Mitogen-induced phosphorylation of human B-lymphocyte proteins

Relationship to protein kinase C activation

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We have investigated the rapid phosphorylation of proteins in B-lymphocytes incubated with the tumour-promoting phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), anti-Ig and combinations of TPA and the Ca^{2+} ionophore ionomycin. Two-dimensional electrophoretic analysis was used to identify the proteins phosphorylated in cells preincubated with [^{32}P]P_i. TPA induced a characteristic pattern of labelled proteins, four of which (pp85, pp76, pp66 and pp63) showed a dose-dependent incorporation of ^{32}P on serine residues. The phosphorylation of pp63 and pp66, in particular, correlated with the mitogenic dose-response curve. Addition of the Ca^{2+} ionophore ionomycin to B-cells also stimulated a characteristic incorporation of ^{32}P into proteins, which included pp63 and pp66. With combined doses of TPA and ionomycin, these two proteins show an enhanced phosphorylation, which correlated well with the synergistic enhancement of proliferation shown by this combination of agents. Protein kinase C (PKC) was partially purified from B-cells and separated into α and β subtypes. The activation of both PKCs was assessed with increasing doses of TPA and concentrations of Ca^{2+} of 0.1 μM and 2 μM . For both forms of PKC, in particular the β form, higher concentrations of Ca^{2+} shifted the dose-response curve for TPA to the left and increased the maximum activation. Anti-Ig, which stimulated B-cells by cross-linking surface immunoglobulin and causing hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$, also caused increased phosphorylation of several proteins, which again included pp63 and pp66. These data suggest that PKC, particularly the β form, is involved in the early part of the proliferation cascade for human B-lymphocytes. It is most probably activated in a synergistic manner by the increased Ca^{2+} and diacylglycerol levels which result from the earlier hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$.

INTRODUCTION

The activation of lymphocytes by specific antigens or polyclonal mitogens induces numerous changes in intracellular biochemical processes which culminate in co-ordinated cell division and differentiation. A closely regulated series of biochemical events is triggered by stimulating agents binding their respective receptor(s). An early response to antigen cross-linking of surface immunoglobulin on B-lymphocytes is the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ and the subsequent mobilization of Ca^{2+} , which is released initially from intracellular stores by $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol (DAG) (reviewed by Guy *et al.*, 1988). DAG is a cofactor in the activation of the ubiquitous protein kinase C (PKC). These two products have been postulated to enhance synergistically, by an undetermined mechanism, the proliferation of cells where such polyphosphoinositide hydrolysis is an early corollary of receptor binding (Nishizuka, 1984). Such a postulate is strengthened by data on T- or B-lymphocytes showing that a synergistic proliferative response is achieved by co-stimulation of resting cells with individually sub-mitogenic doses of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and calcium ionophore (Guy *et al.*, 1988).

The secondary signals generated in stimulated cells control the phosphorylation of a number of cell proteins (Greengard, 1978; Rubin & Rosen, 1975). Protein kinases and phosphatases have been identified in lymphocytes (Murray *et al.*, 1972; Byus *et al.*, 1977), and alterations in the activities of these may phosphorylate key cellular proteins that play a role in mitogenic signalling (Chaplin *et al.*, 1980).

The stimulation of T- or B-lymphocytes by various mitogenic agents results in the phosphorylation of a number of plasma-membrane receptors: HLA antigens (A and B) in lymphocytes stimulated by TPA (Feuerstein *et al.*, 1985), the interleukin-2 receptor and the lymphocyte-specific membrane glycoprotein (T200) in normal T-lymphocytes, again stimulated by TPA (Shackelford & Trowbridge, 1986), the gp140 (CD21) protein, which is the receptor for Epstein-Barr virus, in human peripheral B-lymphocytes stimulated by cross-linking surface immunoglobulins with anti-IgM (μ -chain-specific) or fixed *Staphylococcus aureus* Cowan strain (Barel *et al.*, 1986), the T₃/T-cell antigen complex of human T-lymphocytes by DAG analogues (Cantrell *et al.*, 1985), and the human B-cell-associated antigen CD20 by TPA (Valentine *et al.*, 1987).

Protein phosphorylation in whole cells has been docu-

Abbreviations used: PKC, protein kinase C; DAG, diacylglycerol; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast-derived growth factor; f.p.l.c., fast protein liquid chromatography; $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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mented for mouse and human T-lymphocytes stimulated with mitogens, and to a lesser degree in human and mouse B-lymphocytes (reviewed by Guy *et al.*, 1988). Several previous studies have used total lymphocyte preparations and a selection of polyclonal mitogens (see Dasch & Stavitsky, 1985). A number of proteins showed enhanced phosphorylation in these studies, but none of the proteins have as yet been identified.

In the present study we investigated which proteins are kinase substrates when B-lymphocytes in the resting G_0 phase were stimulated by some of these activating factors. Cells were labelled with [^{32}P]P_i and stimulated with mitogenic doses of TPA, by sub-mitogenic doses of TPA in combination with Ca^{2+} ionophore (ionomycin) and anti-Ig, which stimulates $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis and the subsequent activation of the Ca^{2+} and DAG cofactors and putative pathway activators.

MATERIALS AND METHODS

Materials

1,2-Diolein, phosphatidylserine, histone type III-S and silica-gel 60 plates were from Sigma, Poole, Dorset, U.K. The monoclonal antibody for PKC was obtained from Amersham International, Amersham, Bucks., U.K., and was specific to one of the isoenzymes.

Cells

Purified human B-cells were prepared from human tonsils obtained at routine tonsillectomy. Resting cells were prepared by the method outlined by Gordon *et al.* (1985). At least 98% of the cells were B-lymphocytes, according to studies with pan-B monoclonal antibodies. All the cells were ascertained to be in the G_0 phase of the cell cycle, as analysed on a fluorescence-activated cell sorter by the Acridine Orange staining method of Krishan (1975).

Radiolabelling of cells

Before radiolabelling, the B-cells were washed twice in phosphate-free medium (150 mM-NaCl/5 mM-MgCl₂/5 mM-KCl/1.6 mM-CaCl₂/0.5% glucose/10 mM-Tris, pH 7.4) and then resuspended in 1 ml of phosphate-free medium containing 5% (v/v) dialysed fetal-calf serum and 1 mCi of [^{32}P]P_i (Amersham International) for 4 h. Agonists were added in the last 1–60 min of radiolabelling immediately before harvesting. The cells were then harvested and washed rapidly with chilled phosphate-buffered saline (1.15 mM-KH₂PO₄/8.45 mM-NaH₂PO₄/146 mM-NaCl, pH 7.4) in a micro-tube. Buffer A (50 mM-Tris/HCl, pH 7.3, containing 50 mM-NaF, 5 mM-sodium pyrophosphate, 2 mM-EDTA and 2 mM-phenylmethanesulphonyl fluoride), which inhibits phosphatase activity, was then added at 200 μl /20 $\times 10^6$ cells, and the lysates were stored at -80°C until further use.

Preparation of lysates for two-dimensional gel electrophoresis

Cells were labelled with ^{32}P as above, the pelleted cells were resuspended in buffer A, disrupted with passage of the suspension 30 times through a 26-gauge disposable needle, and centrifuged at 200 g for 5 min and then at 100 000 g for 30 min to obtain the nuclear, cytosolic and membrane fractions. The fractions were lysed in buffer B [50 mM-Tris/HCl, pH 7.0, containing 0.25% SDS, 5 mM-MgCl₂, 1% 2-mercaptoethanol, 1 mM-phenyl-

methanesulphonyl fluoride, DNA (100 $\mu\text{g}/\text{ml}$) and RNAase A (50 $\mu\text{g}/\text{ml}$) (Sigma)]. The lysates were kept on ice for 1 min and then mixed with dry urea to give a concentration of 9.5 M-urea. To this lysate was added an equal amount of 9.5 M-urea, 4% Nonidet P-40, 5% 2-mercaptoethanol and 2% Ampholine pH 5–7 (LKB-Produkter AB, Bromma, Sweden). The final volume of the sample was 200 μl /10⁶ cells. The lysates were loaded on to the first-dimension gels or frozen rapidly and stored at -70°C .

Two-dimensional gel electrophoresis

Portions of the samples prepared as above were subjected to two-dimensional gel electrophoresis. Isoelectric focusing in 9.5 M-urea for 10 000 V·h with an Ampholine mixture of 80% pH 5–7 and 20% pH 3.5–10 was used in the first dimension, and SDS/polyacrylamide-gel electrophoresis on 7.5% or 10% gels in the second dimension. The gels were fixed and stained in Coomassie Brilliant Blue R-250 and dried. Polypeptides labelled with ^{32}P were located by autoradiography, by using intensifying screens. Marker proteins for pI (BDH, Poole, Dorset, U.K.) were used as markers in the first-dimension stick gels, and blue-linked markers (Sigma) were used as molecular-mass markers on the polyacrylamide slab gels.

The extent of the phosphorylation was determined from the densities of spots in the autoradiograms measured with a densitometer, and/or the spots were cut from the gels, eluted and counted for radioactivity by liquid-scintillation spectroscopy. All experiments were repeated at least three times, and the autoradiographs shown are representative of all the experiments.

Culture of cells and measurement of DNA synthesis

Human B-lymphocytes were cultured and assessed for active DNA synthesis by the method described by Gordon *et al.* (1985).

Phosphoamino acid analysis

Spots of interest were excised from two-dimensional gels and incubated in 5.8 M-HCl for 1 h at 110°C to extract and hydrolyse phosphoproteins as described by Ishii *et al.* (1985). The phosphoamino acids were recovered on Dowex AG1-X8 resin (Cl⁻ form; Bio-Rad Laboratories) and separated by thin-layer electrophoresis on cellulose plates in pyridine/acetic acid/water (1:10:189, by vol.), pH 3.5, at 1000 V for 60 min. Samples containing about 2000 d.p.m. were applied to the plates. Phosphoamino acid markers were detected by ninhydrin staining, and ^{32}P -labelled phosphoamino acids were detected by autoradiography using Amersham Hyper-MP autoradiographic film (Amersham International) and intensifying screens.

ATP specific-radioactivity determination

The specific radioactivity of [^{32}P]ATP in cells was measured by the method of Hawkins *et al.* (1983), to check that treatment with mitogens did not increase the incorporation of ^{32}P into the γ -phosphate of ATP.

Purification of protein kinase C

Rat brain. Within 90 s of decapitation, whole brains from 12 rats were homogenized for 45 s with a motor-driven homogenizer (1000 rev./min) and a tight-fitting Teflon pestle. The buffer was ice-cold 10 mM-Tris/HCl,

Fig. 1. Agonist-induced phosphorylation in B-lymphocyte cell lysates

Cell lysates were prepared from 1×10^6 purified resting B-cells and subjected to two-dimensional polyacrylamide-gel electrophoresis as described in the Materials and methods section. Gels were fixed, stained, dried and exposed to X-ray film for 24 h at -70°C , with use of intensifying screens. Panels: (a) control cells; (b) cells treated with 10 ng of TPA/ml for 10 min before termination of ^{32}P labelling; (c) cells treated with 0.8 μg of ionomycin/ml; (d) map of the major proteins that are phosphorylated by the agonists; (e) cells treated with anti-Ig (15 $\mu\text{g}/\text{ml}$). Marked spots on panels: a, pp85; b, pp76; c, pp66; e, pp63; f and g are spots that alter with TPA treatment with moderate changes in ^{32}P incorporation, whereas d and h are phosphoproteins that change only with ionomycin. The pI values were determined with standard proteins by pH-meter probe, and the molecular-mass markers (kDa; centre of the diagram) were standard proteins incorporated into the second dimension of the electrophoretic run.

pH 7.5, containing 10 mM-EGTA, 5 mM-EDTA, 0.1 % 2-mercaptoethanol, 0.1 % aprotinin and 4 μ g of leupeptin/ml. All subsequent operations were performed at 4 °C. The homogenate was centrifuged at 10000 *g* for 60 min. The supernatant was dialysed overnight against the above buffer but containing 1 mM-EDTA and 1 mM-EGTA. The dialysed supernatant was applied to a Mono Q column (1.6 cm $\times$ 10 cm) fitted to a f.p.l.c. system (LKB-Pharmacia, Uppsala, Sweden) and eluted with a NaCl gradient from 0 to 500 mM. Fractions (1 ml) were assayed for phospholipid-stimulated histone kinase activity, and the peak activity was pooled and dialysed overnight against 20 mM-sodium phosphate, pH 7.4, in 50 mM-2-mercaptoethanol/1 mM-EDTA/0.5 mM-EGTA, containing 0.1 % aprotinin and 4 μ g of leupeptin/ml.

A hydroxyapatite column (1.6 cm $\times$ 12 cm) previously equilibrated with 20 mM-phosphate buffer (above) was used to separate PKC into sub-species. The active dialysed fraction from the Mono Q separation was loaded on this column and eluted with a linear gradient of 20–500 mM-potassium phosphate, pH 7.4.

Human B-cells. Purified human B-cells were collected and processed until a combined fraction representing the cytosol from 10^9 cells was collected and purified as outlined above.

Protein kinase C assay

PKC was assayed in 100 μ l of 50 mM-Tris/HCl, pH 7.7, containing 10 mM-MgCl₂, 50 μ M-ATP, 1 μ Ci of [γ -³²P]-ATP, 2 mM-dithiothreitol, 100 μ M-CaCl₂, 120 μ g of phosphatidylserine/ml, 20 μ M histone H1, 5 μ g of dioleoin/ml and 20 μ l of the fraction for assay. For assessment of TPA activation, dioleoin was omitted and replaced by variable doses of TPA. The free Ca²⁺ concentration was measured with a calcium electrode and adjusted to values representing the resting intracellular value (0.1 μ M) and the stimulated value (2 μ M). The assay was terminated by placing the tubes on ice, spotting portions of the radioactive mixture on squares of filter paper and washing these in ice-cold 10 % (w/v) trichloroacetic acid before drying and counting of radioactivity by liquid-scintillation spectroscopy.

RESULTS

TPA-stimulated protein phosphorylation

Protein phosphorylation in TPA-stimulated human B-lymphocytes was assessed with a range of doses from 0.01 to 30 ng/ml and analysed by two-dimensional polyacrylamide-gel electrophoresis. This resolved over 50 spots on the average autoradiograph at optimum doses of TPA. Minor spots only became apparent as the autoradiographs were exposed for longer periods of time. Phosphorylation of a number of cellular proteins increased in a dose-responsive manner. The autoradiograph of treated cells (20 ng of TPA/ml) compared with the untreated control is shown in Figs. 1(a) and 1(b). Four proteins showed a marked increase in phosphorylation, which was optimal at 15–20 min after application of the activator. They were: an 85/87 kDa protein (pI 5.7) (designated 'a' on the map in Fig. 1d), a 76 kDa protein (pI 5.5) ('b' in map), a 66 kDa protein (pI 5.8) ('c' in map) and, the most striking, a 63 kDa protein(s) (pI 4.9–5.5) ('e' in map). Protein spots were excised from

Fig. 2. TPA dose-response of pp63, pp66, pp76 and pp80 phosphorylation

The phosphorylation of major substrates in B-lymphocytes at various doses of TPA was examined as described in Fig. 1. The extent of phosphorylation of each substrate protein was measured with a densitometer and normalized to an invariant protein spot. Analyses of proteins excised from gels and quantified by scintillation counting gave similar results. Each point is the mean of two determinations, and similar results were obtained in three separate experiments.

the series of gels and counted for radioactivity by liquid-scintillation to determine the relative increase in agonist-induced phosphorylation over basal values. The pp63 and pp66 proteins showed increased phosphorylation over their basal values at a similar dose of TPA (2–5 ng/ml), and it rose in a dose-dependent manner, with the pp63 showing the largest increase. pp76 and pp85 were only apparent when doses of over 10 ng of TPA/ml were added (Fig. 2).

The 85 kDa protein was difficult to separate, and showed some inconsistency in its appearance, so subsequent analysis was performed on the other three proteins.

Cycloheximide (100 ng/ml) was added to the cultures 30 min before agonist treatment, to determine whether the amount of substrate protein increased or whether there was an increased kinase (or decreased phosphatase) activity on an existing substrate. As the degree of phosphorylation was unaltered (results not shown), the latter situation appeared to be the case.

Kinetics of TPA-stimulated phosphorylation

A time course of incorporation of ³²P into the three major phosphoproteins was performed over the range 1–60 min after agonist addition. All three proteins increased rapidly in incorporation of label in the first 5 min, reaching a peak in 10–20 min (Fig. 3). As the incorporation of radioactivity increased in a number of proteins during the time course, the increase over basal

Fig. 3. Time course of pp63, pp66 and pp76 phosphorylation

The rate of TPA (10 ng/ml)-stimulated phosphorylation into the major phosphoproteins in B-lymphocytes was examined by adding the agonist to cells pre-labelled with [32 P]P_i for 4 h. Samples for two-dimensional gel analysis of the labelled proteins were taken and processed at the times indicated after the addition of the agonist. Key: ○, pp63; ■, pp66; ●, pp76. Phosphorylation was quantified by densitometer scanning and counting radioactivity of excised gel spots.

was normalized to the autoradiographic intensity of a 65 kDa (pI 4.9) protein that did not seem to vary with any agonist treatment. No other major phosphoproteins were evident during the course of the analysis, although moderate changes in phosphorylation of two peptides (designated 'a' and 'g') are apparent in Figs. 1(a) and 1(b).

Phosphoamino acid analysis

The phosphorylated residues in pp63, pp66 and pp76 were examined by electrophoresis on cellulose of hydrolysed phosphoamino acids. All three proteins were phosphorylated on serine residues only (results not shown).

Effect of Ca²⁺ ionophore on the TPA-induced phosphorylation

The Ca²⁺ ionophore ionomycin at the optimal concentration for mitogenic co-stimulation (0.8 μ g/ml) was added to increasing doses of TPA, and the pre-labelled cells were incubated for a further 20 min. Ionomycin when added alone resulted in a characteristic pattern of phosphorylated proteins, some different from those induced by TPA, but pp63 (e) and pp66 (c) were common to both agonists (Fig. 1c). pp63 (e) and pp66 (c) showed a moderate increase over basal levels when cells were treated with ionomycin alone. When added to ionomycin, doses of TPA between 0.1 and 1 ng/ml caused a further steady rise in the phosphorylation, whereas doses of TPA above this resulted in a more moderate increase, but substantially higher than that with TPA alone (Fig. 4). Neither pp76 nor pp85 was phosphorylated by ionomycin treatment alone, and they did not show an increase when this agonist was added with TPA.

Fig. 4. TPA dose-responses for pp63 and pp66 phosphorylation with increasing [Ca²⁺]

The increase in protein phosphorylation was examined in pp63 (a) and pp66 (b) for increasing doses of TPA, which was co-incubated alone (□) or with (■) 0.8 μ g of the Ca²⁺ ionophore ionomycin/ml. Cells were incubated with combinations of the agonists for the final 10 min of a 4 h 32 P labelling. Lysates were prepared as described in the text for analysis on two-dimensional polyacrylamide-gel electrophoresis. The pp63 and pp66 spots were measured by densitometry and compared with an invariant spot and by scintillation counting of radioactivity of excised gel spots.

Protein phosphorylation induced by cross-linking surface Ig immunoglobulin

Anti-Ig (μ -chain-specific) was added to B-lymphocytes at an optimal activating concentration for 30 s before the processing of the cells for two-dimensional electrophoretic analysis (Fig. 1e). The two-dimensional phosphoprotein pattern showed an increase in a number of spots over the control autoradiograph, but notably the pp63 and pp66 spots were substantially increased. Phosphoproteins in Fig. 1(e) designated 'f' also showed increased incorporation of [32 P]P_i, and those marked 'g' showed a decrease. A number of proteinases and phosphatases are known to be activated by Ca²⁺, and this may explain these decreases.

Immunoblotting of PKC

PKC is known to be autophosphorylated when cells are treated with TPA (Woodgett & Hunter, 1987). It is possible that one of the proteins phosphorylated during B-cell mitogenic stimulation is PKC itself. However, immunolocalization of PKC did not coincide with the major phosphorylated proteins (results not shown).

Determination of ATP specific radioactivity

It is possible, although unlikely, that the increase in phosphorylation of proteins in stimulated cells is due to increased incorporation of ^{32}P into cellular ATP. The specific radioactivity of ATP was measured in stimulated cells at all time points and doses of added agonist, and was found to be the same as the control values at each of these time points (results not shown).

PKC in B-cell cytosol

Since the studies on intact cells presented above are consistent with the involvement of PKC in mediating the activation of protein phosphorylation by several lipid-linked stimuli, the lipid-dependent PKC was characterized in human B-lymphocytes. Extracts from resting B-cells were fractionated and assayed on a Mono Q ion-exchange system with a 0–500 mM-NaCl gradient. Lipid-dependent histone kinase activity was eluted as a peak at 0.18 mM-NaCl, and a second peak of lipid-independent activity was observed at 0.38 mM-NaCl. This latter activity could represent the 50 kDa protein kinase M, the 67 kDa proteolytically cleaved polypeptide that has been immunologically characterized in various rat tissues, or an unrelated kinase that uses histone as a

Fig. 5. Hydroxyapatite chromatographic separation of PKC subspecies

PKC from human B-lymphocytes was prepared as described in the Material and methods section. Peak activity fractions from a Mono Q ion-exchange column were pooled and run on a hydroxyapatite column attached to an f.p.l.c. system. The thin continuous line is the relative protein concentration, and the heavier line is the PKC activity. The broken line shows the concentration of NaCl used to elute the protein. The peaks are designated β and α , in accordance with the nomenclature of Nishizuka (1988).

Fig. 6. Activation of human B-lymphocyte PKC subtypes

The standard PKC assay, where the transfer of ^{32}P is measured from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to histone substrate, was modified to use variable doses of TPA together with two concentrations of Ca^{2+} as co-activators of the enzyme. Increasing doses of TPA were added to the two subtypes of PKC together with Ca^{2+} concentrations of $0.1\ \mu\text{M}$ ($\circ$, $\square$) or $2\ \mu\text{M}$ ($\bullet$, $\blacksquare$). Data shown are means of triplicate experiments and are representative of two separate experiments.

substrate under the conditions of the assay. The lipid-dependent activity was further fractionated on a hydroxyapatite column, and was eluted as two distinct peaks (Fig. 5), as opposed to the three peaks seen with rat brain (results not shown). These two fractions were assayed at Ca^{2+} concentrations of $0.1\ \mu\text{M}$ and $2\ \mu\text{M}$, which represent the intracellular concentrations of Ca^{2+} previously seen in antibody-stimulated B-cells (G. R. Guy, unpublished work).

The data in Fig. 6 show that the addition of Ca^{2+} at higher concentrations shifts the TPA dose-response curve for the activation of both forms of PKC to the left. This gives a TPA (+ionophore or Ca^{2+}) activation profile for PKC which is qualitatively similar to that for the increased phosphorylation of pp63 (see Fig. 4) and the enhancement of DNA synthesis in whole cells (Guy *et al.*, 1985).

DISCUSSION

The mechanism controlling eukaryotic-cell proliferation is among the major problems in biology that remain poorly understood. A variety of polypeptide growth factors (e.g. epidermal, platelet-derived, fibroblast-derived etc.) have been shown to play a crucial role in the regulation of cell division. Quiescent G_0/G_1 -arrested cells recommence DNA synthesis and enter mitosis when exposed to these growth factors. Among the variety of changes induced upon addition of these growth factors to quiescent cells are changes in phosphorylation of cellular proteins, which occur extremely rapidly. Such studies also draw attention to the kinase and/or phosphatase enzymes responsible for the degree of phosphorylation of these proteins and thus the possible transfer of the proliferative signal to the cell genome.

The present study has demonstrated that a range of phosphoproteins are the substrate for endogenous kinases during the early stages of the proliferative response in the human B-lymphocyte. Most previous analyses had used polyclonal stimulators on heterogeneous lymphocyte populations. Several other studies, using different cell types (see below), have described the stimulated phosphorylation of similar phosphoproteins, some of which were also substrates for PKC, but as yet few of these phosphoproteins have been identified. It is noteworthy that three of the phosphoproteins that have been assigned a function are involved in the phosphatidylinositol pathway. A platelet protein of 40 kDa first described by Nishizuka's group (Nishizuka, 1984) was later purified (Connolly *et al.*, 1985) and then identified by Connolly *et al.* (1986) as the InsP_3 5-phosphomonoesterase that hydrolyses $\text{Ins}(1,4,5)\text{P}_3$ and thus abolishes the Ca^{2+} -mobilization period in stimulated cells. Tyers *et al.* (1988), however, have shown that p45 and the phosphomonoesterase activity can be separated. Another protein, variously known as B-50, GAP 43 or F-1, has been purified by several groups (Zwiers *et al.*, 1980; Skene, 1984; Chan *et al.*, 1986) and is thought to modulate the activity of $\text{PtdIns}4\text{P}$ kinase (Van Dongen *et al.*, 1985). A third protein, an 85 kDa phosphoprotein observed in 3T3 cells stimulated by platelet-derived growth factor or transformed by polyoma middle-T antigen, is phosphorylated on tyrosine residues, and immunoprecipitates show enhanced PtdIns kinase activity (Kaplan *et al.*, 1987).

A protein (or proteins) in the 80–87 kDa range has been seen as a common kinase substrate in cells stimulated with phorbol esters or synthetic diacylglycerol analogues that are active as PKC stimulators (Albert *et al.*, 1987). Several groups have recently purified this polypeptide from bovine brain (Albert *et al.*, 1987) and found it to be an extremely elongated monomer which is phosphorylated on serine residues by purified PKC. The 87 kDa polypeptide was first described as a component of rat brain synaptosomes (Wu *et al.*, 1982), where increased phosphorylation was induced by phorbol esters, acetylcholine or by depolarization-induced Ca^{2+} influx (Wu *et al.*, 1982). The 87 kDa protein has been previously demonstrated to be phosphorylated in lymphocytes in response to treatment with concanavalin A or Ca^{2+} ionophores (Denis *et al.*, 1986). There was, however, no Ca^{2+} -stimulated increase of pp85 in this study.

The two major phosphoproteins that become highly labelled during B-cell activation also fit the descriptions given by other workers in different signal-transduction systems. Ishii *et al.* (1987) noted stimulation of phosphoproteins of 63 and 67 kDa in primary T-lymphocytes and derived cell lines when these were activated by TPA or interleukin-2. These are possibly the 63 and 66 kDa proteins described in the present work. Four novel 63 kDa proteins with pI values ranging from 5.3 to 6.1 were phosphorylated strongly after TPA treatment, and to a lesser degree by interleukin-2. Very similar phosphoproteins were previously detected in rodent fibroblasts stimulated with a variety of growth factors (Kohno, 1985; Kohno & Pouyssegur, 1986). Pocotte *et al.* (1985) also noted a moderate increase in phosphorylation of a swarm of pp63 proteins on two-dimensional gels of TPA-stimulated adrenal-medullary cultured cells. Ishii *et al.* (1987) postulated that proteins like pp63s, which are common to fibroblasts and lymphocytes, could contribute to intracellular signal transmission induced by various growth factors.

While this manuscript was being prepared, a paper by Hornbeck *et al.* (1988) described a protein of approx. 67 kDa that was phosphorylated in human B-cells activated by TPA. This protein was identified by immunanalysis as lamin B, which runs at a similar location to pp66 shown in the autoradiographs in Fig. 1. It was also interesting that lamin B was also a substrate of protein kinase M, which has been previously shown to be produced in cells at TPA concentrations above 10 ng/ml. Work is now needed to identify positively pp66 (pp67) and the other major substrates to ascertain their role in mitogenesis.

We have shown here, however, that there is a link between signal transduction involving inositol-lipid hydrolysis, protein kinase C activation, phosphorylation of specific proteins (pp63 and pp66) and mitogenesis in human B lymphocytes. Whether any of the proteins that show increased phosphorylation are themselves involved in inositol phospholipid metabolism awaits further investigation.

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