The association of type 1, type 2A and type 2B phosphatases with the human T lymphocyte plasma membrane

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Several putative plasma-membrane-associated components of the T-lymphocyte signal-transduction pathway are phosphorylated during the initial events of cellular activation. Little is known about the control of dephosphorylation of these components. We have shown by immunoblotting that the type 1 phosphatase, the type 2A phosphatase and type 2B phosphatase (calcineurin) are associated with the plasma membrane of normal human T lymphoblasts and the human T leukaemic cell line Jurkat 6. The type 1 phosphorylase phosphatase activity is present in a latent form which can be stimulated synergistically by dephosphorylation and *p*-nitrophenyl phosphate. The PCS_H form of the type 2A phosphatase appears to be the predominant oligomer in the plasma-membrane fraction. All three phosphatases can be extracted from membranes with Nonidet P40, but whereas the type 1_c and type 2A_c phosphatases separate into the detergent-poor phase of Triton X-114, calcineurin separates into both detergent-rich and -poor phases. It is probable that one or more of these three plasma-membrane-associated phosphatases play regulatory roles in determining the phosphorylation state of membrane-bound proteins involved in human T-cell activation.

INTRODUCTION

Rapid phosphorylation and dephosphorylation of several plasma-membrane-associated proteins occurs during the early events of T-lymphocyte activation. In view of the putative role of the CD3 antigen in transducing signals via the antigen–receptor complex (Brenner *et al.*, 1985), it is of particular interest that the CD3 γ subunit is phosphorylated on addition of specific antigen, phorbol esters or mitogenic lectin to human T cells (Cantrell *et al.*, 1985, 1987) and also in response to T-cell activation through the CD2 pathway (Breitmeyer *et al.*, 1987). Other T-lymphocyte proteins which are phosphorylated *in vivo* in response to either antigen or phorbol ester treatment include CD4 (Acres *et al.*, 1986, 1987) and CD8 (Acres *et al.*, 1987; Hoxie *et al.*, 1988).

The level of phosphorylation of any isolated protein reflects the dual regulation of both kinases and phosphatases (Cohen, 1982). The activation of several kinases has been implicated in the phosphorylation of the human (Davies *et al.*, 1987) and murine (Patel *et al.*, 1987; Klausner *et al.*, 1987) T-cell antigen–receptor complexes. However, little is known concerning the phosphatases involved in dephosphorylating the various components of the T-cell activation pathway. A role for membrane-associated phosphatase(s) has recently been suggested by the finding that the two serine phosphorylation sites on the CD3 antigen γ -chain are in close proximity to the plasma membrane (Davies *et al.*, 1987). The aim of the present study, therefore, was to identify which phosphoserine phosphatases are associated with the human T-cell plasma membrane.

The protein phosphatases of mammalian cells comprise the various forms of type 1 phosphatase [(ATP + Mg²⁺)-dependent], the type 2A phosphatases (polycation-stimu-

lated), calcineurin (type 2B) and the Mg²⁺-stimulated phosphatases (type 2C) (Ingebritsen & Cohen, 1983; Ballou & Fischer, 1986). Phosphatase characterization has largely been confined to cytosolic forms, though microsomal fractions have been reported to contain both type 1 and type 2A phosphatase activities (Alemany *et al.*, 1986; Mieskes & Söling, 1987; Goris & Merlevede, 1988a). The type 1_c and 2A_c enzymes are strikingly homologous (Green *et al.*, 1987; Silva *et al.*, 1987; Berndt *et al.*, 1987), so antibodies prepared against them have frequently shown cross-reactivity (Speth *et al.*, 1984; Brautigan *et al.*, 1986). In order to use immunological methods for identifying these phosphatases in T-cell membrane fractions, we have prepared a series of polyclonal antisera against the 1_c and 2A_c phosphatases, and selected two antisera which showed no cross-reactivity. We present evidence that at least three phosphatases are associated with purified preparations of lymphocyte plasma membrane: a dephosphorylation stimulated form of the type 1 phosphatase, the type 2A phosphatase and calcineurin.

MATERIALS AND METHODS

Materials

Purified skeletal-muscle type 1_c and 2A_c phosphatases were obtained from Professor P. Cohen (University of Dundee, Dundee, Scotland, U.K.), purified brain calcineurin and the VA₁ monoclonal antibody from Dr. J. Wang (University of Alberta, Calgary, Alberta, Canada), immunoaffinity-purified polyclonal antibodies against calcineurin from Professor C. Klee (Bethesda National Institutes of Health, MD, U.S.A.), immunoaffinity purified sheep antibodies against inhibitor-2

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; PNPP, *p*-nitrophenyl phosphate; 1_c and 2A_c, the catalytic subunits of type 1 and type 2A phosphatases; PCS, polycation-stimulated; PBS, phosphate-buffered saline (10 mM-sodium phosphate/150 mM-NaCl, pH 7.2); BSA, bovine serum albumin; e.l.i.s.a., enzyme-linked immunosorbent assay.

from Dr. D. Brautigan (Brown University, Providence, RI, U.S.A.), and dephosphorylase, phosphoinhibitor-1 and the purified skeletal-muscle PCS_H, PCS_M and PCS_L phosphatases from Dr. J. Goris (Catholic University of Leuven, Leuven, Belgium). Peptides were synthesized as described previously (Davies *et al.*, 1987). The A3 peptide (Gln-Asp-Gly-Val-Arg-Gln-Ser-Arg-Ala-Ala-Asp-Lys-Gln-Thr-Leu), an analogue of the CD3 antigen γ -chain, and the T4 peptide (Arg-Arg-Ala-Thr-Val-Ala), a peptide analogue of inhibitor 1, were phosphorylated as described by Davies *et al.* (1987) and Agostinis *et al.* (1987) respectively (phosphorylated residues are italicized). Immunological materials and pre-stained molecular-mass markers were from Amersham, except for horseradish peroxidase-conjugated rabbit anti-rat immunoglobulin G, which was from Dako. Cellulose nitrate sheets were from Schleicher and Schüll, Dassel, Germany. [³²P]ATP was from Amersham International, and other biochemicals were from Sigma Chemical Co., Poole, Dorset, U.K.

Cell culture and membrane fractionation

Cells were cultured (Cantrell *et al.*, 1985, 1987), and microsomes were prepared from them as previously described by Brown *et al.* (1987). The washing and shearing of cells was carried out using PBS without added Mg²⁺ or Ca²⁺. Plasma membrane was purified from the microsomal fraction using well-established methods (Allan & Crumpton, 1970; Crumpton & Snary, 1974; Snary *et al.*, 1974). Membrane separating at the 37%/25% (w/v) interface of a stepwise sucrose gradient was defined as the plasma-membrane fraction, and the pellet found below the 37%-sucrose layer was defined as the endoplasmic-reticulum fraction. As marker enzymes, 5'-nucleotidase, glucose-6-phosphatase and lactate dehydrogenase were assayed in each fraction as described by Widnell (1974), Kitcher *et al.* (1978), and Lee *et al.* (1982) respectively. In a typical preparation, 5'-nucleotidase levels were 6-fold greater in plasma membrane compared with endoplasmic reticulum, lactate dehydrogenase activity in plasma membrane was 3% that found in cytosol, and glucose-6-phosphatase activity was 3-fold greater in endoplasmic reticulum than in plasma membrane. Protein assays were by the method of Bradford (1976), with BSA as the standard protein. Yields of plasma membrane protein were in the range 0.7–1 mg/3 × 10⁹ T lymphoblasts and 0.5–1 mg/10⁹ Jurkat 6 cells.

Preparation of antisera

Two groups of three Wistar rats each were hyper-immunized with either type 1_c or type 2A_c phosphatases. Rats were injected at multiple subcutaneous sites with 20 µg of protein in Freund's complete adjuvant, followed by three injections of 10 µg of protein in Freund's incomplete adjuvant at 14–20-day intervals. Rats were killed 10 days after the final injection, exsanguinated and the blood allowed to clot overnight at 4 °C.

E.l.i.s.a.

Microtitre 72-well plates were precoated with purified phosphatase by incubation overnight at 4 °C with 100 ng of protein/well in 10 µl of 0.1 M-Na₂CO₃ buffer, pH 9.5. The solution was removed and blocking was performed by using 1% BSA in PBS for 1 h at 37 °C, and the wells then developed sequentially using antisera, biotinylated

Fig. 1. Micro-e.l.i.s.a. immunoassays of antisera to type 1_c and 2A_c phosphatases

Wells were precoated with 100 ng aliquots of purified antigen. Values shown are from a single microtitre plate. ■, □, R-2A_c antisera; ●, ○, R-1_c antisera; antigens used: □, ○, type 1_c phosphatase; ■, ●, type 2A_c phosphatase.

rabbit anti-rat IgG (diluted 1:500) and streptavidin-conjugated horseradish peroxidase (diluted 1:1000). Wells were rinsed twice with PBS/0.1% Tween-20 between each procedure, except for the final washes, when only PBS was used. Horseradish peroxidase was detected by using 2,2'-azinobis-(3-ethylbenzthiazoline-sulphonic hydrochloride), and the A₆₆₀ was measured using an automated Genetic Systems Micro EIA reader, as described in the manufacturer's manual.

E.l.i.s.a. assays using the purified 1_c and 2A_c phosphatases as antigen (Fig. 1) showed that one rat antisera out of each group of three rats had relatively high affinity for the injected antigen, with non-detectable levels of cross-reactivity towards the corresponding enzyme under the conditions of the assays. These antisera against the 1_c and 2A_c phosphatases were termed 'R-1'_c and 'R-2A'_c respectively, and were selected for further study by immunoblotting (see the Results section).

Immunoblotting

Proteins were separated by reducing SDS/PAGE on 12%-(w/v)-polyacrylamide slab gels as described by Laemmli (1970) before being transferred to 0.1 µm-pore-size nitrocellulose sheets overnight at 0.25 A. Blocking was carried out by using 5% Marvel (low-fat milk powder) in PBS for 4 h at 4 °C. Nitrocellulose sheets were then incubated in a 1:100 dilution of antisera in 0.5% BSA in PBS overnight at 4 °C, washed three times with 0.5% BSA in PBS, followed by incubation with 1:200 horseradish peroxidase-conjugated rabbit anti-rat or rabbit anti-sheep IgG in 0.5% BSA in PBS for 1 h at 4 °C. After three further washes with PBS, bound horseradish peroxidase was detected with diaminobenzidine (0.5 mg/ml)/0.01% H₂O₂/20 mM-Tris/HCl, pH 7.4.

Enzyme assays

^{32}P -labelled phosphorylase *a* was prepared as described by Krebs *et al.* (1958), and phosphorylase phosphatase activity was assayed by the method of Yang *et al.* (1980) with ^{32}P -labelled phosphorylase *a* (1 mg/ml) as substrate and 5 min incubations. Phosphoinhibitor 1 and de-inhibitor were preincubated with enzymes for 10 min at 30 °C before starting assays by addition of substrate; PNPP was added without preincubation. De-inhibitor was used at a concentration of 3.34 units/ml, twice the concentration required to give maximum stimulation of the de-inhibitor-stimulated phosphatase (Goris & Merlevede, 1988a). Phosphopeptide phosphatase activities were measured by using 50–150 nM [^{32}P]-phosphopeptide (3000–4500 c.p.m./pmol) in 20 mM-Tris/HCl (pH 7.0)/BSA (1 mg/ml) at 30 °C for 5 min in a final volume of 50 μl . Reactions were stopped, and the [^{32}P]P_i released was determined by using 1.0 ml columns of AG50W-X2 (H⁺ form) resin as described by Manalan & Klee (1983). All assays were performed in duplicate.

Membrane extraction

Plasma membrane (1.0 mg of protein/ml) was extracted with 1% (v/v) Nonidet P40/150 mM NaCl/20 mM-Tris/HCl, pH 7.2, for 30 min at 0 °C, then centrifuged for 30 min at 50000 *g*. The insoluble pellet was further extracted for 15 min in the same buffer containing 5 mM-EGTA in place of detergent, before centrifuging as described above. Protein from supernatants was recovered by precipitation overnight with 70% (v/v) ethanol at -20 °C.

Triton X-114 phase separation

Triton X-114 was precondensed three times and the phase separation of membrane proteins performed essentially as described by Bordier (1981). Briefly, plasma membrane (0.7 mg in 1 ml of 20 mM-Tris/HCl, pH 7.0) was solubilized in 1% Triton X-114/150 mM-NaCl/1 mM-benzamidine/20 mM-Tris/HCl, pH 7.0, at 0 °C for 10 min. After centrifugation for 30 min at 50000 *g* at 4 °C, two 0.5 ml aliquots of supernatant were layered on to two 300 μl cushions of 6% sucrose/0.06% Triton X-114 in the same buffer at 0 °C. The samples were then incubated for 3 min at 30 °C, causing clouding of the detergent extract, and centrifuged in an MSE Microcentaur instrument at 6500 rev./min for 3 min. The material was separated into a detergent-rich phase, which formed an oily droplet in the bottom of the tube, and a detergent-poor phase above the sucrose cushion. This condensation was repeated, and the detergent-rich and -poor phases were then subjected to a final condensation from buffer and 2% Triton X-114 respectively. The protein in these final samples was then precipitated overnight from 70% (v/v) ethanol at -20 °C in preparation for SDS/PAGE and immunoblotting analyses.

RESULTS

Immunoblotting of phosphatases in membrane fractions

Immunoblotting of purified type 1_c and 2A_c phosphatases demonstrated a lack of cross-reactivity between the R-1_c and R-2A_c antisera (Fig. 2a, panels 1 and 2, tracks A and B). Mild proteolysis is known to convert the cytosolic 37 kDa type 1_c enzyme into a 34 kDa product which is very resistant to further proteolysis

Fig. 2. Characterization of anti-phosphatase sera by SDS/PAGE and immunoblotting

(a) Tracks: A, 500 ng of purified type 1_c phosphatase; B, 500 ng of purified type 2A_c phosphatase; C, 60 μg of Jurkat-6-cell plasma membrane. Staining was with antisera to type 1_c (panel 1) or type 2A_c (panel 2) phosphatases. (b) Each track contained 300 ng of purified type 1_c (panel 1) or 500 ng of purified type 2A_c phosphatase (panel 2), treated with (tracks B and D) or without (tracks A and C) trypsin (50 ng, 37 °C, 2 min). Staining was with antisera to type 1_c (panel 1) or type 2A_c (panel 2).

(Tung *et al.*, 1984; Price *et al.*, 1986). After brief trypsin treatment, both the proteolytically stable form of 1_c (Fig. 2b, track B) and the proteolytic fragments of 2A_c (Fig. 2b, track D) were detected by the corresponding antisera, showing that immunoblotting of crude fractions would detect not only the intact enzymes, but also forms derived from them by proteolysis. Immunoblotting of Jurkat-6-cell plasma membrane revealed a 36 kDa band staining with R-1_c antisera running in the same position as the pure type 1_c enzyme (Fig. 2a, panel 1, track C). A major 35 kDa band staining with R-2A_c antisera was found in the same position as the pure type 2A_c enzyme (Fig. 2a, panel 2, track C).

In order to compare the distribution of phosphatases in T-cell subcellular fractions, immunoblotting was carried out by using equivalent amounts of protein from each fraction. Fig. 3(a) panel 2 shows that some type 1_c phosphatase was associated with each fraction studied, including plasma membrane. The appearance of a doublet in each fraction was presumably due to partial proteolysis of the 1_c 36 kDa subunit. Though panel 2, track A of Fig. 3(a) gives the impression that cytosolic staining for 1_c was less than in membranes, in two other immunoblots the staining density was equivalent (results not shown). Since the cytosolic form of the type 1 phosphatase is found in association with inhibitor-2 (modulator; Ballou & Fischer, 1986), it was of interest to determine whether inhibitor-2 was also present in T-

Fig. 3. Localization of phosphatases in human T-cell subcellular fractions

(a) Jurkat-6-cell fractions (50 μ g of protein per track): cytosol (track A); microsomes (track B); plasma membrane (track C) and endoplasmic reticulum (track D). Staining was with antisera to type 1_c (panel 2) or type 2A_c (panel 4) phosphatases, and with the appropriate preimmune sera (panels 1 and 3 respectively). (b) T-lymphoblast fractions (50 μ g of protein per track): cytosol (track A); endoplasmic reticulum (track B) and plasma membrane (track C). Staining was with rabbit antibodies to calcineurin (panel 1) or with normal rabbit serum as control (panel 2).

lymphocyte plasma membrane. Immunoaffinity-purified sheep antibodies to inhibitor-2 (Grupposo *et al.*, 1985) revealed a strongly staining 32 kDa band in the cytosol (Fig. 4, track A), but a much weaker band in plasma membrane (Fig. 4, track C). This contrasts with the approximately equivalent levels of staining intensity of the cytosolic and membrane type 1_c phosphatase (Fig. 3a, panel 2), and is not consistent with the 1_c subunit existing in T-cell membranes in association with inhibitor-2.

When T-cell subcellular fractions were stained with R-2A_c antisera, a 35 kDa band was found in plasma membrane, though it was consistently less dense than in the cytosol (Fig. 3a, panel 4). In addition, two bands of 40 and 70 kDa were noted in the cytosol (Fig. 3a, panel

4, track A), the 70 kDa band also being present on using pre-immune sera (Fig. 3a, panel 3, track A). The 40 kDa band was invariably found when using R-2A_c antisera with cytosol, but never with membranes (Fig. 3a, panel 4; cf. track A with tracks B–D), which is consistent with the low level of cytosolic contamination of the plasma-membrane fraction (see the Materials and methods section). Immunoblotting using antibodies to calcineurin revealed a major 16 kDa band in all fractions, corresponding to the B subunit, and a 61 kDa band in the position of the A (catalytic) subunit (Fig. 3b, panel 1). Since the anti-calcineurin antibodies used were less active against the A subunit than they were against the B subunit (C. Klee, personal communication), it was found necessary to increase the antibody concentration during the development of immunoblots in order to reveal the A subunit. In the process, some non-specific staining was obtained, though no bands corresponding to either the A or B subunits were observed on using normal rabbit serum (Fig. 3b, panel 2). The presence of calcineurin was confirmed by immunoblotting Jurkat-6-cell plasma membranes using a monoclonal antibody against subunit B (results not shown).

All the experiments shown in Figs. 2 and 3 were repeated for both normal T lymphoblasts and Jurkat 6 cells, with essentially the same results (not shown), demonstrating that the membrane association of phosphatases was not due to some abnormality in leukaemic cells.

Phosphatase activities of membrane fractions

It has recently been shown that PNPP specifically stimulates the active catalytic subunit of the type 1 phosphatase, as well as any active form of the holoenzyme (Goris & Merlevede, 1988b). Furthermore, when present together, PNPP and the deinhibitor protein (Goris *et al.*, 1984) stimulate synergistically. Since the phosphorylase phosphatase activity of mammalian cells is due to the

Fig. 4. Immunoblotting of inhibitor 2 in T-lymphoblast subcellular fractions

Fractions were (50 μ g of protein per track): cytosol (track A), endoplasmic reticulum (track B) and plasma membrane (track C). Staining was with an affinity-purified anti-inhibitor 2 antibody.

Table 1. Phosphorylase phosphatase activities of Jurkat-6-cell fractions

Assays were carried out as described in the Materials and methods section. Each value is the mean $\pm$ s.d. for four assays carried out on fractions from the same cultured cells. Values in parentheses are percentages of control values.

Additions	Phosphorylase phosphatase activity (pmol/min per mg)	
	Cytosol	Plasma membranes
None	32 $\pm$ 6 (100)	88 $\pm$ 5 (100)
30 nM-Phosphoinhibitor	43 $\pm$ 2 (134)	95 $\pm$ 5 (108)
Deinhibitor	81 $\pm$ 2 (253)	309 $\pm$ 23 (351)
1 mM-PNPP	33 $\pm$ 6 (103)	64 $\pm$ 12 (73)
Deinhibitor + 1 mM-PNPP	94 $\pm$ 4 (294)	493 $\pm$ 32 (560)

type 1 and type 2A phosphatases (Ingebritsen & Cohen, 1983), phosphorylase *a* was used as substrate for assays on Jurkat-6-cell fractions (Table 1). The basal activity was not inhibited by phosphoinhibitor 1 and is therefore due to the type 2A phosphatase. Addition of deinhibitor revealed a latent activity that was further stimulated on addition of PNPP. The increment of activity revealed by this synergistic stimulation was 7-fold greater in plasma membranes (429 pmol/min per mg) than in cytosol (61 pmol/min per mg).

The type 2a phosphatase catalytic subunit is common to various oligomeric forms which have been called 'phosphatases 2A₀, 2A₁ and 2A₂' (Tung *et al.*, 1985) or 'PCS_H-, PCS_M- and PCS_L-phosphatases' (Waelkens *et al.*, 1987). In order to assess which of these oligomers predominates in T-cell membranes, a new technique was developed which was based on the selectivity of the T4 phosphopeptide for the PCS_H phosphatase (Agostinis *et al.*, 1987), in contrast with the A3 phosphopeptide, which shows little selectivity between the PCS oligomers (D. Alexander, unpublished work). Neither T4 nor A3 phosphatase activities in the T-cell fractions under study were inhibited by 30 nM-phosphoinhibitor 1, nor did these activities show the Mg²⁺-dependence characteristic of the type 2C phosphatases (results not shown). Assays were carried out in the presence of 1 mM-EGTA in order to inhibit any calcineurin activity towards the phosphopeptides. The activities of T4 and A3 were compared as substrates for purified skeletal-muscle type 2A (PCS) phosphatases, confirming the selectivity of T4 for the PCS_H oligomer (Table 2a). T4/A3 phosphopeptide phosphatase ratios in T-cell fractions (Table 2b) suggested that the PCS_H phosphatase was the predominant oligomeric form present, particularly in Jurkat 6 cells. The type 2A phosphopeptide phosphatase activities were comparable in membranes and cytosol.

Detergent extraction of phosphatases

A sequential extraction of plasma membrane with non-ionic detergent followed by calcium chelator revealed that most of the type 1_c and 2A_c phosphatases and calcineurin could be solubilized by iso-osmotic 1% Nonidet P40 (Fig. 5, cf. tracks A and B). However, all

Table 2. Phosphopeptide T4 and A3 phosphatase activities of purified enzymes and cell fractions

Assays were carried out as described in the Materials and methods section. Activity values shown in (b) are means $\pm$ s.d. for assays carried out in duplicate on fractions from the number of cell preparations shown in parentheses.

(a) Purified enzymes

Purified phosphatase	T4/A3 phosphopeptide phosphatase ratio
PCS _H	9.6
PCS _M	1.3
PCS _L	0.8
PCS _C	0.2

(b) Cell fractions

Fraction	Phosphopeptide phosphatase activity (pmol/min per mg)		T4/A3 ratio
	A3	T4	
T-lymphoblast			
Cytosol	19 $\pm$ 2 (3)	104 (2)	5.5
Microsomes	16 $\pm$ 8 (4)	70 (2)	4.4
Plasma membrane	15 (2)	100 (1)	6.7
Jurkat 6			
Cytosol	10 $\pm$ 2 (3)	82 (2)	8.2
Microsomes	16 $\pm$ 6 (8)	114 $\pm$ 35 (5)	7.1
Plasma membrane	11 (2)	78 (1)	7.1

three phosphatases were also detectable in the detergent-insoluble fraction (Fig. 5, tracks C), operationally defined as the cytoskeleton (Davies *et al.*, 1984). It should be noted that the protein loading of tracks C in Fig. 5 was only 20% that of tracks A and B. No evidence was obtained for a Ca²⁺-dependent association of types 1_c and 2A_c with detergent-insoluble material (Fig. 5, panels 1 and 2, tracks D), though some B subunit of calcineurin was detected in the EGTA extract (panel 3, track D), so distinguishing these proteins from those previously found associated in a Ca²⁺-dependent manner with lymphocyte cytoskeleton fractions (Owens & Crumpton, 1984).

It has been shown by Bordier (1981) that integral membrane proteins can be distinguished from hydrophilic proteins by separation into the detergent-rich or detergent-poor phases respectively during the temperature-induced phase separation of Triton X-114. Phase separation of Jurkat-6-cell plasma membrane revealed that a distinctly different set of proteins separated into the two phases (Fig. 6b). Both the type 1_c and 2A_c phosphatases separated into the detergent-poor phase (Fig. 6a, panel 1 and 2, tracks E), whereas the calcineurin B subunit was detected in both phases (Fig. 6a, panel 3, tracks D and E). A significant proportion of the type 1_c phosphatase was not solubilized in Triton X-114 (Fig. 6a, panel 1, track C). It is possible that the

Fig. 5. Extraction of Jurkat-6-cell plasma-membrane-associated phosphatases

Plasma membrane (600 μg of protein, track A) was extracted with 1% (v/v) Nonidet P40 as described in the Materials and methods section to produce a detergent-soluble supernatant (400 μg of protein; track B) and an insoluble cytoskeletal pellet (150 μg of protein; track C). This pellet was further extracted with 5 mM-EGTA to produce an EGTA extract (50 μg of protein; track D) and an insoluble pellet (100 μg of protein; track E). Protein loadings were 100, 100, 20, 10 and 30 μg for tracks A–E respectively, the yields of protein being too low at various stages to allow equivalent loading. Staining was with antisera to type 1_c (panel 1) or type 2A_c (panel 2) phosphatases, or calcineurin (panel 3). The 70 kDa band in panel 2, tracks A and B was also observed on using pre-immune sera (cf. Fig. 3a, panel 3, track A); the identity of the 55 kDa band in panel 2, tracks A and B is not known.

doublets running at about 30 kDa in panels 1 and 2, tracks C (and not normally observed) represent degradation products. It was not found possible to assay the dephosphorylation-stimulated type 1 phosphatase in each phase, since Triton X-114 interfered with the dephosphorylation stimulation. However, the T4 phosphopeptide phosphatase activity in the detergent-poor phase was 17.9 pmol/min per mg, in contrast with 0.1 pmol/min per mg in the detergent-rich phase, confirming that the type 2A phosphatase was in only one phase.

DISCUSSION

The major conclusion from the study is that calcineurin and the type 1 and type 2A phosphatases are associated with the plasma-membrane fraction of human T lymphoblasts and the Jurkat-6 human leukaemia T cell line. This finding was based upon the availability of novel rat antisera which were specific for the type 1 and 2A catalytic subunits and which did not cross-react with other phosphatases. As judged by immunoblotting of SDS/PAGE electrophoretograms, the sizes of the type 1_c and 2A_c subunits associated with T-lymphocyte plasma membrane were as expected from the corresponding cDNA clones (Silva *et al.*, 1987; Berndt *et al.*, 1987). The identity of the cytosolic 40 kDa band detected with antisera to the type 2A_c phosphatase is unknown

(Fig. 2), but presumably this band does not represent a precursor of the 35.6 kDa subunit predicted by the cDNA clone (Silva *et al.*, 1987). The band could, however, represent the product of one of the family of related type 2A_c genes described by Stone *et al.* (1987) that is expressed only in cytosol. The apparent plasma-membrane association of the three phosphatases studied is unlikely to be due to contamination by cytosolic phosphatases for three reasons. First, the density of staining of the bands in the membrane fraction is inconsistent with the 3% contamination with cytosol estimated from the respective lactate dehydrogenase assays (Fig. 3). Secondly, the 40 kDa band detected in the cytosol fraction with the type 2A_c antiserum (see above) was not also detected in the membrane fractions (Fig. 3). Thirdly, the specific activities of the type 1 and 2A phosphatases in plasma-membrane fractions were comparable with those determined for the cytosol fractions (Table 2). Type 1 phosphatase was reported previously to be the major phosphorylase phosphatase activity in microsomal fractions (Alemany *et al.*, 1986; Mieskes & Söling, 1987). However, the present study provides for the first time evidence that the type 1 as well as the type 2A enzymes are associated with the plasma membrane of cultured cells. Calcineurin was shown previously to be present in human placental membranes (Pallen *et al.*, 1985) and pig mesenteric lymphocyte plasma membrane (Chantler,

Fig. 6. Triton X-114 phase separation of plasma-membrane-associated phosphatases

(a) Jurkat-6-cell plasma membrane (700 μ g of protein; track A) was extracted with 1% (v/v) Triton X-114 to yield a detergent-soluble supernatant (560 μ g of protein; track B) and an insoluble pellet (140 μ g of protein; track C). The supernatant was then subjected to a temperature-dependent phase separation to produce a detergent-rich phase (230 μ g of protein; track D) and a detergent-poor phase (230 μ g of protein; track E). Proteins were precipitated with 70% (v/v) ethanol and subjected to SDS/PAGE and immunoblotting. Protein loadings were 50 μ g per track. Staining was with antisera to type 1_c (panel 1), or type 2A_c (panel 2) phosphatases, or calcineurin (panel 3). Further details are given in the Materials and methods section. (b) SDS/PAGE and Coomassie Blue staining of the detergent-poor phase (track A) and the detergent-rich phase (track B). Protein loadings were 50 μ g/track.

1985). The present studies extend this list to include the plasma-membrane fraction of T-lymphocytes.

The mode of attachment of the type 1 and type 2A phosphatases, and calcineurin, to the plasma membrane of T cells has not been defined. Our data appear to exclude a hydrophobic anchor for the type 1_c and 2A_c subunits because, on Triton X-114 extraction of the plasma-membrane fraction, both subunits partitioned into the detergent-poor phase (Fig. 6). It is possible that the catalytic subunits are targeted to the membrane by association with other subunits in the holoenzyme, as has been suggested previously for the type 1 phosphatase (Alemany *et al.*, 1986; Villa-Moruzzi *et al.*, 1987). Since the antisera recognize the catalytic subunits only, the possibility that the holoenzymes dissociate in Triton X-114, with the more hydrophobic non-catalytic subunits passing into the detergent-rich phase, cannot be excluded. In contrast with the type 1_c and 2A_c phosphatases, about half of the calcineurin B subunit was associated with the detergent-rich phase (Fig. 6a, panel 3, track D). Since the B subunit of calcineurin is myristoylated (Aitken *et al.*, 1982), it appears possible that the attached myristoyl group mediates membrane binding.

The deinhibitor-stimulated form of the type 1 phosphatase detected in the plasma-membrane fraction had properties similar to those of the type 1 enzyme purified from liver by Goris & Merlevede (1988a) (Table 1). The plasma-membrane fraction contained only trace amounts of inhibitor 2 (Fig. 4), to which type 1_c is complexed in cytosol (Ballou & Fischer, 1986). This implies that the type 1_c membrane-associated subunit is associated with a

different regulatory subunit, as in the deinhibitor-stimulated form described in liver (Goris & Merlevede, 1988a) and in the form of the type 1 enzyme associated with myosin in skeletal muscle (Chisholm & Cohen, 1988). Presumably, inhibition by this putative subunit is counteracted by deinhibitor. Whether or not deinhibitor is present in T cells is unknown.

We have shown recently that the CD3 T-cell surface antigen is selectively dephosphorylated by type 2A phosphatases, the PCS_H and PCS_M oligomeric forms showing the highest activities (D. Alexander, unpublished work). The relatively high level of the PCS_H-phosphatase activity present in the T-cell plasma-membrane fraction (Table 2) is therefore of particular interest. Furthermore, the oligomeric PCS-phosphatases have been reported to selectively dephosphorylate protein kinase C (Parker *et al.*, 1986), and may therefore also be involved in regulating protein kinase C activity (Parker *et al.*, 1987).

Calcineurin is known to be regulated by Ca²⁺ and calmodulin [reviewed by Pallen & Wang (1985)] and by specific phospholipid interactions (Politino & King, 1987). As a result, it is presumably activated by the rise in cytosolic Ca²⁺ concentration which occurs on T-cell activation (Alcover *et al.*, 1987; Imboden & Weiss, 1987). This may in turn lead to the modulation of cyclic AMP levels, since the regulatory subunit of type II cyclic AMP-dependent protein kinase and phosphodiesterase isoenzymes are known to be particularly good substrates for calcineurin (Sharma & Wang, 1985; Blumenthal *et al.*, 1986).

The role of phosphatases in regulating the phosphorylation status, and thereby the functions, of the CD3, CD4 and CD8 cell-surface antigens remains a challenge for further investigation.

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