Phosphorylated lymphocyte plasma-membrane proteins

Alan P. JOHNSTONE,* John H. DuBOIS† and Michael J. CRUMPTON‡

National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K., and Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, U.K.

(Received 17 June 1980/Accepted 29 September 1980)

Lymphocytes were labelled by incubation with [32 P] P_i and their plasma membranes isolated. Analysis by one-dimensional and two-dimensional gel electrophoresis revealed a small number of strongly phosphorylated polypeptides. Two of these were especially prominent; they had molecular weights of about 52 000 and 90 000, were acidic and were apparently not glycosylated. Similar patterns were obtained for quiescent T- and B-lymphocytes from different species and for cultured lymphoblastoid cells, although the relative amounts of the labelled polypeptides varied. Immunoprecipitation analyses of the detergent-solubilized 32 P-labelled plasma membranes indicated that the glycosylated polypeptide of the human major transplantation (HLA-A and HLA-B) antigens and its mouse and pig counterparts are phosphorylated. In contrast, no phosphorylation of the membrane-associated immunoglobulin, the mouse Thy-1 antigen or the human HLA-DRw(1a) antigen was detected. The phosphorylation patterns of human peripheral blood and nude-mouse spleen lymphocytes did not change during the period 5–30 min after mitogen stimulation. Therefore a change in the phosphorylation of plasma-membrane protein(s) is probably not an early biochemical event in the initiation of T-lymphocyte and B-lymphocyte growth, although a rapid transient change cannot be ruled out. Similar plasma-membrane phosphorylation patterns were also obtained by incubating the purified plasma membrane with [γ - 32 P]ATP. The phosphorylation of the 90 000-mol.wt. polypeptide was particularly rapid and was stimulated by the addition of cyclic AMP.

Enzymic phosphorylation and dephosphorylation of proteins is a well-documented way of regulating their biological activity. Interest in this regulatory mechanism has been stimulated by the finding that cyclic AMP, a well-characterized intracellular mediator of the action of various hormones, activates several protein kinases (Walsh *et al.*, 1968). The overall activity of many phosphorylation–dephosphorylation systems, comprising both specific kinases and phosphatases, is controlled by a diverse collection of factors in addition to cyclic AMP, including other cyclic nucleotides, Ca^{2+} and steroid hormones. In view of this close association with well-established intracellular regulators, it is not

surprising that phosphorylation–dephosphorylation has been ascribed profound roles in mediating cell behaviour. Greengard (1978) has proposed that protein phosphorylation is a common intracellular regulatory pathway that is utilized by many biologically active extracellular ligands, and, more specifically, phosphorylation of membrane proteins has been implicated in the mechanism of action of many hormones and neurotransmitters (Walton, 1978). In addition, the expression of the transformed cell phenotype of some virally transformed cells has been directly associated with the synthesis of a phosphorylated protein kinase (Collett & Erickson, 1978).

Quiescent lymphocytes are stimulated to grow, divide and differentiate by a specific antigen, or non-specifically by various mitogens (Crompton *et al.*, 1977). The biochemical basis of the initiation of lymphocyte growth has not been delineated, although cyclic nucleotides and Ca^{2+} have been separately implicated as second messengers in the transmission of the extracellular signal to the cell

* Present address: Department of Immunology, St. George's Hospital Medical School, London SW17 0RE, U.K.

† Present address: Institute of Neurology, National Hospital, Queen Square, London WC1N 3BG, U.K.

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

interior (Wedner & Parker, 1976). The possibility that the initiation of growth also involves changes in the degree of phosphorylation of lymphocyte plasma-membrane proteins is attractive (Chaplin *et al.*, 1979b), especially since stimulation of fibroblast growth has been associated with the increased phosphorylation of a 33 000-mol.wt. membrane protein (Nilsen-Hamilton & Hamilton, 1979). Although it has been reported that mitogen activation of human lymphocytes induces increased phosphorylation of non-nuclear proteins (Wedner & Parker, 1975; Chaplin *et al.*, 1980), and that lymphocytes possess various cyclic AMP-dependent and -independent protein kinases (Kemp *et al.*, 1975; Chaplin *et al.*, 1979a), little actual data are available on plasma-membrane phosphoproteins, and whether their degree of phosphorylation changes after mitogenic stimulation. The human major histocompatibility (HLA-A and HLA-B) antigens represent a notable exception; the C-terminal intracellular portion of these transmembrane glycoproteins is phosphorylated (Pober *et al.*, 1978).

The present study is concerned with characterizing lymphocyte plasma-membrane phosphoproteins and determining whether there is any gross change in their pattern or degree of phosphorylation on mitogenic stimulation. The results indicate that the plasma membrane has a simple pattern, comprising a small number of strongly phosphorylated polypeptides. No changes were detected during the initiation of T-lymphocyte and B-lymphocyte activation. A preliminary report of this work has been presented (Johnstone *et al.*, 1980).

Experimental

Materials

Antisera against human β_2 -microglobulin, human HLA-DRw(Ia) antigens, mouse immunoglobulin and BRI-8-cell plasma membrane were raised in rabbits by intramuscular and intraperitoneal injections of the purified protein or membrane emulsified in complete Freund's adjuvant. The antisera against purified proteins were shown to be specific for the respective antigen by a combination of cytotoxicity, immunofluorescence and immune-precipitation analyses. Rabbit antisera against the mouse Thy-1 differentiation antigen were donated by Dr. R. Morris (National Institute for Medical Research, London NW7 1AA, U.K.) and Professor C. Bron (Institut de Biochimie, Université de Lausanne, Lausanne, Switzerland). A rabbit antiserum against mouse major transplantation (H-2^b) antigens was donated by Professor S. G. Nathenson (Albert Einstein College of Medicine, Bronx, NY, U.S.A.); this antiserum recognizes lymphocyte cell-surface components in addition to the H-2 antigens. [³²P]P_i (carrier-free), [γ -³²P]ATP (2000 Ci/mmol), Na¹²⁵I

(carrier-free), [³⁵S]methionine and NaB³H₄ (7 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). *Phaseolus vulgaris* phytohaemagglutinin (leucoagglutinin) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and *Escherichia coli* (0550:B5) lipopolysaccharide from Difco Laboratories (Detroit, MI, U.S.A.). *Lens culinaris* phytohaemagglutinin was prepared and attached to Sepharose as described previously (Hayman & Crumpton, 1972). Carbonyl iron (grade SF) was obtained from GAF Ltd. (Manchester, U.K.); it was washed once in 70% (v/v) ethanol, four times in water and dried before use.

Preparation of lymphocytes and membranes

Pig mesenteric-lymph-node lymphocytes were prepared as described previously (Perlès *et al.*, 1977), except that the nylon wool step for B-lymphocyte depletion was omitted. Spleens or thymuses from freshly killed 6–10-week-old mice were dispersed by passage through stainless-steel wire mesh by using the plunger from a plastic syringe. The lymphocytes were separated from erythrocytes and dead cells as described for pig lymphocytes by Perlès *et al.* (1977). Human peripheral-blood lymphocytes were isolated from the buffy coat from 1 unit of blood after depletion of erythrocytes by thorough mixing with 0.8 vol. of 3.5% (w/v) Dextran 250 (Sigma Chemical Co., Poole, Dorset, U.K.) in saline (0.9% NaCl) containing 10 i.u. of heparin/ml followed by sedimentation at 1 g for 30 min at 37°C. Platelets were removed from the supernatant by centrifuging at 120 g for 10 min and resuspension of the pellet in 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]-buffered Eagle's BHK medium (Perlès *et al.*, 1977) containing 10 i.u. of heparin/ml; this procedure was repeated twice. Phagocytic cells were removed by resuspending the pellet in Hepes-buffered Eagle's BHK medium containing 10 i.u. of heparin/ml and 5% (v/v) foetal-calf serum (10⁷ cells/ml) and incubating with carbonyl iron (10 mg per 10⁸ cells) at 37°C for 30 min with periodic mixing (McCullagh, 1970). A magnetic stirring bar was then added, rotated slowly for a few minutes and removed, and the adherent cells were wiped off. The latter procedure was repeated five or six times, and the entire carbonyl iron procedure was repeated twice more. Any remaining erythrocytes and dead cells were removed by layering 10⁸ cells in 2–4 ml on to 2.5 ml of Ficoll-Paque (Pharmacia Fine Chemicals) and centrifuging at 400 g for 30 min at 20°C. The final lymphocyte preparation contained less than 1% erythrocytes, 1% polymorphonuclear leucocytes and 5% platelets.

Pig and human cells could be kept overnight at 20°C before use, but mouse cells were used on the day of preparation. All cell suspensions were at least 95% viable as judged by exclusion of Eosin.

Lymphoblastoid B-cell lines (BRI-8 and Daudi) and T-cell lines (MOLT 4) were maintained by culturing in RPMI 1640 medium (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) containing 10% (v/v) foetal-calf serum. The BRI-8 cells were initially obtained from G. D. Searle (High Wycombe, Bucks., U.K.), and the Daudi and MOLT 4 cells were initially provided by Dr. W. F. Bodmer (Imperial Cancer Research Fund, London WC2A 3PX, U.K.).

Microsomal and plasma-membrane fractions were separated after mechanical disruption of the cells as described previously (Snary *et al.*, 1974).

Incorporation of ^{32}P into whole cells

Lymphocytes were incubated in phosphate-free RPMI 1640 medium containing 10% (v/v) dialysed foetal-calf serum (10^6 – 10^7 cells/ml) with [^{32}P]P_i (1–3 mCi/ 10^8 cells) for 3 h at 37°C or 16 h at 20°C. The cells were washed twice with normal medium and were then either stimulated to transform by using various mitogens (see below) or immediately broken and the microsomal fraction was isolated.

Incorporation of ^{32}P into isolated plasma membranes

Phosphorylation of purified BRI-8-cell plasma membrane (50 µg of membrane protein) was conducted at 20°C in a final volume of 50 µl of 25 mM-sodium phosphate buffer, pH 7.5, containing 5 mM-MgCl₂ and 0.5 mM-EGTA; cyclic AMP (1 µM) and theophylline (3 mM) were added to some samples as indicated. The mixtures were kept on ice and then equilibrated for 1 min at 20°C before initiation of phosphorylation by the addition of 20 µCi of [γ - ^{32}P]ATP (0.2 µM). After various times (5 s–1 h), the reaction was terminated by adding 50 µl of 2% (w/v) sodium dodecyl sulphate in 80 mM-Tris/HCl buffer, pH 6.8, containing 10% (w/v) glycerol and heating at 100°C for 1 min; the samples were stored frozen at –20°C before analysis.

Mitogenic stimulation of lymphocytes

After [^{32}P]P_i incorporation, the washed cells were resuspended in RPMI 1640 medium containing 10% (v/v) foetal-calf serum (10^7 cells/ml) at 37°C, and mitogen was then added (20 µg of *Phaseolus* phytohaemagglutinin/ 10^7 human peripheral-blood lymphocytes or 100 µg of lipopolysaccharide/ 10^7 nude-mouse spleen lymphocytes). These conditions were known to induce optimal stimulation, as judged by incorporation of [^3H]thymidine into DNA after

about 44 h of culture (Perlès *et al.*, 1977; J. H. DuBois, unpublished work). After incubation for 5–30 min at 37°C the cells were washed twice with phosphate-buffered saline (0.15 M-NaCl/15 mM-sodium phosphate buffer, pH 7.3) and immediately broken, and the microsomal fraction was isolated.

Other labelling procedures

Lactoperoxidase-catalysed radioiodination was performed as detailed by Hubbard & Cohn (1976), with glucose oxidase and glucose. Periodate-mediated ^3H -labelling of sialic acid residues was as described by Gahmberg & Andersson (1977), with NaB³H₄. Biosynthetic labelling of lymphocyte protein was done at 37°C for 16 h in methionine-free RPMI 1640 medium containing 10% (v/v) foetal-calf serum (10^6 cells/ml) and [^{35}S]methionine (20 µCi per 10^6 cells).

Immunoprecipitation

Immunoprecipitation of membrane solubilized in 0.5% sodium deoxycholate or of whole cells lysed in 0.5% Nonidet P40 was performed with rabbit antisera and fixed *Staphylococcus aureus* organisms, as detailed in Owen *et al.* (1980).

Analytical methods

Incorporation of radioactivity into protein was assessed by counting the radioactivity of a measured sample dried on to a Whatman GF/A filter disc before and after sequential washing with 7% (w/v) trichloroacetic acid, ethanol and diethyl ether. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed on slab gels (10%, w/v, acrylamide) (Laemmli, 1970), after reduction of the samples in 1% sodium dodecyl sulphate/0.1 M-dithiothreitol / 5% (w/v) glycerol / 40 mM-Tris/HCl buffer, pH 6.8, at 100°C for 1 min. Two-dimensional polyacrylamide-gel electrophoresis was performed as described by O'Farrell (1975) by using 2% (w/v) Ampholine (comprising 1.6% of pH range 5–7 and 0.4% of pH range 3–10) in 3.8%-(w/v)-acrylamide gel for the isoelectric-focusing dimension and 10%-(w/v)-acrylamide gel (Laemmli, 1970) for the second sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis dimension. The samples contained less than 20 µg of protein and radioactivity between 10^5 and 3×10^5 trichloroacetic acid-insoluble c.p.m. Radiolabelled proteins were detected, after drying of the gel, by direct radioautography for ^{125}I and ^{32}P , and fluorography for ^3H (Bonner & Laskey, 1974).

Results

Incubation at 37°C for 3 h or at 20°C overnight

Fig. 1. Comparison of phosphoproteins, total proteins and glycoproteins in the microsomal fraction from human

B-lymphoblastoid BRI-8 cells

BRI-8 cells were incubated in the presence of (a) $[^{32}\text{P}]\text{P}_i$, (b) $[^{35}\text{S}]\text{methionine}$ or (c) 1 mM-periodate followed by NaB^3H_4 (see the text for details). The microsomal fractions were then isolated and analysed by two-dimensional gel electrophoresis. The positive and negative poles of the isoelectric-focusing dimension are indicated at the top and the positions of marker proteins of known molecular weight ($\times 10^{-3}$) on the left. In (a) the dotted spot indicates the position of actin and the two-dimensional gel is aligned with the left-hand one-dimensional gel. Radioactivity was detected by direct radioautography (a and b) or fluorography (c).

of 10^8 quiescent human, mouse or pig lymphocytes with 1–3 mCi of $[^{32}\text{P}]\text{P}_i$ resulted in the incorporation of between 3×10^5 and 9×10^5 c.p.m. into the microsomal membrane fraction. As anticipated from their increased growth rate, cultured lymphoblastoid cell lines (BRI-8, Daudi and MOLT 4) incorporated much more radioactivity into the microsomal membrane fraction, under the same conditions (between 1×10^7 and 2×10^7 c.p.m.). The

Fig. 2. Comparison of the phosphorylated polypeptides of various subcellular fractions

MOLT 4 cells were incubated in the presence of $[^{32}\text{P}]\text{P}_i$ and disrupted. The subcellular fractions were isolated and then analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. A, Purified plasma membrane isolated from the microsomal fraction by sucrose-density centrifugation; B, microsomal fraction (i.e. 20000 g pellet of cell homogenate); C, ribosomal fraction (i.e. 100000 g pellet of cell homogenate); D, cytosol fraction (i.e. 100000 g supernatant). Radioactive bands were detected by radioautography. The positions of marker proteins of known molecular weight ($\times 10^{-3}$) are indicated on the right.

above values did not change significantly when longer incubation periods were employed. On average, 35% of the incorporated radioactivity was precipitated by trichloroacetic acid, and was thus most probably attached to protein. During the incubation and subsequent washing procedure, between 10 and 40% of the cells were routinely lost. The viability of the recovered cells was always greater than 80%.

Two-dimensional gel-electrophoretic analysis of the ^{32}P -labelled microsomal membrane fraction from BRI-8 cells (Fig. 1a) revealed two major and approximately five minor radioactive spots. The mobilities of the two major spots corresponded to acidic pI values and molecular weights of about 90 000 and 52 000. In contrast with this simple labelling pattern, a large number of radioactive spots was given by the microsomal membrane fraction of BRI-8 cells that had been biosynthetically labelled with [^{35}S]methionine (Fig. 1b). It was concluded that only a small fraction of the membrane proteins of BRI-8 cells were labelled by [^{32}P]P_i to high specific radioactivity under the above conditions.

The two ^{32}P -labelled polypeptides of about 90 000

and 52 000 molecular weight were observed in every lymphocyte plasma-membrane preparation studied, irrespective of the species of origin (human, mouse or pig) and the lymphocyte type (T- and B-cells; quiescent, stimulated or cultured), although their relative amounts varied. For example, in MOLT 4 cells the radioactive intensity of the 52 000-mol.wt. band was markedly decreased relative to the 90 000-mol.wt. band (Fig. 2, track A) compared with BRI-8 cells. The minor ^{32}P -labelled polypeptides of BRI-8 cells (Fig. 1a) varied for the different lymphocyte populations, but no band could be related to a particular species of origin or type of lymphocyte.

In the case of one cell line, namely MOLT 4, the ^{32}P -labelled polypeptide compositions of the sub-cellular fractions obtained during the preparation of the plasma membrane were compared by one-dimensional gel electrophoresis. As shown in Fig. 2, the purified plasma-membrane fraction possessed a similar composition to the microsomal fraction (comprising approx. 70% plasma membrane), whereas the ribosomal and cytosol fractions each contained more than 20 radioactive bands distri-

Fig. 3. Immunoprecipitation of cell-surface proteins from BRI-8 cells and human peripheral-blood lymphocytes. Peripheral-blood lymphocytes (tracks A and B) and BRI-8 cells (tracks C–F) were incubated in the presence of [^{32}P]P_i and disrupted. The microsomal fractions were isolated and solubilized in 0.5% sodium deoxycholate in 10 mM-Tris/HCl buffer, pH 8.2. Plasma membrane was also isolated from unlabelled BRI-8 cells, solubilized as above and then radioiodinated (Greenwood *et al.*, 1963) (tracks G and H). The solubilized proteins were precipitated with normal rabbit serum (tracks A, F and G), anti-(plasma membrane) serum (tracks B, D and H), anti-(HLA-DRw antigen) serum (track C) or anti- β_2 -microglobulin serum (track E), and the precipitates were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Radioactive bands were detected by radioautography. The positions of marker proteins of known molecular weight ($\times 10^{-3}$) are indicated.

buted throughout the molecular-weight range of the gel (from about 250 000 to 10 000 molecular weight).

Characterization of ^{32}P -labelled proteins

The ^{32}P -labelled microsomal fractions from various lymphocytes were analysed after solubilization in detergent (sodium deoxycholate or Nonidet P40) by immunoprecipitation with antisera against defined lymphocyte surface antigens. Antisera against human β_2 -microglobulin and BRI-8 plasma membrane that recognize the human major transplantation (HLA-A and HLA-B) antigens precipitated a ^{32}P -labelled polypeptide of about 43 000 molecular weight from the microsomal fractions of BRI-8 cells and human peripheral-blood lymphocytes (Fig. 3). In contrast, although the anti-(BRI-8-cell plasma-membrane) serum contained antibodies against immunoglobulin M and HLA-DRw(Ia) antigens, no radioactivity was detected (Fig. 3) in the positions of these polypeptides (about 80 000 and 25 000 mol.wt. for immunoglobulin M, and 33 000 and 28 000 mol.wt. for HLA-DRw antigen). The anti-(β_2 -microglobulin) serum also precipitated a ^{32}P -labelled polypeptide of about 43 000 molecular weight from pig lymphocytes, and an antiserum recognizing the mouse major transplantation H-2^b antigen precipitated a radioactive band of about 47 000 molecular weight from the microsomal fractions of mouse spleen and thymus cells (Fig. 4). With the latter fractions, no detectable radioactivity was precipitated by antisera against mouse immunoglobulin or the Thy-1 antigen, even though these antisera precipitated the predicted radioactive bands from ^{125}I -labelled cells (Fig. 4). It was concluded that the major transplantation antigens are strongly phosphorylated, but that the membrane-associated immunoglobulin or the human (HLA-DRw)Ia and mouse Thy-1 antigens are not phosphorylated to a significant extent.

The possibility that the two major ^{32}P -labelled polypeptides of 90 000 and 52 000 molecular weight were glycosylated was investigated in two ways. Firstly, the two-dimensional electrophoresis pattern given by BRI-8 cells, whose surface sialic acid residues had been labelled by periodate oxidation followed by NaB^3H_4 reduction, was compared with that of ^{32}P -labelled cells. The results (Figs. 1a and 1c) indicated that neither of the two major ^{32}P -labelled polypeptides co-migrated with a ^3H -labelled spot. Secondly, the glycoproteins of deoxycholate-solubilized ^{32}P -labelled microsomal fractions from MOLT 4 and Daudi cells were depleted by adsorption on *Lens* phytohaemagglutinin attached to Sepharose. The adsorbed glycoproteins were subsequently eluted with 1% sodium dodecyl sulphate and were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. No ^{32}P -labelled

Fig. 4. Immunoprecipitation of cell-surface proteins from mouse lymphocytes

A mixture of spleen and thymus cells from Swiss Neider and C3H mice were either labelled by lactoperoxidase-catalysed iodination (tracks A–D) or incubated with [^{32}P]P_i (tracks E–H). The former cells were solubilized in 0.5% Nonidet P40 and the nuclei were removed by centrifugation. The latter cells were disrupted, and the separated microsomal fraction was solubilized in 0.5% Nonidet P40. Solubilized proteins were precipitated with normal rabbit serum (tracks A and E), anti-(H-2^b antigen) serum (tracks B and F), anti-(Thy-1 antigen) serum (tracks C and G) and anti-(mouse immunoglobulin) serum (tracks D and H), and the precipitates were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The samples used for immunoprecipitation contained either 0.5×10^6 – 1.0×10^6 c.p.m. of ^{125}I or 1.5×10^5 – 3.0×10^5 c.p.m. of ^{32}P attached to protein. Radioactive bands were detected by radioautography. Track B revealed two bands; one was coincident with the μ -chain of immunoglobulin M (see track D), and the second fainter band of 47 000 molecular weight, which did not reproduce well photographically, represented the H-2K and D antigens. The positions of marker proteins of known molecular weight ($\times 10^{-3}$) are indicated.

bands were detected in the eluted fraction (results not shown). In contrast, a large number of radioactive bands was revealed when samples of microsomal fractions from radioiodinated or ^{35}S (biosynthetically)-labelled lymphocytes, containing a similar

amount of radioactivity, were analysed by an identical procedure.

Effect of initiation of lymphocyte growth

Human peripheral-blood lymphocytes and nude-CBA-mouse spleen cells that had been prelabelled by incubation with [32 P]P_i were re-incubated for up to 30 min with *Phaseolus* phytohaemagglutinin and lipopolysaccharide respectively, under conditions

giving rise to maximal lymphocyte transformation; control cells were incubated under the same conditions but without added mitogen. Analysis of the respective microsomal fractions revealed an increased incorporation (1.6–1.9-fold) of 32 P after mitogenic stimulation for 30 min. However, as shown in Fig. 5, a comparison of one-dimensional and two-dimensional gel-electrophoresis patterns of the microsomal fractions failed to reveal any apparent changes in the 32 P-labelled polypeptides on *Phaseolus*-phytohaemagglutinin stimulation of human peripheral-blood lymphocytes (spots below the bottom horizontal line can be attributed to phospholipids). Similar results were obtained on the activation of nude-CBA-mouse spleen lymphocytes by lipopolysaccharide (results not shown).

Fig. 5. Comparison of microsomal fractions from 32 P-labelled human peripheral-blood lymphocytes before and after mitogenic stimulation

Peripheral-blood lymphocytes were incubated with [32 P]P_i and divided into two equal samples. One was stimulated by incubation with *Phaseolus* phytohaemagglutinin for 30 min (gel b) and the other held under the same conditions in the absence of mitogen (gel a). The microsomal fractions were isolated and analysed by one-dimensional and two-dimensional electrophoresis followed by radioautography. The two-dimensional gels are aligned with the right-hand one-dimensional gels. The positive and negative poles of the isoelectric-focusing dimension are indicated at the top and the positions of marker proteins of known molecular weight ($\times 10^{-3}$) on the right. The dotted spots indicate the position of actin.

Fig. 6. BRI-8-cell plasma-membrane polypeptides phosphorylated in whole cells and the isolated plasma membrane

The Figure shows radioautographs of sodium dodecyl sulphate / polyacrylamide - gel electrophoretograms of (A) microsome fraction isolated from whole cells incubated with [32 P]P_i, (B) plasma membrane incubated with [γ - 32 P]ATP for 30 s and (C) plasma membrane incubated with [γ - 32 P]ATP for 30 s in the presence of cyclic AMP and theophylline. The positions of marker proteins of known molecular weight ($\times 10^{-3}$) are indicated on the right.

Phosphorylation of isolated plasma membrane

The purified plasma-membrane fraction from BRI-8 cells that had been incubated with [γ - ^{32}P]ATP in the presence or in the absence of cyclic AMP at 20°C for various periods of time was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The result of one such experiment in which the plasma membrane was incubated for 30 s is shown in Fig. 6. The pattern of ^{32}P -labelled bands included the two major radioactive polypeptides of 90 000 and 52 000 molecular weight that were labelled in whole cells and at least three additional minor bands (mol.wts. about 70 000, 54 000 and 48 000). The lower-molecular-weight ^{32}P -labelled bands of BRI-8 cells were not well represented in the phosphorylated plasma membrane. ^{32}P -labelling of the 48 000-mol.wt. and 70 000-mol.wt. bands was detected only when phosphorylation of the plasma membrane was performed in the absence of cyclic AMP and theophylline, whereas the degree of labelling of the 90 000-mol.wt. band was increased in the presence of cyclic AMP and theophylline. Although the above bands were labelled after only 5 s incubation of plasma membrane with [γ - ^{32}P]ATP, the phosphorylation of most bands increased up to 2 min incubation, remained constant for 15 min and then decreased. The 90 000-mol.wt. band was notable for being maximally phosphorylated after 5 s and for the subsequent complete loss of label after a further 2.5 min incubation.

Discussion

A large number of polypeptides are labelled when quiescent lymphocytes are incubated with [^{32}P]P_i (Wedner & Parker, 1975). In the present study subcellular fractionation has demonstrated that only a small number of these polypeptides are associated with the plasma membrane and that the vast majority are located in other subcellular fractions (Fig. 2). Since further purification of the plasma membrane from the microsomal fraction is associated with a negligible change in the phosphorylated polypeptide pattern (Fig. 2), the microsomal fraction was assumed to be representative of the plasma membrane for the purposes of this study. This assumption is supported by the observation that phosphorylation of the purified plasma membrane gave a pattern of radioactive spots similar to that shown by the microsomal fraction from ^{32}P -labelled lymphocytes (Fig. 6).

The marked conservation of the 52 000-mol.wt. and 90 000-mol.wt. phosphorylated plasma-membrane polypeptides between different types of lymphocytes from various species is consistent with them having an important biological role. This view

is supported by the observation that proteins of similar molecular weight are major phosphorylated components of erythrocyte 'ghosts' and synapses (Avruch *et al.*, 1976; Ueda *et al.*, 1973). Another group (Chaplin *et al.*, 1979b) have reported a much more complex pattern of phosphorylated polypeptides (comprising 12–24 spots) in the plasma membrane of human peripheral-blood lymphocytes than that observed in the present study; also, neither the 52 000-mol.wt. nor the 90 000-mol.wt. acidic polypeptides were observed. The reason for these discrepancies is unknown, but may include different labelling procedures (although the similar patterns obtained in the present study with two different procedures make this unlikely), as well as the degree of purity of the lymphocyte and plasma-membrane preparations employed.

Most of the phosphorylated polypeptides present in the microsomal fraction from ^{32}P -labelled lymphocytes were also detected with purified lymphocyte plasma membrane that had been incubated with [γ - ^{32}P]ATP (Fig. 6). This demonstrates that protein kinase(s) are associated with the membrane. The cyclic AMP-related phosphorylation of the 90 000-mol.wt. polypeptide together with its rapid phosphorylation (within 5 s) and dephosphorylation (2.5 min) makes this polypeptide an attractive candidate for a regulatory role.

The report (Pober *et al.*, 1978) that the 43 000-mol.wt. glycosylated polypeptide chain of human major transplantation (HLA-A and HLA-B) antigens is phosphorylated was confirmed in the present study with both human B-lymphoblastoid BRI-8 cells and peripheral-blood lymphocytes (Fig. 3). Interestingly, the extent of phosphorylation of the HLA-A and HLA-B polypeptide of peripheral-blood lymphocytes appeared to be much greater than that of BRI-8 cells, in spite of the higher antigen content of the latter cells (compare Figs. 1a and 5). The phosphorylation of major transplantation antigens appears to be a general phenomenon, since the mouse and pig analogues of the human HLA-A and HLA-B antigens were also shown to be phosphorylated (Fig. 4). On the other hand, no phosphorylation of several other major lymphocyte surface antigens was detected. Although it is possible that some degree of phosphorylation may have been detected if a greater amount of radioactivity had been added [indeed Kaufman & Strominger (1979) have reported ^{32}P -labelling of HLA-DRw antigen under extreme conditions: 10 mCi/ 10^6 cells compared with 1–3 mCi/ 10^8 cells], it is apparent that these antigens differ significantly in this respect from the major transplantation antigens. An alternative explanation could be a relatively high rate of turnover of the HLA-A and HLA-B phosphate groups compared with the other antigens; however, the long period of equilibration with [^{32}P]P_i used in

the present study makes this unlikely. As judged by their reaction with lentil lectin or periodate, the major phosphorylated polypeptides, apart from the major transplantation antigens, were not glycosylated (Figs. 1a and 1c). These techniques would have detected most, but not all, plasma-membrane glycoproteins (Hayman & Crumpton, 1972).

The primary purpose of the present studies was to determine whether initiation of lymphocyte activation by mitogens is associated with changes in the phosphorylation of plasma-membrane protein(s). The experimental conditions were designed with this specific purpose in mind. Thus the lymphocytes were allowed to equilibrate with [32 P]P_i before mitogen stimulation in order to avoid possible artifact changes in phosphorylation caused by fluctuations in the specific radioactivities of the intracellular [32 P]P_i and [γ - 32 P]ATP pools due to mitogen-induced variations in phosphate uptake (Cross & Ord, 1971). Similarly, studies were restricted to initial events at the level of the lymphocyte surface membrane by analysing the purified plasma membrane recovered from cells that had been incubated with mitogen for less than 30 min. In these conditions, mitogen-induced activation of both human peripheral-blood (T)-lymphocytes and nude-mouse spleen (B)-lymphocytes was associated with an increase in the specific radioactivities of the plasma-membrane fractions. This increase is probably related to the increased rate of turnover (i.e. hydrolysis and resynthesis) of lymphocyte plasma-membrane phosphatidylinositol that is stimulated by the addition of mitogen (Maino *et al.*, 1975), because no changes in either the amounts or the pattern of phosphorylated polypeptides were detected, as judged by two-dimensional gel electrophoresis (Fig. 5). These experiments do not preclude the possibility that transient changes may have occurred within a few minutes of adding the mitogen. Although this possibility is consistent with the very rapid phosphorylation-dephosphorylation cycle (about 2.5 min) of the 90 000-mol.wt. component observed with the purified plasma membrane, the technical difficulty of rapidly removing the added mitogen prevented the isolation of plasma membrane from lymphocytes that had been incubated with mitogen for less than 5 min. However, changes in the phosphorylation pattern of plasma-membrane polypeptides have been revealed on stimulation of other types of quiescent cells during similar time periods (5–20 min) to those used in the current experiments (Rudolph & Greengard, 1974; Nilsen-Hamilton & Hamilton, 1979). The present data therefore suggest that, if changes in protein phosphorylation occur during the stimulation of lymphocyte growth by mitogens, then they either do not involve plasma-membrane-located polypeptides or are rapid and transient.

We thank Mr. R. A. Faulkes for the two-dimensional electrophoretograms. A. P. J. was a recipient of a Medical Research Council Training Fellowship and J. H. D. a Medical Research Council Studentship.

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