

# Mitogen treatment of permeabilized human T lymphocytes stimulates rapid tyrosine and serine phosphorylation of a 42 kDa protein

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Three agents which are mitogenic for T lymphocytes (phytohaemagglutinin, monoclonal antibody UCHT 1 and 12-*O*-tetradecanoylphorbol-13-acetate) stimulated rapid phosphorylation of a 42 kDa protein in permeabilized T lymphocytes. Phosphorylation occurred on tyrosine and serine residues. A non-mitogenic monoclonal antibody (RFT11) did not stimulate phosphorylation of this protein. Furthermore, the dose response of 42 kDa protein phosphorylation and of mitogenesis to increasing amounts of phytohaemagglutinin were closely similar. We therefore propose that mitogen-stimulated phosphorylation of the 42 kDa protein is part of the mechanism for transduction of mitogenic signals in lymphocytes. To our knowledge, this is the first report of rapid, ligand-stimulated tyrosine protein phosphorylation in T lymphocytes.

*T lymphocyte    Tyrosine protein kinase    Protein kinase C    Mitogenesis*

## 1. INTRODUCTION

The antigen receptor of T lymphocytes consists of two clone-specific disulfide-linked transmembrane glycoprotein chains,  $\alpha$  and  $\beta$  [1]. In vivo, the binding of antigens (in association with products of the major histocompatibility complex genes) triggers mitotic activation of specific T lymphocyte clones [2]. The receptor-associated T3 complex, consisting of three monomorphic transmembrane polypeptides,  $\gamma$ ,  $\delta$  and  $\epsilon$  [3], is also important in the transduction of mitogenic signals from the antigen receptor to the cell [4]. In vitro, the receptor-binding lectin PHA or the monoclonal antibody UCHT 1, which binds the T3 complex, are strongly mitogenic for T lymphocytes [5,6]. The phorbol

ester TPA, which stimulates protein kinase C directly [7], is mitogenic for T lymphocytes, when added together with  $\text{Ca}^{2+}$  ionophores [8].

The cell surface receptors for several growth factors (e.g. platelet-derived and epidermal growth factors, insulin) possess cytoplasmic domains with tyrosine protein kinase activity. Addition of the growth factor to membrane preparations carrying the appropriate receptor stimulates the tyrosine kinase activity [9–11]. The importance of tyrosine phosphorylation in the regulation of proliferation is also indicated by the observation that an increase in cellular phosphotyrosine content caused by inhibition of phosphotyrosine phosphatases is mitogenic for fibroblasts [12]. It is therefore believed that phosphorylation of proteins on tyrosine residues is a key event in mitogenic signal transduction [13]. Furthermore, tyrosine kinase-containing growth factor receptors are down-regulated following signal transduction [14–16]. Although the cytoplasmic domains of the T cell receptor and T3 complex proteins are too small to

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**Abbreviations:** PHA, phytohaemagglutinin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

contain kinase activity [1,3], we have observed that a novel 38 kDa tyrosine protein kinase found in the membrane fraction of quiescent T lymphocytes is down-regulated following PHA-stimulated mitogenesis [17], suggesting that tyrosine phosphorylation may nevertheless play a role in mitotic signalling in lymphocytes. We therefore sought evidence for tyrosine phosphorylation of proteins in response to stimulation of lymphocytes by mitogens. The use of permeabilized cells has proved useful in the detection of mitogen-stimulated protein phosphorylation [18,19] and has advantages over the use of isolated membranes, since this system allows the detection of mitogen-stimulated events which are dependent on interactions between cytoplasmic and membrane-bound proteins [18]. We have therefore used permeabilized lymphocytes and the results show that three different mitogens stimulate the phosphorylation of a 42 kDa protein on both tyrosine and serine residues.

## 2. MATERIALS AND METHODS

PHA was from Wellcome Reagents, and TPA from Sigma. Monoclonal antibody UCHT 1 was kindly given by Drs Peter Beverley and Kieran O'Flynn and RFT11 by Professor George Janossy and Dr Dario Campana.

Quiescent lymphocytes were obtained from the peripheral blood of healthy volunteers and freed of monocytes and platelets. Protein phosphorylation was detected by permeabilizing the lymphocytes with a brief treatment with lyssolecithin followed by incubation with [ $\gamma$ - $^{32}$ P]ATP in the buffer system detailed elsewhere [18]. Following labelling (usually for 3 min) cells were fractionated into Nonidet P-40 soluble and insoluble fractions and analyzed by SDS-PAGE under reducing conditions. Phosphorylated proteins were identified by autoradiography of dried gels. These procedures have been previously described in detail [18]. Autoradiograms were scanned using a Joyce-Loebl Chromoscan equipped with an electronic integration facility.

Phosphoamino acid analysis was carried out by hydrolyzing individual labelled protein bands in 6 N HCl followed by high voltage paper electrophoresis in 7% formic acid, pH 1.7 [20].

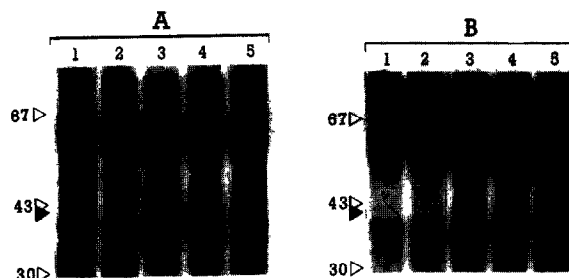


Fig.1. Mitogen stimulated phosphorylation of a 42 kDa protein in permeabilized lymphocytes. Lymphocytes were permeabilized and labelled with [ $\gamma$ - $^{32}$ P]ATP. Nonidet P-40 soluble (A) and insoluble (B) fractions were analyzed by SDS-PAGE and autoradiography. Lanes: 1, no additions; 2, plus 2  $\mu$ l RFT11; 3, plus 1  $\mu$ l PHA; 4, plus 5  $\mu$ l TPA (1  $\mu$ M); 5, plus 2  $\mu$ l UCHT 1. Figures indicate molecular masses of marker proteins in kDa. The filled triangle indicates the position of the 42 kDa protein.

## 3. RESULTS

Permeabilized lymphocytes were labelled with [ $\gamma$ - $^{32}$ P]ATP in the presence or absence of PHA and the Nonidet P-40 soluble protein fraction analyzed

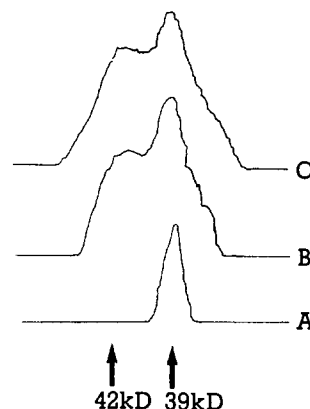


Fig.2. Time course of PHA-stimulated phosphorylation of the 42 kDa protein. Permeabilized lymphocytes were labelled with [ $\gamma$ - $^{32}$ P]ATP. Curves: A, no addition; B, 1  $\mu$ l, PHA added, 2 min incubation; C, 1  $\mu$ l PHA added, 5 min incubation. The Nonidet P-40 soluble fraction was analyzed by gel electrophoresis. Densitometric scans of the relevant region of the autoradiograms are shown. Molecular masses of the bands are shown in kDa.

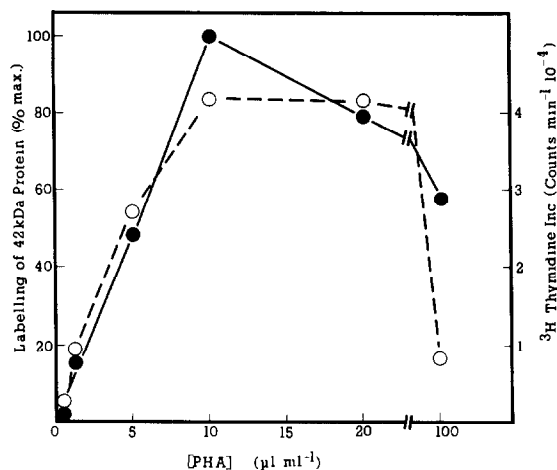


Fig.3. Dose response of the 42 kDa protein phosphorylation and [<sup>3</sup>H]thymidine incorporation to increasing concentrations of PHA. Permeabilized lymphocytes were labelled with [ $\gamma$ -<sup>32</sup>P]ATP in the presence of increasing concentrations of PHA. Nonidet P-40 soluble fractions were analyzed by gel electrophoresis. Autoradiograms were scanned, and areas under the 42 kDa peak assessed by integration (●). The mitogenic response of lymphocytes to increasing concentration of PHA was estimated by measuring the incorporation of [<sup>3</sup>H]thymidine 3 days following mitogen addition (○) (mean of triplicate determinations).

by SDS-PAGE. The phosphorylation of a 42 kDa protein was markedly stimulated by the addition of PHA (fig.1A, lanes 1 and 3). Two other agents which are also mitogenic for T lymphocytes, the monoclonal antibody UCHT 1 and the phorbol

ester TPA, also stimulated the phosphorylation of a 42 kDa protein (fig.1A, lanes 4 and 5). Identical results were obtained in fourteen consecutive experiments. By contrast, the non-mitogenic monoclonal antibody RFT11 (which binds the T11 glycoprotein on the lymphocyte surface) did not stimulate 42 kDa protein phosphorylation (fig.1A, lane 2).

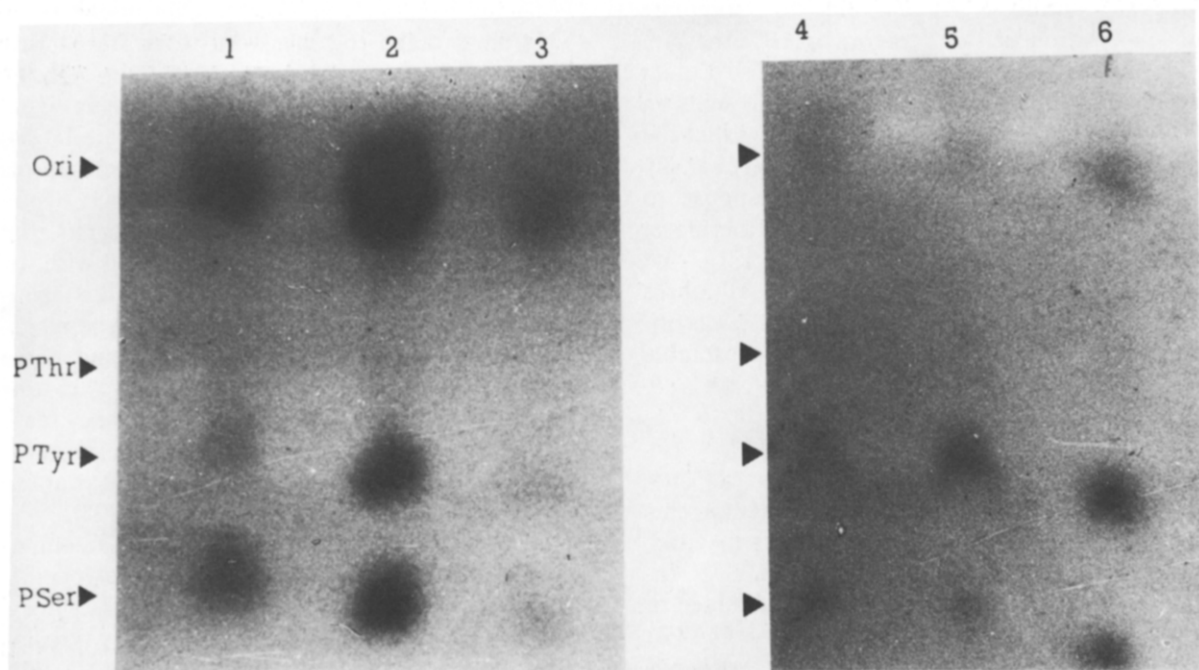


Fig.4. Phosphoamino acid analysis of 42 kDa bands. Individual 42 kDa bands from Nonidet P-40 soluble (lanes 1–3) and insoluble (lanes 4–6) lymphocyte fractions were excised from gels, and the composition of labelled phosphoamino acids was analyzed as described in section 2. Ori, origin; PThr, phosphothreonine; PTyr, phosphotyrosine; PSer, phosphoserine. Lanes: 1,4, plus 1 µl PHA; 2,5, plus 5 µl TPA (1 µM); 3,6, plus 2 µl UCHT 1.

Stimulation of the 42 kDa protein phosphorylation by PHA was rapid, being maximal within 2 min of addition of PHA (fig.2). Furthermore, the dose response of PHA-stimulated phosphorylation closely paralleled the mitogenic response of intact lymphocytes to PHA, as measured by the incorporation of [ $^3$ H]thymidine into DNA (fig.3), strongly suggesting that this phosphorylation event was an important part of the mitogenic signalling mechanism initiated by PHA. In an attempt to characterize the 42 kDa protein, we initially studied its subcellular localization. Although this phosphorylated protein was initially detected in the Nonidet P-40 soluble fraction of labelled lymphocytes (fig.1A), the phosphorylation of this protein was also clearly observed by electrophoretic analysis of Nonidet P-40 resistant cytoskeletal material from permeabilized cells which were labelled in the presence of PHA, UCHT 1 or TPA (fig.1B, lanes 1,3,4 and 5). This suggested that the 42 kDa protein was at least partially associated with the detergent resistant cytoskeletal material of the cell. The non-mitogenic antibody RFT11 did not stimulate phosphorylation of this protein (fig.1B, lane 2).

We further characterized the 42 kDa protein phosphorylation by identifying the amino acids on which it was phosphorylated. Fig.4 shows that the protein was phosphorylated approximately equally on tyrosine and serine residues in response to PHA, UCHT 1 or TPA stimulation. Furthermore, the identical amino acid specificity of phosphorylation was observed with all three mitogens and whether the protein analyzed was obtained from either the Nonidet soluble or insoluble cell fraction (fig.4).

#### 4. DISCUSSION

The results here show that three different agents which are mitogenic for T lymphocytes *in vitro*, stimulated rapid phosphorylation of a 42 kDa protein on tyrosine and serine residues when added to permeabilized cells in the presence of [ $\gamma$ - $^{32}$ P]ATP. We suggest that this phosphorylation event is important in transduction of mitogenic signals, since it was elicited by three mitogens of completely different classes (a lectin, a monoclonal antibody and a phorbol ester) but not by the non-mitogenic monoclonal antibody RFT11. The similarity of the

dose-response curves for PHA-stimulated protein phosphorylation and for PHA-stimulated mitogenesis reinforces this conclusion.

Rapid tyrosine phosphorylation of 42 kDa proteins in response to treatment of mouse [21,22], chick [23,24] and human [24] fibroblasts by a variety of growth stimulatory agents has been reported. Although direct evidence implicating this phosphorylation in the mitogenic response has not been obtained, the observation that this phosphorylation was elicited by different natural growth factors and other mitogenic agents suggests that tyrosine phosphorylation of the 42 kDa protein plays an important role in mitosis [22]. We are currently attempting to characterize the 42 kDa tyrosine phosphorylated protein of lymphocytes in order to evaluate its relationship to the 42 kDa proteins described in fibroblasts.

We have recently employed a quantitative immunoassay using an antiphosphotyrosine antibody to demonstrate that the content of phosphotyrosine in cellular proteins increased three-fold when intact lymphocytes were stimulated with PHA for 10 min (Hall, B. et al., *in preparation*). This data, taken together with work from other laboratories which suggest that protein tyrosine phosphorylation is a key event in mitogenic signal transduction in non-lymphoid cells [9-13,21-24] strongly support the view that similar mechanisms operate in T lymphocytes.

Subcellular distribution studies indicated that the 42 kDa protein is partially associated with the detergent-resistant cytoskeleton of the lymphocyte. It is therefore of interest that the receptors for epidermal growth factor [25] and nerve growth factor [26] are also associated with this structure. Furthermore the intrinsic tyrosine kinase activity of the epidermal growth factor receptor was shown to phosphorylate cytoskeletal proteins in a ligand-stimulated reaction *in vitro* [25].

The 42 kDa protein is unlikely to be a component of the antigen receptor, since the polypeptide chains of the receptor have molecular masses in excess of 50 kDa [1] and do not contain any tyrosine residues in their cytoplasmic domains [27]. The polypeptide chains of the T3 complex are also ruled out, since their molecular masses are below 30 kDa [1]. Therefore, the 42 kDa protein may be a novel component of the T lymphocyte mitogenic signalling mechanism.

Although the experiments described here point strongly to a crucial role for tyrosine phosphorylation in mitogenic signalling in lymphocytes, we are uncertain as to the identity of the enzyme catalyzing this event. We have previously observed that a 38 kDa membrane-bound tyrosine kinase found in quiescent T lymphocytes is down-regulated following PHA-stimulated entry into the cell cycle [17]. Separation of cell extracts by gel filtration has shown that this enzyme is the predominant tyrosine kinase in lymphocytes (Hall, B. et al., in preparation). However, we and others have also described a 55–60 kDa tyrosine kinase in particulate fractions from normal [17,28] and malignant [29] lymphoid cells. The cDNA encoding this enzyme has been cloned and its gene has been shown to be preferentially expressed in lymphoid tissues [30]. Although the level of the 55–60 kDa enzyme did not appear to vary during the lymphocyte cell cycle [17] we cannot exclude a role for this kinase in catalyzing the mitogen-stimulated phosphorylation described here.

Since TPA activates protein kinase C directly [7], stimulation of tyrosine phosphorylation by TPA suggests that initial stimulation of protein kinase C activates a tyrosine kinase which phosphorylates in turn the 42 kDa protein. (Kinase C itself does not phosphorylate tyrosine residues [7].) TPA, which is mitogenic for fibroblasts, also stimulated tyrosine phosphorylation of a 42 kDa protein in these cells [22,24,30]. TPA treatment of the human promyelocytic leukaemia cell line HL60, which stimulates differentiation to macrophages, also stimulated tyrosine phosphorylation of a 42 kDa protein [31]. These authors have also proposed a mechanism whereby activation of kinase C activates in turn a tyrosine kinase [22,31]. However, as in the present work, the identity of the tyrosine kinase involved and the mechanism of its activation by kinase C remain unknown.

A role for kinase C in T lymphocyte mitogenesis is also strongly supported by the observation that stimulation of T lymphocytes by PHA causes the rapid breakdown of inositol lipids [32], with the generation of diacylglycerol, the physiological activator of kinase C [7]. Furthermore, stimulation of T lymphocytes by either PHA or anti-T3 monoclonals activates kinase C by stimulating its rapid translocation to the membrane fraction

[33,34]. The observations described here, together with the additional evidence for the involvement of kinase C in the mitogenic response of T lymphocytes, suggest that a cascade of biochemical events, initiated by the breakdown of inositol-containing lipids and involving subsequent protein phosphorylation events, may be important in the activation of T lymphocytes. However, inositol lipid breakdown also generates inositol 1,4,5-trisphosphate [7] which causes an increase in cytoplasmic  $\text{Ca}^{2+}$  [35] by stimulating release of  $\text{Ca}^{2+}$  from intracellular stores [36]. This increase in cytoplasmic  $\text{Ca}^{2+}$  is also an essential component of the mitogenic response of lymphocytes [37]. The manner in which  $\text{Ca}^{2+}$ -mediated events and protein phosphorylation events synergize to secure commitment to mitosis remain unclear. The identification of tyrosine protein kinases and their substrates which are phosphorylated in response to mitogen stimulation will contribute to the unravelling of the complex pathways of mitogenesis in T lymphocytes.

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