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MULTIPLE TYROSINE PROTEIN KINASES STRUCTURALLY RELATED TO $p56^{lck}$ ARE DOWN-REGULATED FOLLOWING MITOGENIC STIMULATION OF HUMAN T LYMPHOCYTES

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Summary: $p56^{lck}$ is a well-characterized tyrosine protein kinase (TPK) which is thought to play a role in mitogenic signal transduction in T lymphocytes. Immunoblot analysis of human lymphocyte proteins using an antiserum cross-reactive with phosphotyrosine resulted in the detection of a 55-60kDa protein band (presumably $p56^{lck}$) as well as several additional phosphotyrosyl proteins in lymphocyte extracts. All of these phosphotyrosyl proteins were down-regulated following mitotic stimulation. Autophosphorylation of lymphocyte microsomal fractions in the presence of [γ-^{32}P] ATP resulted in the labelling of $p56^{lck}$ as well as other proteins of different molecular weights. Analysis of these labelled proteins by tryptic digestion resulted in strikingly similar peptide maps. The data suggest that lymphocytes may contain a family of TPKs structurally related to $p56^{lck}$. The down-regulation of the putative TPKs following mitogenic stimulation of lymphocytes with phytohaemagglutinin suggests that this family of TPKs may participate in mitotic signalling events, followed by their down-regulation.

Tyrosine protein kinases (TPKs) are key elements in the regulation of cell proliferation [1,2]. The mitotic activation of T lymphocytes following the binding of antigens, monoclonal antibodies or lectins to the antigen receptor complex or to a number of other cell-surface structures [3] is thought to involve the phosphorylation of proteins on tyrosine residues [4-6]. However, the polypeptides comprising the T cell antigen receptor complex do not express TPK activity. It is therefore likely that the binding of ligands to these cell-surface molecules activates TPKs which are discrete molecular entities (non-receptor TPKs).

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Abbreviations: TPK, tyrosine protein kinase; PHA, phytohaemagglutinin; ABP, azobenzylphosphonate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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The best characterised non-receptor TPK of lymphocytes is $p56^{lck}$ [7,8], a 55-60kDa member of a group of closely related enzymes known as the src family [9]. Its association with the cell-surface molecules CD4 and CD8 [10,11], both modulators of the T cell response to antigen, and its rapid activation by specific antibodies to CD4 [12], provide strong evidence for a role in T cell activation. In addition, mitogenic stimulation with phorbol myristate acetate and concanavalin A resulted in the modification of $p56^{lck}$, also consistent with a role in some aspect of lymphocyte activation [13]. In the present communication we have analyzed the TPKs and phosphotyrosine-containing proteins of T lymphocytes and provide evidence for the existence of multiple TPKs which are structurally related to $p56^{lck}$. Their down-modulation following mitogenic stimulation suggests that, like $p56^{lck}$ itself, the other members of this family may also play a role in the transduction of mitogenic signals in T lymphocytes.

MATERIALS AND METHODS

Antibodies. Anti-azobenzylphosphonate (ABP) antibodies cross-reactive with phosphotyrosine were prepared in a goat by injection of ABP haptenized to bovine serum albumin [14]. Immunoblot analysis was carried out as previously described [15]. Equal amounts of protein (50μg) were loaded in each lane.

Lymphocyte preparation and stimulation.
Human peripheral blood lymphocytes were prepared and stimulated with phytohaemagglutinin as previously described [16,17]. Cells were lysed by boiling in a solution of 2% SDS, 20mM Tris HCl pH 7.9, 10mM EDTA, 100μM $ZnCl_2$, 100μM sodium orthovanadate and 1mM PMSF. For subcellular fractionation studies, cells were subjected to hypotonic lysis in 5mM Hepes pH 7.4, 1mM $MgCl_2$, 5mM 2-mercaptoethanol, 2mM EGTA and 1mM phenylmethylsulfonyl fluoride. After restoration of tonicity by the addition of 0.25M sucrose, nuclei were removed by sedimentation at 800 x g. Mitochondria and lysosomes were obtained by sedimentation at 10,000 x g for 10 min and the microsomal fraction was prepared by centrifugation for 1 hr at 100,000 x g. All procedures were carried out at 4°C.

Autophosphorylation.
The autophosphorylation of proteins was carried out as described [16]. Proteins were separated on an 8.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel. Gels were treated with hot KOH prior to drying and autoradiography in order to detect proteins phosphorylated on tyrosine [16].

Peptide mapping. Autophosphorylated protein bands were excised from gels, washed in acetone, equilibrated in 50mM $NaHCO_3$ and incubated for 20h at 37°C in two 2ml aliquots of 200μg/ml^{-1} of TPCK-trypsin in 50mM $NaHCO_3$. The digests were removed, lyophilised and taken up in 50μl SDS-PAGE load buffer. The peptides were separated on a 20% SDS-PAGE gel and detected by autoradiography.

RESULTS AND DISCUSSION

The subcellular distribution of tyrosine phosphorylated proteins in normal human lymphocytes was studied by immunoblotting with an anti-ABP serum cross-reactive with phosphotyrosine [14]. Protein bands with apparent molecular weights ranging from 30 to 150kDa were detected in the cytosolic, lysosomal/mitochondrial and detergent-soluble and insoluble microsomal fractions (Fig. 1A). The overall pattern of phosphotyrosyl proteins was similar in each fraction, although amounts of individual proteins was variable. The mitogenic stimulation of lymphocytes for 72 hours with PHA led to a decrease in all of the tyrosine phosphorylated protein bands in all fractions (Fig. 1B). This decrease is not attributable to an increase in phosphatase or protease activity in the PHA stimulated cells, since the mixing of triton extracts from treated and untreated cells did not affect detectability (data not shown). Furthermore, the same mitogen induced loss of tyrosine phosphorylated protein bands was observed when whole cells were lysed rapidly in boiling SDS in order to avoid protease or phosphatase action during cell lysis (Fig. 1C). This additionally suggests that the multiple phosphotyrosyl protein bands were present in intact unstimulated lymphocytes and were not generated by proteolytic cleavage during cell fractionation (Fig. 1C, lane 1).

$p56^{lck}$ is known to be phosphorylated on tyrosine residues in intact, unstimulated lymphocytes [18], and the 55-60kDa phosphotyrosyl protein band of

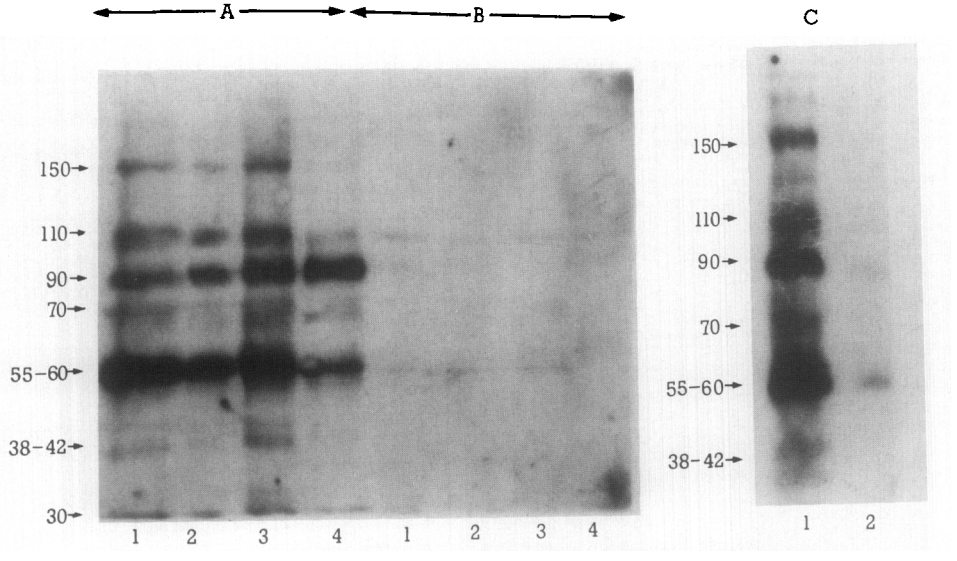

Fig. 1. Immunoblots of subcellular fractions from resting (A) or PHA-treated (B) lymphocytes were probed with anti-ABP serum. Lane 1, cytosol, lane 2, lysosomal + mitochondrial fraction, lane 3, 1% triton X-100 extract of microsomes, lane 4, triton X-100 insoluble fraction of microsomes, (C) anti-ABP immunoblotting of whole cell proteins from untreated (lane 1) or PHA-treated (lane 2) lymphocytes.

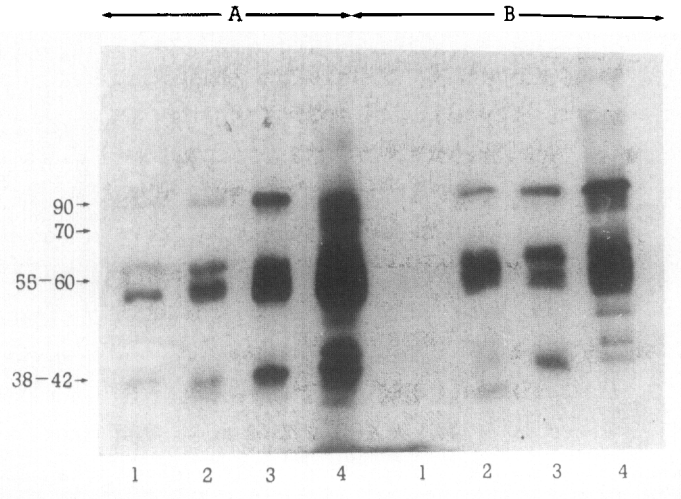

Fig. 2. Autophosphorylation of subcellular fractions from resting (A) or PHA-treated (B) lymphocytes. Lane 1, cytosol, lane 2, mitochondria + lysosomes, lane 3, triton X-100 solubilized microsomes, lane 4, triton X-100 insoluble fraction of microsomes. Gels were treated with N KOH to enhance detection of phosphotyrosine. 2μg protein were analyzed in each lane.

Fig. 1 probably represents this TPK. The additional bands detected by blotting with anti-ABP serum may represent other autophosphorylated TPKs. On the other hand, they may represent substrates of TPKs. Several TPKs, including $p56^{lck}$ [7], are known to autophosphorylate on tyrosine residues when incubated *in vitro* with [^{32}P] ATP. In an attempt to determine whether additional TPKs related to $p56^{lck}$ were expressed in T lymphocytes, we incubated subcellular fractions from these cells with [^{32}P] ATP. The resulting radiolabelled proteins were resolved by SDS-PAGE and the gels treated with hot N KOH in order to detect proteins phosphorylated on tyrosine residues [19]. Several KOH resistant phosphoproteins were detected in all fractions (Fig. 2A). We have verified by direct phosphoaminoacid analysis of acid hydrolysates of

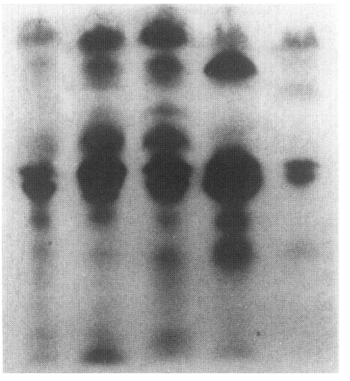

Fig. 3. Proteolytic mapping of autophosphorylated protein bands using trypsin. Molecular weights of the original protein bands (see Fig. 2) are shown under the relevant lane. Gels were treated with glutaraldehyde and with N KOH at 56°C to enhance detection of labelled phosphotyrosine.

each of these bands that >95% of the radiolabel was in fact covalently bound to tyrosine residues [5]. Evidence suggesting that some of the in vitro radiolabelled bands are themselves TPKs is presented later (Fig. 3).

Bands of 55-60kDa, which represent autophosphorylated $p56^{lck}$ [7,8] were detected in all of the fractions. Other bands, whose molecular weights also co-incided with those of some of the phosphotyrosyl proteins detected by the anti-ABP serum (Fig. 1A) were also prominently labelled on tyrosine residues in vitro. These include 70 and 90kDa bands and a closely spaced triplet of bands at 38, 40 and 42kDa (compare Fig. 2A, lane 4 and Fig. 1A, lane 3).

The bands other than $p56^{lck}$ which were detected by in vitro labelling may either be TPKs or substrates phosphorylated by endogenous TPKs. In order to clarify this issue we investigated the structural relationship between the autophosphorylated proteins by tryptic proteolytic mapping (Fig. 3). The marked similarity between the peptide maps of the 42, 70 and 90kDa proteins and of $p56^{lck}$ (lane 4) suggests that they may also be TPKs, since the major in vitro autophosphorylation site of $p56^{lck}$ (Tyr 394) lies within the kinase catalytic domain, which is highly conserved among TPKs [1]. While it is possible that the putative TPKs with molecular weights lower than that of $p56^{lck}$ may have been derived from the latter or from the 70 and/or 90kDa forms by proteolysis during cell fractionation [20], we consider this unlikely, since the subcellular fractionations were carried out in the presence of protease inhibitors. The same fractionation protocol was used in the immunoblot studies depicted in Fig. 1, which shows clearly that the phosphotyrosyl proteins were not degraded in the subcellular fractions compared to the whole cell extracts.

With the exception of the lysosomal/mitochondrial fraction, the intensity of the protein bands detected by in vitro radiolabelling decreased following PHA treatment of T lymphocytes (Fig. 2B), suggesting that the putative TPK species detected in this manner were down-regulated following mitogenic activation. It is unlikely that the reduced intensity of autophosphorylated bands in PHA-treated lymphocytes was attributable to increased phosphatase or ATPase activity, since we have found that both these activities were lower in PHA-treated cells than in untreated lymphocytes [16,21]. Down-modulation of TPK-containing growth factor receptors following mitogenic activation is a well-documented phenomenon [22,23] and we suggest by analogy that, like $p56^{lck}$ itself [10,12], the additional putative TPKs may also play a role in T cell activation by catalyzing tyrosine phosphorylation events in parallel to or in series with those mediated by $p56^{lck}$. The recent description of a 47kDa TPK which itself phosphorylates tyrosine 527 of the c-src-encoded TPK, with consequent modulation of its enzymic activity [24] lends credence to the concept of cascades of TPK catalyzed regulatory reactions.

The correspondence in molecular weight distribution between many of the T lymphocyte tyrosyl phosphoproteins detected by immunoblotting and the putative TPKs detected by *in vitro* radiolabelling (compare Fig. 1A, lane 3 and Fig. 2A, lane 4) suggest tentatively that the former proteins are, in fact, TPKs autophosphorylated on tyrosine residues in intact cells and that their loss on PHA stimulation represents their down-modulation consequent to mitogenic stimulation. Attempts to establish this identity by immunoprecipitation of phosphotyrosyl proteins followed by autophosphorylation by [^{32}P] ATP *in vitro* have been unsuccessful, due to the poor recovery in immunoprecipitation. Casnellie [25] has also reported the poor recovery of $p56^{lck}$ by immunoprecipitation of T lymphocyte extracts.

In conclusion, we have presented evidence that T lymphocytes contain multiple TPK species structurally related to $p56^{lck}$. While it has been reported that the molecular weight of $p56^{lck}$ can be increased to 70kDa by phosphorylation on serine residues, these slowly migrating species were only observed in phorbol ester-treated cells [25], whereas the higher molecular weight species described here were clearly seen in resting cells. On the other hand, the 70 and 38-42kDa TPKs described here may correspond to the 72 and 40kDa enzymes previously detected by their ability to phosphorylate exogenous substrates [20,21]. However, the structural relatedness of these TPKs to $p56^{lck}$ has not previously been investigated. Tentative evidence suggests that these TPK species may represent the majority of the tyrosine phosphorylated proteins detected by immunoblotting of extracts from intact, unstimulated lymphocytes. The down-modulation of the TPKs and of the phosphotyrosyl proteins following mitogenic stimulation of T lymphocytes emphasizes the importance of tyrosine phosphorylation events in lymphocyte activation, and suggests that TPKs other than $p56^{lck}$ may also play a role in mitogenic signalling in these cells.

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