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Two major tyrosine protein kinases of resting human T lymphocytes are down-regulated following mitotic stimulation

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Human lymphocyte tyrosine protein kinases (TPKs) have been analyzed by gel-filtration chromatography. The major TPK species with activity towards an exogenous tyrosine-containing peptide had molecular masses of 70–100 kDa (TPK I) and 35–40 kDa (TPK II). TPKs I and II were distinct from the well-characterized autophosphorylating lymphoid cell TPK, pp56^{lck} [(1983) J. Biol. Chem. 258, 10738–10742]. Both TPK I and TPK II were down-regulated following mitogenic stimulation of lymphocytes with phytohaemagglutinin. By contrast, pp56^{lck} remained clearly detectable in stimulated lymphocytes. We suggest that TPKs I and II may play a role in the regulation of the lymphocyte cell cycle.

Tyrosine protein kinase; Lymphocyte mitogenesis

1. INTRODUCTION

TPK activity was originally discovered in the products of viral oncogenes, but is now implicated in the control of normal as well as malignant cell proliferation [1]. The receptors for several growth factors and hormones, including platelet-derived and epidermal growth factors and insulin, are TPKs whose activity is regulated by the binding of their respective ligands [1]. However, little is known of the pathways linking changes in membrane TPK activity to changes in cell behaviour.

Peripheral blood lymphocytes have a high level of TPK activity [2,3], which decreases following mitogenic activation by the lectin phytohaemagglutinin (PHA) [4], a polyclonal mitogen which binds the T cell antigen-receptor complex [5,6]. Although the known components of the receptor are not TPKs, mitogenic stimulation of murine [7] or human [8,9] T cells causes rapid tyrosine phosphorylation of specific proteins.

The enzymes responsible for mitogen-stimulated tyrosine phosphorylation in lymphocytes have not been identified. The major autophosphorylating kinase, pp56^{lck}, a lymphocyte-specific protein closely related to pp60$^{c\text{-}src}$, has been extensively studied [10–13] and its cDNA has been cloned and sequenced [14]. Recently 35 and 40 kDa TPKs from rat spleen [15] and a 40 kDa soluble TPK from bovine thymus [16] have been partially or completely purified, but it has not been established whether they bear any relationship to pp56^{lck} or play a role in the transduction of mitotic signals. We therefore attempted to identify the T lymphocyte TPKs whose activities vary during the PHA-stimulated cell cycle. We show that two

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Abbreviations: TPK, tyrosine protein kinase; FPLC, fast protein liquid chromatography; RRsrc, synthetic peptide of sequence RRLIGEDAEYAARE; PMSF, phenylmethylsulphonyl fluoride; EGF, epidermal growth factor; PDGF, platelet-derived growth factor

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highly labile TPKs of 70–100 and 35–40 kDa respectively, detected by their ability to phosphorylate a synthetic tyrosine-containing peptide [11], are the major TPKs in lymphocyte particulate fractions and are distinct from $pp56^{lck}$. Both these enzymes are almost completely down-regulated when resting cells are stimulated to enter the cell cycle. By contrast, we could not detect a change in the level of $pp56^{lck}$.

2. MATERIALS AND METHODS

2.1. *Lymphocyte isolation and culture*

Lymphocytes were isolated from the blood of healthy volunteers by sedimentation on a Lymphoprep gradient (Nyegaard, Oslo) and were freed of platelets and monocytes [4,8]. The cells were either lysed immediately or cultured at 37°C for 72 h with 1% PHA (Wellcome, Beckenham) at 10^6 cells/ml in RPMI medium (Gibco) containing 8% fetal calf serum and antibiotics.

2.2. *Enzyme extraction*

Preparation of the particulate fraction was carried out as in [4] except that 1 mM PMSF and 1% aprotinin were included in all stages. TPK activity was extracted with 10 mM glycine-NaOH, pH 9.1, 1 mM EDTA, 20 mM NaCl, 1% Triton X-100, 30% glycerol, 20% sucrose, 5 mM 2-mercaptoethanol, 1 mM PMSF and 1% aprotinin. 80% of the particulate TPK activity was solubilized in this manner.

Zn^{2+}-dependent phosphatases were removed from the protein extract by affinity chromatography with Zn^{2+}-bound aminodiacetic acid Sepharose 6B [17]. The resulting solution was applied to an FPLC Superose 12 gel-filtration column (Pharmacia) and eluted at a rate of 0.3 ml/min with 20 mM Tris-Cl (pH 8), 1 mM EDTA, 20 mM NaCl, 20% glycerol (w/v), 1% Triton X-100 (w/v) and 5 mM 2-mercaptoethanol. Fractions of 0.5 ml were collected and 1 mg/ml BSA was added immediately to stabilize the highly labile TPK activity.

2.3. *Enzyme assays*

TPK activity was quantitated [4] by measuring the incorporation of label from [γ-^{32}P]ATP into the synthetic peptide RRsrc [11]. Autophosphorylation reactions were carried out by incubation of enzyme fractions with [γ-^{32}P]ATP in the absence of exogenous substrates. Proteins phosphorylated on tyrosine residues were identified by analysis of reaction mixtures on 8.5% polyacrylamide gels followed by hydrolysis in 1 N KOH and autoradiography [3,4].

3. RESULTS

3.1. *Chromatographic resolution of TPKs in resting lymphocytes*

Extracts of particulate fractions from resting T lymphocytes were freed from Zn^{2+}-dependent tyrosine protein phosphatases by affinity chromatography and then fractionated by gel filtration and the fractions assayed for TPK activity using RRsrc as substrate. Two major peaks were detected, with apparent molecular masses in the range 70–100 kDa (TPK I) and 30–40 kDa (TPK II) (fig.1, open triangles). TPK I showed an apparent K_m for RRsrc of 0.77 mM and TPK II a K_m of 1.56 mM. The apparent K_m values for ATP were 80 and 16 μM, respectively, for TPK I and II (not shown). Attempts at further purification and characterisation of TPKs I and II have been hindered by the rapid loss of activity on storage or further chromatography. T lymphocyte TPK II has a similar molecular mass to the 40 kDa TPK isolated from bovine thymus [16], and both enzymes are extremely labile suggesting that they may be related. However, the thymus TPK is a soluble enzyme where TPK II is particulate.

3.2. *Relationship of TPKs I and II to $pp56^{lck}$*

$pp56^{lck}$ is readily detected in particulate fractions of lymphocytes by its ability to autophosphorylate on tyrosine residues [10–13]. We therefore assayed the Superose 12 fractions for $pp56^{lck}$ by incubating them with [γ-^{32}P]ATP. Proteins phosphorylated on tyrosine residues were detected by gel electrophoresis, followed by incubation of the gel in alkali in order to eliminate phosphate esterified on serine residues. We have established by acid hydrolysis followed by electrophoretic analysis that autophosphorylating lymphocyte proteins detected in this manner are predominantly phosphorylated on tyrosine residues [3,4]. $pp56^{lck}$ eluted between 12 and 13 ml (fig.2). Comparison with fig.1 shows that $pp56^{lck}$ eluted between the peaks of TPK I and II strongly

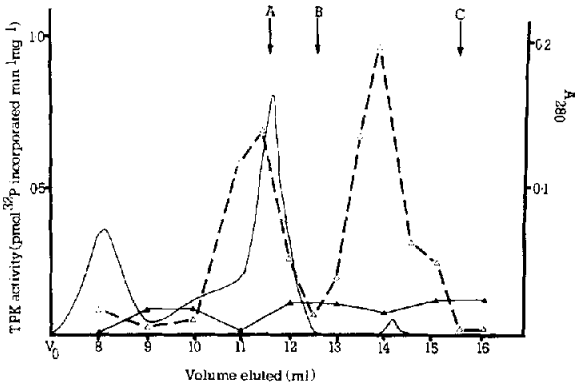

Fig.1. Superose 12 elution profile of solubilized particulate TPK activity from untreated (Δ---Δ) and PHA treated (▲——▲) lymphocytes. The activity shown is the rate of $^{32}PO_4$ incorporation into RRsrc per mg protein loaded. Each point represents the average of duplicate assays. The unbroken line is a typical protein elution profile. A similar pattern was obtained for both PHA-treated and untreated lymphocytes. Arrows indicate elution positions of molecular weight markers: A, bovine serum albumin, 67 kDa; B, ovalbumin, 43 kDa; C, chymotrypsinogen, 25 kDa.

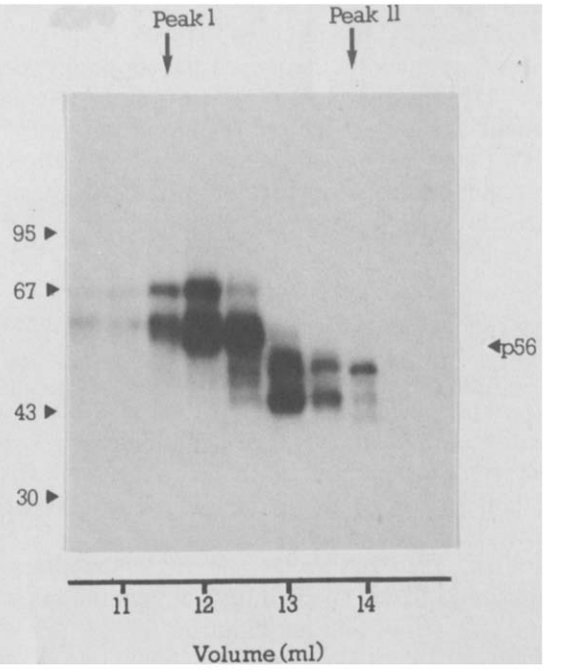

Fig.2. In vitro autophosphorylation of Superose 12 column fractions from untreated lymphocyte particulate fraction separated by SDS-PAGE (see section 2 for details). Arrows on the left indicate molecular masses, those on the right denote apparent molecular masses of labelled proteins. The peak fractions for TPK I and II are indicated.

suggested that this enzyme, detected by its ability to autophosphorylate, was a separate entity from the two TPKs detected by their ability to phosphorylate an exogenous tyrosine-containing peptide. The $pp56^{lck}$ of normal lymphocytes appeared to have a relatively low reactivity towards RRsrc.

No tyrosine-phosphorylated protein band corresponding to TPK I was detected (fig.2, lanes 2,3), suggesting that this enzyme either did not autophosphorylate or did so at a level too low to be detectable. A number of phosphorylated protein bands were seen in the fractions corresponding to TPK II. However, we cannot at present be certain whether any of these bands corresponds to autophosphorylated TPK II, due to the promiscuity of TPKs in the phosphorylation of protein substrates.

3.3. *TPK I and II are down-regulated following PHA stimulation of lymphocytes*

Extracts of particulate fractions prepared from lymphocytes 3 days following PHA-stimulated mitogenesis were analyzed by Superose 12 column chromatography. Assay of the column fractions with RRsrc showed that both TPK I and II were almost undetectable (fig.1, solid triangles). By contrast, $pp56^{lck}$, revealed by autophosphorylation, was readily detectable (fig.3) at intensities comparable to those in fractions from resting lymphocytes (fig.2). Therefore, we conclude that both TPK I and TPK II were down-regulated following mitotic stimulation of T lymphocytes whereas $pp56^{lck}$ was unaffected. This latter conclusion is in agreement with our previous observations on $pp56^{lck}$ autophosphorylation in unfractionated lymphocyte particulate preparations [4]. We stress that the down-regulation of TPK I and II was strictly a consequence of PHA stimulation, since incubation of lymphocytes in culture medium for 3 days resulted in an increase in particulate TPK activity [4]. In addition, down-regulation of particulate TPK following PHA treatment is not due to translocation of the enzyme to another cellular compartment [4]. Assays of the Superose column

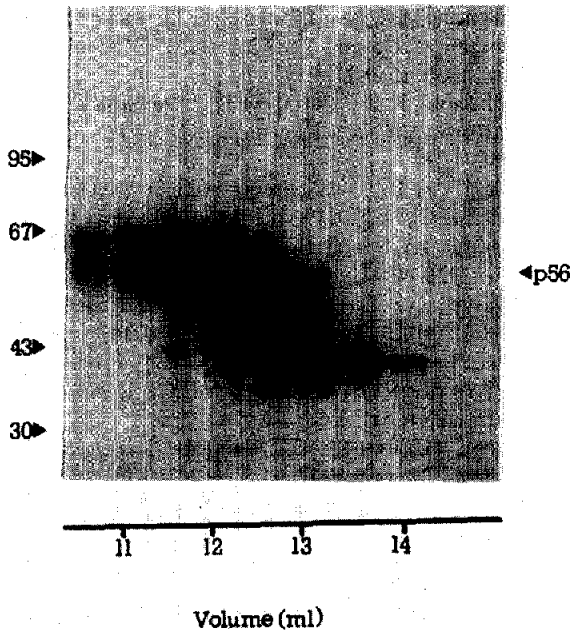

Fig.3. In vitro autophosphorylation of Superose 12 column fractions from PHA treated lymphocyte particulate fractions (see legend to fig.2 for details).

fractions showed that the observed decrease in activity of TPK I and II could not be explained by an increase in either ATPase or phosphatase activity, which could potentially have interfered with the TPK assay (not shown).

4. DISCUSSION

Growing evidence suggests that the phosphorylation of proteins on tyrosine residues contributes to the process of mitotic signal transduction in T lymphocytes [7–9]. We therefore attempted to identify TPK species in lymphocytes whose activity varied during the cell cycle. Following gel filtration of cell extracts we have identified two peaks of TPK activity, TPK I and II, which eluted in different fractions from the well-characterized $pp56^{lck}$ [10–14].

Clarification of the relationship between TPK I and II and their biochemical characterization have been frustrated by the extreme lability and low yield of these activities, which are lost completely on overnight storage. However, we have observed a 2-fold difference in their apparent K_m values towards RRsrc and a 5-fold difference in the K_m towards ATP. However, we cannot conclude that they are distinct species, since TPK I may be a dimer of TPK II, with consequently altered kinetic parameters. It is unlikely that TPK II is a proteolytic degradation product of TPK I, since protease inhibitors were used during enzyme extraction.

TPKs I and II were down-regulated following mitotic stimulation of lymphocytes, whereas $pp56^{lck}$ was not. $pp56^{lck}$ is a member of a closely related family of TPKs which have strong sequence homology to c-src, the cellular homologue of the transforming gene of Rous sarcoma virus [14,18,19]. The expression of several members of this TPK family (c-src, hck) in post-mitotic cells has led to the suggestion that these may be involved in maintenance of the differentiated state of cells rather than in mitotic signalling [18,19]. Given the strong sequence homology of $pp56^{lck}$ the products of c-src and hck [14,18,19] and the invariance of $pp56^{lck}$ during the lymphocyte cell cycle, we suggest that $pp56^{lck}$ may also be involved in maintaining the differentiated state of lymphocytes rather than in the control of proliferation.

By contrast, the down-regulation of TPK I and II following mitotic activation is reminiscent of the loss of the TPK-containing receptors for EGF [20] and PDGF [21] following stimulation with the appropriate growth factor. Ligand-stimulated down-regulation is not of itself proof of involvement of TPK in regulation of the cell proliferation. However, taken with the evidence for involvement of tyrosine phosphorylation in lymphocyte mitotic signalling [7–9], down-regulation of TPK I and II prompts us to suggest that they may be candidates for a role in transduction of the PHA signal. We note, however, that lymphocyte TPK activity is lost between 24 and 48 h following activation of T lymphocytes [4], whereas the EGF and PDGF receptors are lost within hours of mitogen stimulation of fibroblasts [20,21]. However, the G_1 phase of the lymphocyte has a duration of 1–2 days, whereas that of fibroblasts is only several hours long. Therefore, the longer half-life for down-regulation of lymphocyte TPK [4] may be related to the extended time scale of pre-replicative events in these cells.

By contrast with the receptors for EGF and

PDGF, which include intrinsic ligand-stimulated TPK activity [1], TPKs I and II are not an integral part of the known components of the T cell receptor or its associated CD3 complex, since the cytoplasmic domains of these proteins are too small to include an enzymic activity [22]. Mitogen stimulation of T lymphocytes triggers rapid breakdown of inositol-containing lipids [23] and the consequent generation of diacylglycerol [24] activates the serine/threonine-specific protein kinase C, which is rapidly translocated from the cytosol to the membrane fraction [25,26]. Furthermore, tyrosine phosphorylation of the same 42 kDa protein is elicited both by PHA or TPA treatment of lymphocytes [8]. Since TPA activates kinase C directly [24], we reasoned that activation of lymphocyte TPKs by PHA occurs indirectly as a consequence of inositol lipid breakdown and activation of kinase C [8]. Clarification of the details of this pathway are the subject of continuing investigations.

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