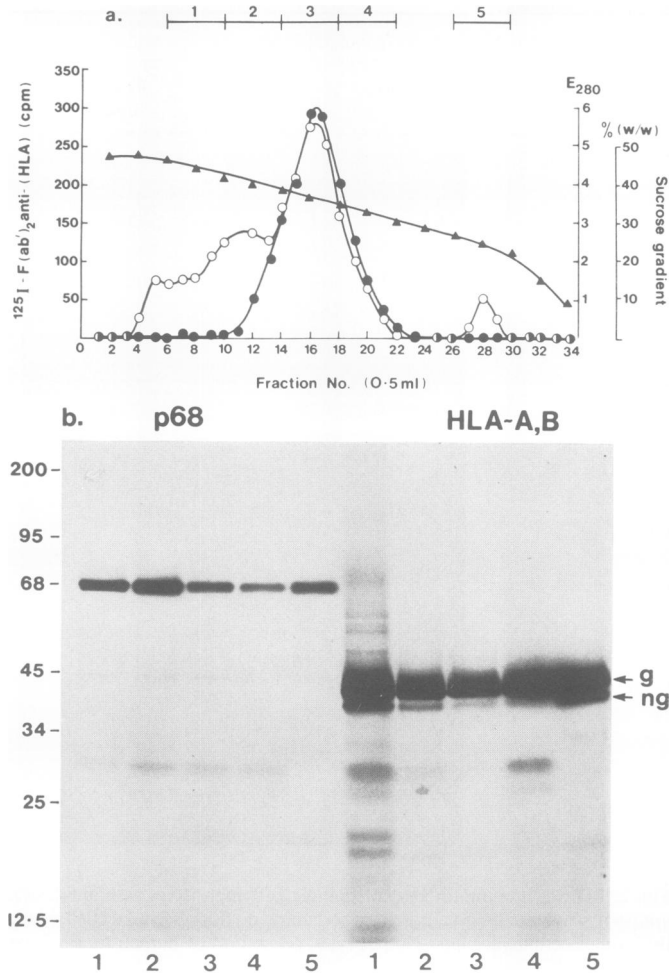

Fig. 4. Fractionation of lymphoblastoid cell membranes. **(a)** BRI 8 cells were incubated with ^{125}I-labelled F(ab′)$_2$ fragments of the W6/32 monoclonal antibody as a marker for the surface membrane. A microsomal fraction was prepared (see Materials and methods) and fractionated on a 25–45% (w/w) continuous sucrose gradient. The gradient was fractionated into 0.5 ml aliquots and each aliquot was assayed for E280 nm (○), radioactivity (●) and sucrose concentration by refractive index (▲). **(b)** A microsomal fraction prepared from [^{35}S]methionine-labelled BRI 8 cells was fractionated on a 25–45% (w/w) continuous sucrose density gradient. The five membrane sub-fractions indicated in **(a)** were separately combined and analysed by immunoprecipitation for the presence of p68 and HLA-A,B antigens. Immunoprecipitates were run on 10% polyacrylamide SDS gels under reducing conditions. Autoradiograph of immunoprecipitates from membrane sub-fractions 1–5 using anti-(p68) **(left hand panel)** and W6/32 antibodies **(right hand panel)**. In some of the anti-(p68) immunoprecipitates, p68 appeared as a very close doublet (e.g., **track 1**). The reason for this is unclear, though the slightly lower mol. wt. band may have been generated by proteolysis. ng indicates the non-glycosylated HLA-A,B heavy chain; g indicates the N-glycosylated HLA-A,B heavy chain.

Detection of p68 in non-lymphoid cells

Having established the sub-cellular distribution of p68 in lymphocytes an important question is whether the protein occurs exclusively in lymphoid cells. To investigate whether p68 has a wider cellular distribution, a number of lymphoid- and non-lymphoid-derived cell lines in culture were biosynthetically labelled with [^{35}S]methionine and whole cell lysates subjected to immunoprecipitation with anti-(p68). The results, summarised in Table II indicate that p68 is present not only in lymphocytes but also in other haemopoietic cells. In addition, the protein was detected in fibroblast and epithelial cells of both human and mouse origin, indicating a

Table II. Presence of p68 in different cell types

Cell type	Description	Reactivity with anti-(p68)
Human		
'Hairy' cell	Late B cell leukaemia	+
BRI 8	B lymphoblastoid	+
Maja	B lymphoblastoid	+
Nalm 6	pre B cell leukaemia	+
HPB-ALL	T cell leukaemia	+
Reh	non B/T cell leukaemia	+
HL60	promyelocytic	+
K562	erythroblastoid	+
Butler	fibroblastic	+
HeLa	epithelial	+
Other		
3T3k (mouse)	fibroblastic	+
CHO (hamster)	fibroblastic	+

Cells were biosynthetically labelled with [^{35}S]methionine (20 μCi/10^6 cells/ml or 250 μCi/90 mm dish) for 4 h and then lysed in NP-40. The lysates were pre-cleared with pre-immune immunoglobulin G and then subjected to immunoprecipitation using the affinity-purified rabbit anti-(p68) antibody. Immunoprecipitates were analysed on 10% polyacrylamide gels in SDS.

degree of conservation between species. The distribution of p68 in fibroblasts was studied further by immunofluorescent staining.

Immunofluorescent staining of fibroblasts

The rabbit anti-(p68) antibody gave a diffuse staining of the whole cytoplasm of mouse 3T3k fibroblasts that had been fixed and permeabilised with cold acetone (Figure 5a). In contrast, when cells were extracted with a non-ionic detergent (NP-40) to give a cytoskeletal preparation (Lenk and Penman, 1979) and then fixed and stained, a reticulate pattern was observed (Figure 5b and c).

The anti-(p68) antibody staining of fibroblasts was clearly distinct from the patterns obtained with antibodies to myosin and intermediate filament proteins (Figure 5d and e). The anti-(myosin) antibody stained transcellular stress fibres (Weber and Groschel-Stewart, 1974). The anti-(intermediate filament) antibody gave a staining pattern characteristic of intermediate filaments which in cultured cells of mesenchymal origin consist of vimentin (Lazarides, 1980).

Discussion

This study was undertaken to investigate the cellular distribution of a new 68 000 mol. wt. calcium-binding protein, termed p68. This protein was originally identified as a major component of a non-ionic detergent-insoluble fraction of purified lymphocyte plasma membrane (Davies *et al.*, 1984; Owens and Crumpton, 1984). By analogy to studies on erythrocytes (Yu *et al.*, 1973) it has been suggested that this detergent-insoluble fraction may represent a sub-membranous 'cytoskeleton' in lymphocytes (Davies *et al.*, 1984). p68 has been purified from lymphocyte plasma membrane and was used to selectively purify the homologous antibodies present in an antiserum prepared against the entire detergent-insoluble fraction. The results of the immunofluorescence and immunoprecipitation experiments reported here, show that p68 is an intracellular protein in lymphocytes and is preferentially located on the cytoplasmic face of the lymphocyte plasma membrane. This localisation of p68 together with its insolubility in NP-40 lends support to the idea that the protein is part of a membrane-cytoskeleton complex.

Subcellular fractionation studies on lymphocytes indicated that p68 is not only a component of the plasma membrane but is also associated with other endomembranes including the endoplasmic reticulum (ER). At present, it is not possible to distinguish whether p68 is synthesised in association with membranes, i.e., on ER-bound polysomes, or whether it is synthesised on free polysomes and subsequently incorporated into endomembranes. The detection of a small soluble pool of p68 would, however, favour the latter situation. In this respect, the synthesis of p68 may be similar to that proposed for the tyrosine kinase, $pp60^{src}$. This protein appears to be synthesised on free polysomes (Lee *et al.*, 1979) and then rapidly becomes associated with a particulate fraction (Levinson *et al.*, 1981). Immunocytochemical (Willingham *et al.*, 1979) and cell fractionation (Courtneidge *et al.*, 1980) studies suggest that the kinase is localised to the cytoplasmic face of the plasma membrane.

Lymphocytes are not the only haemopoietic cells which synthesised p68. The protein was immunoprecipitated from an erythroblastoid and a promyelocytic (undifferentiated monocyte) cell line. In addition, the protein was detected in epithelial cells and fibroblasts suggesting that p68 may have a common function in different cell types, possibly as a Ca^{2+} receptor (Owens and Crumpton, 1984). The distribution of p68 in fibroblasts, as judged by immunofluorescence, was similar to that observed for fodrin (Burridge *et al.*, 1982) and other spectrin-related molecules (Glenney *et al.*, 1982; Greenberg and Edelman, 1983; Lehto *et al.*, 1983) as well as a phosphotyrosine-containing protein, p34/39, (Greenberg and Edelman, 1983; Lehto *et al.*, 1983; Nigg *et al.*, 1983) that acts as a substrate for $pp60^{src}$ kinase (Erikson and Erikson, 1980). All these antigens demonstrated diffuse or reticulate staining patterns depending upon the conditions used to prepare the cells for staining. The reticulate pattern was obtained with fibroblasts that had been extracted with non-ionic detergents. This yields a cytoskeletal preparation from which most cellular proteins have been removed, but which retains certain cell surface membrane proteins (Ben-Ze'ev *et al.*, 1979; Lehto, 1983). This suggests that p68 together with fodrin and p34/39 are associated with the cytoskeleton in these cells. However, the distribution of these antigens in fibroblasts does not correspond to that of recognised cytoskeletal structures such as stress fibres (Figure 5; Greenberg and Edelman, 1983).

Sub-cellular fractionation experiments indicate that p34/39 is associated with the plasma membrane in fibroblasts (Greenberg and Edelman, 1983). In view of this localisation and the association of fodrin with membranes (Bennett *et al.*, 1982), the immunofluorescence distribution of p34/39 has been taken to indicate a submembranous/cytoskeletal localisation of the protein in fibroblasts (Greenberg and Edelman, 1983; Lehto *et al.*, 1983; Nigg *et al.*, 1983). We have already indicated that p68 is most probably located on the cytoplasmic face of the lymphocyte plasma membrane in association with a putative membrane-cytoskeletal complex. It thus seems likely that p68 (together with fodrin and p34/39) is part of a similar complex in fibroblasts and possibly other non-lymphoid cells.

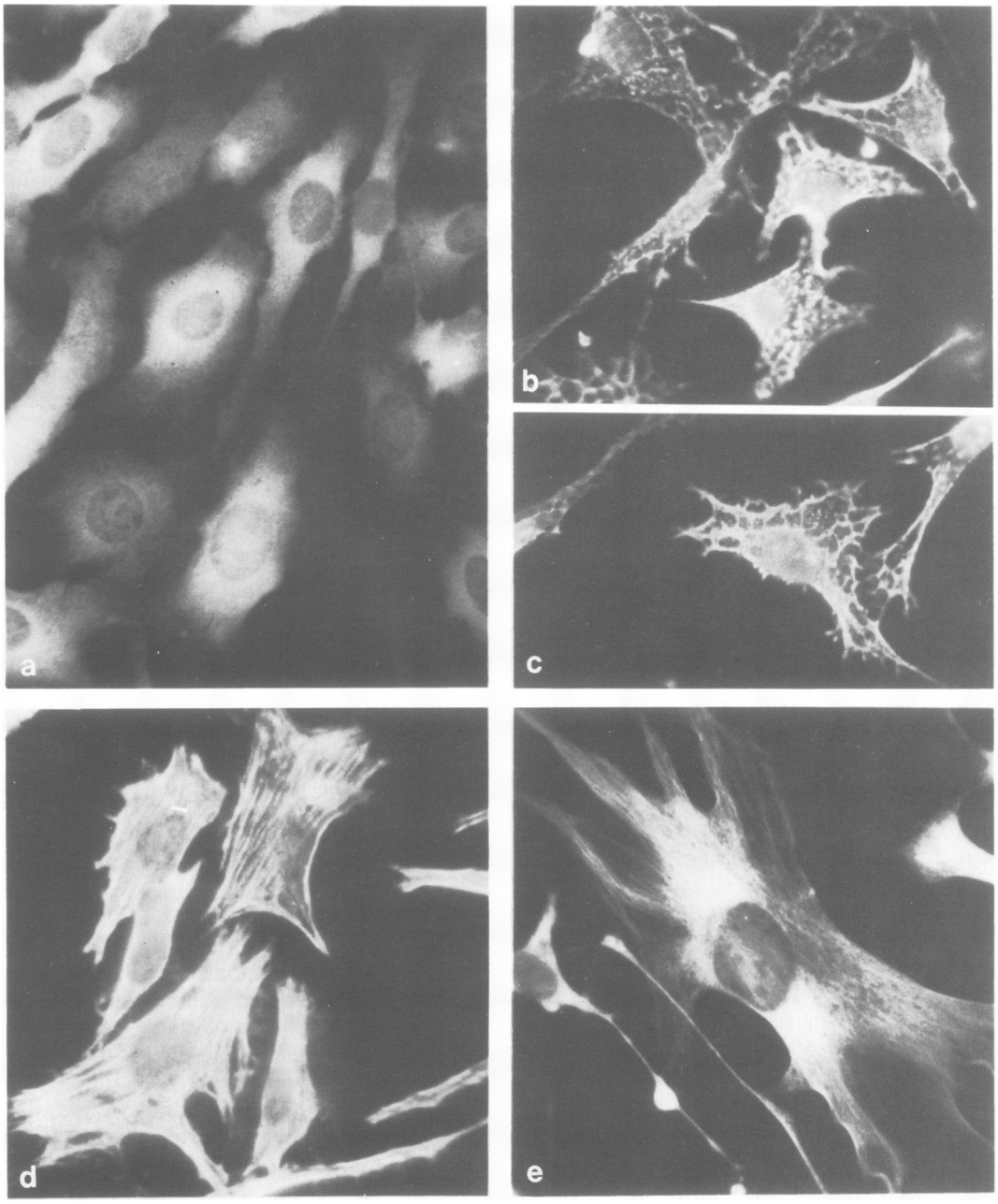

Fig. 5. Indirect immunofluorescent staining of mouse 3T3k fibroblasts with anti-(p68), anti-(myosin) and anti-(intermediate filament)(IFA). Mouse 3T3k fibroblasts were either fixed and permeabilised with cold acetone **(a, d, e)** or extracted with 1% NP-40 and then fixed with 2% *p*-formaldehyde **(b,c)**. Cells were incubated with anti-(p68) **(a, b, c)**, anti-(myosin) **(d)** and anti-(IFA) **(e)**. Specifically bound antibody was visualised with either rhodamine-labelled goat anti-rabbit immunoglobulin G) **(a, b, c, d)** or fluorescein-labelled rabbit anti-(mouse immunoglobulin G) **(e)**. Magnifications **a, b, c** and **d**, x 270; **e** x 400.

Materials and methods

Cells

The following human cell lines were cultured in RPMI 1640 medium, containing penicillin (100 units/ml), streptomycin (50 μg/ml) and 10% (v/v) foetal calf serum: BRI 8, Maja, CLL, Nalm 6, HPB-ALL, HL60 and K562. Mouse 3T3k fibroblasts, CHO cells, HeLa cells and human fibroblasts ('Butler') were cultured in Eagle's medium together with the above supplements.

Human peripheral blood lymphocytes were separated from platelet-depleted blood by layering onto an equal volume of Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuging at 4 x 10^4 *g* min; the cells collecting at the interface were washed twice with phosphate buffered saline (PBS: 3 mM KCl, 170 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2).

Antibodies

The NP-40 insoluble fraction (referred to as the '20-K pellet') of the plasma membrane of human B lymphoblastoid cells (BRI 8) was prepared as previously described (Davies *et al.*, 1984). One rabbit was injected at multiple sites with 1 mg of the 20-K pellet, suspended in 1 ml of Freunds complete adjuvant. The immunisation was repeated 8 weeks later using 1 mg of the 20-K pellet in Freunds incomplete adjuvant and the rabbit was bled after a further 8 weeks. Thereafter, the rabbit was injected i.v. with 0.5 mg of the 20-K pellet (in 0.5 ml of PBS) and bled 2 weeks following each injection. The serum from each bleed was stored at −70°C without further processing.

The 68 000 mol. wt. calcium-binding protein (p68) of the 20-K pellet was purified from BRI 8 cells as previously described (Owens and Crumpton, 1984). Purified p68 (130 μg) was conjugated to 0.4 ml CNBr-activated Sepharose CL-4B (Pharmacia, Uppsala, Sweden) in 0.1 M-$NaHCO_3$, pH 8.5 at 4°C for 16 h (March *et al.*, 1974). Excess reactive groups were blocked by incubating the gel in 0.5 M ethanolamine at 4°C for 4 h. The product was used for the preparation of a monospecific antibody namely anti-(p68). Anti-(20-K pellet) serum (0.5 ml) was applied to the p68-Sepharose packed into a column. After sequential washing with 10 mM Na phosphate buffers, pH 7.2, containing 150 mM and 500 mM NaCl, bound antibody was recovered by

elution with 1 ml of 3.5 M KCNS. Approximately 50 μg of immunoglobulin G was eluted.

A mouse hybridoma which produces a monoclonal antibody against all classes of intermediate filaments [anti-(IFA); Pruss *et al.*, 1981] was kindly provided by Professor M.C.Raff, University College, London. The antibody was purified from culture supernatants by precipitation with 45% saturated ammonium sulphate followed by affinity chromatography on Protein A-Sepharose (Pharmacia) (Goding, 1976).

An affinity purified rabbit antibody to bovine thymus myosin (Drenckhahn *et al.*, 1983) was kindly provided by Dr.J.Kendrick-Jones, MRC Laboratory of Molecular Biology, Cambridge. W6/32 is a mouse monoclonal antibody which recognises a common epitope expressed by all HLA-A,B,C antigens provided the heavy (i.e., 43 000 mol. wt.) chain is associated with β_2-microglobulin (Barnstable *et al.*, 1978b). It was used as purified immunoglobulin prepared using Protein A-Sepharose. $F(ab')_2$ fragments of W6/32 were produced by pepsin digestion and were kindly donated by C.E.Rudd.

Immunofluorescence

Lymphocytes were fixed with 2% (w/v) *p*-formaldehyde in PBS for 15 min at 20°C, washed twice with PBS, resuspended in a small volume of 10% (v/v) foetal calf serum in Hanks balanced salt solution and smeared onto microscope slides. Cells were permeabilised with 0.2% (v/v) NP-40 in Hanks medium for 10 min at 20°C.

Biopsies of human tonsil were immediately frozen in Tissue-Tek II O.C.T. Compound (Miles,IL, USA) with liquid N_2. Frozen sections (3 – μm thick) were mounted on glass slides, air dried and then permeabilised with acetone (−20°C, 10 min).

Mouse 3T3k fibroblasts, grown on glass coverslips were fixed and permeabilised in acetone (−20°C, 10 min). Fibroblast cytoskeletal preparations were produced by extracting the cells, grown on coverslips, with 1% (v/v) NP-40 in 10 mM Pipes buffer, pH 6.8, containing 100 mM KCl, 300 mM sucrose, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma Chemicals, Poole, Dorset, UK) and 0.1 mM N-*p*-tosyl-L-lysine chloromethylketone (Sigma), for 5 min at 4°C (Greenberg and Edelman, 1983). The cells were washed twice with the above buffer without NP-40, twice with PBS and then fixed for 15 min in 2% (w/v) *p*-formaldehyde.

Staining was by indirect immunofluorescence. The samples were incubated with the first antibody [anti-(p68), 40 μg/ml; anti-(IFA), 50 μg/ml; anti-(myosin), 2.5 μg/ml] for 1 h at 20°C and then washed five times with PBS containing 0.05% (v/v) Tween 20 (BDH Chemicals, Poole, Dorset, UK). The preparations were incubated with the second antibody, either fluorescein-labelled goat anti-(rabbit immunoglobulin G) or fluorescein-labelled rabbit anti-(mouse immunoglobulin G) (25 μg/ml, Miles, Slough, UK) for 1 h at 20°C and then again washed five times with PBS/Tween 20. Preparations were mounted in 50% (v/v) glycerol and examined under a Leitz epifluorescence microscope. Controls were performed in which the first antibody was replaced with either pre-immune rabbit or mouse immunoglobulin G (40 μg/ml).

Radioactive labelling

[^{35}S]methionine (600 Ci/mmol) and Na [^{125}I] (350–600 mCi/ml) were from Amersham International, UK. Cells grown in suspension were biosynthetically labelled by incubating (10^6 cells/ml) with [^{35}S]methionine (25 μCi/ml) in methionine-free RPMI 1640 medium containing 2% (v/v) foetal calf serum for 4 h or 20 h at 37°C. Cells grown on 90 mm plastic dishes were labelled with 250 μCi [^{35}S]methionine as above. BRI 8 cells and plasma membrane vesicles were labelled by lactoperoxidase-catalysed iodination (Walsh and Crumpton, 1977). Labelled cells were washed twice with PBS before processing for immunoprecipitation or subcellular fractionation.

Protein A (Pharmacia) and $F(ab')_2$ W6/32 were iodinated using chloramine T (McConahey and Dixon, 1980); 75–80% of the added radioactivity was incorporated.

Immunoprecipitation and sub-cellular fractionation

Cells were lysed (10^7 cells/ml) in 10 mM Tris/HCl buffer, pH 7.4, containing 1% (v/v) NP-40, 1 mM EDTA, 150 mM NaCl, 1 mg/ml ovalbumin and 1 mM PMSF for 30 min at 4°C. Lysates were centrifuged at 800 *g* for 5 min at 4°C and the supernatants recentrifuged at 100 000 *g* for 30 min at 4°C. Immunoprecipitation was carried out as previously described (Owen *et al.*, 1980) using 2.0 μg antibody/ml lysate and fixed *Staphylococcus aureus* organisms to precipitate antigen-antibody complexes. Immunoprecipitates were resuspended in 80 mM Tris/HCl buffer, pH 8.6, containing 4% (w/v) SDS, 4% (w/v) glycerol, 0.1 M dithiothreitol and 0.02% bromophenol blue (SDS sample buffer), heated at 100°C for 5 min and were analysed by SDS-polyacrylamide gel electrophoresis.

BRI 8 cells in PBS containing 10 mM iodoacetamide and 1 mM PMSF were broken and subcellular fractions separated as previously described (Crumpton and Snary, 1974; Snary *et al.*, 1976). The microsomal pellet was further fractionated on a continuous 25–45% (w/w) sucrose gradient (Snary *et al.*, 1974). After centrifuging at 65 000 *g* for 16 h at 4°C, the gradient was divided into 0.5 ml aliquots and each fraction was assayed for radioactivity and E280 nm. Peak fractions were combined, diluted with homogenisation buffer and centrifuged at 100 000 *g* for 30 min at 4°C. Sub-cellular fractions were solubilised in lysis buffer supplemented with 0.5% (w/v) sodium deoxycholate and processed for immunoprecipitation as described above. For quantitative immunoprecipitation, 1 ml of the lysates was subjected to three cycles of precipitation with an excess of antibody (5 μg/ml). The amount of labelled polypeptide precipitated was determined by cutting out the relevant bands from the SDS-polyacrylamide gel and solubilising in H_2O_2 and counting by liquid scintillation (Owens and Northcote, 1981).

Assays

5′-Nucleotidase activity was assayed as described by Stanley *et al.* (1980) and protein was estimated according to the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Electrophoretic analysis

SDS-polyacrylamide gel electrophoresis was carried out in 0.1% (w/v) SDS in Tris/glycine buffer on 10% (w/v) acrylamide slab gels (Laemmli, 1970). Gels were stained with 0.01% Coomassie Blue in methanol/water/acetic acid (41:52:7 by vol). Autoradiography was performed on the dried gels using Kodak SB-5 X-ray film. The following mol. wt. markers were used: myosin heavy chain (200 000); 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).

For immunoblotting, polypeptides were separated by SDS-polyacrylamide gel electrophoresis using a discontinuous sulphate/borate buffer (Neville, 1971). The polypeptides were electrophoretically transferred to diazophenylthioether paper (Reiser and Wardale, 1981) which was probed with either anti-(20-K pellet) serum (1 mg/ml) or anti-(p68) (10 μg/ml). Bound immunoglobulin was visualised by using ^{125}I-labelled protein A.

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