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Phosphorylation of T-lymphocyte plasma membrane-associated proteins by ectoprotein kinases: implications for a possible role for ectophosphorylation in T-cell effector functions

Frank A. Redegeld *, Pat Smith, Sergey Apasov, Michail V. Sitkovsky

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

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Abstract

Extracellular adenosine triphosphate (ATPo) has been suggested to play a role in lymphocyte effector functions. Recently, it has been suggested that $MgATP^{2-}$ may be the molecular species which is involved in modulating the lytic interaction between cytotoxic T-lymphocytes (CTL) and their target cells. In this study, we provide evidence that ATPo mediates the phosphorylation of extracellular proteins on T-lymphocytes through the action of ectoprotein kinases. The ectophosphorylation is temperature-dependent, supported by Mg^{2+} and Mn^{2+}, and both ATP and GTP, whereas kinase activity and/or substrates were removed by pretreatment of intact lymphocytes with trypsin. We show the presence of extracellular ATP/GTP-binding sites, indicating the presence of ectoenzymes on intact lymphocytes. The major ectoprotein kinase was identified as a casein kinase II-like protein kinase and could be inhibited by heparin, whereas its activity was enhanced by spermine. The ectoprotein kinase showed remarkable substrate specificity, phosphorylating the serum protein vitronectin, but not fibronectin. In experiments with the cell-impermeable protein kinase inhibitor K-252b, we demonstrate the possible functional importance of ectoprotein kinase in CTL-mediated cytotoxicity, i.e., target cell death was completely blocked by K-252b without affecting intracellular phosphorylation. These results suggest that ectoprotein phosphorylation may possibly be an important event in immunologically relevant cell–cell interactions. © 1997 Elsevier Science B.V.

Keywords: Cell mediated cytotoxicity; Extracellular ATP; Ectoprotein kinase; K-252b; Ectophosphorylation; T lymphocyte

Abbreviations: ATP, adenosine triphosphate; ATPo, extracellular ATP; GTP, guanosine triphosphate; AMP-CPP, α,β-methyleneadenosine 5′-triphosphate; $^{32}P_i$, radiolabeled inorganic phosphate; PSA, polyanethole sulfonate; DCBF, 5,6-dichlorobenzimidazole furanoside; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; Ag, antigen; CTL, cytotoxic T-lymphocytes; FCS, fetal calf serum; TC, target cell; mAb, monoclonal Ab; CKII, casein kinase II

* Corresponding author. Present address: Department of Pharmacology, Faculty of Pharmacy, Utrecht University, P.O. Box 80.082, 3508 TB Utrecht, The Netherlands. Fax: (31) (30) 253-7420.

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1. Introduction

Extracellular ATP (ATPo) was strongly implicated as a fast excitatory transmitter in synaptic transmission [1] and in neuronal events associated with memory formation, probably by interaction with purinergic receptors, ecto-ATPases and phosphorylation by ectoprotein kinases. In addition, ATPo was shown to mediate cell–cell spread of calcium signals between mast cells through purinergic receptors [2]. Moreover, purinergic receptors, as well as extracellular phosphorylation, were suggested to be involved in the cell–cell contacts leading to activation of T-lymphocytes (T-cell-mediated cytotoxicity) [3–7].

Recently, antigen receptor-triggered accumulation of ATPo [3] was demonstrated in both T-helper and cytotoxic T-lymphocytes (CTL). Understanding the mechanism of this ATPo accumulation has been furthered with the finding that a multi-drug resistance (mdr1) gene product served as an ATP channel, mediating the release of intracellular ATP into the extracellular space [8]. In the past few years, ATPo was proposed as an important component of an alternative cytotoxic pathway in the mechanism of cell-mediated cytotoxicity. It was found that ATPo can induce target cell death through interaction with purinergic receptors inducing colloid-osmotic lysis and DNA fragmentation [8,9] or possibly through other ectoenzyme systems [5], whereas the presence of highly active ecto-ATPases was shown to explain the refractoriness of the effector T-cells to the lytic properties of ATPo [4].

Comparative studies on the molecular requirements for the CTL-mediated cytotoxic effector functions revealed the requirement of extracellular Mg^{2+}, even in an adhesion molecule-independent retargeted cytotoxicity assay. This pointed to the importance of $MgATP^{2-}$ as a likely participant in the induction of cell-mediated cytotoxicity [5]. While originally ATPo was considered as a candidate lytic intermediate in CTL's effector functions [4,6,7], it is likely that ATPo is involved in more complex and tightly regulated processes of lymphocyte function. This, in turn, attracted our attention to the possible involvement of $MgATP^{2-}$-utilizing ectoprotein kinases (plasma membrane-associated and/or releasable into extracellular space).

In this report, we describe the presence of ectoprotein kinase activity on T-cells. In the presence of ATPo, these kinases are able to phosphorylate lymphocyte surface proteins. The major ectoprotein kinase, which utilized ATPo in lymphocytes, is identified as a casein kinase-II (CKII)-like protein kinase. The effect of cell membrane-impermeable protein kinase inhibitors suggest the involvement of these enzymes in CTL-mediated cytotoxicity, likely on the level of cell–cell contacts. Thus, according to our current model, cell–cell contacts during the immunologically relevant interactions, e.g. thymocyte differentiation, CTL/target cell and T-helper cell/antigen presenting cells interactions, can be regulated by ATPo at the level of purinergic receptors (S. Apasov, M. Koshiba and M. Sitkovsky, manuscript in preparation) or through the ectophosphorylation of functionally important plasma membrane proteins.

2. Experimental procedures

2.1. Cells

CTL clone OE4 (anti H-2^d) was maintained as described earlier [3], using irradiated stimulator cells and an IL-2-containing supernatant from PMA-stimulated EL-4 cells. Peritoneal exudate CTL and alloimmune peritoneal exudate lymphocytes (PEL) with BALB/c anti-EL-4 specificity were obtained 5 days after secondary i.p. immunization of BALB/c mice with 25×10^6 EL-4 tumor cells (grown as ascites in syngeneic C57BL/6 mice). PEL were purified from crude peritoneal exudate cells by removal of macrophages and other adherent cells on nylon wool columns (60 min, 37°C) followed by elution of the non-adherent PEL. EL-4 cells (T-cell lymphoma) and 2B4 cells (T-helper hybridoma) were maintained in RPMI 1640 supplemented with 10% FCS (Biofluids, Inc., Rockville, MD). To maintain cells in serum-free conditions, RPMI 1640 media (supplemented with 1% Nutridoma (Boehringer, Indianapolis, IN)) and 1% bovine serum albumin or AIM-V medium (Gibco-BRL, Gaithersburg, MD) were used. Cytotoxic T-cell hybridomas PN37, MD45, MD90, and MD45-27J were cultured in RPMI 1640 supplemented with 10% FCS. Cytotoxic T-cell hybridomas were kindly provided by Dr. G. Berke (Weizmann Institute, Israel). Shortly before each experiment, cells

were centrifuged over Ficoll/Hypaque to remove dead cells and cell debris. Cells were washed three times with HBSS, 10 mM HEPES, 10 mM $MgCl_2$, pH 7.4, before assay for ectokinase activity, since it was shown that FCS could contain protein kinase activity. Moreover, no differences in ectokinase activity nor in the phosphorylation pattern were found in 2B4 cells grown in 10% FCS or in serum-free medium (data not shown), excluding the possible interference of soluble serum-derived kinases.

2.2. Ectoprotein kinase assay

Cells (10×10^6) were resuspended in 200 μl HBSS, 10 mM HEPES, 10 mM glucose, 10 mM $MgCl_2$ (or concentration indicated in figures), pH 7.4 (reaction buffer), and preincubated for 5 min at 37°C. Ectophosphorylation was started by addition of 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol) (Amersham, Arlington Heights, IL) or [γ-^{32}P]GTP (3000 Ci/mmol) or [α-^{32}P]ATP (3000 Ci/mmol, as a control) (final NTP conc. 16.6 nM), and the reaction mixture was incubated for 10 min at 37 or 4°C. 10 μM cold ATP was also included in some experiments. To stop the reaction, samples were placed on ice, centrifuged (5 min, 1200 rpm, $240 \times g$), and the supernatant removed. To analyze the released phosphorylated proteins, 5 × Laemmli sample buffer was added to the supernatant.

The cell pellets were washed twice by centrifugation (5 min, 1200 rpm, $240 \times g$) with reaction buffer supplemented with or without phosphatase inhibitors (0.4 mM vanadate, 10 mM NaF, 10 mM orthophosphate). Cells were lysed in 50 μl Laemmli sample buffer and subsequently sonicated for 10 s. Before analysis by SDS–PAGE, samples were reduced with 100 mM DTT and boiled for 3 min. Samples were analyzed on 4–20% or 10–20% polyacrylamide gels (ISS/Enprotech, Natick, MA). Following electrophoresis, gels were stained with Coomassie Brilliant Blue, destained, dried, and subjected to autoradiography using Kodak XAR-2 film. In these experiments, we used 2B4 or CTL-OE4 cloned cells in parallel experiments. Mouse spleen or thymus cells were also tested immediately after harvesting in vivo. Very similar results were obtained with both cell types. In some experiments, the intensity of phosphorylation of different bands on the same autoradiograph was evaluated using computer-assisted densitometry (Image QuantJ, Molecular Dynamics, Sunnyvale, CA).

To demonstrate the substrate specificity of ectokinases, intact CTL OE4 or 2B4 (5×10^5 cells/200 μl) were mixed with 10 μg vitronectin (Gibco-BRL) or 20 μg fibronectin (Telios, San Diego, CA) in serum-free AIM-V medium (Gibco-BRL) supplemented with 10 mM $MgCl_2$. Phosphorylation was started by adding 10 μCi of [γ-^{32}P]ATP (final ATP conc. 16.6 nM), and samples were incubated for 10 min at 37°C. After incubation, the cells were centrifuged (5 min, 1200 rpm, $240 \times g$), and supernatants were collected. The cell pellets were washed twice with HBSS and supplemented with cold ATP and phosphatase inhibitors as described above. Supernatants and cell pellets were mixed with Laemmli sample buffer and subjected to SDS–PAGE under reducing conditions. Gels were stained to confirm the presence of vitronectin or fibronectin and dried. The extent of phosphorylation was determined by autoradiography using XAR-2 film (Kodak). To check for autophosphorylation of vitronectin or fibronectin, identical incubations were carried out in the absence of cells.

2.3. Control experiments for use of extracellular ATP during ectokinase assay and for ectodomain localization of phosphorylated amino acid residues

To deplete extracellular ATP, cells (5×10^6 to 10×10^6) were resuspended in 200 μl of reaction buffer with 10 mM $MgCl_2$ and were preincubated for 5 min in the presence of 80 U/ml of hexokinase (Boehringer) or 100 U/ml apyrase (Sigma, St. Louis, MO). The reaction was started by adding 10 μl of [^{32}P]ATP (final ATP conc. 16.6 nM) to the reaction mixture and continuing the incubation for an additional 10 min. The reaction was stopped, and samples were treated as described above.

To measure the possible contribution of intracellular phosphorylation, 10 μCi of ^{32}P-orthophosphate or 10 μCi of ^{32}P-pyrophosphate was added to the samples to start the reaction. Measurement of the effect of competition with cold ATP analog on ectophosphorylation were performed by adding 100 μM AMP-CPP (Boehringer) to the incubation mixture.

In another series of control experiments, incubations were done in a physiological buffer with millimolar concentrations of inorganic phosphate (P_i) to compete with labeled $^{32}P_i$, which can be derived from hydrolyzed [γ-^{32}P]ATP. Indeed, ecto-ATPases – which we have shown to be present in lymphocytes [3] – can hydrolyze [γ-^{32}P]ATP, and the resulting $^{32}P_i$ could then pass through the cell membrane leading to phosphorylation of intracellular proteins, which, in turn, could be misinterpreted as being due to ectophosphorylation. As would be expected if phosphorylating enzymes did indeed utilize extracellular ATP, the presence of high concentrations of inorganic phosphate did not affect phosphorylation (data not shown).

2.4. Trypsin treatment

Before labeling, cells were treated for 3 min with 5 mg/ml trypsin (Worthington, Freehold, NJ) at 37°C. Trypsinization was stopped by adding 30 mg/ml trypsin inhibitor to the cell suspensions. Cells were washed and centrifuged (5 min, 1200 rpm, $240 \times g$), checked for viability, and taken into the ectophosphorylation assay as described above. Trypsinization did not affect viability of cells as measured by Trypan blue exclusion (data not shown).

2.5. Measurement of cation dependency of ectophosphorylation

Cells were resuspended in Ca^{2+}- and Mg^{2+}-free HBSS, 10 mM HEPES, 10 mM glucose, pH 7.4. To measure the Ca^{2+} and Mg^{2+} dependency of the ectophosphorylation, cations were added to the reaction mixture at a final concentration of 1.0 mM. The ectophosphorylation assay was carried out as described above. In other experiments, $MgCl_2$, and $MnCl_2$ were added to achieve the concentrations indicated in the figures.

2.6. Detection of ecto-ATP / GTP-binding sites

Labeling of ATP/GTP-binding sites of extracellular portions of membrane proteins was done using a modified procedure originally described by Peter et al. [10]. Cells were isolated over Ficoll/Hypaque and washed with HBSS 10 mM HEPES, 10 mM $MgCl_2$, pH 7.4. Cells (2×10^6) were then resuspended in 0.5 ml and incubated with 10 μCi [α-^{32}P]ATP or [α-^{32}P]GTP (final ATP/GTP conc. 16.6 nM) for 10 min at 37°C. To crosslink ATP or GTP to the binding site of the protein, ribose moiety was oxidized by incubation with 1 mM $NaIO_4$ for 1 min on ice and subsequently reduced with 1 mM $NaCNBH_3$ during the 1 min on ice. The reaction was stopped by addition of 20 mM $NaBH_4$ and incubation for 5 min on ice. Cells were centrifuged for 5 min at 1200 rpm ($240 \times g$) and lysed in 50 μl SDS sample buffer. Proteins were run on SDS–PAGE and analyzed by autoradiography. To detect intracellular ATP/GTP-binding sites prior to labeling, cells were permeabilized with 10 μg/ml lysophosphatidylcholine in permeabilization buffer (20 mM HEPES, 140 mM KCl, 10 mM NaCl, 2.5 mM $MgCl_2$, pH 7.8) for 5 min. Cells were washed and resuspended in HBSS/HEPES/$MgCl_2$.

2.7. Inhibitors and activators of ectophosphorylation

To inhibit ectokinase activity, intact cells were incubated in the presence of various protein kinase inhibitors. Staurosporine (Gibco-BRL) was dissolved in DMSO and added to a final concentration of 5 μM. Heparin from porcine intestinal mucosa (Sigma, cat. no. H3393), a specific casein kinase II (CKII) inhibitor [11], was dissolved in reaction buffer and used at concentrations as indicated in the figures. Dichlorobenzoimidazole furanoside (DCBF) (Sigma), another specific inhibitor of CKII, but also CKI [12], was dissolved in a reaction buffer and used at a final concentration of 100 μM.

To measure the activation of the ectokinase activity, spermine (Sigma), an activator of CKII [13], was added to the samples at a final concentration as indicated in the figures. All these reagents were shown not to affect viability of the cells (data not shown).

2.8. Phosphorylation of exogenous substrates

For detection of phosphorylation of exogenous substrates, casein (Sigma) at a concentration of 1 mg/ml was added to cell suspensions in reaction buffer. Casein was used as a substrate in most experiments described here after preliminary experiments

established the preferential ectophosphorylation of casein by ectokinases. Phosphorylation of casein was started by the addition of 10 μCi [γ-^{32}P]ATP (final ATP conc. 16.6 nM), and the mixture was incubated for 10 min at 4°C or 37°C. Following incubation, cells were centrifuged (5 min, 1200 rpm, $240 \times g$), and part of the supernatant was mixed with 5H SDS sample buffer. Samples were analyzed by SDS–PAGE using 10–20% gradient gels as described above. After the gels were stained, destained, and dried, the protein bands corresponding to casein were cut out and counted in a scintillation counter (Beckman, Columbia, MD). As a control for non-specific binding of radiolabel by the substrate, casein was incubated with [γ-^{32}P]ATP in the absence of cells.

2.9. Ectophosphorylation of CKII specific peptide

A casein kinase II assay (Upstate Biotechnology, Lake Placid, NY) was used to measure specific ectokinase activity on 2B4 cells. Briefly, 2B4 cells (10^5 cells) were incubated in HBSS buffer containing 10 mM MOPS, 12.5 mM glycerol phosphate, 2.5 mM EGTA, 0.5 mM Na- orthovanadate, 0.5 mM DTT, 0.25 mM casein kinase II specific peptide (RRRDDDSDDD), 20 mM $MgCl_2$, 125 μM cold ATP, and 10 μCi [γ-^{32}P]ATP for 20 min at 37°C. After the incubation, cells were centrifuged for 10 min at 1000 rpm ($180 \times g$) and 20 μl was spotted on phosphocellulose paper. The phosphocellulose paper was washed 5 times in cold 1% (v/v) phosphoric acid solution and 2 times with H_2O. The phosphocellulose paper was subsequently counted in a scintillation counter (Beckman, Columbia, MD).

2.10. $^{32}P_i$ labeling of cells in the presence and absence of K-252a and K-252b kinase inhibitors

One million cells (5.0×10^6 cell/ml) were incubated for 30 min at 37°C with 10 μCi of $^{32}P_i$ in 200 μl of phosphate-free RPMI 1640 media (Biofluids, Inc.) supplemented with 10% (v/v, dialyzed 3 times against phosphate-free RPMI 1640) fetal calf serum. Cell labeling was performed in the presence or absence of water-soluble and -insoluble kinase inhibitors at concentrations indicated in the figure legends. K-252a and K-252b protein kinase inhibitors were purchased from Kamiya (Thousand Oaks, CA). Cells were washed twice with HBSS containing 10 mM $MgCl_2$ and preincubated for 1 h in phosphate-free RPMI 1640 media (Biofluids, Inc.) prior to labeling in a CO_2 incubator. After labeling, the reaction mixtures were centrifuged (1000 rpm, 10 min, $180 \times g$) and the supernatant removed; the cells were washed with 200 μl phosphate-free media, then lysed with 0.5% (v/v) NP-40 and analyzed by SDS–PAGE and autoradiography.

2.11. Cytotoxicity assay

^{51}Cr-labeled target cells (P-815) were resuspended in assay buffer consisting of DMEM supplemented

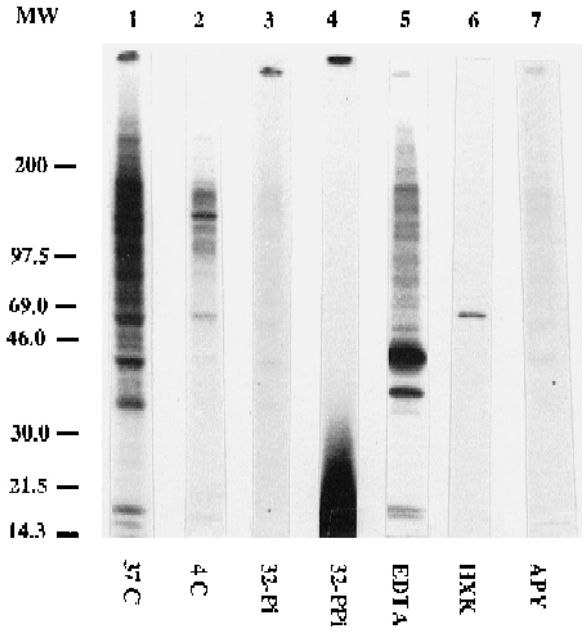

Fig. 1. Demonstration of ectophosphorylation of lymphocytes. CTL-OE4 lymphocytes were incubated with [γ-^{32}P]ATP or $^{32}P_i$ or $^{32}PP_i$ for 10 min according to the standard ectophosphorylation assay described in Section 2: Experimental procedures. To ensure that phosphorylation of extracellular portions of surface proteins was detected, the following control experiments were performed. A: lanes 1 and 2, CTL-OE4 were incubated with 10 μCi [γ-^{32}P]ATP for 10 min at 37 and 4°C, respectively; lanes 3 and 4, incubation with 10 μCi $^{32}P_i$ or 10 μCi $^{32}PP_i$, correspondingly, for 10 min at 37°C; lane 5, 10 μCi [γ-^{32}P]ATP and 10 mM EDTA; lane 6, 10 μCi [γ-^{32}P]ATP and 80 U/ml hexokinase; lane 7, 10 μCi [γ-^{32}P]ATP and 100 U/ml apyrase; representative example from 3 to 4 experiments.

with 5% FCS and 5 mM HEPES. To promote cell-mediated lysis, ^{51}Cr-labeled target cells (10^4 cells/well) were mixed with CTL at different effector : target (E:T) ratios as previously described [5] in triplicate in 96-well, V-bottom plates. After incubation, the 96-well plate was centrifuged (5 min, 800 rpm), and 50 μl of the supernatants were harvested. Radioactivity in the samples was counted using a Beckman gamma counter (Beckman Instruments, Inc., Fullerton, CA), and the mean value of each triplicate was determined. The percentage of specific ^{51}Cr release was calculated as $100 \times (a - b)/(t - b)$, where a is ^{51}Cr release from target cells in the presence of CTL, b is spontaneous release from labeled target cells in the absence of CTL, and t is the total number of cpm in the target cells. Spontaneous ^{51}Cr release from target cells was in the range of 10–15% of the total cpm.

3. Results

3.1. Ectophosphorylation of extracellular domains of cell membrane proteins

Incubation of intact T-cells (CTL-OE4) (Fig. 1) with membrane impermeable [γ-^{32}P]ATP at 16.6 nM readily resulted in a temperature-dependent and time-dependent phosphorylation of cell membrane proteins (e.g., Fig. 1, lanes 1 and 2). Similarly, cytotoxic T-cell hybridomas (PN37, MD45, MD90, and MD45-27J), T-helper hybridoma 2B4, T-cell thy-

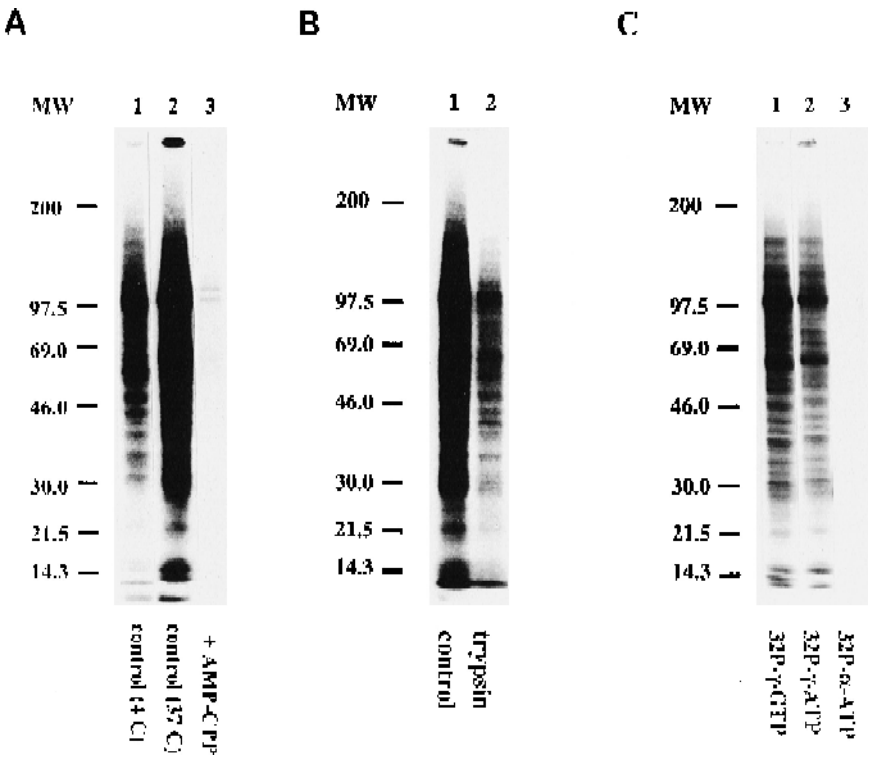

Fig. 2. Ectolabeling of lymphocytes is due to covalent binding of phosphate and can be inhibited by inclusion of cold AMP-CPP or removal of ectoproteins by trypsin pretreatment. 2B4 lymphocytes were incubated with 10 μCi [γ-^{32}P]ATP for 10 min according to the standard ectophosphorylation assay described in Section 2: Experimental procedures. A: inhibition of 2B4 phosphorylation in the presence of ATP analog AMP-CPP (100 μM) (lane 3). B: effect of trypsin treatment on the phosphoprotein pattern in 2B4 cells. Cells were pretreated with trypsin as described in Section 2. Pretreated cells or control cells were subsequently incubated for 10 min at 37°C with 10 μCi [γ-^{32}P]ATP. Lane 1, control; lane 2, trypsin pretreatment. C: both [γ-^{32}P]GTP (lane 1) and [γ-^{32}P] ATP, but not [α-^{32}P] ATP can serve as phosphate donors in ectophosphorylation of 2B4. Cells were incubated for 10 min at 37°C in the presence of 10 μCi of the indicated nucleotide. Data are representative examples from 3 experiments.

moma EL-4, and freshly isolated PELs, as well as P-815 mastocytoma cells, all had many easily detectable, ectophosphorylated surface proteins ranging in molecular weight from 14 to 200 kDa. Both quantitative and qualitative differences were observed in the ectophosphorylation patterns of these cells (data not shown).

In order to prove the 'ecto' nature of the phosphorylation after incubation with [γ-^{32}P]ATP, we performed extensive control experiments. Intracellular labeling of proteins due to hydrolysis of [γ-^{32}P]ATP and intracellular formation of ^{32}P-labeled ATP was prevented by using millimolar concentrations of inorganic phosphate (P_i) in the standard ectophosphorylation incubation. In addition, millimolar concentrations of unlabeled ATP present intracellularly will also compete with the labeling of intracellular proteins. Indeed, no labeling of cell proteins occurred when $^{32}P_i$ or $^{32}PP_i$ was added to cell suspensions (Fig. 1, lanes 3 and 4) during a 10-min incubation period. This excludes the possibility of phosphorylation of intracellular proteins by $^{32}P_i$ or $^{32}PP_i$ possibly formed through the hydrolysis of extracellular [γ-^{32}P]ATP.

Furthermore, the labeling is not the result of strong ATP binding and is, indeed, due to phosphorylation since, when [α-^{32}P]ATP was used instead of [γ-^{32}P]ATP, no labeling of any protein was observed (Fig. 2C, lane 3), reflecting that only transfer of γ-phosphate to ectosubstrates is detected under our assay conditions.

Phosphorylation by ectokinases is expected to be inhibitable by the presence of ATP-consuming enzymes, whereas intracellular phosphorylation should not be affected. This was indeed the case, since we observed inhibition of labeling of proteins by ATPo in the presence of hexokinase or apyrase (Fig. 1, lanes 6 and 7) (the only labeled band in lane 6 is due to autophosphorylation of hexokinase itself).

Labeling of ectosubstrates was strongly inhibited by water-soluble, cell-impermeable ATP analog, AMP-CPP, a hydrolyzable ATP analog, most likely due to competition with [γ-^{32}P]ATP for binding at the ATP-binding sites of ectokinase(s) and phosphorylation (Fig. 2A).

Additional evidence that the observed phosphorylation of most of labeled proteins is due to phosphorylation of extracellular portions of surface proteins was provided by the use of proteases to remove cell-surface proteins (Fig. 2B). Treatment with trypsin prior to incubation with [γ-^{32}P]ATP substantially reduced ectophosphorylation of cells either due to the removal of ectoprotein kinases or their ectosubstrates.

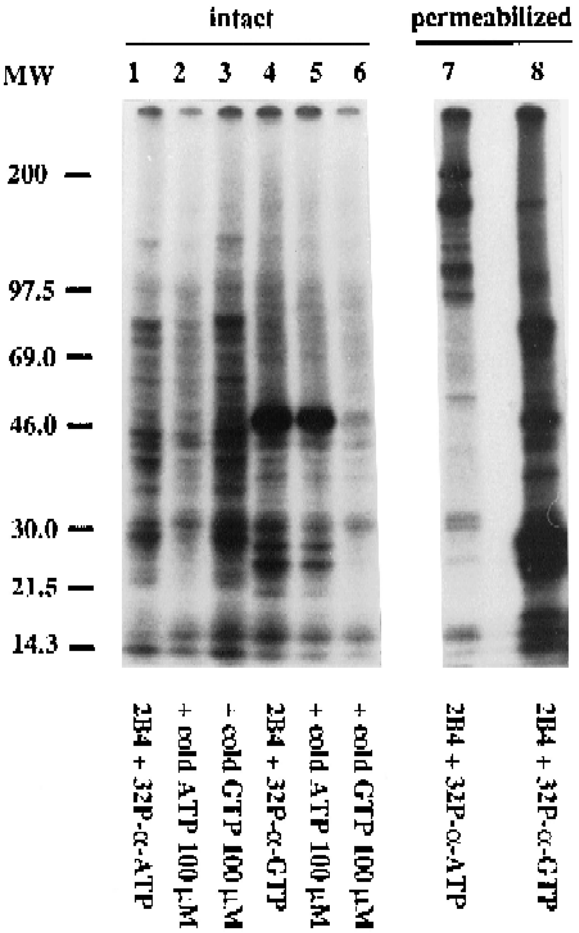

Fig. 3. Labeling of the extracellular ATP- and GTP-binding proteins on lymphocyte surface. Lanes 1–3: labeling of ATP-binding proteins in 2B4 cells. Cells were incubated with [α-^{32}P]ATP for 10 min at 37°C, followed by covalent linkage of the nucleotide to its binding site through periodate oxidation (lane 1), as described in Section 2: Experimental procedures. Competition for labeling of ATP-binding site (which reflects the specificity of interactions) was studied by inclusion of 100 μM cold ATP (lane 2) or GTP (lane 3). Lanes 4–6: labeling of GTP-binding proteins in 2B4 cells. Cells were incubated with [α-^{32}P]GTP for 10 min at 37°C, followed by covalent linkage of the nucleotide to its binding site through periodate oxidation (lane 4); lanes 5 and 6, effect of inclusion of 100 μM cold ATP (lane 5) or 100 μM cold GTP (lane 6) on labeling of GTP binding sites; lanes 7 and 8, labeling of lysophosphatidyl choline-permeabilized 2B4 cells with [α-^{32}P]ATP (lane 7) or [α-^{32}P]GTP (lane 8). Data are representative examples from 4 to 5 experiments.

3.2. ATP- and GTP-binding sites at the cell surface

Important evidence for the presence of ectoenzymes in lymphocytes is provided by the demonstration of ATP-binding proteins and GTP-binding proteins by labeling of extracellular ATP- and GTP-binding sites (Fig. 3) on the surface of T-cells. These ATP-binding proteins are expected to include ectokinases, ecto-ATPases, and purinergic ATP receptors. Their expression is demonstrated here using chemical crosslinking of [α-^{32}P]ATP [10]. More than 20 major cell-surface proteins of 2B4 were labeled with [α-^{32}P]ATP (Fig. 3, lane 1). Labeling was specific for ATP, since an excess of cold ATP reduced incorporation of label by approximately 35% (e.g., radiolabeling of 25 kDa protein), whereas cold GTP did not (Fig. 3, lanes 2 and 3). Labeling with [α-^{32}P]GTP revealed GTP-binding proteins on the cell surface of 2B4 cells. Excess cold GTP (lane 6), but not ATP (lane 5) inhibited the incorporation of label, showing the specificity of the labeling. A 56% inhibition of labeling of radiolabeled 50 kDa protein in presence of cold GTP was observed, whereas cold ATP reduced labeling only by 8%. The pattern of GTP-labeled proteins is different from that after ATP labeling. Prominent GTP-labeled proteins have molecular weights of approximately 50, 27, and 24 kDa.

Control labeling of intracellular ATP/GTP-binding sites in permeabilized cells resulted in a completely different labeling pattern (Fig. 3, lanes 7 and 8) when compared to labeling of intact cells. Most notably, among the intracellular proteins that were extensively labeled in permeabilized cells, but were not detected amongst ectolabeled proteins, are at least five proteins, with molecular weights ranging from 90 to 200 kDa. Since we loaded approximately ten times less protein obtained from the permeabilized cells on the gels, the specific ATP-labeling of these intracellular proteins is much higher than that of the extracellular portions of proteins from intact cells. Similar results were obtained with the CTL clone

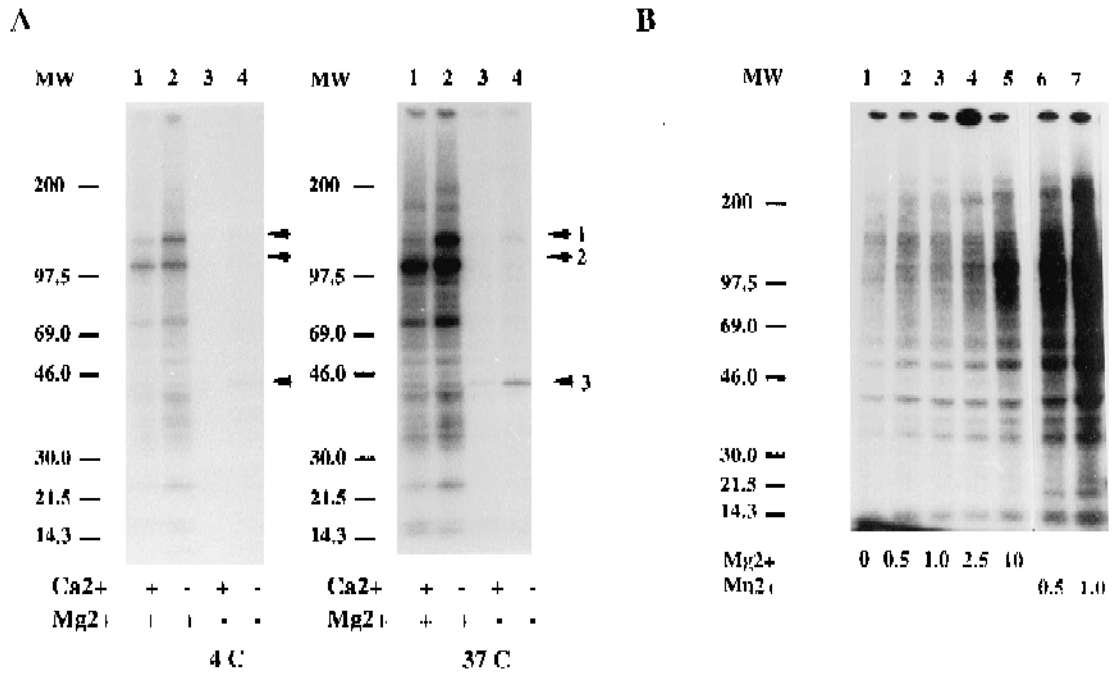

Fig. 4. Cation dependency of ectophosphorylation. A: effect of different combinations of Ca^{2+} and Mg^{2+} on ectophosphorylation of 2B4 cells at 4 and 37°C. Arrows 1, 2, and 3 next to lane 4 (at 4 and 37°C) indicate proteins that are ectophosphorylated in a Ca^{2+}- and Mg^{2+}-independent manner. B: Mn^{2+} and Mg^{2+} concentration dependency of the ectophosphorylation of CTL OE4; lanes 1–5, effect of increasing concentrations Mg^{2+} on phosphorylation, lane 6 and 7, effect of increasing concentrations of Mn^{2+} (mM) on ectophosphorylation of 2B4. Incubation was performed in the presence of 10 μCi [γ-^{32}P]ATP for 10 min at 37°C. Data are representative examples from 3 to 4 experiments.

OE4 (data not shown). The extensive qualitative and quantitative differences between intact and permeabilized cells suggest that the observed labeling is indeed due to the presence of ATP-binding sites of proteins that are exposed on the extracellular side of the plasma membrane of the lymphocyte.

3.3. Characterization of ectoprotein kinase on T-cells: cation dependency of ectophosphorylation

Extracellular phosphorylation of T-cells was Mg^{2+} dependent (Fig. 4) and increasing the concentration of Mg^{2+} augmented the labeling. Addition of Ca^{2+} to the medium not only did not increase ectophosphorylation, but the labeling of some proteins was diminished (compare labeling pattern form lane 1 with lane 2 in Fig. 4A). This indicated the presence of specific, but minor, Ca^{2+}-dependent ectophosphatase activity and, possibly, specific phosphorylation/dephosphorylation of some ectophosphoproteins.

The addition of EDTA to chelate Mg^{2+} and Ca^{2+} resulted in a greatly decreased phosphorylation of most proteins in a short-term ectophosphorylation assay. Of special interest are two ectophosphorylated proteins, with apparent MW of 38 and 43 kDa, which were extensively labeled even in the presence of EDTA (Fig. 1, lane 5). Labeled proteins with similar molecular weights were prominent after incubation in the absence of Ca^{2+} (Fig. 4A; compare bands marked by arrows in lanes 3 and 4), indicating that these proteins are phosphorylated in a Ca^{2+}- and Mg^{2+}-independent manner. The nature of these phosphoproteins is not yet known and warrants further investigation.

Addition of Mn^{2+} greatly enhanced ectophosphorylation (Fig. 4B). Interestingly, Mn^{2+} has an inhibitory effect on CTL function, possibly due to interference with a Ca^{2+}-dependent step, but not due to inhibition of the CTL–target cell adhesion [5]. Our observation of strong Mn^{2+} dependency of ectokinase activity is in agreement with the observation of Skubitz et al. [20], in neutrophils, but is in contrast to Kinzel et al. [24], who found that Mn^{2+} inhibited the activity of ectokinase of HeLa cells. It remains to be established if the observed effect of Mn^{2+} is also linked to ectotyrosine kinase activity, since similar enhancing effects of Mn^{2+} were observed with ectoprotein tyrosine kinase activity in epidermoid cells [19].

3.4. Ectokinase has properties of a casein kinase II (CKII)

The results of preliminary experiments (data not shown), in which different substrates and inhibitors were tested in ectophosphorylation assays, suggested that one of the major ectokinases in T-lymphocytes is

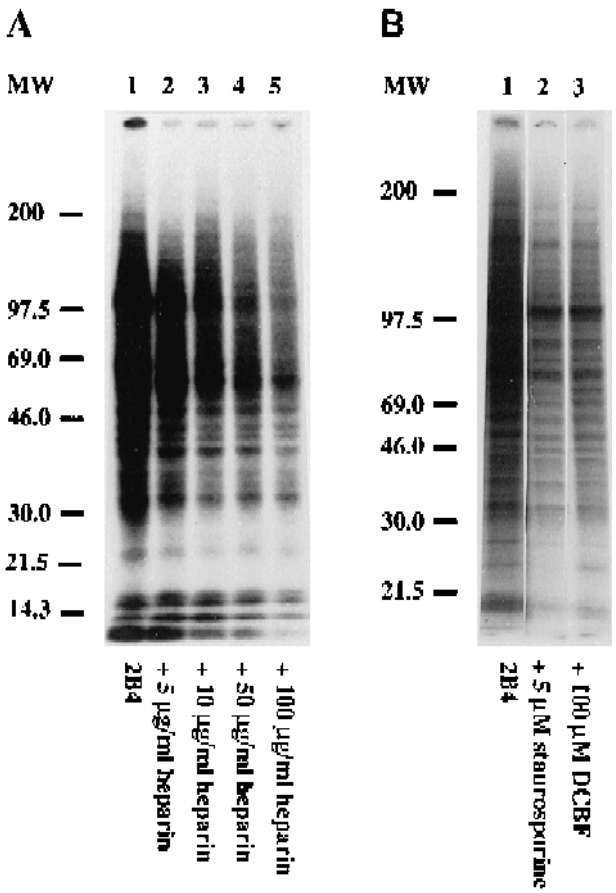

Fig. 5. Effect of different protein kinase inhibitors on phosphorylation of surface proteins of T-cells. A: effect of heparin, staurosporine, and 5,6-dichlorobenzimidazole furanoside (DCBF) on the ectophosphorylation of surface proteins of 2B4 cells. Cells were incubated for 10 min at 37°C in presence of 10 μCi [γ-^{32}P]ATP. Left panel: lane 1, ectophosphorylation of 2B4 cells, no heparin present; lanes 2, 3, 4, and 5, ectophosphorylation of 2B4 in the presence of 5, 10, 50, and 100 μg/ml heparin, respectively. Right panel: lane 1, ectophosphorylation of 2B4 cells for 10 min at 37°C, no inhibitors present; lane 2, 10 μM staurosporine; lane 3, 100 μM DCBF. Data are representative examples from 2 to 3 experiments.

similar to CKII. A broad-spectrum protein kinase inhibitor, staurosporine, inhibited total ectophosphorylation at a relatively low concentration for 36% (Fig. 5B, lane 2). Other inhibitors, such as heparin and 5,6-dichlorobenzimidazole furanoside (DCBF) (Fig. 5B, lane 3), both inhibitors of CKII activity [11,12], have also been shown to be very effective blockers of ectophosphorylation. DCBF (100 μM) reduced the total incorporated label for 35%. Heparin caused a concentration-dependent inhibition of ectophosphorylation, and as little as 5 μg/ml of heparin (Fig. 5A, lane 2, left panel) was sufficient to effectively decrease the incorporated label into cell membrane proteins of 2B4 cells. Total ectophosphorylation of 2B4 was reduced by 45% at 100 μg/ml heparin. In addition, the recently developed inhibitor of ectoprotein kinases, the membrane-impermeable compound K-252b [26,27], strongly inhibited ectophosphorylation in our assays, while having no effect on intracellular phosphorylation (see Fig. 8).

Phosphorylation of the exogenously added protein substrate casein was inhibited by heparin in a dose-dependent fashion (Fig. 6A) at both 4 and 37°C. At 5 μg/ml, heparin inhibited casein phosphorylation more than 60%. At 4°C, the intracellular uptake of heparin by active mechanisms is very unlikely, providing strong support for the inhibitory effect on an extracellular process (Fig. 6A). Other sulfated polysaccharides (e.g., dermatan sulfate, hyaluronic acid) were much less inhibitory or not at all effective in blocking phosphorylation of either exogenous or endogenous ectosubstrates in accordance with their CKII inhibitory potency [13] (data not shown).

Addition of cAMP or cGMP (0.5 mM) into the ectokinase assay of T-cells did not influence the degree or pattern of phosphorylation of the membrane proteins (data not shown), suggesting that, in contrast to other cells [28], no cAMP- or cGMP-dependent ectoprotein kinases are present at the T-cell surface.

Our data suggested that one of the most active ectoprotein kinases on the T-cell surface has similarities to CKII. A similar kinase has been reported to be present on the surface of other cell types, including

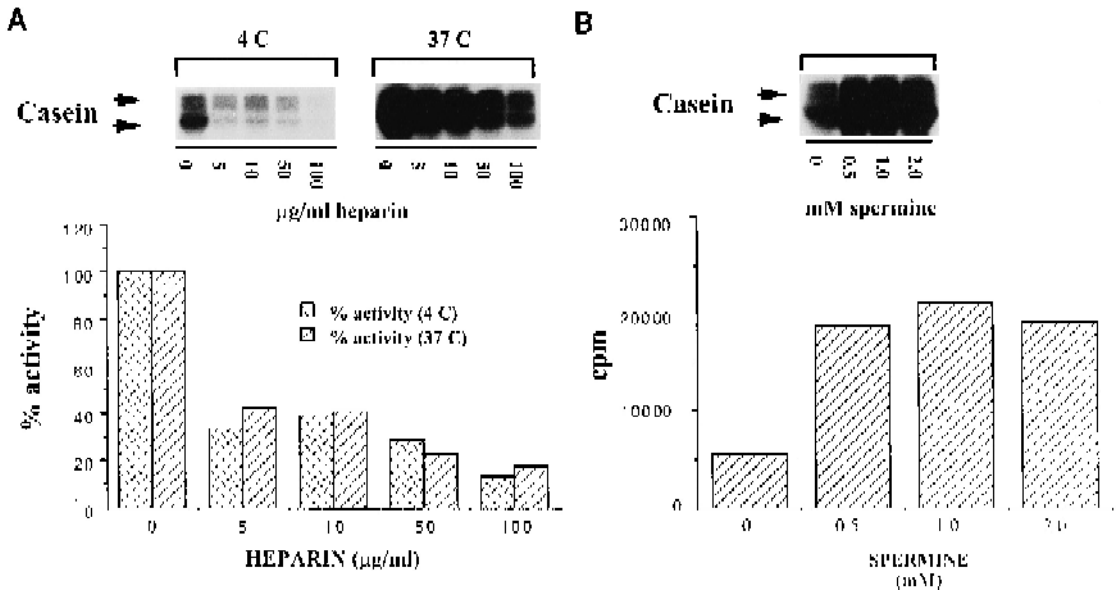

Fig. 6. Heparin inhibits and spermine enhances the phosphorylation of extracellular casein by 2B4 lymphocytes. A: effect of heparin on the phosphorylation of casein. Substrate (casein) was added to intact 2B4 cells at 4 and 37°C and incubated for 10 min after addition of 10 μCi [γ-^{32}P]ATP in absence or presence of increasing concentrations of heparin (0, 5, 10, 50, and 100 μg/ml). After the incubation, supernatant was collected, subjected to SDS–PAGE and radioactivity of the casein bands was quantitated. B: effect of casein kinase II activator, spermine (0, 5, 1.0, and 2.0 mM) on the ectophosphorylation of casein by 2B4. Substrate (casein) was added to intact 2B4 cells at 37°C and incubated for 10 min after addition of 10 μCi [γ-^{32}P]ATP in the presence of increasing concentrations of spermine. Data are representative examples from 3 to 4 experiments.

Table 1
Phosphorylation of casein kinase II specific peptide by intact 2B4 lymphocytes

	Incorporated label [a,b]
2B4 + CKII peptide	5048 ± 101
2B4 + CKII peptide + 1 mM cold ATP	1360 ± 249 [c]
2B4 + CKII peptide + 1 mM cold GTP	696 ± 264 [c]

[a] Corrected for no peptide addition.
[b] Results represent mean from 3 separate experiments with 4 replicates.
[c] Significantly different from 2B4 + CKII peptide, $P < 0.05$.

neutrophils and HeLa cells [21]. One of the identifying properties of CKII-like kinases is their ability to efficiently use both ATP and GTP as phosphate donors [13]. In agreement with the identification as CKII-like protein kinase, the ectokinases from 2B4 and OE4 are shown here (Fig. 2C, lanes 1 and 2) to utilize both [γ-^{32}P]GTP and [γ-^{32}P]ATP equally well.

Another property of CKII protein kinase is that polycations such as spermine can stimulate kinase activity [13,14]. An increase of up to four times in the phosphorylation of casein by 2B4 cells was observed in the presence of 0.5 mM spermine (Fig. 6B). Similarly, spermine enhanced phosphorylation of casein by CTL-OE4. No effect of spermine on cell viability was observed, thus excluding the possibility of the increased kinase activity being caused by leakage of intracellular protein kinases (data not shown). Furthermore, a specific peptide substrate for CKII protein kinase is rapidly phosphorylated by intact 2B4 lymphocytes (Table 1). Phosphorylation of the peptide is inhibited by adding both cold ATP or GTP (1 mM). Thus, these results suggest that T-cells express at least one protein kinase with casein kinase II-like properties.

3.5. Substrate specificity of the ectoprotein kinase in phosphorylation of serum proteins

The exact physiological role of ectokinases in T-cells is not clear, but clues may come from the identification of specific substrates of ectokinases. Cell surface-located ectoprotein kinases that can phosphorylate endogenous cell-surface proteins or exogenous substrates (e.g., fibroblast growth factor) [14], when submicromolar concentrations of extracellular ATP are present, have been detected in different intact cells [14–19]. An important serum protein vitronectin was shown to be a specific substrate for human neutrophil ectoprotein kinase [20].

A comparison of phosphorylation of purified vitronectin and fibronectin was done in experiments where T-cells were incubated with these protein substrates in the presence of [γ-^{32}P]ATP. Results (Fig. 7) reveal the remarkable substrate specificity of the ectokinase for vitronectin (lanes 1–3), but not for

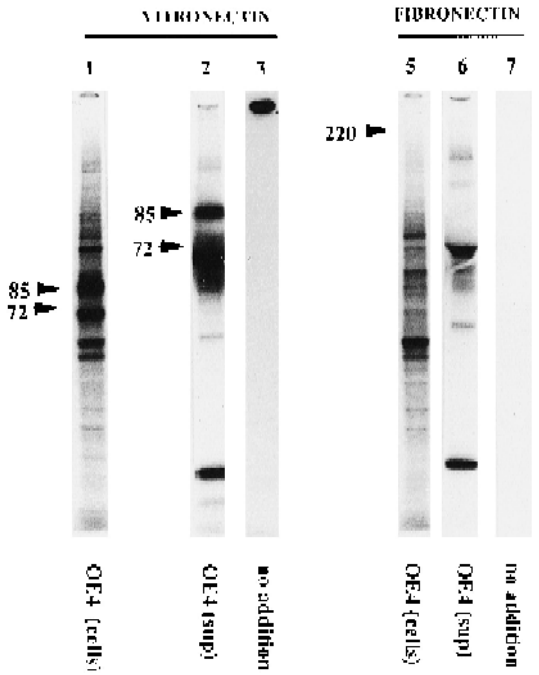

Fig. 7. Substrate specificity of ectoprotein kinase. Vitronectin, but not fibronectin, is a substrate for phosphorylation by the T-cell surface ectoprotein kinases. Intact cells were incubated with [γ-^{32}P]ATP in presence of vitronectin (50 μg/ml) or fibronectin (100 μg/ml) for 10 min at 37°C as described in Section 2: Experimental procedures. The cells were centrifuged after incubation, and cell-associated proteins (cell pellet, lane 1) and supernatants were analyzed for the composition of phosphorylated proteins. Lane 1, CTL-OE4 cell pellet-associated proteins. Vitronectin chains (85 and 72 kDa) are indicated by arrows; lane 2, phosphorylated chains of vitronectin in supernatant of CTL-OE4. Arrows indicate the positions of the 85- and 72-kDa vitronectin chains; lane 3, incubation of vitronectin with [γ-^{32}P]ATP in the absence of cells; lanes 5–7, no detectable phosphorylation of fibronectin by T-cells; lane 5, CTL-OE4 cell pellet-associated proteins; lane 6, phosphorylated proteins in cell supernatants after incubation with fibronectin; lane 7, incubation of fibronectin in the absence of cells. Arrow indicates position of 220-kDa fibronectin band on protein-stained gel. Data are representative examples from 3 experiments.

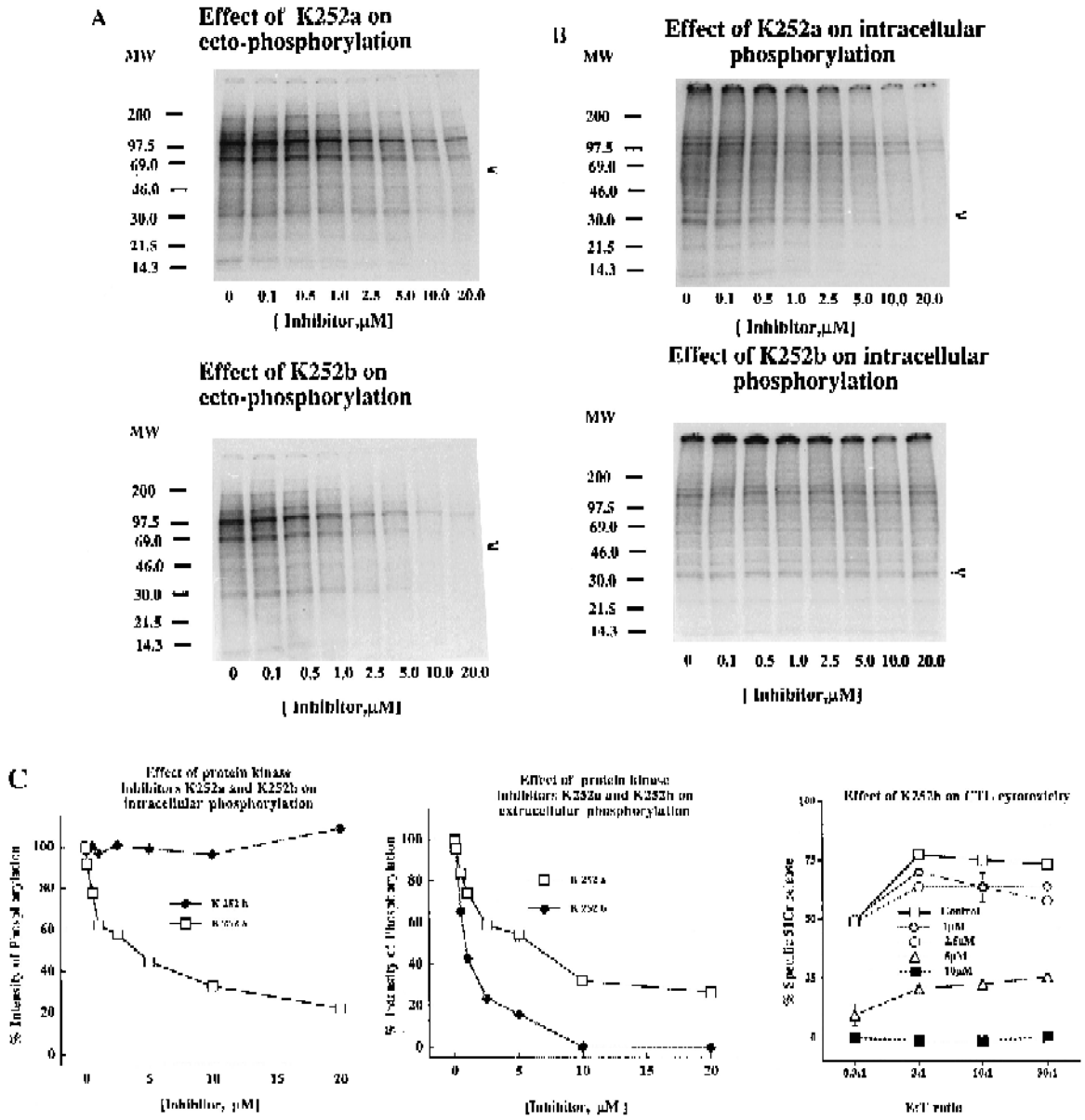

Fig. 8. Effect of cell membrane-impermeable ectoprotein kinase inhibitor K-252b on T-cell ectophosphorylation and effector functions. Comparison of effects of K-252a and K-252b on ecto- and intracellular protein phosphorylation of CTL-OE4. A: both K-252a and K-252b inhibit *extracellular* phosphorylation. Ectophosphorylation assay of intact CTL-OE4 cells was performed in the presence of increasing concentrations of cell-permeable kinase inhibitor K-252a (upper panel) or the cell-impermeable inhibitor K-252b (lower panel) as described in Section 2: Experimental procedures. B: K-252a, but not K-252b, is efficient in the inhibition of *intracellular* phosphorylation. CTL-OE4 cells were metabolically labeled with $^{32}P_i$ for 30 min at 37°C (see Section 2) in the presence of increasing concentrations of cell-permeable kinase inhibitor K-252a (upper panel) or the cell-impermeable inhibitor K-252b (lower panel). C: densitometer analysis of the inhibitory effect of K-252a (open symbols) and K-252b (closed symbols) on extracellular (left figure) and intracellular (middle figure) phosphorylation of CTL-OE4. Arrows identify arbitrarily chosen protein bands (indicated by arrows in A and B), that are compared using a computerized densitometer. Right figure, K-252b inhibits CTL cytotoxicity at concentrations, which effectively block extracellular phosphorylation, but not intracellular phosphorylation. CTL-OE4 were incubated with ^{51}Cr-labeled allogeneic target cells P-815 at different effector to target ratios for 4 h at 37°C. The cell impermeable kinase inhibitor K-252b was added to the incubation at increasing concentrations. After 4 h, target cell death was measured by determining the ^{51}Cr release in the supernatant, as described in Section 2. Data are representative examples from 2 experiments.

fibronectin (lanes 5–7), since phosphorylated chains of vitronectin (85 and 72 kDa) are observed both cell-associated (lane 1) and in supernatants after incubation with OE4 (lanes 2). Both vitronectin and fibronectin were present in the assay as evidenced by protein staining of gels (data not shown). No phosphorylation of vitronectin was observed when incubation with [γ-^{32}P]ATP was performed in the absence of cells (lanes 3 and 7).

3.6. Correlations of inhibition of ectokinase and cellular cytotoxicity by general and CKII-specific protein kinase inhibitors

Important evidence supporting the role of ectophosphorylation in T-cell effector functions was provided by experiments where the water-soluble and membrane-impermeable inhibitor K-252b [21,22] was able to inhibit CTL cytotoxicity (Fig. 8C, right figure). This ability to interfere with functions of intact cells without entering the cell interior is interpreted as evidence for the involvement of ectoprotein kinases.

We confirmed the ability of this inhibitor to affect ectophosphorylation of T-lymphocytes (but not intracellular phosphorylation) in our experimental system (Fig. 8). Both K-252a (membrane-permeable, chemically closely related protein kinase inhibitor) and K-252b were able to inhibit ectophosphorylation (Fig. 8A, lower panel) at concentrations similar to those described in ectophosphorylation studies using neuronal cells [33]. As predicted, K-252b was indeed unable to block intracellular protein phosphorylation using $^{32}P_i$ (Fig. 8B, lower panel).

Strong inhibition of CTL-mediated cytotoxicity by cell membrane-impermeable ectokinase inhibitors K-252b (Fig. 8, right figure) was observed at concentrations that correlate with the ability of K-252b to block ectophosphorylation without interference in intracellular phosphorylation (Fig. 8C, left figure). This inhibition is likely to be exerted on the level of the cell–cell contacts since, e.g., 5 μM K-252b strongly inhibited CTL-mediated cytotoxicity during cell–cell interactions, while no effect of 5 μM K-252b on anti-TCR mAb-induced granule exocytosis from CTL was detected (data not shown).

In addition to the recently developed inhibitor of ectokinases K-252b, we tested other available inhibitors of CKII-kinase. Target cell lysis induced by CTL-OE4 was also inhibited by various inhibitors of CKII protein kinase. Heparin at 100 μg/ml strongly inhibited ectophosphorylation and inhibited CTL-induced TC lysis. Importantly, highly inhibitory concentrations of heparin in cytotoxicity assays against nucleated TC did not affect lysis of sheep red blood cells at all. These data serve as an important control that heparin is not interfering in the TCR-triggered transmembrane activation of CTL, but inhibiting interactions leading to nucleated TC lysis. Inclusion of other mucopolysaccharides, such as hyaluronic acid or dermatan sulfate, which do not have a strong inhibitory effect on CKII activity [11], did not have a significant effect on OE4-induced TC lysis. Polyanethole sulfonate (PSA), a highly charged anion that was shown to block cell-mediated killing as reported earlier [23], also blocked retargeted killing of EL-4 by CTL-OE4. PSA was used here, since it was found to be a potent inhibitor of the ectoprotein kinase activity [12]. Another inhibitor of CKII, DCBF, at 100 μM caused strong inhibition of the TC lysis by CTL-OE4. Addition of casein, acting as a competitive substrate for phosphorylation by CKII, resulted in a nearly complete inhibition of TC lysis at 5 mg/ml (data not shown).

4. Discussion

Our present working hypothesis is based on the assumption that after TCR-triggered accumulation of ATPo (as $MgATP^{2-}$ but not ATP^{4-}) on the surface of the T-cell, the lymphocyte's ectoprotein kinases/phosphatases could phosphorylate/dephosphorylate extracellular portions of plasma membrane proteins (including recognition molecules, growth factor receptors, growth factors, and cell–cell adhesion proteins), changing their conformation, affinity to ligands, ability to propagate transmembrane signals, and capacity to form multimolecular complexes. Such changes may play a role in the regulation of molecular interactions during cell–cell contacts between, e.g., CTL and target cells, T-helper and APC, and T-helper and B-cells [7].

In this study, we show the presence of ectoprotein kinase activity on the surface of murine T-lymphocytes. Several cell-surface proteins of the T-cells are

phosphorylated in the presence of ATPo. Extensive control studies [24] confirmed that labeling was due to ectophosphorylation and was not the result of labeling of proteins by intracellular protein kinases due to uptake of hydrolyzed [γ-^{32}P]ATP. Since in our experiments we used nanomolar concentrations of ATP (final conc. 16.6 nM) and reported plasma concentrations of ATP range from 200 to 1150 nM [25], ectophosphorylation in vivo likely to occur. Further characterization of the ectoprotein activity revealed properties of a CKII-like kinase, which phosphorylation activity could be supported by both ATP and GTP as phosphate donor and could be inhibited by classical CKII inhibitors as heparin, DCBF or enhanced by spermine. Recently, the presence of a heparin-inhibitable CKII-like ectoprotein kinase has also been described for human neutrophils [20].

The potential role of these ectoprotein kinase activity in the effector function of the lymphocytes was examined using the hydrophilic protein kinase inhibitor K-252b [26]. Ehrlich and collaborators found that K-252b inhibited nerve growth factor-induced neurite outgrowth in PC12 cells, which was mediated through the inhibition of ectoprotein kinase activity [27]. Previous studies showed that K-252b does not permeate the cell membrane [21,22], although some controversy exists about the impermeability [36]. Our study showed that K-252b at concentrations which greatly inhibited extracellular phosphorylation, was a potent inhibitor of the killing of P-815 TC by CTL-OE4 in a cytotoxicity assay. Since K-252b did not affect intracellular phosphorylation at these concentrations, interference with intracellular events is not likely to be the cause of inhibition of CTL effector function. Moreover, similar results were obtained using heparin, DCBF, other ectoprotein kinase inhibitors or by the inclusion of casein as a competitive substrate for the ectoprotein kinases (unpublished results). These results strongly suggest the importance of the ectoprotein kinase activity in the effector function of CTL.

The mechanism by which ectophosphorylation of cell-surface proteins is involved in modulating the effector function of T-cells is presently unknown. Firstly, the possibility is considered that ectodomains of important surface proteins could be phosphorylated while inside cells and subsequently be dephosphorylated by ectoprotein phosphatases. Indeed, since many secretory proteins are Ser/Thr-phosphorylated [28], it was proposed that ectoprotein kinases can phosphorylate secreted proteins during their presence in the lumen of ER or Golgi complex [29] before the ectoprotein kinase is transported to the cell surface. Recently, we have found that ectodomains domains of the T-cell antigen receptor is constitutively phosphorylated at serine and threonine intracellularly. In addition, phosphorylation of the extracellular domains could also be achieved by extracellular ATP, suggesting that ectophosphorylation of T-cell receptor domains can reversibly modified by extracelluar protein phosphatases and kinases [30]. Secondly, it is also possible that – by autophosphorylation or phosphorylation of (neighboring) cell-surface proteins – the ectoprotein kinase transduces transmembrane signals. This is evidenced by the observation of changes in activity of PC-1, a threonine-specific ectoprotein kinase present on plasma cells, in response to fibroblast growth factor (FGF) [29]. Thirdly, ATPo may also modulate the functioning of recognition and/or adhesion molecules by phosphorylating their cytoplasmic domains. This is supported by the demonstration that ATPo mediated the phosphorylation of CD31, CDw32, and HLA-class I proteins on the surface of human neutrophils [31]. Lastly, phosphorylation/dephosphorylation of ectoproteins can have substantial effects on their binding affinity for other proteins. For instance, phosphorylation of the ectodomains of CD36, a membrane receptor of thrombospondin, malaria-infected erythrocytes and collagen, increased its affinity for collagen and resulted in a loss of trombospondin binding. Whereas, dephosphorylation of CD36 induced opposite binding reactivity towards these substrates [32]. Our study shows that CTL-OE4 and 2B4 lymphocytes exhibit a clear substrate specificity, mediating the phosphorylation of the serum protein vitronectin, but not fibronectin. It is presently unknown if phosphorylation of vitronectin modulates its ability to bind to its receptor. It is interesting to note that we have also found the presence of ectoprotein phosphatase activity on the lymphocyte's cell surface (unpublished results), indicating that there is a complete system of phosphorylation and dephosphorylation present at the cell surface.

According to ongoing studies, ATPo may, in addi-

tion, act by transmitting transmembrane signals through purinergic receptors [33]. Effects of ATPo on the thymocyte development in the fetal thymus organ culture are consistent with a role for the p2x ATPo receptor in thymocyte maturation [8]. The recent discovery of identity between the p2x ATPo purinergic receptor [34] and the thymocyte apoptosis related RP2 gene [35] suggests a functional importance of these receptors in T-cell development. Moreover, we recently cloned a p2u ATPo receptor from a T-lymphocyte's cDNA library and we are currently investigating the functional role of this purinergic receptor (M. Koshiba, S. Apasov, and M.V. Sitkovsky, manuscript in preparation).

In conclusion, the results described here point to the presence of a functional ectophosphorylation system on the lymphocyte surface. Phosphorylation and possibly dephosphorylation of ectosubstrates may be important in the regulation of cell–cell interactions. Since cell–cell interactions are of decisive importance in the functioning of the immune system, our experiments point to the need to study specific ectophosphorylated substrates among functionally important cell-surface proteins to understand the exact contribution of ATPo and ectoenzymes in lymphocyte differentiation, development, and effector responses.

References

[1] F.A. Edwards, A.J. Gibb, D. Colguhoun, Nature 359 (1992) 144–147.
[2] Y. Osipchuk, M. Cahalan, Nature 359 (1992) 241–244.
[3] A. Filippini, R.E. Taffs, M.V. Sitkovsky, Proc. Natl. Acad. Sci. USA 87 (1990) 8267–8271.
[4] A. Filippini, R.E. Taffs, T. Agui, M.V. Sitkovsky, J. Biol. Chem. 265 (1990) 334–340.
[5] F.A. Redegeld, A. Fillippini, M.V. Sitkovsky, J. Immunol. 147 (1991) 3638–3645.
[6] F.A. Redegeld, A. Fillippini, G. Trenn, M.V. Sitkovsky, in: M.V. Sitkovsky, P.A. Henkart (Eds.), Cytotoxic Cells, Birkhauser, Boston, 1993, pp. 307–313,
[7] E.H. Abraham, A.G. Prat, L. Gerweck, T. Sneveratne, R.J. Arceci, R. Kramer, G. Guidotti, H.F. Cantiello, Proc. Natl. Acad. Sci. USA 90 (1993) 312–316.
[8] S. Apasov, M. Koshiba, F. Redegeld, M. Sitkovsky, Immunol. Rev. 146 (1995) 5–19.
[9] F. Di Virgilio, V. Bronte, D. Collava, P. Zanovello, J. Immunol. 143 (1989) 1955–1960.
[10] M.E. Peter, J. She, L.A. Huber, C. Terhorst, Anal. Biochem. 210 (1993) 77–85.
[11] G.M. Hathaway, T.H. Lubben, J.A. Traugh, J. Biol. Chem. 255 (1980) 8038–8041.
[12] L.A. Pinna, Biochim. Biophys. Acta 1054 (1990) 267–284.
[13] F. Meggio, B. Boldyreff, O.G. Issinger, L.A. Pinna, Biochemistry 33 (1994) 4336–4342.
[14] K.M. Skubitz, D.D. Ehresmann, T.P. Ducker, J. Biol. Chem. 147 (1991) 638–650.
[15] D. Kubler, W. Pyerin, V. Kinzel, J. Biol. Chem. 257 (1982) 322–329.
[16] F. Amano, T. Kitagawa, Y. Akamatsu, Biochim. Biophys. Acta 803 (1984) 163–173.
[17] Y.H. Ehrlich, T.B. Davis, E. Bock, E. Kornecki, R.H. Lenox, Nature 320 (1986) 67–70.
[18] U.P. Naik, E. Kornecki, Y.H. Ehrlich, Biochim. Biophys. Acta 1092 (1991) 256–264.
[19] E.S. Kang, S. Postlethwaite, S. Shaeffer, B. Sawhney, Cell Immunol. 87 (1984) 319–326.
[20] K.M. Skubitz, D.D. Ehresmann, T.P. Ducker, J. Immunol. 147 (1991) 638–650.
[21] K. Muramota, K. Kobayashi, S. Nakanishi, Y. Matsuda, Y. Kuroda, Proc. Jpn. Acad. 64 (1988) 319–322.
[22] K. Nagashima, S. Nakashini, Y. Matsuda, FEBS Lett. 293 (1991) 19–23.
[23] E. Martz, B. Benacerraf, Clin. Immunol. Immunopathol. 1 (1973) 533–546.
[24] V. Kinzel, D. Kubler, E. Burow, W. Pyerin, in: Kreutzenberg, G.W. et al. (Eds.), Cellular Biology of Ectoenzymes, Springer Verlag, Berlin, 1986, pp. 179–190.
[25] L.M. Ryan, J.W. Rachow, B.A. McCarty, D.J. McCarty, J. Rheumatol. 23 (1996) 214–219.
[26] H. Kase, K. Iwahashi, S. Nakanishi, Y. Matsuda, K. Yamada, M. Takahashi, C. Murakata, A. Sato, M. Kaneko, Biochem. Biophys. Res. Commun. 142 (1987) 436–440.
[27] Z. Pawlowska, M.V. Hogan, E. Kornecki, Y.H. Ehrlich, J. Neurochem. 60 (1993) 678–686.
[28] K.M. Skubitz, S.A. Gouelin, Biochem. Biophys. Res. Commun. 174 (1991) 49–55.
[29] Y. Oda, M.D. Kuo, S.S. Huang, J.S. Huang, J. Biol. Chem. 266 (1991) 16791–16795.
[30] S.A. Apasov, P.T. Smith, M.T. Jelonek, D.H. Margulies, M.V. Sitkovsky, J. Biol. Chem. 271 (1996) 25677–25683.
[31] K.M. Skubitz, S.A. Goueli, J. Immunol. 152 (1994) 5902–5911.
[32] A.S. Asch, I. Liu, F.M. Briccetti, J.W. Barnwell, F. Kwakye-Berko, A. Dokun, J. Goldberger, M. Pernambuco, Science 262 (1993) 1436–1440.
[33] G. Burnstock, Annu. New York Acad. Sci. 603 (1990) 1–19.
[34] A. Brake, M.J. Wagenbach, D. Julius, Nature 371 (1994) 519–524.
[35] G.P. Owens, W.E. Hahn, J.J. Cohen, Mol. Cell. Biol. 11 (1991) 4177–4188.
[36] A.H. Ross, C.A. McKinnon, M.C. Daou, K. Rattcliff, D.E. Wolf, J. Neurochem. 65 (1995) 2748–2756.