Specific Protein Kinases Modulated during T Cell Mitogenesis

ACTIVITY OF A 55-kDa SERINE KINASE IS ASSOCIATED WITH GROWTH ARREST IN HUMAN T CELLS*

(Rceived for publication, January 6, 1992)

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The intracellular events which are involved in controlling the G1 to S phase transition during the eucaryotic cell cycle are important to define in order to understand the mechanisms by which mitogenic and growth arrest-inducing agents control cell growth. Because a change in protein kinase activity is associated with the initial response of cells to mitogenic stimulants and growth factors, we used a kinase renaturation assay to identify specific protein kinases which are modulated as human T cells make the G1 to S phase transition after mitogenic stimulation with lectin. We identified four protein serine/threonine kinases of 180, 97, 85, and 38 kilodaltons which are increased in activity as these cells enter S phase. A 55-kDa serine/threonine kinase (PK55) was shown to have maximal activity during G0 and its activity was reduced by 95% upon movement into S phase. PK55 is inducible in human T cells by removal of interleukin 2 and low serum incubation which arrests cells in G1 phase, indicating that it is closely associated with G1 phase growth arrest. Furthermore, a similar PK55 activity was induced upon growth arrest in HL-60 cells treated with dimethyl sulfoxide and in Daudi cells treated with interferon α. Because the CAMP-dependent protein kinase (PK-A) family has been shown to be antiproliferative to lectin-stimulated T cells, we were interested in determining whether PK55 was in fact an isozyme of PK-A. Comparative analysis using a specific peptide inhibitor of PK-A activity revealed that PK55 is catalytically distinct from PK-A. This data suggest that increases in PK55 may be associated with the growth-arrested state and further that PK55 is distinct from PK-A.

A major control point for the regulation of the eucaryotic cell cycle is the G1/S phase boundary. Mitogenic growth factors drive the cell from G1 into S phase, whereas growth arrest- or differentiation-inducing agents stop cell growth by halting cell cycle progression in G1 phase prior to the G1/S phase boundary. Therefore understanding the factors involved in controlling the G1 to S phase transition is of considerable importance in deducing the mechanisms by which mitogenic growth factors or growth arrest-inducing agents control cell growth. Resting human T cells present an excellent model for the study of these mechanisms. In human T cells, cell cycle progression and movement from G1 to S phase is governed by the interaction of antigen (or lectin) with the T cell receptor followed by the production of IL-2 and expression of high affinity IL-2 receptors (1). Autocrine production of IL-2 followed by IL-2/IL-2 receptor interaction forces the T cell from G1 to S phase and onward through the cell cycle (2, 3). Although several protein kinases have been shown to have increased activity as cells make this transition (4–7), the overall kinase signaling mechanisms which control mitogen-stimulated cell cycle progression in human T cells are not well defined. In an attempt to identify the major cellular kinase activities which are modulated as T cells make the G1 to S phase transition following lectin stimulation, we analyzed cell lysates using a kinase renaturation assay which primarily identifies autophosphorylating protein kinases after separation by SDS-PAGE. Using this assay we present evidence that several, potentially novel, protein kinases are modulated during the G1 to S phase transition. Additionally we show that a protein kinase of 55 kilodaltons is associated with growth arrest in human T cells and that a similar activity can be stimulated with growth arrest-inducing agents in other non-T cell lineage hematopoietic cell lines.

MATERIALS AND METHODS

Tissue Culture Media and Reagents—Normal RPMI 1640 medium, 100 × penicillin-streptomycin, and 100 × glutamine were purchased from Mediatech, Washington, D. C. Phosphate-free RPMI 1640 medium was purchased from Advanced Biotechnologies Inc., Columbia, MD. Fetal calf serum was purchased from GIBCO, Life Technologies Inc., bovine heart cAMP-dependent protein kinase, PK-A inhibitor peptide, and DMSO was purchased from Sigma. INFα and phytohemagglutinin were purchased from Wellcome Diagnostics, Temple Hill, Dartford, Great Britain. Immobilon PVDF paper was purchased from Millipore Corp., Bedford, MA. All other chemicals and reagents were from standard vendors.

Lymphocyte Preparation and Cell Culture—Human peripheral blood mononuclear cells were obtained by leukapheresis of normal volunteers at the blood bank of the National Institutes of Health. After density sedimentation of the mononuclear cells with lymphocyte separation medium (Organon Teknika, Durham, NC), the lymphocytes were purified by counterflow centrifugal elutriation as described previously (8), except that pyrogen-free phosphate-buffered saline was used in the elutriation procedure. The purified lymphocytes

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* This project was funded at least in part with Federal funds from the Department of Health and Human Services under Contract N01- CO-74102 with Program Resources, Inc./DynCorp. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: IL-2, interleukin 2; PHA, phytohemagglutinin; SDS, sodium dodeyl sulfate; PAGE, polyacrylamide gel electrophoresis; MeSO, dimethyl sulfoxide; INFα, interferon α; PVDF, polyvinylidene difluoride; PK-A, cAMP-dependent protein kinase.
RESULTS

Specific Kinases Are Modulated during T Cell Mitogenesis—In an attempt to identify protein kinases which are modulated as T cells progress through the cell cycle, we treated resting T cells with PHA for up to 96 h and assayed cell lysates for specific kinase activity using a kinase renaturation assay following SDS-PAGE and Western transfer to PVDF paper. Using this assay we have identified protein kinases of 180, 97, 85, 55, and 38 kilodaltons which are modulated by 48 h after PHA treatment (Fig. 1, A and C). Thymidine uptake data on PHA-treated T cells show that entrance into S phase occurs by 48 h and is maximal by 72 h after PHA treatment (Fig. 1B). Based on the thymidine uptake data and kinase renaturation analysis, we are excited to conclude that increased activity of these kinase is much more tightly associated with growth arrest in early G1 phase as compared with changes in kinase activity which are observed in G0-G1 phase transition (Fig. 1C). This indicates a much tighter association of increased activity of these kinase with the G1 to S phase transition compared with changes in PK180 activity (Fig. 1C). Interestingly, PK55 has its highest level of activity during the growth-arrested state in early G1 phase.
were cut from the renaturation assay blot and hydrolyzed in 6 N HCl phosphoamino acid standards, and subjected to one-dimensional thin layer electrophoresis. Standards were stained with ninhydrin, and radiolabeled phosphoamino acids were identified by autoradiography. The migration of phosphoamino acid standards is identified to the G1 to S phase transition (PK180, PK97, PK85, PK55, PK38) and achieves base-line activity of only 5-7% of that in the quiescent cell by 72 h (Fig. 1C). A number of serine/threonine protein kinases have been shown to have increased activity as the cell moves through G1 phase in response to mitogen. Some of these include p34CDK2 (4, 5), raf-1 (6), PK-C iso-zymes (7), and others; however, the observation of PK55 having maximal activity during growth arrest and decreased activity as the cell moves from G1 to S phase (Fig. 1) is unique in human T cells. Given that a kinase activity associated with growth arrest may be potentially regulated by a variety of different stimuli, including mitogenic, growth arrest-inducing, or differentiating agents as well as being potentially antion-cogenic, we chose to focus our attention on the study of PK55.

PK55 Is Modulated during the Cell Cycle and Is Distinct from PK-A—In order to determine if PK55 activity can be induced upon inducing growth arrest in dividing human T cells, we recovered actively dividing 72-h cultured PHA-treated T cells, washed the cells free of IL-2 with low pH media, and incubated these cells for 24 h in 1% fetal calf serum. This allowed us to recover a G1-enriched T cell population that had been cultured for 96 h after PHA treatment. These cells were then compared with a dividing 96-h population and assayed for PK55 activity. Thymidine uptake of the G1-enriched T cell population was less than 10% of that observed in an actively dividing 96-h population verifying the growth-arrested state of these cells (data not shown). Analysis of total cellular kinase activity by renaturation assay revealed that by 96 h after PHA treatment, PK55 activity is less than 5% of that seen in freshly explanted cells and can be increased by approximately 9-fold by IL-2 removal and growth arrest in G1-enriched cells (Fig. 3 and densitometric analysis). These results indicate that PK55 is directly associated with a growth-arrested physiology in human T cells and appears to be regulated in a cell cycle-dependent manner.

PK55 is associated with growth arrest and is inducible upon growth factor removal in human T cells. Human T cells were treated with PHA for up to 96 h. A 96-h G1-enriched population was obtained by washing 72-h-treated cells with RPMI media made slightly acidic with CO2 bubbling and then incubating with 1% fetal calf serum in RPMI for 24 h. This resulted in a G1-enriched growth-arrested cell population by removal of the principal T cell growth factor IL-2. Cell lysates were obtained from normal PHA-treated and PHA-treated/growth-arrested cells and analyzed using the kinase renaturation assay. The protein kinase band corresponding to PK55 is indicated to the left.

PK55 is catalytically distinct from cAMP-dependent protein kinase. Ten μg of a G0 T cell lysate and 1 μg of purified PK-A from bovine heart was subjected to the kinase renaturation assay in the presence or absence of 10 or 50 μg/ml of a synthetic peptide inhibitor of PK-A catalytic activity, lanes 1, 3, and 5, T cell lysate containing an active PK55. Lanes 2, 4, and 6, 1 μg of bovine heart PK-A. Lanes 1 and 2, no inhibitor; lanes 3 and 4, 10 μg/ml inhibitor; lanes 5 and 6, 50 μg/ml inhibitor. PK55 and PK-A are indicated on the left.

PK55 is associated with growth arrest in other hematopoietic cell lines—From the data, it is clear that PK55 is a serine/threonine protein kinase which is catalytically distinct from PK-A, is associated with growth arrest, and is regulated in a cell cycle-dependent manner in human T cells. In an attempt to determine the extent to which PK55 activity is associated with growth arrest in other cell types, we chose several hematopoetic cell models of growth arrest or differentiation in which to look for inducible PK55 activity. For these studies, we used HL-60 cells, a promyeloid-promonocyctic cell line and treated these cells with Me2SO (18) to promote granulocyte differentiation and thus growth arrest and Daudi cells (a B cell line) which can be growth-arrested with interferon α (19). Fig. 5 shows thymidine incorporation data of HL-60 cells treated with 1.25% Me2SO and Daudi cells treated with 100 units/ml IFNα for from 0–96 h. This data shows that HL-60 cells can be very effectively growth-arrested by greater than 90% with Me2SO by 72-h treatment (Fig. 5). Daudi cells were growth-arrested by approximately 85% by 96-h treatment with IFNα (Fig. 5). In order to during ATP addition in the kinase renaturation assay. Fig. 4 shows the results of an experiment of this type using a freshly explanted T cell preparation and purified PK-A catalytic subunit from bovine heart. In this experiment, PK-A was inhibited by 80 and 90% with 10 and 50 μg/ml inhibitor peptide, respectively, whereas PK55 activity was unaffected (Fig. 4 and densitometric analysis). This clearly shows that PK55 is distinct from PK-A.

It has been shown that increases in cAMP-dependent protein kinase (PK-A) activity can prove to be antiproliferative in T cells (15, 16). Because of this, we were interested in determining whether PK55 represented a novel catalytic subunit of PK-A. We tested this by incubating PK55 with a well characterized peptide inhibitor of PK-A catalytic activity (17),
Induction of growth arrest in HL-60 cells treated with Me$_2$SO and Daudi cells treated with interferon $\alpha$. Thymidine incorporation was measured at 24-h intervals for up to 96 h on HL-60 cells treated with 1.25% Me$_2$SO and on Daudi cells treated with 100 units/ml interferon $\alpha$. The data represent the average of three independent experiments ± S.D. □ HL-60 no treatment; ■ HL-60 Me$_2$SO; Δ, Daudi no treatment; △, Daudi IFNa.

Fig. 5. Induction of growth arrest in HL-60 cells treated with Me$_2$SO and Daudi cells treated with interferon $\alpha$. Thymidine incorporation was measured at 24-h intervals for up to 96 h on HL-60 cells treated with 1.25% Me$_2$SO and on Daudi cells treated with 100 units/ml interferon $\alpha$. The data represent the average of three independent experiments.

Table I Relative induction of a PK55-like activity in growth-arrested hematopoietic cells

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Discussion

The use of an assay which identifies distinct protein kinase activity has allowed the identification of several protein serine/threonine kinases that are modulated during the G1 to S phase transition in human T cells (Fig. 1). Several of these kinases, PK180, PK97, PK85, and PK38, have increased activity after mitogen stimulation. Interestingly, a 55-kDa serine-specific protein kinase activity was found to have its highest activity during growth arrest and was down-modulated upon PHA treatment in human T cells (Figs. 1 and 2). Based on the relative molecular weight and pattern of activity, one potential candidate for PK55 may be cAMP-dependent protein kinase (15, 16). However using a specific inhibitor of PK-A (17) we have shown that PK55 is catalytically distinct from this class of kinases (Fig. 4). These data suggest that PK55 represents a novel activity in human T cells associated exclusively with growth arrest. Further analysis of several other cellular models of growth arrest showed that a similar activity can be induced in HL-60 and Daudi cells (Fig. 6).

Although we have clearly shown that PK55 activity is associated with growth arrest using a kinase renaturation assay, we do not know the factors involved in regulating intracellular activity. The method of assay which has allowed the identification of these kinases involves denaturation and SDS-PAGE prior to the assay of kinase activity. Because of this we can primarily identify only distinct catalytic proteins and are unable to ascertain the direct mechanisms which allow regulation of this activity. Any apparent modulation of activity must be associated with either the direct posttranslational modification of the catalytic protein or more likely with changes in the concentration of specific catalytic protein within the cell. We are presently developing the reagents necessary to make that determination.

In mammalian systems there is no identified cell cycle controlling protein kinase activity which governs the attainment of growth arrest. However, in the yeast system the protein product of the fks3 gene is a distinct protein serine kinase which controls G1 arrest (24). The manner in which this kinase induces G1 arrest is not well understood but may center on the control of cyclin production/degradation (24). Because of the high degree of conservation of function of the proteins which control cell cycle progression in yeast and mammalian systems, it seems plausible that there may be a similar G1 arrest-associated protein kinase in mammalian
systems comparable with FUS3. From the data presented it seems that PK55 may be a candidate human homolog of FUS3. Its activity is highest during the point in the cell cycle when cyclin production is at a minimum and is apparently inactivated or degraded at the point in the cell cycle which has been reported to be associated with initial increases in mitogen stimulated cyclin production (25, 26). This may suggest a functional role for PK55 in inducing and/or maintaining G1 arrest by regulating via phosphorylation the stability, synthesis, or degradation of cyclin proteins.

Like the antioncogenes so far identified, PK55 appears to have highest activity during G1 phase of the cell cycle. The retinoblastoma susceptibility protein (RB) is active as a cell cycle suppressor during early to late G1 until it is phosphorylated, which results in the inhibition of this function (20–22). PK55 is also strictly controlled in its apparent activity during the cell cycle. Activity is virtually eliminated at the G1/S phase boundary and closely parallels the phosphorylation and inactivation of RB (23). The close parallel cell cycle association of PK55 activity with antioncogene activity further substantiates the tight association with the growth-arrested state and may also suggest a possible role of PK55 as an antioncogene. If PK55 functions as an antioncogene, it will be the first identification of a catalytic protein functioning in this manner. In order to substantiate this, it must be shown that purified PK55 can directly induce growth arrest. We are presently purifying PK55 for microinjection studies aimed at answering this question.

By incorporating a kinase renaturation assay into our studies of mitogen signaling and cell cycle regulation, we have easily identified mitogen-stimulated as well as mitogen-inhibited kinase activities. The identification of PK55 has opened new possibilities in the study of mitogen, growth arrest-inducing, and differentiating agents by providing a new and potentially very important target for cell cycle regulation by these agents. Although the direct role that PK55 has in controlling growth arrest and cell cycle progression is not known, it represents a novel activity associated with growth arrest in human T cells.

Acknowledgments—We thank Drs. Joost Oppenheim, Dan Longo, and Jeff Rossio for critical reviews of the manuscript and Dr. Larry Wahl for providing freshly isolated human T cells.

REFERENCES