Protein Phosphorylation in Human Peripheral Blood Lymphocytes

PHOSPHORYLATION OF ENDOGENOUS PLASMA MEMBRANE AND CYTOPLASMIC PROTEINS

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Phosphorylation of endogenous proteins in subcellular fractions of human peripheral-blood lymphocytes was studied by one- and two-dimensional polyacrylamide-gel electrophoresis. Studies using extensively purified subcellular fractions indicated that the endogenous phosphorylating activity in the particulate fractions was derived primarily from the plasma membrane. Electrophoresis of ^{32}P -labelled subcellular fractions in two dimensions [O'Farrell (1975) *J. Biol. Chem.* **250**, 4007–4021] provided much greater resolution of the endogenous phosphoproteins than electrophoresis in one dimension, facilitating their excision from gels for quantification of ^{32}P content. More than 100 cytoplasmic and 20 plasma-membrane phosphorylated species were observed. Phosphorylation of more than 10 cytoplasmic proteins was absolutely dependent on cyclic AMP. In the plasma membrane, cyclic AMP-dependent phosphoproteins were observed with mol.wts. of 42000, 42000, 80000 and 90000 and pI values of 6.1, 6.3, 6.25 and 6.5 respectively. Phosphorylation of endogenous cytoplasmic and plasma-membrane proteins was rapid with $t_{\frac{1}{2}} = 5\text{--}12\text{ s}$ at 25°C . Between 40 and 70% of the ^{32}P was recovered as phosphoserine and phosphothreonine when acid hydrolysates of isolated plasma-membrane phosphoproteins were analysed by high-voltage paper electrophoresis. The presence of cyclic AMP-dependent protein kinase and endogenous phosphate-acceptor proteins in the plasma membranes of lymphocytes provides a mechanism by which these cells might respond to plasma-membrane pools of cyclic AMP generated in response to stimulation by mitogens or physiological modulators of lymphocyte function.

In the preceding paper (Chaplin *et al.*, 1979), we demonstrated that subcellular fractions prepared from human peripheral-blood lymphocytes contained cyclic AMP-dependent protein kinase activities and that the soluble and particulate activities were functionally distinct. All of the subcellular fractions were also found to contain endogenous phosphate-acceptor proteins. Phosphorylation of these proteins was measured by incubating samples with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence and absence of cyclic AMP, precipitating the samples with trichloroacetic acid and determining the ^{32}P content of the precipitated material. In the soluble fraction, phosphorylation of endogenous substrates was dramatically stimulated by cyclic AMP. However, in the particulate fractions, we were unable to observe clear-cut cyclic AMP-dependent phosphorylation of endogenous substrates, despite the evidence for cyclic AMP-dependency when phosphorylation was measured with exogenous substrates. It seemed

possible, however, that cyclic AMP-mediated phosphorylation of a fraction of the endogenous substrates could be occurring and might be revealed if the individual substrates were analysed individually. In this paper, phosphorylation of endogenous proteins is studied by one- and two-dimensional polyacrylamide-gel electrophoresis. In the two-dimensional system (O'Farrell, 1975), a combination of isoelectric focusing and electrophoresis in SDS is used to resolve at least 1000 separate proteins in human lymphocytes. Using this powerful technique, unique cyclic AMP-dependent phosphorylation patterns are demonstrated in lymphocyte plasma membranes as well as in other lymphocyte subcellular fractions.

Materials and Methods

Materials

Reagents and their sources were as follows: $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ($>100\text{ Ci/mmol}$; ICN Pharmaceuticals, Irvine, CA, U.S.A.), Na^{125}I (carrier-free; Amersham/

Abbreviation used: SDS, sodium dodecyl sulphate.

Searle, Arlington Heights, IL, U.S.A.), ATP, cyclic AMP, cyclic GMP, *Escherichia coli* β -galactosidase, rabbit skeletal-muscle phosphorylase, bovine serum albumin, bovine liver catalase, porcine heart fumarase, ovalbumin, bovine pancreatic α -chymotrypsinogen and lactoperoxidase (Sigma, St. Louis, MO, U.S.A.), glucose oxidase (Worthington Biochemical Corp., Freehold, NJ, U.S.A.), SDS (Gallard-Schlesinger, Carle Place, NY, U.S.A.), acrylamide, *NN'*-methylenebisacrylamide, ammonium persulphate, *NNN'*-tetramethylethylenediamine and Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA, U.S.A.), carrier ampholytes (LKB, Rockville, MD, U.S.A.), theophylline and urea (Schwarz/Mann, Orangeburg, NY, U.S.A.), Protosol and Econofluor (New England Nuclear, Boston, MA, U.S.A.) and Nonidet P-40 (Particle Data Laboratories, Elmhurst, IL, U.S.A.). Chicken skeletal-muscle myosin was generously given by Dr. E. Richard Bischoff, Department of Anatomy, Washington University School of Medicine.

Cell purification and subcellular fractionation

Human peripheral-blood lymphocytes were isolated from freshly drawn heparinized blood, and subcellular fractions were prepared as described in the preceding paper (Chaplin *et al.*, 1979). Human polymorphonuclear leucocytes were recovered from the pellets of Ficoll/Hypaque gradients, and freed of erythrocytes by hypo-osmotic lysis as described by Atkinson *et al.* (1976). Subcellular fractions were prepared as for lymphocytes. Membrane fractions were routinely washed free of sucrose by dilution with ten times their volume of Mg/acetate buffer (2mM-magnesium acetate/50mM-sodium acetate, pH 7.3), followed by centrifugation at 100000g for 60min in a Beckman L-65 preparative ultracentrifuge in an SW 27.1 rotor. Sedimented membranes were resuspended in a small volume of Mg/acetate buffer (0.2–0.5ml) with three to five strokes of a 2ml tight-fitting Dounce homogenizer.

Gel electrophoresis

One-dimensional. SDS/polyacrylamide-slab-gel electrophoresis was performed by the method of Laemmli (1970). The stacking gel was 3% (w/v) acrylamide with 2.7% (w/w) *NN'*-methylenebisacrylamide (bisacrylamide). The separating gel was 7.5% (w/v) acrylamide with 2.7% (w/w) bisacrylamide. Samples were prepared as described under 'Phosphorylation of subcellular fractions'. Gels were fixed and stained in a solution containing 12% (w/v) trichloroacetic acid, 50% (v/v) methanol and 0.03% (w/v) Coomassie Blue R-250. Destaining was in several changes of 7% (v/v) acetic acid/10% (v/v)

methanol. Gels were soaked in 7% (v/v) acetic acid/1% (w/v) glycerol before being dried on filter paper under vacuum. Radioautographs were prepared by exposing the dried gels to Kodak XR-5 medical X-ray film followed by processing in a Kodak RP X-Omat developer. Radioautographs were scanned on a spectrophotometer equipped with a linear transport mechanism (Gilford Instrument Laboratories, Oberlin, OH, U.S.A.).

Two-dimensional. Two-dimensional polyacrylamide-gel electrophoresis was performed by the method of O'Farrell (1975). Isoelectric focusing was carried out in rods (105mm $\times$ 5mm) containing 9.2M-urea, 2% (w/v) Nonidet P-40 and 2% (w/v) Ampholines (1.6% pH range 5–8, 0.4% pH range 3.5–10), and consisting of 3.8% (w/v) acrylamide and 5.8% (w/w) bisacrylamide. Samples were prepared for electrophoresis as described under 'Phosphorylation of subcellular fractions'. Isoelectric focusing was carried out at 400V for 10–14h followed by 1h at 800V. After isoelectric focusing, rod gels were equilibrated for 45min in 15ml of 10% (w/v) glycerol/5% (v/v) 2-mercaptoethanol/2.3% (w/v) SDS/0.0625M-Tris/HCl, pH 6.8 (equilibration buffer). Equilibrated gels could be frozen at -80°C before electrophoresis in the second dimension. The second dimension was the discontinuous SDS/polyacrylamide-gel system of Laemmli (1970). The separating gel was 160mm $\times$ 140mm $\times$ 1mm and consisted of a linear gradient of polyacrylamide from 20% (w/v) to 6% (w/v) acrylamide with 2.7% (w/w) bisacrylamide. The stacking gel was 160mm $\times$ 20mm $\times$ 1mm and consisted of 3% (w/v) acrylamide with 2.7% (w/w) bisacrylamide. The equilibrated rod gels were held in place over the stacking gel by 3ml of 1% agar in equilibration buffer. Tracking dye [0.01% Bromophenol Blue in running buffer containing 10% (w/v) glycerol] was layered over the agar-embedded rod gels. Electrophoresis was at 125V until the tracking dye reached the bottom of the gel (approx. 10h).

Gels were fixed, stained and destained as above. Gradient gels were dried in one of two ways. For radioautography, they were soaked in 50% (v/v) methanol/2% (w/v) glycerol/7% (v/v) acetic acid. For quantification of ^{32}P in specific regions of the gel, they were soaked in 98% (v/v) methanol/2% (v/v) glycerol. All gels were dried on filter paper as described above.

Gels treated in 98% methanol/2% glycerol were only partially dried, leaving the gel easily separable from the filter-paper backing and facilitating complete solubilization for scintillation counting. Gel slices were rehydrated in a minimal volume of water and were incubated overnight at 37°C in 10ml of 7% (v/v) Protosol in Econofluor before liquid-scintillation counting in a Beckman LS-8100 (Beckman Instruments, Anaheim, CA, U.S.A.).

For estimation of the molecular weights of lymphocyte proteins in SDS/polyacrylamide gels (Weber & Osborn, 1969), the following standards were employed (molecular weights in parentheses): chicken skeletal-muscle myosin (220000), *E. coli* β -galactosidase (130000), rabbit skeletal-muscle phosphorylase (94000), bovine serum albumin (68000), bovine liver catalase (58000), porcine heart fumarase (49000), ovalbumin (43000) and bovine pancreatic α -chymotrypsinogen (25700).

Iodination of intact lymphocytes

Lymphocytes were surface-iodinated with ^{125}I by the lactoperoxidase method (Phillips & Morrison, 1970) by using glucose oxidase to generate H_2O_2 (Hubbard & Cohn, 1972). ^{125}I was measured in a Beckman model 300 gamma counter.

Phosphorylation of subcellular fractions

Phosphorylation of subcellular fractions was carried out at 25°C in a final volume of 0.1 ml containing 1 mM-magnesium acetate, 3 mM-theophylline, 50 mM-sodium acetate, pH 7.3, and lymphocyte protein in the amounts indicated. Cyclic AMP ($2\mu\text{M}$) or cyclic GMP ($1\mu\text{M}$) was present as indicated. Subcellular fractions were preincubated with the reaction constituents for 30 s. Phosphorylation was started by the addition of 2–200 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (150–250 Ci/mmol) and was allowed to proceed for the indicated times (usually 45 s). For one-dimensional electrophoresis, phosphorylation was terminated by the addition of 50 μl of 6% (w/v) SDS/30% (w/v) glycerol/15% (v/v) 2-mercaptoethanol/0.003% (w/v) Bromophenol Blue/0.75 M-Tris/HCl, pH 6.8, followed immediately by incubation at 100°C for 5 min. For

two-dimensional electrophoresis, phosphorylation was terminated by the addition of either 100 μl of lysis buffer [9.5 M-urea, 2% (w/v) Nonidet P-40, 2% (w/v) Ampholines (1.6% pH range 5–8, 0.4% pH range 3.5–10) and 5% (v/v) 2-mercaptoethanol] or 1 ml of cold 7% trichloroacetic acid. Samples precipitated with trichloroacetic acid were kept at 4°C for 30 min, and centrifuged for 4 min in a Beckman Microfuge B. Supernatants were aspirated, and sedimented material was solubilized in 200 μl of lysis buffer. All samples could be kept frozen overnight before electrophoresis.

In selected experiments, the phosphorylation reaction was carried out at 37°C . Under these conditions, phosphorylation was maximal within less than 10 s, hampering an analysis of the time course of the reaction. Since the phosphoprotein pattern observed in two-dimensional gels was the same regardless of whether phosphorylation was carried out at 25 or 37°C , experiments were routinely carried out at 25°C .

Acid hydrolysis

The presence of ^{32}P in phosphoserine and phosphothreonine residues in proteins was verified by elution of the phosphoproteins from gel slices followed by limited acid hydrolysis and high-voltage paper electrophoresis by the method of Allerton & Perlmann (1965).

Results

Subcellular fractionation and one-dimensional polyacrylamide-gel electrophoresis

Subcellular fractions were obtained from human peripheral-blood lymphocytes by Dounce homo-

Table 1. ^{32}P content of phosphoproteins in the 16000g supernatant

Cytosol protein (30 μg) was incubated for 45 s with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, $\pm 2\mu\text{M}$ -cyclic AMP and the indicated concentrations of magnesium acetate and CaCl_2 . Samples were electrophoresed in one dimension in SDS/polyacrylamide gels. Radioautographs were prepared and scanned as described in the Materials and Methods section. Data are expressed in arbitrary units of peak height as determined from scans. pp80 and pp50 indicate phosphoproteins of 80000 and 50000 mol.wt. respectively. Similar results were obtained in two additional experiments.

	Peak height (arbitrary units)					
	pp80			pp50		
	–cyclic AMP	+cyclic AMP	+cyclic AMP –cyclic AMP	–cyclic AMP	+cyclic AMP	+cyclic AMP –cyclic AMP
1 mM- Mg^{2+}	15.1	25.4	1.68	12.8	22.5	1.76
+0.1 mM- Ca^{2+}	15.4	23.0	1.49	13.8	23.0	1.67
+0.5 mM- Ca^{2+}	13.4	19.7	1.47	12.1	20.7	1.71
+1.0 mM- Ca^{2+}	11.2	15.9	1.42	10.5	18.7	1.78
+5.0 mM- Ca^{2+}	5.7	6.2	1.09	5.8	9.4	1.64
5 mM- Mg^{2+}	23.5	28.5	1.21	20.2	28.8	1.43
+5.0 mM- Ca^{2+}	16.9	16.8	0.99	13.5	17.0	1.26

genization and sucrose-density-gradient centrifugation. The preparation and characterization of these fractions are discussed in detail in the preceding paper (Chaplin *et al.*, 1979). In preliminary experiments, endogenous protein kinase substrates were analysed by one-dimensional SDS/polyacrylamide-gel electrophoresis. ^{32}P content of phosphoproteins was quantified by densitometric tracing of radioautographs of the gels. A complex phosphoprotein pattern was obtained for all fractions (results not shown). ^{32}P content of at least 14 phosphoproteins in the cytoplasmic fraction ranging in mol.wt. from 25000 to greater than 200000 was increased by phosphorylation in the presence of cyclic AMP. Cyclic AMP-dependent phosphoproteins were detected in the particulate fractions, but were inadequately resolved from proteins whose phosphorylation was insensitive to cyclic AMP to permit quantification of ^{32}P content.

Effect of Ca^{2+} on phosphorylation of endogenous substrates

There have been many reports that Ca^{2+} can modulate protein phosphorylation (Avruch & Fairbanks, 1974; Malkinson, 1975; Chacko *et al.*, 1977; Krueger *et al.*, 1977; Small & Sobieszek, 1977). When Ca^{2+} (0.1–5.0 mM) was present during phosphorylation of the 16000g supernatant fraction, it produced a dose-dependent inhibition of phosphorylation of all the major phosphoprotein bands (Table 1). The 80000-mol.wt. band is representative of most endogenous substrates. Ca^{2+} inhibited phosphorylation in the absence of added cyclic AMP. High doses of Ca^{2+} also eliminated the ability of cyclic AMP to enhance phosphorylation of all endogenous substrate proteins, except a band with mol.wt. approx. 50000. High Mg^{2+} concentrations were able partially to restore basal phosphorylation, but did not restore cyclic AMP responsiveness. The absolute amount of phosphorylation of the 50000-mol.wt. protein was decreased in the presence of Ca^{2+} ; however, the ratio of cyclic AMP-stimulated/unstimulated phosphorylation remained constant.

Addition of Ca^{2+} to the other subcellular fractions inhibited phosphorylation of all of the substrate proteins, both in the absence and presence of cyclic AMP (results not shown).

Since the one-dimensional radioautograph patterns were so complex, and were unable adequately to resolve the cyclic AMP-dependent phosphoproteins from those that were unresponsive to cyclic AMP, further studies were performed by the two-dimensional gel electrophoresis system of O'Farrell (1975).

Protein-staining patterns of two-dimensional gels

Protein-staining patterns of two-dimensional gels of the 16000g supernatant (cytoplasmic), plasma-

membrane and microsomal/mitochondrial fractions are shown in Fig. 1. Proteins with isoelectric points ranging from 4.2 to 7.5 and mol.wts. in SDS of 15000–400000 are fractionated by this technique. Several experiments were performed using isoelectric-focusing gels spanning the range 3.5 to 9.5, but no phosphoproteins were detected with pI outside the range of 4.4–7.1.

Samples containing up to 150 μg of protein could be electrophoresed on a single gel by this technique; however, optimal resolution was obtained when less than 75 μg of protein was used.

The staining patterns in Fig. 1 show that the protein compositions of the various subcellular fractions are distinctly different. One protein of

Fig. 1. Coomassie Blue-staining patterns of two-dimensional gels

(a) 16000g Supernatant (cytosol) fraction (60 μg of protein); (b) 30–40% sucrose interface (plasma-membrane) fraction (50 μg of protein); (c) 40–55% sucrose interface (microsomal/mitochondrial) fraction (40 μg of protein).

mol.wt. 40000–42000 and pI 5.2–5.4 is present in all fractions. On the basis of Coomassie Blue staining, it is the most abundant protein in each of the fractions. This protein has been tentatively identified as actin on the basis of agreement with published values for its molecular weight and isoelectric point (Garrels & Gibson, 1976).

¹²⁵I-labelling of intact lymphocytes

Surface labelling of lymphocytes with ¹²⁵I by using lactoperoxidase and glucose oxidase allowed an analysis of the partitioning of plasma membranes among the several subcellular fractions (Table 2 and Fig. 2). When subcellular fractions prepared from iodinated cells were analysed for protein-bound ¹²⁵I (Table 2), 1.4% of the total protein-bound ¹²⁵I was truly soluble, representing either labelling of cytoplasmic proteins or surface proteins solubilized during homogenization. Some 88% of the protein-bound ¹²⁵I was recovered in the crude particulate fraction. ¹²⁵I content of the 20–30% (w/v) sucrose-gradient interface and 30–40% sucrose-gradient interface fractions (plasma membrane) was enriched 5.2- and 7.5-fold over that of the crude homogenate. The 40–55% sucrose-gradient interface fraction (microsomal/mitochondrial) was enriched 1.6-fold, indicating significant contamination of this fraction with plasma membrane. These results confirm a marker-enzyme analysis of the subcellular fractions (Chaplin *et al.*, 1979).

Two-dimensional gel patterns of subcellular fractions obtained from [¹²⁵I]iodinated lymphocytes are shown in Fig. 2. Equivalent amounts of protein

were applied to each gel. Few iodinated proteins are present in the 16000g supernatant. All the iodinated proteins with pI less than 5.9 seem to be also present in the plasma-membrane fraction. The pair of proteins with mol.wt. approx. 25000 and pI approx. 6.3 are not present in the plasma-membrane fraction. The plasma-membrane fraction contains many iodinated proteins. Groups of iodinated species are prominent, with apparently identical molecular weights but fairly broad charge heterogeneity. This may represent single proteins with various degrees of glycosylation, phosphorylation or iodination, or various degrees of artifactual amidation or deamidation. Iodinated proteins are present, spanning the full range of isoelectric points and with mol.wts. as high as 200000. The microsomal/mitochondrial fraction contains many iodinated proteins which can be found in the plasma-membrane fraction. However, the relative quantities of the several proteins are very different. For example, the group of iodoproteins with mol.wts. approx. 42000 and pI approx. 4.6 are present in apparently equal quantities in the two fractions, but the protein with mol.wt. approx. 110000 and pI 4.6 is much more strongly represented in the plasma-membrane fraction than in the microsomal/mitochondrial fraction. Thus the plasma-membrane fragments contaminating the microsomal/mitochondrial fraction are not representative of the plasma membrane as a whole, indicating that Dounce homogenization produces a heterogeneous population of plasma-membrane fragments, which can be fractionated into distinct subpopulations by sucrose-gradient centrifugation.

Table 2. *¹²⁵I-labelling of intact human lymphocytes*

Purified human lymphocytes (6×10^8) were incubated for 10 min at room temperature in 10 ml of Gey's balanced-salt (GBS) solution containing 5 mCi of Na¹²⁵I, 2.5 mg of glucose/ml, 30 μ g of lactoperoxidase/ml and 5 μ g of glucose oxidase/ml. A 10-fold excess of ice-cold GBS solution containing 2 μ M-tyramine was added to terminate labelling. The cells were washed 8 times with GBS solution, broken by Dounce homogenization, and fractionated on sucrose gradients as described in the Materials and Methods section.

Fraction	$10^{-8} \times$ Total ¹²⁵ I (c.p.m.)	Percentage recovery*	$10^{-4} \times$ Specific radioactivity (c.p.m./ μ g of protein)	Relative specific radioactivity†
Crude homogenate	7.60	—	4.22	1
16000g Supernatant	0.52	6.8	0.69	0.16
100000g Supernatant‡	0.11	1.4	0.15	0.03
16000g Pellet	6.69	88.0	11.15	2.64
20% Sucrose	0.16	2.1	0.80	0.19
20–30% Interface	0.22	2.9	22.00	5.2
30–40% Interface	0.57	7.5	31.67	7.5
40–55% Interface	0.79	10.4	6.58	1.56
55% Pellet	0.69	9.1	5.75	1.36

* Expressed as percentage of trichloroacetic acid-precipitable radioactivity in the crude homogenate.

† Specific radioactivity of fraction/specific radioactivity of crude homogenate.

‡ Prepared from the 16000g supernatant by recentrifugation for 30 min at 33000 rev./min in a Beckman L-65B ultracentrifuge in an SW 50.1 rotor.

Fig. 2. Radioautographs of two-dimensional gels of subcellular fractions from ^{125}I -surface-labelled lymphocytes (a) 16000g Supernatant; (b) 30–40% sucrose interface; (c) 40–55% sucrose interface; 50 μg of protein was applied to each gel.

Analysis of phosphorylation in subcellular fractions by two-dimensional gels

Electrophoresis of ^{32}P -labelled subcellular fractions in two dimensions resulted in a complete separation of phosphoproteins that were poorly resolved in one-dimensional gels. More than 100 phosphoproteins were observed in radioautographs of the 16000g supernatant fraction (Figs. 3a and 3b). The phosphorylation of more than 20 endogenous substrates was absolutely dependent on the presence of cyclic AMP. Particularly prominent was a group of three proteins with mol.wts. approx. 190000, 210000 and 240000 and pI approx. 4.5–4.8. Many other proteins were phosphorylated equally in the presence and absence of cyclic AMP (e.g. those of mol.wt. 15000, pI 7.0).

Cyclic AMP-dependent phosphorylation also occurred in the plasma-membrane fraction (Figs. 3c and 3d). The number of phosphoproteins was small compared with the 16000g supernatant fraction, ranging from 12 to 24 in different experiments and the overall extent of labelling was lower owing to the limited amount of sample obtained from one unit of blood. A pair of phosphoproteins with mol.wt. approx. 42000 and pI 6.1 and 6.3 were the most prominent in 12 out of 12 experiments. A pair of phosphoproteins of mol.wt. approx. 24000 and similar pI were present in 10 out of 12 experiments. Phosphorylation of this pair of proteins was minimally stimulated by 2 μM -cyclic AMP. Cyclic AMP-dependent phosphorylation of two proteins with mol.wts. 80000 and 90000 and pI 6.25 and 6.5 respectively was also observed in 9 out of 12 experiments.

A comparison of the plasma-membrane phosphoprotein pattern seen in one- and two-dimensional gels showed that several cyclic AMP-independent phosphoproteins that were prominent in one-dimensional gels were not seen in the two-dimensional system, even when gels spanning a range of pI from 3.5 to 9.5 were used. Trichloroacetic acid precipitation of the sample before solubilization in lysis buffer resulted in recovery of several of the cyclic AMP-independent phosphoproteins in the two-dimensional pattern (Fig. 4), presumably by enhancing solubilization of the sample. Trichloroacetic acid precipitation of microsomal/mitochondrial fractions was routinely required to obtain consistent electrophoresis of both cyclic AMP-dependent and -independent phosphoprotein species.

Two-dimensional gels of phosphorylated and trichloroacetic acid-precipitated microsomal/mitochondrial samples produced the radioautography pattern seen in Fig. 3 (e and f). All of the major cyclic AMP-dependent and -independent phosphoproteins observed in the plasma-membrane fraction were found in the microsomal/mitochondrial fraction. A phosphoprotein of mol.wt. less than 20000 and pI approx. 4.5 was inconsistently found in the microsomal/mitochondrial fraction (5 out of 12 experiments). This protein was not observed in the plasma-membrane fraction.

The major contaminating cells in the purified lymphocyte preparations were polymorphonuclear leucocytes comprising 2–8% of the total nucleated cells. When subcellular fractions were prepared from purified polymorphonuclear leucocytes by the fractionation scheme used for lymphocytes, no phosphorylating activity was detected in the 30–40% sucrose-gradient-interface fraction. On the basis of ^{32}P incorporation/mg of protein, less than 20% of the phosphorylating activity found in the microsomal/mitochondrial fraction of lymphocytes could be detected in the equivalent fraction prepared from

Fig. 3. Radioautographs of phosphorylated lymphocyte subcellular fractions

(a), (c) and (e) Phosphorylated in the absence of added cyclic AMP; (b), (d) and (f) phosphorylated in the presence of $2\mu\text{M}$ -cyclic AMP. (a), (b) 16000g supernatant fraction ($70\mu\text{g}$ of protein); (c), (d) 30–40% sucrose interface fraction ($50\mu\text{g}$ of protein); (e), (f), 40–55% sucrose interface fraction ($50\mu\text{g}$ of protein). Arrows indicate prominent cyclic AMP-dependent phosphorylation.

purified polymorphonuclear leucocytes (results not shown). Thus it seems unlikely that contaminating polymorphonuclear leucocytes contribute significantly to the phosphoprotein patterns observed in the lymphocyte particulate fractions.

Lyons *et al.* (1975) reported the presence of a

40000-dalton protein in human platelets whose phosphorylation was regulated by thrombin and which in broken platelets was phosphorylated by cyclic AMP-dependent protein kinase. In view of the fact that several of the lymphocyte phosphoproteins are in the 40000–42000mol.wt.

range and since platelets comprise a significant portion of the cells contaminating our lymphocyte preparations, we examined the role of platelets in producing the phosphorylation pattern seen in two-dimensional gels. Lymphocytes were isolated by the

usual methods and were found to contain 25 platelets/100 lymphocytes. A portion of the cells was subjected to five additional low-speed centrifugations (700 rev./min for 7 min) yielding a population with two to five platelets per 100 lymphocytes. Subcellular fractions were prepared from each population and endogenous phosphorylation was studied by two-dimensional gels. For all subcellular fractions, the phosphoprotein pattern was unaltered by platelet depletion (results not shown).

Fig. 4. Radioautograph of two-dimensional gel of plasma membranes phosphorylated in the presence of $2\mu\text{M}$ -cyclic AMP and precipitated with trichloroacetic acid before solubilization and electrophoresis

Two of the prominent cyclic AMP-independent phosphoproteins which require trichloroacetic acid precipitation in order to penetrate the isoelectric-focusing gel are indicated by arrows; $50\mu\text{g}$ of protein was applied to the gel.

Further purification of particulate fractions

Since most of the endogenous phosphoproteins of the microsomal/mitochondrial fraction were also present in the plasma-membrane fraction and since the microsomal/mitochondrial fraction contained plasma-membrane marker-enzyme activity, it seemed possible that much, if not all, of the microsomal/mitochondrial phosphorylation might be due to plasma-membrane contamination. Purified plasma-membrane and microsomal/mitochondrial fractions were repurified on a more complex discontinuous sucrose density gradient, consisting of 55, 50, 45, 40, 30 and 20% (w/v) sucrose layers (Table 3). When the plasma-membrane-rich fraction was repurified, 70% of the original protein was recovered, of which 77% was at the 30–40% interface. This fraction contained essentially all the plasma-membrane-marker-enzyme activity (5'-nucleotidase) and 88% of the endogenous phosphorylating activity. The endogenous phosphorylating activity recovered at the 40–45% and 45–50% interfaces represents only 7 and 5% of the

Table 3. Repurification of plasma-membrane and microsomal/mitochondrial fractions

Plasma-membrane and microsomal/mitochondrial fractions prepared in the usual way were diluted with an equal volume of Mg/acetate buffer and were refractionated on discontinuous sucrose gradients consisting of 0.7 ml each of 55, 50, 45 and 40% (w/v) sucrose, 1.4 ml of 30% (w/v) sucrose, and the sample, all in Mg/acetate buffer. Determination of protein content and marker-enzyme activities were performed as described in the preceding paper (Chaplin *et al.*, 1979).

Fraction	Total protein (μg)	5'-Nucleotidase (units/mg)	NADH-ferricyanide oxidoreductase (units/min)	β -Glucuronidase (units/mg)	Specific activity of endogenous phosphorylation (units/30s per mg)	
					Control	+Cyclic AMP
First gradient						
30–40% Interface	256	27340	14	56.8	1000	1020
Second gradient						
30–40% Interface	134	28730	4	49.4	700	860
40–45% Interface	12	500	1.6	59.1	755	850
45–50% Interface	9	420	2.4	14.1	0	640
First gradient						
40–55% Interface	1710	4670	151.2	132.9	84	111
Second gradient						
30–40% Interface	15	14470	0.8	32.6	434	618
40–45% Interface	37	15760	2.4	54.9	821	908
45–50% Interface	150	6270	17.6	125.0	314	339
50–55% Interface	1140	2630	139.2	143.7	56	58

total recovered activity. The apparent high cyclic AMP responsiveness of the 45–50% interface fraction is probably not significant. This is the fraction with the lowest protein content and therefore subject to the greatest experimental error. Repurification of the microsomal/mitochondrial fraction resulted in recovery of 80% of the protein, of which 83% was at the 50–55% interface. The microsomal enzyme activity in this fraction was 1.4-fold enriched over the crude microsomal/mitochondrial fraction, whereas the 5'-nucleotidase and endogenous phosphorylating activities were depleted to 56 and 52% respectively of that in the original crude fraction. By contrast, in the lighter fractions from the repurified microsomal/mitochondrial fraction, NADH-ferricyanide oxidoreductase activity was decreased, and phosphorylating activity and 5'-nucleotidase activity were increased. Moreover, the changes in phosphorylating activity also showed an inverse correlation with changes in lysosomal-marker-enzyme activity. These observations argue strongly that the major phosphorylating activity in these particulate fractions is contained in the plasma membrane.

Phosphoserine/phosphothreonine analysis and time course of membrane phosphorylation

³²P content of individual phosphoproteins was quantified by elution of phosphoproteins from gel slices. Acid hydrolysis of eluted samples followed by high-voltage paper electrophoresis demonstrated that both phosphoserine and phosphothreonine were present in all of the cyclic AMP-dependent membrane phosphoproteins, suggesting that these proteins are multiply phosphorylated. Phosphorylation was very rapid both in the absence and presence of cyclic AMP with a $t_{1/2}$ at 25°C of approx. 12s.

Discussion

As an initial step in elucidating the role of plasma-membrane kinases in the control of plasma-membrane function in lymphocytes, the endogenous substrates for phosphorylation were investigated by one- and two-dimensional electrophoresis. Since the phosphorylation patterns we obtained were very complex, two-dimensional gel electrophoresis proved to be considerably more advantageous in making the analysis than one-dimensional gel electrophoresis. When the plasma-membrane fraction was treated with [γ -³²P]ATP in the presence of cyclic AMP, precipitated with trichloroacetic acid, and analysed in two-dimensional gels, at least 20 distinct areas of ³²P radioactivity were present (Fig. 4). When the trichloroacetic acid-precipitation step was omitted, eight of these bands were no longer demonstrable,

presumably because acid denaturation facilitates their penetration into the isoelectric-focusing gel (Fig. 3d). Even though cyclic AMP failed to stimulate phosphorylation convincingly in the plasma-membrane fraction as a whole, four bands, corresponding to apparent mol.wts. 42000, 42000, 80000 and 90000, were clearly cyclic AMP-dependent when the individual phosphorylated species were resolved. When the microsomal/mitochondrial fraction was analysed, all of the bands present in the plasma membranes were identifiable, as would be expected if the phosphorylating activity in both fractions was dependent on their plasma-membrane content (see above). However, in contrast with the plasma membrane, the microsomal/mitochondrial fractions frequently contained phosphorylated proteins with pI values of 4.4–5.0, migrating in the 15000–20000-mol.wt. range. These acidic phosphorylated proteins may correspond to phosphorylated proteins of similar size described in purified ribosomes (Traugh & Porter, 1976; Horak & Schiffmann, 1977).

It appears certain that lymphocyte plasma membranes contain a cyclic AMP-dependent protein kinase; however, the nature of the cyclic AMP-independent activity remains to be resolved (Figs. 3c and 4). This activity could be due to a true cyclic AMP-independent enzyme, but the possible presence of an activated catalytic subunit of cyclic AMP-dependent protein kinase, perhaps produced as an artifact at the time that the cells were disrupted, has yet to be excluded. Cyclic AMP-independent phosphorylation has been described in several tissues in the central nervous system where univalent (Gordon *et al.*, 1977) and bivalent (Krueger *et al.*, 1977) cation fluxes appear to be important in their regulation. In view of the studies indicating that Ca²⁺ may have an important regulatory function in the control of lymphocyte metabolism (Parker, 1974; Wedner & Parker, 1976), a more detailed study of cyclic AMP-independent phosphorylation in lymphocyte plasma membranes is required.

A prominent feature of the two-dimensional gel phosphoprotein patterns was that many phosphorylated species appeared to exist as doublets with the same molecular weights, but with pI values differing by approx. 0.15–0.2 pH unit [for example, the 42000-mol.wt. protein that undergoes cyclic AMP-dependent phosphorylation (Fig. 3d)]. Using two-dimensional gel electrophoresis, Steinberg *et al.* (1977) studied variations in the regulatory subunit of protein kinase in mutants of the S49 lymphoma cell line and showed that phosphorylation of the subunit resulted in an alteration in the pI of approximately this magnitude. This raises the possibility that labelled doublets in lymphocyte plasma membranes represent two phosphorylated species of the same protein differing only by one phosphorylated group. However, no direct evidence for or against this possibility is

presently available. The observed charge heterogeneity could involve a number of charged moieties other than esterified phosphate.

Protein phosphorylation has been studied in many different tissues (Rubin & Rosen, 1975; Greengard, 1978). However, speculation as to the relationship of the lymphocyte phosphoproteins to phosphorylated proteins in other cell types is hampered both by the absence of data gathered in analogous two-dimensional gel systems and the frequently anomalous behaviour of glycoproteins in SDS/polyacrylamide gels (Weber & Osborn, 1975), making comparisons of molecular weights difficult. Ueda & Greengard (1977) have demonstrated that in isolated synaptic membranes from bovine brain, proteins with mol.wts. 48000, 80000 and 86000 undergo cyclic AMP-dependent phosphorylation. These proteins correspond surprisingly closely (but quite possibly fortuitously) to the cyclic AMP-dependent phosphorylated protein species present in lymphocyte plasma membranes (approx. mol.wts. 42000, 80000 and 90000). In addition to the similarity to the synaptic membrane phosphoproteins, work by Pober *et al.* (1978) has demonstrated that human histocompatibility antigens HCA-A and -B (mol.wt. 44000) are phosphorylated both in intact cells and in lymphocyte homogenates. Further studies are required to examine the possibility that these or other lymphocyte plasma-membrane phosphoproteins may be involved in the transduction of mitogenic or immunoregulatory signals generated at the cell surface.

The functional properties of the lymphocyte phosphoproteins remain unclear. However, it is apparent that lymphocytes possess a highly sophisticated protein kinase system capable of phosphorylating many different endogenous protein substrates, both in the cytoplasm and plasma membrane. The complexity of the system appears to provide the high degree of flexibility that may be needed for the many different modalities that regulate lymphocyte function to exert their various effects.

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