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CORRELATION BETWEEN PHOSPHORYLATION AND KINASE ACTIVITY OF A TYROSINE PROTEIN KINASE : P56 *lck*.

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P56 *lck* is the product of a cellular oncogene highly expressed in lymphoid cells. Tyrosine kinase activity was measured by using an exogenous substrate: polyamino acid glutamic acid-tyrosine (4:1) (PGT). Different levels of phosphorylation of p56 *lck* were achieved by the utilisation of SH reagents and different lengths of incubation time. The phosphorylation of PGT was proportional to the level of phosphorylation of p56 *lck*. Identical results were obtained with crude membrane preparations and with p56 *lck* partially purified on immunecomplexes.

A large number of oncogene products are tyrosine protein kinases. This family can be schematically divided in two groups : Those with growth factor receptor activity and those which are membrane bound without receptor functions, for example the gene products of *src, abl, lck* (1).

The lck gene product is a tyrosine protein kinase that was detected in lymphatic cells. It is overexpressed in a murine lymphoma (LSTRA), generated by chronic infection of Balb/c mice with the Moloney Murine Leukemia Virus (MoMuLV) which carries no oncogene (2,3). Overexpression of the lck gene product (p56 *lck*) may be the consequence of the insertion of MoMuLV sequences upstream of the gene (4). The murine *lck* gene is known and the deduced amino acid sequence shows no transmembrane domain, the N-terminal amino acid is a glycine which is the mirystilation site necessary for membrane binding (5). A similar gene

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is present in different human and murine lymphatic cell lines . The sequence shows over 70% homology with p60 $^{c\text{-}src}$ (6).

Previous work from our group and others showed that the p56 ^{lck} of LSTRA is a very active protein kinase (7,3). It is present in T lymphocytes and barely detectable in B lymphocytes, it was also found in human acute myeloblastic leukemias (8).

We have studied the phosphorylation of p56 ^{lck} in LSTRA cells membranes and in immunecomplexes generated with an antibody specific to the N-terminal end of the protein. P56 ^{lck} phosphorylated to different levels was used in kinase assays with an exogenous substrate to determine whether kinase activity of p56 ^{lck} correlates with its extent of phosphorylation.

MATERIALS AND METHODS

Cell lines and membrane preparation :

LSTRA and MbL2 cells were grown in RPMI-1640 supplemented with 10 % foetal calf serum and 10 µM β-mercaptoethanol.

Membrane preparation (9) : Cells were washed in PBS then lysed (at 10^7 cells per ml) in, mM : Hepes 10 (pH 7,4) ; $MgCl_2$ 2 ; EDTA 1 ; PMSF 1 ; Aprotinine 100 KIU/ml. They were left for 20 min. on ice and homogenized 20 times with a tight fitting dounce homogenizer. The post nuclear fraction was centrifuged at 105,000 x g and the pellet resuspended in the above buffer containing 10 % glycerol and stored in liquid nitrogen.

Solubilization of membrane proteins:

Membranes were solubilized in a modified RIPA buffer (DOC 1%, NP40 1%, NaCl 150 mM, Tris-HCl 10 mM (pH 7,4), Vanadate 50 µM, PMSF 1 mM, Aprotinine 0,1%) for 1 hour at 4°. After 20 strokes in a tight fitting dounce homogenizer they were centrifuged at 15000xg for 15 min. and the supernatant collected.

Immunoprecipitation :

Solubilized membranes were incubated with antiserum (diluted 1/25) for 3 hours at 4°, protein A-sepharose (10%) was then added and incubated for 30 min. The immunecomplexes were washed 3 times with NP40 1% containing : PMSF 1%, Aprotinine 1%, EDTA 1 mM, followed by a final wash with the incubation solution , containing in mM : Hepes 20 (pH 7,2), $MgCl_2$ 10, $MnCl_2$ 10, PMSF 1, Aprotinine 1%.

Phosphorylation assays :

Phosphorylation of p56 lck

Membranes (50 to 80 µg of proteins) were incubated for different periods of time (from 30 sec. up to 5 min.) at 30°, in a total volume of 50 µl,in (mM) : Hepes 20 (pH 7,2), $MgCl_2$ 10, $MnCl_2$ 10. 10 µCi of [γ-^{32}P] ATP (5000 Ci/m mole, Amersham) were added. The immuneprecipitated p56 lck was similarly processed but in different concentrations of ATP were added (1-800 µM).

Kinase activity using exogenous substrate:

After phosphorylation, PGT (75 µg) and different concentrations of non labelled ATP were added and incubated for one minute. The reaction was stopped by addition of SDS-sample buffer. The samples were heated (95° for 3 min. or 60° for 20 min. with immunecomplexes) and resolved on 10% polyacrylamide-SDS gels, followed by autoradiography (with X-AR Kodak film). Bands were excised and counted by Cerenkov radiation.

Phosphate transfer activity :

Immuneprecipitated p56 lck was phosphorylated for 2 min at 30° with high specific activity [γ-^{32}P] ATP (5000 Ci/mmole, Amersham). To remove labelled ATP the immunecomplexes were washed 3 times at 2° with Tris 10 mM (pH 6,8) containing : PMSF 1mM, EDTA 1mM, Aprotinine 1%. Incubations (30µl), contained in mM : Hepes 20, $MgCl_2$ 10, $MnCl_2$ 10, PMSF 1, Aprotinine 1%, ADP 1, ATP 0,2 in the presence or absence of EDTA 20, for 5 to 45 min. at 30°. After addition of EDTA to 20mM, aliquots (1 µl) were spotted onto polyethyleneimine thin layer cellulose and chromatography were performed in 0,5M KH_2PO_4 (pH 3,5) and [γ-^{32}P] ATP formation was detected by autoradiography (14).

RESULTS

Relationship between phosphorylation of membrane bound p56lck and kinase activity: To achieve different phosphorylation levels , membrane preparations were phosphorylated with high specific activity [γ-^{32}P] ATP for differerent periods of time (from 10 secondes to 5 minutes). Under our conditions, the level of phosphorylation of p56 lck increases during the first five minutes, and thereafter remains constant (Fig 1).

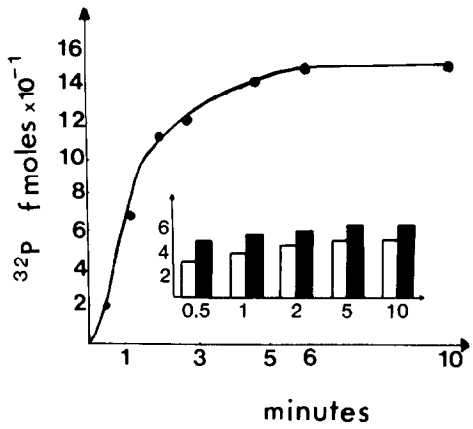

FIGURE 1

Kinetics of phosphorylation and kinase activity of membrane p56 *lck*

The graphics express incorporation of radioactivity in function of time. The curve represent the phosphorylation kinetic of p56 *lck* using high specific activity ATP (5000 Ci/m mole) and histogram the correlation between the label in p56 *lck* (□) and in PGT (■) using low specific activity ATP (40 Ci/m mole).

To quantify the kinase activity, membranes were preincubated with low specific activity [γ-^{32}P] ATP for different periods of time followed by the addition of PGT for one minute. Figure 1 shows that the later PGT is added to incubations the more it becomes phosphorylated, in the same time p56 *lck* becomes more phosphorylated. Indeed, incorporation of the radioactivity in PGT is proportionnal to the extent of phosphorylated p56 *lck*.

Effect of N-ethylmaleimide and β-mercaptoethanol on p56 *lck* phosphorylation and kinase activity : N-ethylmaleimide (NEM) and β-mercaptoethanol (β-ME) react with disulfide bonds , modifying the activity of several kinases (10,11)

NEM at 10 mM inhibits p56 *lck* phosphorylation in membranes by a factor 4 , while β-ME at 50 µM brings about a two fold stimulation of p56 *lck* phosphorylation (fig. 2 A).

In membranes preincubated with NEM, phosphorylation of PGT decreased, whereas if membranes were preincubated with β-ME the

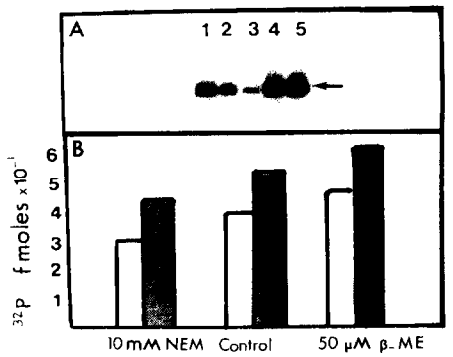

FIGURE 2

Effect of NEM and β-ME on p56^{lck} kinase activity

A : Membranes were phosphorylated for 5 min. at 20° with high specific ATP (5000 Ci/m mole), in presence or absence of SH reagents in the incubation mixture (at constant volume).

1 : control, 2 : NEM 2mM, 3 : NEM 10mM, 4 : β-mercaptoethanol 50µM,

5 : β-mercaptoethanol 100µM

The arrow indicates the migration of p56^{lck}, the figure is an autoradiogramm.

B : effect of NEM and β-ME on the incorporation of labelled phosphate in p56^{lck} (□) and in PGT (■). The phosphorylation reactions were performed with low specific activity ATP (40 Ci/m mole).

phosphorylation of PGT increased (fig 2 B). Similar results have been obtained in 4 separate experiments.

Relationship between phosphorylation of p56^{lck} in the immunecomplex and kinase activity : To avoid possible interference of membrane structures in p56^{lck} phosphorylation, we examined the relationship between p56^{lck} phosphorylation and exogenous kinase activity by the use of partially immunepurified p56^{lck} obtained with a specific antibody directed against the N-terminal part of the protein (aa 38-62).

P56^{lck} in the immunecomplex can phosphorylate itself as well as exogenous substrates (12). Phosphorylation kinetics reached a maximum after 2 minutes, followed by a plateau (fig 3 A). Phosphorylation of PGT was directly proportional to the level of autophosphorylation (fig 3 B).

We have verified that phosphorylated p56^{lck} is responsible for the phosphorylation of PGT by using immuneprecipitates obtained i) with

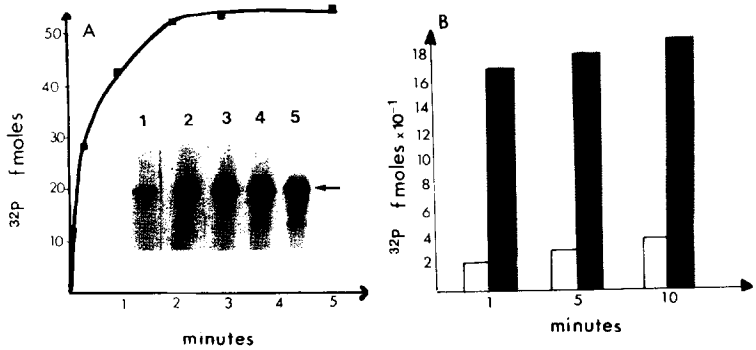

FIGURE 3

Kinetics of phosphorylation and kinase activity of partially immunepurified p56 *lck*.

A : Phosphorylation in presence of 10µM ATP
1 : 30 sec., 2 : 1 min., 3 : 2 min., 4 : 5 min., 5 : 10 min.
The arrow indicates the migration of p56 *lck* and the curve represents the kinetics.
B : Incorporation of radioactivity in p56 *lck* (□) and in PGT (■).

preimmune serum and LSTRA extracts or ii) with N-terminal antibody used in extracts from a murine lymphoma (MbL2) that does not express p56 *lck*. In both cases the phosphorylation of PGT was undetectable (data not shown).

Phosphate transfer activity : Some kinases can transfer their esterified phosphate yielding ATP (14,15,16). Such an activity was detected in p56 *lck*. Partially immunepurified p56 *lck* was phosphorylated for two minutes and washed 3 times to remove [γ-^{32}P] ATP . The formation of labelled ATP was measured in the presence of ADP and ATP. Phosphate transfer was measurable from 5 and up to 45 minutes and was inhibited by EDTA (20 mM). The amount of labelled ATP synthesized was 7 to 10 fold above residual labelled ATP (fig 4-A). Several control experiments were performed to demonstrate that phosphate transfer activity was due to p56 *lck* : No ATP synthesis was detectable when p56 *lck* was not phosphorylated, or with immunecomplexes from MbL2 cells (fig 4-B).

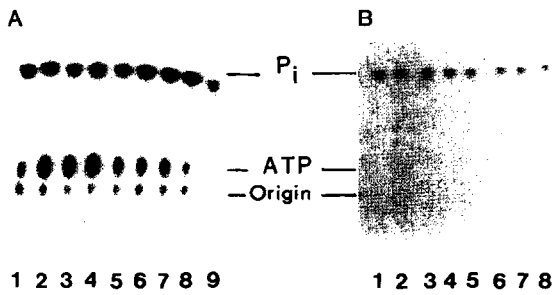

FIGURE 4

Reversibility of phosphorylation in partially immunepurified p56^{lck}

A : Incubations of immunecomplexed p56^{lck} contained : ADP (1mM), ATP (0.2mM) and either EDTA (20mM) : lanes 5,6,7; or no EDTA : lanes 2,3,4. Lanes 8,9 : no additions.
1 : time zero; 2,5 : 5 min.; 3,6,8 : 30 min.; 4,7,9 : 45 min.
B : Controls . Lanes 1, 2, 3, 4 : immunecomplexes obtained with cellular extract of MbL2. Lanes 4, 5, 6, 8 : with immunecomplexes obtained from non phosphorylated p56^{lck}.
1, 5 : 5 min.; 2, 6 : 30 min.; 3, 7 : 45 min.; 4, 8 : 90 min.

DISCUSSION

We have shown that in membranes from LSTRA cells or in immune-precipitates, the extent of p56^{lck} autophosphorylation is correlated with the kinase activity measured with an exogenous substrate. However, the experimental set up used here does not allow to verify what would be the level of exogenous substrate phosphorylation when p56^{lck} is not phosphorylated.

As judged from two-dimensional peptide mapping of in vitro labelled p56^{lck} the only tyrosine phosphorylated is tyrosine 394 of the autophosphorylated site (not shown) , in agreement with previous results (13). The phosphorylation of this tyrosine might modulate the TPK activity. We cannot assess the significance of our results in intact cells, but findings obtained with others TPKs, like p60$^{v\text{-}src}$, showed that the deletion of the autophosphorylated site (tyr 416) is not absolutly essential for its activity but modulates substrate phosphorylation (17,18,19), suggesting that autophosphorylation is a physiological modulator of the kinase activity. The high degree of homology between

these two proteins suggests that p56 *lck* might have similar properties. In addition to this, p56 *lck* possesses SH groups situated at positions that are important for its activity ; indeed, NEM and β-ME which both react with SH groups modulate the activity of the kinase.

Previous studies have shown that the N-terminal antibody reduces autophosphorylation and kinase activity of the p56 *lck* if compared with immunecomplexes generated from antibodies against the autophosphorylation site (12). However as shown here, the positive correlation between the autophosphorylation and exogenous kinase activity is not affected by N-terminal antibody. The kinase activity of p56 *lck* was tested on PGT; the cellular substrates and the function of p56 *lck* phosphorylation are not known . However, phosphorylation of many TPKs, e.g. the growth factor receptors (1), is part of their physiological response.

Labelled p56 *lck* in immunecomplexes is capable to transfer its phosphate for the synthesis of ATP, indicating that autophosphorylation of p56 *lck* is reversible. Others TPKs, like the *abl* gene product have this particularity (14). As suggested (14), one can postulate that autophosphorylation is an equilibrium between protein phosphorylation which produces ADP and dephosphorylation of the protein which synthesizes ATP . We have observed that in the absence of ADP there is no synthesis of labelled ATP (not shown), suggesting that the reaction is a transfer of the esterified phosphate of the protein to ADP rather than the exchange of the γ-phosphate of ATP with the labelled phosphate present on the enzyme. This implies that the phosphotyrosine residue on p56 *lck* contains a high energy bond as was suggested for the Abelson kinase (14). This activity is not well characterized and we ignore its role in vivo .

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