Vol. 162, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1989 Pages 51-57

PHOSPHORYLATION OF p56^{lck} BY EXTERNAL ATP IN INTACT CELLS

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Received May 8, 1989

Recent studies have suggested a role for extracellular ATP. In this report we show that extracellular labelled ATP crosses the plasma membrane of intact lymphoma cells and peripheral blood lymphocytes and phosphorylates p56^{lck} a tyrosine protein kinase specific of lymphoïd cells. Two other phosphoproteins of 92Kd and 35Kd become detectable on alcali treated gels. Phosphorylation occurs within minutes following addition of ATP. ATP, GTP, ADP and an ATP analog prevent phosphorylation but not AMP nor P_i; trypsinization of cells abolishes labelling. The possible involvement of P_2 Purinergic receptors is discussed.

Adenosine Triphosphate (ATP) has been known for several decades to play a central role in cell intermediary metabolism acting as an energy transporter. More recently, ATP was shown to act as transmitter of intercellular signals (3) such as neurotransmission, vascular tone, muscle contraction and formation of prostacyclins in medullary gland cells, and several cell types were found to release ATP (reviewed in 13). In lymphoïd cells, extracellular ATP enhances the proliferation of thymocytes (14) and is mitogenic for differentiated T cells of peripheral blood (17). These effects are thought to be mediated by receptors specific for ATP, the P_2 purinergic receptors (3); these receptors seem to be coupled to intracellular second messengers (Ca^{++}, inositolphospholipids) (6, 10, 19, 23). ATP, under certain conditions, can also permeabilize cells and allow entry of large molecules (1, 25, reviewed in 13 and 15). The interaction of membrane protein kinases with extracellular ATP is not clearly understood; ectoprotein kinases have been described (7, 8, 18) and external ATP was shown to stimulate phosphorylation of intracellular proteins in aortic endothelial cells (5). A class of tyrosine protein kinases has been described whose cellular location is restricted to the inner face of the plasma membrane (16); among those, p56^{lck} is expressed only in lymphocytes (12, 20). We have studied the effect of extracellular ATP on p56^{lck} by analysing its phosphorylation by exogenously added (γ^{32}P)-ATP to mouse lymphoma cells (LSTRA) expressing p56^{lck} at a high level (11, 20).

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0006-291X/89 $1.50

METHODS

Incubation of cells with (γ^{32}P-ATP) : 5.10^5 LSTRA cells were suspended in medium A (50 µl) containing : Bovine Serum Albumin (1mg/ml), Mg^{++} 10mM, glucose 5mM and PIPES 20mM pH 6.8 or 7.4 as indicated in the legends; (γ^{32}P)-ATP (10 µCi) (5000 Ci/mmole, Amersham) was added for different periods of time and incubation performed at 37° or 10°. In some experiments, cells were preincubated with nucleotides (ADP, GTP, AMP) or the nucleotide analog (AMPCPP) prior to labelling with (γ^{32}P)-ATP. After labelling, cells were washed in a 20 mM pH 7.4 phosphate buffer containing : NaF 50mM, EDTA 50mM, NaCl 150mM, PMSF 1mM, Aprotinine 2% and Vanadate 1mM; lysed in SDS-sample buffer and analysed on 8% acrylamide-SDS gels, stained, dried and autoradiographed. Alcali treatment of the gels was performed with 1N KOH for 2 hours at 55°. For ^{32}P labelling, 10^6 cells were incubated 3 hours with 2 mCi/ml ^{32}Pi (Amersham) in phosphate free medium (Flow) supplemented with 10% dialysed foetal calf serum. Cells were washed in PBS and lysed in SDS sample buffer.

Measurement of ^{3}H-adenosine release : This was performed as described (25),10^6 cells were incubated for 2 hours with 1µCi/ml of ^{3}H-adenosine (Amersham) at 37°. Cells washed in medium A pH 6.8 were incubated for 10 minutes at 37° in 800ml of the same medium with or without ATP; the supernatant was counted in Aquasol (New England Nuclear).

Cell surface trypsinization : Before labelling, 10^6 cells were incubated with 0.01% trypsin (Sigma) at 37° for different times. Cells were then washed in the incubation medium (1ml) and centrifuged. The viability of cells checked by trypan blue exclusion was superior to 95%.

Preparation of human peripheral blood lymphocytes : human PBL were prepared from heparinized venous blood by dextran sedimentation followed by Ficoll-Hypaque centrifugation.

In vitro phosphorylation : Phosphorylation of membrane extracts was performed as previously described (2). Immunoprecipitation of p56^{lck} was performed as described (9) using an antiserum specific to the N-terminal end of the protein.

Tryptic peptide analysis : Phosphoproteins were excised from gels and subjected to exhaustive digestion with 50mg/ml trypsin (Sigma) for 24 hours at 37°. Samples were analysed on 40% acrylamide gels as previously published (26) and autoradiography performed.

RESULTS

Proteins phosphorylated with external ATP : The addition of (γ^{32}P)-ATP to intact LSTRA cells in the presence of Mg^{++} (10 mM) resulted in the incorporation of ^{32}P in several proteins (figure 1), a 56 Kd phosphoprotein was detected after 1 minute at 37° or 10°, a 92 Kd phosphoprotein was detected after 5 minutes, and several others including a 35 Kd after 20 minutes. Comigration with the major phosphoprotein from LSTRA membranes (figure 1, panel A, lane 9) and immunoprecipitation with a specific antiserum (figure 1, panel C) revealed that the 56 Kd phosphoprotein was p56^{lck}, an intracellular tyrosine protein kinase whose expression is restricted to lymphoïd cells (12, 20). In contrast, incubation of cells with ^{32}Pi resulted in a different phosphorylation pattern (figure 1, lane 10): the 56 Kd phosphoprotein was undetectable, the 92 Kd and 35 Kd bands were less phosphorylated. Labelling with ATP followed by treatment of the gel with KOH revealed alcali resistant phosphoproteins migrating at 56 Kd 92 Kd and 35 Kd (figure 2B, lane 3).

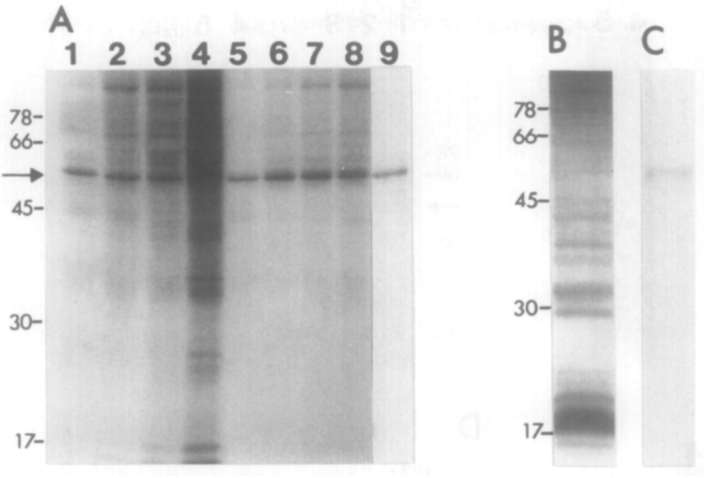

Figure 1 : Phosphorylation of proteins by exogenous (γ^{32}P)-ATP applied to intact LSTRA cells : A : cells (5.10^5) were incubated with 10 µCi of (γ^{32}P-ATP) at 37° (lanes 1 to 4) or at 10° (lanes 5 to 8) for 1 minute (lanes1 and 5), 5 minutes (lanes 2 and 6), 10 minutes (lanes 3 and 7) and 20 minutes (lanes 4 and 8); lane 9 : phosphoprotein pattern of LSTRA cell membrane phosphorylated with (γ^{32}P-ATP) for 5 minutes at 30°. 5 hours exposure B : phosphoprotein pattern of LSTRA cells incubated with ^{32}P (2 mCi/ml) for 3 hours, 12 hours exposure. C : phosphoprotein pattern of p56*lck* immunoprecipitated from (γ^{32}P-ATP) labelled LSTRA cells (5 minutes at 37°), 4 hours exposure.

Optimal conditions for the phosphorylation of proteins with external ATP :
Permeabilization of cells with ATP requires : low Mg^{++}, alcaline pH, low glucose and low Ca^{++} (1). In contrast, as shown in figure 2 maximum phosphorylation of LSTRA cells proteins with external (γ^{32}P)-ATP was observed with : Mg^{++} (10mM) or Ca^{++} (10mM), glucose (5mM) and pH 6.8. Absence of permeabilization was confirmed by the absence of (^{3}H)-adenosine release (figure 2, panel D) and of trypan blue uptake of cells in the presence of external ATP.
Phosphopeptide pattern of p56*lck* : Phosphorylation of p56*lck* in vitro results in the preferential label of tyrosine 394 and a very low level of phosphorylation of tyrosine 505 (figure 3, lane 3) (4, 21). In vivo phosphorylation occurs mostly on tyrosine 505 (figure 3, lane 2) (4, 21). By analysing the phosphopeptide profile of p56*lck* labelled with external ATP we observed that tyrosine 394 and tyrosine 505 were both highly phosphorylated, as well as a third phosphopeptide (Figure 3, lane 1).
Analysis of other lymphoïd cells : A murine lymphoma cell line (MbL2) showed not to express p56*lck* (11) was found to contain phosphorylatable proteins upon treatment with external ATP but no 56 Kd phosphoprotein; the human lymphoma cell line CEM expressing p56*lck* (22) displayed phosphoproteins including a 56 Kd band, peripheral blood cells express p56*lck* and were found to display a 56 Kd alcali resistant phosphoprotein corresponding to p56*lck* (Figure 4).
Specificity of ATP labelling : Labelling of proteins with external ATP was blocked by : AMPCPP (100µM), GTP (100µM) and unlabelled ATP itself (100µM) (Figure 5). AMP

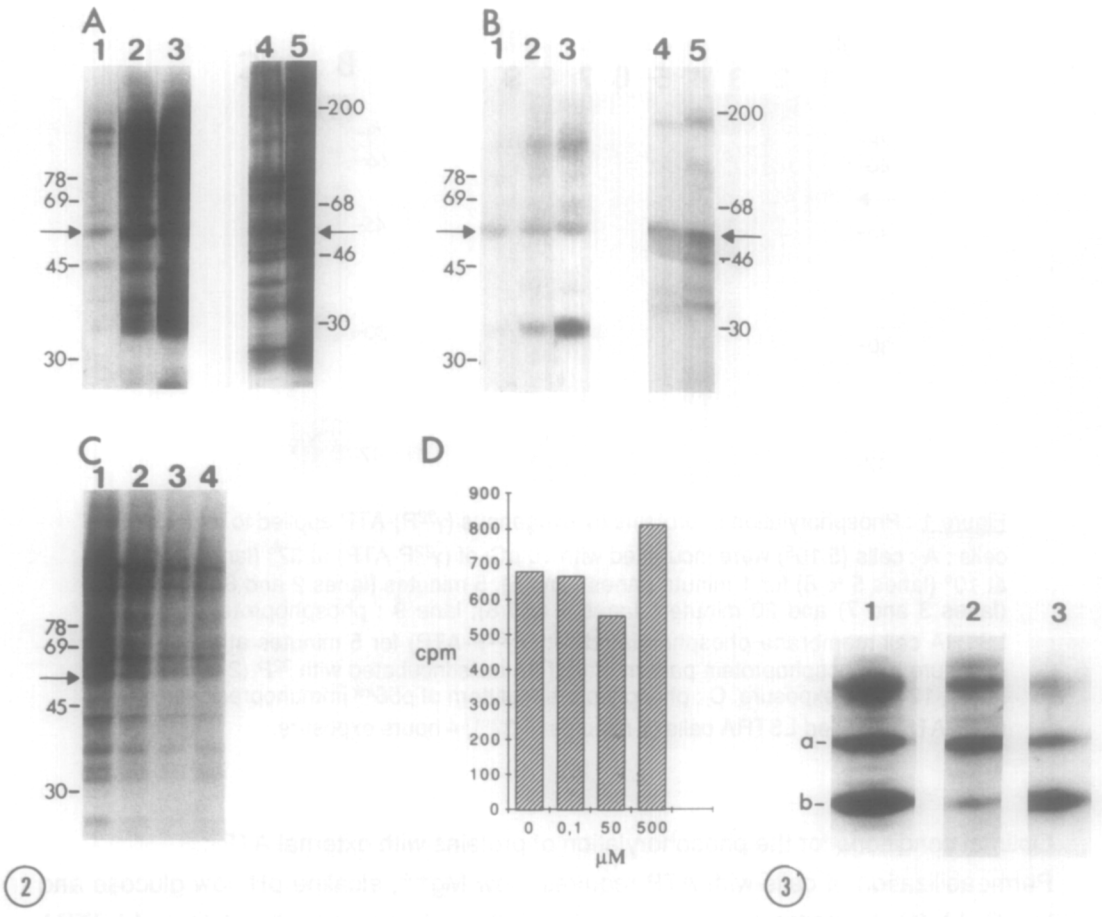

Figure 2 : Optimal conditions for the phosphorylation of proteins with external ATP : A : effect of Mg^{++} and Ca^{++}concentrations (lane 1 : no Mg^{++}, lane 2 : 0.15mM Mg^{++}, lane 3 : 10 mM Mg^{++}, lane 4 : no Ca^{++}, lane 5 : 10 mM Ca^{++}) exposure : 6 h at room temperature. B : same experiment as A, the gel was treated with KOH for 2 hours, exposed for 72 h with intensifying screen. C : effect of pH and glucose : lane 1 : pH 6.8, 5 mM glucose, lane 2 : pH 6.8, no glucose, lane 3 : pH 7.4, 5 mM glucose, lane 4 : pH 7.4, no glucose,exposure time 12 hours with intensifying screen. D : measurement of ^{3}H-adenosine efflux to evaluate cell permeability : the cells loaded with ^{3}H-adenosine were treated or not with different concentrations of ATP and the release of ^{3}H-adenosine measured in the cell supernatant.

Figure 3 : Analysis of $p56^{lck}$ tryptic phosphopeptide pattern. Gel slices containing $p56^{lck}$ were treated with trypsin and remigrated on a 40% acrylamide gel. Lane 1 : pattern of $p56^{lck}$ from cells labelled with external ATP, lane 2 : $p56^{lck}$ from cells labelled in vivo with ^{32}Pi, lane 3 : $p56^{lck}$ from membranes labelled with ATP. Peptide a corresponds to tyrosine 505 and peptide b to tyrosine 394. 72 hours exposure with intensifying screen.

(100µM) was not inhibitory (not shown); ADP (100µM) was 80% inhibitory (not shown). Treatment with trypsin prior to incubation with external (γ^{32}P)-ATP completely abolished labelling of proteins (figure 6). Preincubation of cells with Pi (10 mM) resulted in no decrease of labelling (not shown) thus ruling out labelling by ^{32}Pi released by degradation of (γ^{32}P)-ATP at the cell surface.

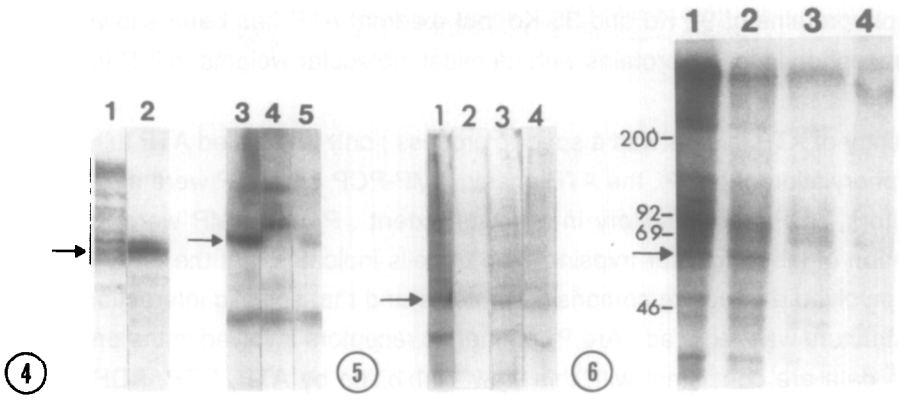

Figure 4 : Labelling of proteins with external ATP in different cell types. Peripheral blood lymphocytes were treated with (γ^{32}P)-ATP and the lysates analysed : lane 1 : phosphoprotein profile(exposed 12 h at -70°C) , lane 2 : phosphoprotein pattern after alcali treatment(60 H at -70°C)L; lane 3 : phosphoprotein pattern of LSTRA cells, lane 4 : pattern of the MBL2 cell line (that does not express p56^{lck}), lane 5 : pattern of the human lymphoma CEM. Lane 3,4,5 are seen after alkali treatment, 4 Days exposed at -70°.

Figure 5 : Specificity of labelling with (γ^{32}P)-ATP. Different compounds structurally related to ATP were tested for their ability to inhibit the labelling of phosphoproteins by (γ^{32}P)-ATP. After labelling, the cell lysates were analysed on SDS acrylamide gels. Lane 1: no inhibitor, lane 2 : ATP (100µM), lane 3 : AMPCPP (100µM), lane 4 : GTP (100µM); 12 hours exposure.

Figure 6 : Effect of treatment of the cells with trypsin on labelling with external ATP : the cell suspensions were treated with trypsin (0.01%) for 3 minutes (lane 2), 5 minutes (lane 3) and 10 minutes (lane 4) or not treated (lane 1) and labelling with external ATP performed. Arrow indicates migration of p56^{lck} . 12 hours exposed at -70° .

DISCUSSION

This work was undertaken to analyse the relationship between extracellular ATP and protein tyrosine kinases located at the inner face of the plasma membrane. To test this, we analysed the phosphorylation of p56^{lck} by extracellular (γ^{32}P)-ATP. The results presented here show that rapid phosphorylation of p56^{lck} occurred. Measurements of ^{3}H-adenosine release showed that ATP in the presence of Mg^{++}, glucose and pH 6.8 did not permeabilize cells, therefore the entry of ATP in the cells and subsequent phosphorylation of p56^{lck} did not result from the formation of pores in the membranes. The tryptic phosphoprotein pattern of p56^{lck} observed with extracellular ATP was different from the pattern observed in vitro and the phosphopeptide pattern observed in vivo, this ruled out a contamination of cells by membranes fragments. p56^{lck} was characterized by two different criteria : immunoprecipitation and phosphopeptide profile; this excluded the possibility that a protein located at the cell surface and migrating at 56 Kd was phosphorylated by external ATP. The 56 Kd phosphoprotein was undetected in MBL2, a cell line that does not express p56^{lck} . However, the possibility remains that other proteins were phosphorylated by ectokinases, particularly the two alcali resistant

phosphoproteins of 92 Kd and 35 Kd, but external ATP has been shown to stimulate the phosphorylation of proteins with identical molecular weights in ^{32}P incubated cells (5).

The entry of ATP in cells was a specific process : only unlabelled ATP fully inhibited the phosphorylation of $p56^{lck}$, the ATP analog AMP-PCP and GTP were also very efficient inhibitors, ADP was inhibitory in a lesser extent , Pi and AMP were not inhibitory. Inhibition of labelling after trypsinization of cells indicates that the entry of ATP in the cells involved a structure comprising proteins and that specific interaction of ATP with this structure was required. Are P_2 purinergic receptors involved in the process? Some of the data are consistent with this view : inhibition by ATP, GTP, ADP and an ATP analog, absence of inhibition by AMP, inhibition by trypsinization of cells. P_2 purinergic receptors have not been shown to transfer ATP into cells but to act as receptors resulting in stimulation of inositol phospholipids metabolism (6, 10); thus, the entry of ATP into cells may have taken place via unknown channels that might be specific for ATP.

ACKNOWLEDGMENTS

This work was supported by grants from "Conseil Economique Européen","Association pour la Recherche contre le Cancer" and "Fondation de France".

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