

Protein Tyrosine Kinases and Phosphatases Control Apoptosis Induced by Extracellular Adenosine 5'-Triphosphate

Vincenzo Bronte,¹ Beatrice Macino, Annalisa Zambon, Antonio Rosato, Susanna Mandruzzato, Paola Zanovello, and Dino Collavo²

Institute of Oncology, Chair of Immunology, Inter-University Center for Cancer Research, Via Gattamelata 64, Padova 35128, Italy

Received November 24, 1995

Extracellular ATP (ATPo) induces apoptosis and osmotic lysis in several cell lines. We investigated the role of protein tyrosine kinases (PTKs) and phosphatases (PTPases) in ATPo-induced apoptosis. The PTK inhibitor genistein prevented DNA fragmentation due to ATPo without affecting cell lysis. Comparison of western blot analysis and in vitro kinase assays of anti-phosphotyrosine immunoprecipitates indicated that ATPo activated PTKs whose activity was tightly regulated by PTPases. In fact, an early increase in tyrosine kinase activity was observed after ATPo-treatment and was prevented by specific PTPase inhibitors. In addition, a rapid dephosphorylation of phosphotyrosyl residues on several proteins was detected in ATPo-treated cells. Accordingly, inhibitors of PTPases, but not of serine/threonine phosphatases, were as effective as PTK-inhibitors in blocking ATPo-mediated DNA fragmentation. We describe the early events occurring in ATPo-induced apoptosis and suggest a role for PTPases in cell death. © 1996 Academic Press, Inc.

The term apoptosis generally refers to a programmed cell death (PCD) phenomenon characterized by a distinctive series of morphological alterations of the cell that are commonly associated with internucleosomal DNA cleavage following activation of endogenous nuclease. Apoptosis occurs during ontogenesis, adult tissue growth and homeostasis, and represents a common event involving numerous immune system reactions, for example negative selection during acquisition of the T and B lymphocyte repertoire, and target cell destruction by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (for review see 1). The induction apoptosis is subject to a complex regulatory process and represents the final outcome of multiple signal-transduction pathways that have been shown to be mediated by different second messengers, including intracellular Ca^{2+} , protein kinase C (PKC), cAMP and ceramide (2).

Moreover, a distinct set of genes, some of which have been identified, are activated and exert a positive or negative control on apoptosis in response to different stimuli or cell differentiation states (3). Numerous physiological signals can trigger PCD, such as T lymphocyte receptor (TCR) engagement, tumor necrosis factor (TNF), Fas/Apo 1 receptor, corticosteroids, irradiation and growth factor withdrawal. Our laboratory and others have demonstrated that extracellular ATP (ATPo) can act as a mediator of lymphocyte cytotoxicity by activating plasma membrane purinergic receptors (4–6). In fact, ATPo is secreted by CTL and induces target cell destruction not only by inducing necrosis but also apoptosis, thus sharing characteristics with TNF and Fas, two relevant mediators of lymphocyte cytotoxicity. Moreover, ATPo and TNF can cooperate in producing a synergistic increase in target cell death (7).

In the present studies we investigated the early cellular signalling events during ATPo-induced

¹ Current address: National Cancer Institute, Surgery Branch, National Cancer Institute, Bethesda, MD 20892.

² Corresponding author. Fax: 39-49-807-2854.

Abbreviations used: PCD, programmed cell death; TNF, tumor necrosis factor; PKC, protein kinase C; ATPo, extracellular ATP; PTK, protein tyrosine kinase; ptyr, phosphotyrosine; PTPase, protein tyrosine phosphatase; PAO, phenylarsine oxide; FBS, foetal bovine serum; DMSO, dimethyl sulfoxide; vanadate, sodium orthovanadate; mAb, monoclonal antibody; BSA, bovine serum albumine; NP-40, Nonidet P-40; DTT, dithiothreitol; CTL, cytotoxic T lymphocytes.

apoptosis. Our results indicate that tyrosine phosphatases might constitute obligate regulators of ATPo-mediated apoptosis, through a pathway also involving PTKs.

MATERIALS AND METHODS

Cells. The murine mastocytoma cell line P815 and the murine leukaemia cell line L1210 were grown in complete DMEM medium (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% Foetal Bovine Serum (GIBCO), 2 mM L-glutamine, 10 mM hepes, and 50 μ M 2-mercaptoethanol.

Reagents and antibodies. Genistein, PAO (Sigma Chemical Co., St. Louis, MO, USA) and herbimycin A (Calbiochem Corp., La Jolla, CA, USA) were dissolved in dimethyl sulfoxide (DMSO); okadaic acid stock solution (Sigma) was prepared in dimethylformamide. ATP (Sigma, A 2383) and sodium orthovanadate (vanadate) (Sigma) were dissolved in water; vanadate concentration was determined according to J. Gordon (8). The solvents alone were included in all control samples at appropriate doses. The anti-phosphotyrosine monoclonal antibody antibodies (anti-ptyr mAb) 4G10 (UBI Inc., Lake Placid, NY, USA) and 1G2 (ATCC, Rockville, MD, USA) were used in these studies.

⁵¹Cr and ¹²⁵IUDR short term release assay. Target cells (2×10^6) were labelled in 5 ml of complete medium with 3 μ Ci/ml of ¹²⁵IUDR (Amersham, Amersham U.K.) for 14 h at 37°C, in a 5% CO₂ incubator, washed, allowed to rest for 1 h, and washed again. Alternatively, cells (1×10^6) were pelleted and labelled with 100 μ Ci ⁵¹Cr (Na₂CrO₄, NEN Boston, MA, USA) for 1 hour at 37°C. Labelled target cells were resuspended in DMEM 3% FBS and plated (5000/well in triplicate) in round-bottom microtiter plates in 100 μ l/well final volume. After incubation at 37°C, 100 μ l/well of DMEM 3% FBS or 2X lysis buffer (20 mM Tris, 10 mM EDTA, 1% Triton X-100, pH 7.4) were added to ⁵¹Cr- and ¹²⁵IUDR-labelled cells, respectively. For the ⁵¹Cr release assay, plates were centrifuged at 200 \times g for 5 min and 100 μ l/well of supernatant were collected for counting. For the DNA fragmentation assay, plates were held at 4°C for 20 min after adding lysis buffer, centrifuged at 800 \times g for 10 minutes at 4°C, and 100 μ l were used for γ -counting. The percentage of specific ⁵¹Cr release or of DNA fragmentation was calculated using the expression:

$100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$, where maximum release represents 100 μ l supernatant of lysed ⁵¹Cr labelled cells or 100 μ l medium after adding lysis buffer to ¹²⁵I labelled cells, and mixing the well contents.

Phosphotyrosine immunoblot analysis. After stimulation, cells were pelleted rapidly (1700 rpm at 4°C), dissolved in 4% SDS, 10 mM Tris-acetate, 0.1 mM EDTA, 10% glycerol, and 5% 2-mercaptoethanol, pH 6.8, and immediately boiled at 100°C for 5 min. Proteins were resolved by SDS-PAGE (3×10^6 cell equivalent per lane) in 10% gels, and electrotransferred onto nitrocellulose membranes. The membranes were blocked with 3% bovine serum albumine (BSA) prepared in 50 mM Tris-Cl, 2 mM CaCl₂, 85 mM NaCl, pH 7.5, and then probed with 1 μ g/ml anti-ptyr mAb (4G10). The blots were washed four times with 50 mM Tris-Cl, 2 mM CaCl₂, 85 mM NaCl, 0.1% BSA, and 0.2% Nonidet P40 (NP-40), pH 8, incubated with ¹²⁵I-protein A (0.7 μ Ci/ml in blocking solution, Amersham), then exposed to Kodak X-Omat-S film with an X-Omatic regular intensifying screen (Kodak).

Immunoprecipitation and in vitro kinase assay. Cells (1.5×10^6 /ml) were incubated for different times with ATPo in medium containing 5 mM EDTA. The reaction was stopped by adding an equal volume of cold PBS containing 200 μ M vanadate. The cells were then pelleted at 1700 rpm for 2 min at 4°C, washed once in cold TBS (25 mM Tris, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol (DTT), 100 μ M vanadate), and solubilized at a concentration of 20×10^6 /ml of lysis buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 1% NP-40, 1mM EDTA, 1 mM DTT, 10 mM NaF, 100 μ M vanadate), to which leupeptin and pepstatin (each at 5 μ g/ml) and 1% aprotinin were freshly added. The lysates were mixed for 20 min at 4°C on a rotating wheel, and then centrifuged at 12,000 \times g for 10 min. The supernatants were placed in an Eppendorf tube containing 100 μ l of 10% *S. aureus* (Calbiochem) in lysis buffer. After 30 min incubation on a rotating wheel, they were centrifuged at 12,000 \times g, and the supernatants were transferred to new tubes containing 10 μ g/ml of 1G2 protein-G-purified mAb, and further incubated for 1.5 h. Samples were centrifuged at 12,000 \times g for 10 min, transferred to tubes containing 10 μ l of 10% *S. aureus* in lysis buffer and incubated for a further 45 min. Immunocomplexes were collected by centrifugation for 3 min at 12,000 \times g, washed three times in RIPA buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 1 mM DTT and 100 μ M vanadate), and once in TBS; they were then resuspended in a total reaction volume of 20 μ l of kinase buffer (20 mM Hepes, pH 7.5, 10 mM MnCl₂, 1 mM DTT) containing 1 μ M of unlabeled ATP and 10 μ Ci of γ -³²P-ATP (4500 Ci/mmol, ICN, Costa Mesa, USA). Samples were incubated for 20 min at 30°C to favour phosphorylation and then stopped by the addition of reducing sample buffer, and boiled. Radiolabeled proteins were resolved on 10% SDS-PAGE gels and detected by autoradiography. Where indicated, alkali treatment of the gel was performed with 1N KOH for 2 h at 56°C.

RESULTS

To define the intracellular signalling pathway leading to DNA fragmentation following ATPo treatment, P815 and L1210 cells were pretreated with increasing concentrations of genistein and then incubated in medium containing a constant ATPo dose (8 mM). As shown in Fig. 1, genistein

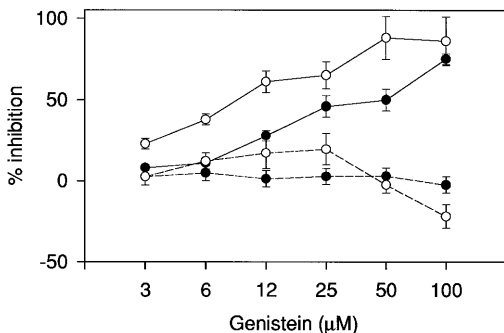


FIG. 1. Dose-dependence inhibition of ATPo-induced DNA fragmentation by genistein. Labeled P815 (open symbols) and L1210 (closed symbols) cells were incubated for 5 h with 8 mM ATPo in the presence or absence of different genistein concentrations. At the end of incubation, the supernatant was withdrawn and counted to evaluate specific release of ¹²⁵IUDR and ⁵¹Cr, as measures of DNA breakdown and cell lysis, respectively. Results are expressed as the percentage of inhibition of ¹²⁵IUDR release (continuous line) and of ⁵¹Cr release (broken line) in the presence of genistein. Negative values indicate an enhancement of isotope release. ¹²⁵IUDR release induced by ATPo treatment varied from 30 to 60%, according to the cell line and the different experiments. Spontaneous release of ¹²⁵IUDR and ⁵¹Cr never exceeded 20%, and was not modified by the presence of genistein (data not shown). Data represent the mean ± SD of three independent experiments.

brought about a dose-dependent inhibition of DNA fragmentation, evaluated as ¹²⁵IUDR release, in both cell lines without affecting the cellular lysis, assessed by ⁵¹Cr-release. Genistein alone did not modify the ¹²⁵IUDR- and ⁵¹Cr-release levels except at concentrations higher than 200 μM. Moreover it did not act as an antagonist for binding to membrane purinoreceptors since even in the presence of genistein concentrations (100 μM) that abrogated DNA fragmentation, ATPo maintained the capability to induce cell lysis. ATPo-induced DNA fragmentation was also prevented by herbimycin A (data not shown), but this PTK-inhibitor showed an autonomous DNA damaging property, probably involving a pathway distinct from its action on PTKs, as previously suggested by others (9).

To evaluate the protein phosphorylation pattern in cells exposed to ATPo, western blot analysis with anti-ptyr mAb was performed (Fig. 2, left panel). Surprisingly, the addition of 8 mM ATPo

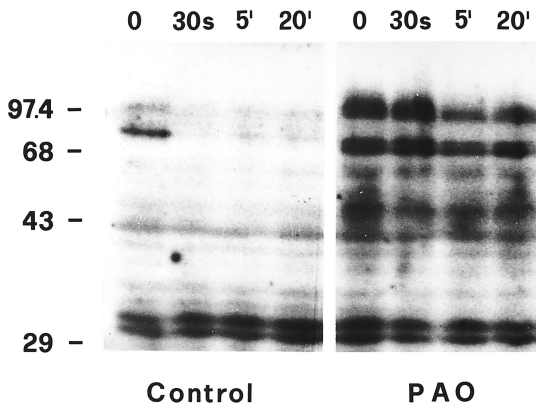


FIG. 2. Effect of ATPo on substrate tyrosine phosphorylation in L1210 lymphoma cells. 3×10^6 L1210 cells were pretreated for 20 min with 40 μM PAO or an appropriate amount of DMSO (control), washed, and incubated at 37°C for the times indicated with medium alone (time 0) or containing 8 mM ATPo. Cells were solubilized, and proteins were separated on 10% SDS-PAGE. Phosphotyrosine-containing proteins were visualized with an anti-ptyr mAb (4G10) and ¹²⁵I-protein-A. The presence of proteins with apparent molecular masses between 32 and 33 kDa, that did not specifically react with ¹²⁵I protein A in the control lanes (not shown), clearly indicated that an equal amount of lysate was loaded for each lane. Molecular weights are shown in kDa. These findings were confirmed in three other independent experiments.

to L1210 cells induced a rapid and marked decrease in the amount of anti-tyr reactivity of several phosphorylated protein bands that were present in untreated cells. This effect was particularly evident in two proteins of about 105 and 76 kDa. Even 20 min after ATPo addition, the initial phosphorylated state was still not restored, especially in the case of the 76 kDa protein.

To test whether ATPo-induced dephosphorylation reflected an enhanced PTPase activity, L1210 cells were pretreated for 20 min with 40 μ M PAO, a concentration that ensured a complete loss of CD45 PTPase function (10). Treated cells showed a marked increase in phosphorylation, most likely due to the imbalance between PTK and PTPase activities consequent to PAO pretreatment. Anti-tyr mAb revealed a strongly phosphorylated 54–56 kDa protein; moreover, the 105 and 76 kDa proteins clearly appeared as doublets (Fig. 2, time 0, right panel), probably reflecting a heterogeneous phosphorylation of the same protein, or the phosphorylation of other proteins of similar size. The addition of ATPo did not cause complete tyrosine dephosphorylation of the 105 and 76 kDa substrates, as observed in the control (Fig. 2, right and left panels, respectively).

The role of PTPase in ATPo-mediated cytotoxicity was further studied using phosphatase inhibitors. Given that a few hours' exposure to PAO caused cell death, vanadate, a less toxic inhibitor (8), was employed. We observed a dose-dependent inhibition of DNA fragmentation in both P815 and L1210 cells and no effect on cell lysis (Fig. 3). Interestingly, okadaic acid, a highly efficient inhibitor of the two major serine/threonine PPases type 1 and 2A (11), was ineffective in P815 cells and produced only a 23% reduction of DNA degradation in L1210 cells at concentrations as high as 200 ng/ml (data not shown).

To rule out the possibility that the phosphatase inhibitors counteracted ATPo binding to its receptors, P815 cells were pretreated with vanadate, washed, and then exposed to 8 mM ATPo. A strong (65%) reduction in DNA breakdown was achieved with 100 μ M vanadate, while ^{51}Cr release was unaffected (data not shown). Therefore, these results excluded the possibility that the blockade in DNA fragmentation was due to ATP sequestration by vanadate, thus confirming that negatively charged vanadate species do not bind to ATP at neutral pH (12).

As newly phosphorylated proteins following ATPo-stimulation were detected only after prolonged autoradiographic exposure, we investigated whether this difficulty simply reflected the exiguity of kinase activity in the cells. Therefore, we evaluated the enzymatic activity of phosphotyrosil proteins by an *in vitro* immune-complex kinase assay. L1210 and P815 cells (Fig. 4 panel A and panel B, respectively) were incubated with 8 mM ATPo for different times, lysed, immunoprecipitated with anti-tyr mAb, and subsequently analyzed for the presence of phosphorylation activity. In cells exposed as briefly as 30 s, we recovered an enhanced kinase activity that

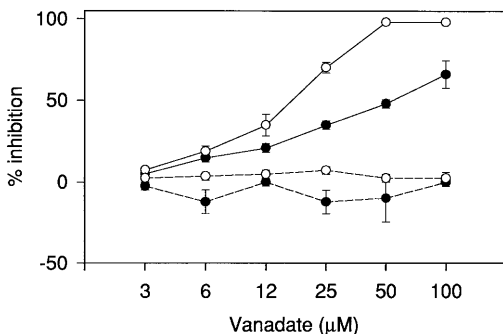


FIG. 3. Vanadate reduces ATPo-triggered DNA breakdown. Labelled P815 (open symbols) and L1210 (closed symbols) cells were incubated for 5 h with 8 mM ATPo in the presence or absence of different vanadate concentrations. At the end of incubation, supernatant was withdrawn and counted to evaluate specific release of $^{125}\text{IUdR}$ and ^{51}Cr . Results are expressed as the percentage of inhibition of $^{125}\text{IUdR}$ release (continuous line) and of ^{51}Cr release (broken line) in the presence of vanadate. Spontaneous release was unaffected by the addition of the indicated vanadate concentrations.

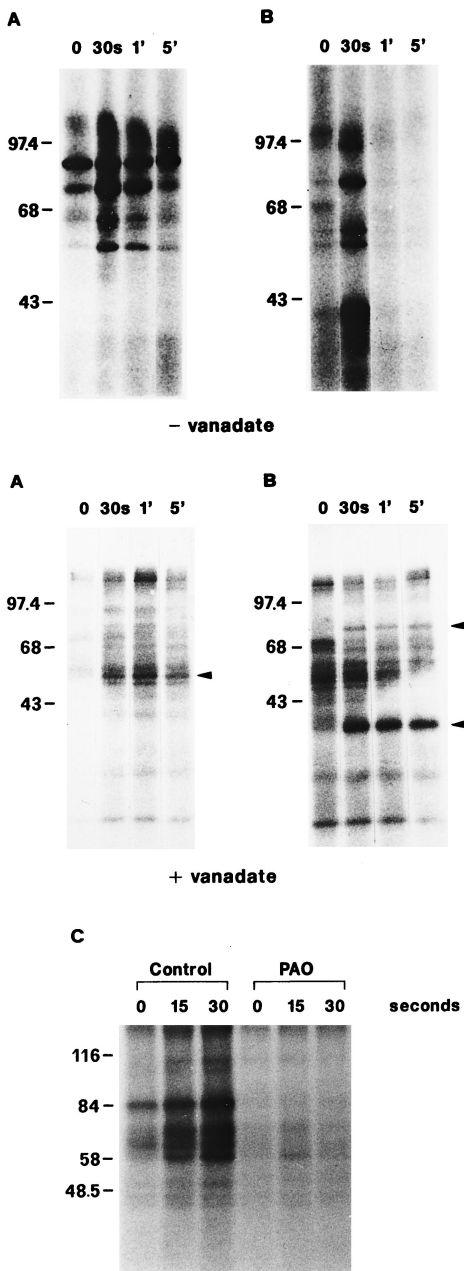


FIG. 4. ATPo activates *in vitro* kinase activity of phosphotyrosine-containing proteins. Detergent-soluble lysates from 20×10^6 L1210 (**A**) and P815 (**B**) cells, incubated at 37°C in medium alone (time 0) or in the presence of 8 mM ATPo for 30 s, 1, and 5 min, as indicated, were immunoprecipitated with anti-ptyr 1G2 mAb. *In vitro* kinase assay with immunoprecipitates was performed under conditions which favored protein autophosphorylation. Proteins were electrophoresed on 10% SDS-PAGE, and the gels were then treated with 1 M KOH prior to visualization by autoradiography (3 d exposure at -70°C). In parallel experiments, 200 μM vanadate (+vanadate) was added to kinase buffer in which anti-ptyr immunoprecipitates from L1210 and P815 cells were resuspended. In this case, the resulting gels were not treated with alkali, and were exposed for shorter time (10 h). Arrowheads indicate proteins discussed in the text: these bands persisted after alkali treatment (not shown). In panel C, P815 cells were treated for 20 min with 40 μM PAO, or an appropriate amount of DMSO (control), before addition of 8 mM ATPo. At the end of the indicated time, cells were lysed and processed as above. The experiment reported is representative of two others.

caused the phosphorylation of different substrates: two protein bands with apparent size of 56 and 75 kDa were present in both L1210 and P815 cells, while other bands were observed only in one or the other cell line (62 kDa in L1210, and 41, 60, and 97 kDa in P815). This particular kinase activity, however, was only transient, because the phosphorylated bands almost completely disappeared 5 min after addition of ATPo to L1210 cells, and after only 1 min of ATPo in P815 cells. By adding 200 μ M vanadate to the kinase-assay buffer, phosphorylation of some proteins was prolonged for as long as 5 min (see bands indicated by arrowheads in panel A and B, +vanadate), thus suggesting that ATPo-activated PTPases were co-precipitated with the anti-tyr-reactive proteins. Indeed, a PTPase that was active on synthetic phosphotyrosine-containing peptides, was recovered in the anti-tyr immunoprecipitates; the characterization of this PTPase is now under way. Finally, PAO pretreatment of P815 (Fig. 4 panel C) and L1210 cells (not shown) completely prevented early ATPo-induced kinase activation.

DISCUSSION

On the whole, the present study shows that both PTKs and PTPases are involved in ATPo-mediated apoptotic cell death. These findings provide further evidence that kinase/phosphatase activity plays a central role in transduction of extracellular signals through the cytoplasm to the nucleus. In addition, the observation that ATPo-induced DNA fragmentation, but not colloid-osmotic lysis, was reduced in a dose-dependent fashion in the presence of genistein and vanadate confirms that ATPo can cause cell death through two different mechanisms, one of which requires the active participation of target cell and leads to apoptosis, while the other may be the result of a non specific perturbation of plasma membrane permeability (13). Although it is not clear if the two pathways can be activated independently, several observations indicate that ATPo may exert lytic effects through at least two subtypes of P2 receptors, P2x and P2z (14,15). The precise assignment of a given ATPo-triggered response to a receptor subtype is as yet uncertain; however, ATPo-induced apoptosis was in the past considered to be principally associated with the function of the P2x receptor, which is a ligand-gated ion channel (16). Support for this hypothesis was recently provided by the cloning of the P2x receptor gene, whose sequence shows extensive homology to a gene called RP-2, which is activated in thymocytes undergoing apoptosis. Although the function of the protein coded by the RP-2 gene is still unknown, it has been suggested that it might be a receptor for ATP or a related metabolite released during PCD (17,18).

The requirement for the coordinate action of PTK and PTPases has been evidenced in various cellular processes. Particular sets of phosphatases are involved in different forms of PCD (19); however, phosphatase activity alone is necessary but not sufficient to initiate apoptosis. On the other hand, PTK inhibition prevents PCD induced by anti-CD3 activation (20), staphylococcal enterotoxin superantigens (21), anti-Fas and anti-Ig B cell lymphoma treatment (22), and glucocorticoid treatment of rat thymocytes (23). A premature termination of PTK activation was reported in other models of PCD, although the mechanism of such "abortive" signal was not investigated. For example, activation of p56^{lck}, lasting only few minutes, has been observed following exposure of a lymphoblastic leukemia B cell line to apoptotic stimuli such as ionizing radiation and engagement of the CD19 receptor (24). Moreover, engagement of Fas antigen on Jurkat cells induces a transient tyrosyl-phosphorylation of some substrates, and genistein and herbimycin A prevent DNA fragmentation (25).

The present data indicate that the intracellular pathway induced by ATPo is characterized by a transient PTK activation. This activation appears to be closely regulated by unidentified PTPases, since the rapid drop in kinase activity was prevented when vanadate was added to the kinase buffer in which anti-tyr immunoprecipitates were resuspended. The presence of PTPases among anti-tyr immunoprecipitated proteins can be explained in two ways. PTPases themselves might be tyrosine phosphorylated, as a consequence of a previous PTK activation; this event was described for

murine Syp and human PTP 1D, two SH2-containing PTPases related to the *Drosophila corkscrew* gene product (26,27). Alternatively, purinergic receptor stimulation might activate PTPases directly, which then associate with the phosphotyrosyl-proteins; thus, an intracellular redistribution of these enzymes could explain the dephosphorylation of the tyrosine-containing substrates observed *in vitro*. This second hypothesis seems more likely, since ATPo exposure caused an early (within 15 s in some experiments) and almost complete dephosphorylation of two major proteins of 105 and 75 kDa visualized in anti-ptyr immunoblots of L1210 cell line lysates; this dephosphorylation was long-lasting, and was specifically prevented by PAO. Both of these hypotheses are based on the assumption that ATPo exerts lytic effects as a ligand for signal-transducing purinoreceptors: ATPo binding to a cell-surface receptor could result in changes in the phosphorylation level of intracellular proteins. Although the order of events in the subsequent kinase/phosphatase activation cascade is unknown, the substrates of this network are likely to ultimately include nuclear transcription factors, as already seen in different models of PCD (28,29). In fact, our preliminary observations indicate that the *c-fos* and *c-jun* protooncogenes are rapidly induced in tumor cells undergoing apoptosis following ATPo treatment. Moreover, expression of these two genes is abrogated by PTK and PTPase inhibitors (Zambon, A., unpublished observations).

An alternative mechanism of ATPo action should be considered, based on its ability to serve as a phosphate donor in phosphorylation reactions. According to this model, ATP-dependent changes in the phosphorylation of plasma membrane proteins could trigger the death pathway in target cell (30). The recent molecular characterization of the P2x purinergic receptor will allow a better definition of the ATPo-stimulated responses, revealing the possible kinases that are physically interacting with a given purinoreceptor. Characterization of the transiently phosphorylated 105 and 75 kDa proteins, identified in our *in vitro* kinase assay of anti-ptyr immunoprecipitated L1210 cell proteins, may provide an alternative strategy for identifying a common intermediate in ATPo-triggered signals.

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