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IDENTIFICATION OF THE HUMAN T-LYMPHOCYTE PROTEIN-TYROSINE KINASE BY PEPTIDE-SPECIFIC ANTIBODIES

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We have recently cloned a cDNA from the human T-cell leukemia, JURKAT, having homology with the src-like family of protein-tyrosine kinases. We have made rabbit polyclonal antibodies against the synthetic peptide CKERPEDRPTFDYLRSVLEDFFTATEGQYQPQP (cys-33-pro) deduced from the carboxy-terminal amino acid sequence predicted by the JURKAT cDNA. In this report, we demonstrate that these antibodies immunoprecipitate the protein-tyrosine kinase activity from solubilized membrane extracts from JURKAT T-leukemia cells and from human peripheral blood T-lymphocytes from normal donors. A 58 kd protein, exhibiting protein-tyrosine kinase activity, was specifically immunoprecipitated in both cases. The antibodies failed to crossreact with $pp60^{c\text{-}src}$ from human platelets, but did crossreact with the murine T-lymphocyte protein-tyrosine kinase, $pp56^{T\text{-}cell}$.

Protein-tyrosine kinases (PTK)s have been implicated in the control of cell proliferation and malignant transformation. High levels of PTK activity have been described in particulate fractions of both normal and transformed T-lymphocytes of either human (1-4) or murine (5-8) origin. Stimulation of T-lymphocytes by mitogenic lectins have been shown to cause increased phosphorylation of membrane (3) and cytosolic (9) proteins on tyrosine residues suggesting a role for protein-tyrosine kinase activity in T-cell activation and/or proliferation. A murine T-lymphocyte PTK has been characterized as a 56 kd membrane associated protein ($pp56^{T\text{-}cell}$) (7,8) and cDNAs encoding the murine $pp56^{T\text{-}cell}$ have been reported (10,11). We have recently cloned a cDNA from the human T-cell leukemia, JURKAT, which shares extensive homology with the murine cDNA encoding, the $pp56^{T\text{-}cell}$ kinase (12). In this report we demonstrate that

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Abbreviations: SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis, PTK, protein-tyrosine kinase.

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rabbit polyclonal antibodies made against a synthetic peptide deduced from the predicted carboxy-terminal amino acid sequence of the JURKAT cDNA, identify a 58 kd membrane associated PTK from JURKAT cells and from human peripheral blood T-lymphocytes from normal donors. The antibodies crossreact with the murine $pp56^{T-cell}$ but do not react with the human $pp60^{c-src}$ PTK, expressed in human platelets (13).

Materials and Methods

Cells. The human T-cell leukemia, JURKAT, was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The murine LSTRA T-lymphoma line was cultured as previously reported (5). To establish primary cultures of human T-lymphocytes, mononuclear cells from the peripheral blood of normal donors were isolated by centrifugation through Ficoll-Paque (Pharmacia), stimulated with irradiated human-B lymphoma cells, IM-9, and expanded for 10 days in the presence of 50 units/ml IL-2. Human platalets were isolated by elutriation of peripheral blood from normal donors. Particulate fractions from these cells were obtained by high speed centrifugation of post-nuclear supernatants as previously described (1).

Protein-tyrosine kinase (PTK) assay. Cell particulate fractions were solubilized for 1 hr on ice in buffer containing 100 mM Tris.HCl pH 7.4/1% sodium deoxycholate/1% Triton X-100/0.1% SDS/150 mM NaCl. Insoluble material was removed by high speed centrifugation and the supernatant assayed for PTK activity using a synthetic substrate homologous to the autophosphorylation site of $pp60^{v-src}$ as previously described (1). Approximately 80% of the PTK activity associated with the particulate fraction was solubilized in this manner.

Rabbit polyclonal antibodies to the human T-lymphocyte PTK. Recently we have cloned a cDNA from the human T-cell leukemia line, JURKAT, having homology with the src-like family of PTKs (12). A synthetic 33 amino acid peptide was made (Peninsula Labs) deduced from the C-terminal amino acid sequence of the JURKAT cDNA, having the following sequence: CKERPEDRPTFDYLRSVLEDFFTATEGQYQPQP. This peptide (cys-33-pro) was coupled to bovine serum albumin through the N-terminal cysteine residue with 3-(2-pyridyldithio) proprionic acid N-hydroxy-succinimide ester as described (13). Rabbits were initially injected with 100 μg BSA-(cys-33-pro) conjugate in Freunds' complete adjuvant at multiple subcutaneous sites, with booster injections, in Freunds' incomplete adjuvant, following at three week intervals. After six weeks, antibody titers greater than 100,000 were detected against the peptide (cys-33-pro) by ELISA. The immune rabbit serum was purified by Protein A Sepharose affinity chromotagrophy, and the IgG fraction coupled to CNBr-activated Sepharose CL-4B® (Pharmacia) at a concentration of 10 mg of immunoglobulin per ml of beads using the manufacturer's directions.

Immunoprecipitation and Immunoblotting. Detergent extracts from JURKAT cells, normal T-lymphocytes and platelets were incubated for 3 hrs at 4°C with antipeptide antibodies covalently coupled to Sepharose CL-4B® The immune complexes on the Sepharose beads were washed 4 times in solubilization buffer, dispersed in SDS/PAGE sample buffer, heated for 2 min in a boiling water bath, subjected to SDS/PAGE in precast 10% polyacrylamide gels (Integrated Separation Systems, Newton, MA) and autoradiographed at -70°C using hypersensitized Kodak XAR-X-ray film (15) and Du Pont Lightning Plus intensifying screens. Immunoblotting was performed essentialy as described (16). Antigen-antibody complexes were visualized by staining with Immunogold Staining (IGS) reagent, according to Moeremans et al (17). IGS reagent consists of colloidal gold particles coated with affinity-purified goat anti-rabbit IgG (Integrated Separation Systems, Newton, MA).

Results and Discussion

We have recently cloned a cDNA from the human T-cell leukemia line, JURKAT, (12) having homology with the src like family of protein tyrosine kinases. Rabbit polyclonal antibodies were produced against the synthetic peptide (cys-33-pro) corresponding to the carboxy-terminal amino acid sequence predicted by the JURKAT cDNA (see Materials and Methods). These antipeptide antibodies were purified by Protein A Sepharose affinity chromotography and coupled to CNBr-activated Sepharose CL-4B® for immunoprecipitation experiments.

Previous studies by us and others have shown that the PTK activity expressed in human T-lymphocytes from peripheral blood (1,2) as well as human T-leukemic lines (2,4) (including JURKAT[1]) localize to the particulate fraction. Approximately 80% of the PTK activity can be solubilized (data not shown) by high concentrations of both ionic and nonionic detergent as described in materials and methods. As shown in Table 1, the PTK activity in detergent extracts from either human JURKAT T-leukemic cells or peripheral blood T-lymphoctyes was efficiently removed (by 88% and 76%, respectively) following a 3 hr incubation with immobilized antipeptide antibodies. The loss of PTK activity was measured in comparison to PTK activity in the same extract incubated with an equivalent concentration of an irrelevant rabbit IgG-Sepharose. Loss of PTK activity in the detergent extracts of both JURKAT cells, and normal T-lymphoctyes, correlated with an increase in PTK activity associated with the immobilized antipeptide antibodies (Table 1). The antipeptide antibodies immunoprecipitated the PTK activity expressed in the murine T-cell lymphoma line LSTRA, which expresses high levels of the $pp56^{T\text{-}cell}$ PTK (6). However, the antipeptide antibodies failed to immunoprecipitate the PTK activity in detergent extracts from human platelets, which have been demonstrated to express high levels of the human $pp60^{c\text{-}src}$ kinase (13) (Table 1).

Antipeptide antibodies immunoprecipitate a 58 Kd phosphoprotein from human T-lymphocytes. Immobilized antipeptide antibodies specifically immunoprecipitated a ^{32}P-labeled pp58 from detergent extracts, from both JURKAT

[1] J. M. Trevillyan, unpublished result.

Table I

Immunoprecipitation of protein-tyrosine kinase activity in solubilized membrane extracts by immobilized antipeptide antibodies

Cell Type	Rabbit IgG conjugated to Sepharose CL-4B®	Protein tyrosine kinase activity following incubation of detergent extracts with Sepharose CL-4B® conjugated antibodies[a]		% of protein-tyrosine kinase activity remaining in supernantant
		Supernatant	Immobilized on antibody-conjugated Sepharose CL-4B®	
		cpm ^{32}P incorporated into peptide substrate		
Antigen, IL-2 stimulated human T-lymphocytes	control rabbit IgG	16,210	293	(100)
	antipeptide IgG	3,943	9,148	24
JURKAT, human T-leukemia cells	control rabbit IgG	10,665	73	(100)
	antipeptide IgG	1,274	7,494	12
LSTRA, murine T-lymphoma cells	control rabbit IgG	65,151	359	(100)
	antipeptide IgG	3,207	23,433	5
Human platelets	control rabbit IgG	31,104	358	(100)
	antipeptide IgG	31,436	941	101

[a]The particulate fraction from 10^8 cells was suspended in 200 µl of solubilization buffer (see Materials and Methods) and incubated for 1 hr on ice with frequent agitation. Insoluble material was removed by high speed centrifugation. Half of the resulting supernatant was incubated with 50 µl of Sepharose CL-4B® coupled with an irrelevant rabbit IgG and half of the supernatant incubated with 50 µl Sepharose CL-4B® coupled with the antipeptide antibodies. Incubations were at 4°C for 3 hr with constant agitation. After the 3 hr incubation 20 µl of supernatant was assayed for protein-tyrosine kinase activity in the presence of 5mM $MgCl_2$, 25 µM [^{32}P] ATP (2,000 cpm/pmol) and 1 mM synthetic peptide substrate as previously described (1). The antibody conjugated Sepharose CL-4B® beads were washed four times in solubilization buffer and resuspended in protein-tyrosine kinase reaction buffer. Protein-tyrosine kinase activity immobilized on the antibody conjugated Sepharose beads was measured essentially as described above, except that the Sepharose CL-4B® beads were kept suspended throughout the reaction by constant agitation. Values represent the average of duplicate assays which differed by less than 10%.

cells and normal human T-lymphocytes (Fig. 1). The reaction between the antipeptide antibodies and pp58 was specific because, incubation of the extracts with an irrelevant rabbit IgG-Sepharose failed to immunoprecipitate pp58 and preincubation of the antipeptide antibodies with the (cys-33-pro) peptide antigen, completely blocked the immunoprecipitation of pp58 (Fig. 1). As predicted from the data in Table 1, incubation of ^{32}P-labeled detergent extracts from murine LSTRA cells with antipeptide antibodies specifically immunoprecipitated a pp56 molecule corresponding to the murine $pp56^{T-cell}$ PTK

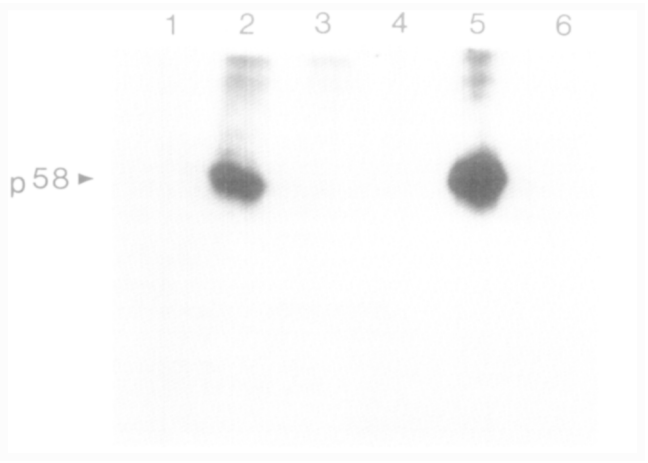

Fig. 1. Immunoprecipitation of ^{32}P-labeled pp58 from human JURKAT T-leukemia cells and normal T-lymphocytes. The particulate fraction pellet from $1x10^8$ JURKAT cells or $1x10^8$ normal T-lymphocytes were resuspended in 100 μl protein-tyrosine kinase buffer (1) and incubated with 25 μM [γ^{32}P] ATP, (4,000 cpm/pmol) for 10 min at 30°C. The ^{32}P-labeled particulate fraction was solubilized by addition of 100 μl of 2X solubilization buffer as described in Materials and Methods. 65 μl aliquots from the detergent extracts from the normal T-lymphocytes (lanes 1-3) or JURKAT cells (lanes 4-6) were incubated with 50 μl of Sepharose beads coupled to 500 μg of (a) an irrelevant rabbit IgG (lanes 1 and 3), (b) antipeptide antibodies (lanes 2 and 5) or (c) antipeptide antibodies preincubated for 30 min with 1 μM (cys-33-pro) peptide antigen (lanes 3 and 6). Immune complexes were boiled in sample buffer, subjected to SDS-PAGE and autradiographed as described in Materials and Methods.

(data not shown.) The antipeptide antibodies failed to immunoprecipitate any phosphoprotein from ^{32}P-labeled extracts from human platelets (data not shown).

pp58 is capable of autophosphorylation. Because PTKs are often capable of autophosphorylation, the data in Table 1 and Fig. 1 suggested that pp58 is the autophosphorylated form of the human T-lymphocyte PTK recognized by the antipeptide antibodies. To more rigorously test this hypothesis, pp58 was first immunoprecipitated from detergent extracts from JURKAT cells and normal human T-lymphocytes, and then the immune complex was incubated with [^{32}P-γ] ATP as described in Table 1. Immune complexes were subjected to SDS/PAGE and immunoblotted as described in Materials and Methods. Following incubation of the immunoblot with 6 μg/ml Protein-A Sepharose-purified antipeptide antibodies, immune complexes were detected colorimetrically with colloidal gold particles coated with goat-anti-rabbit IgG (see Materials and Methods). pp58 was detected by this immunoblot procedure in immunoprecipitates from JURKAT cell and normal human T-lymphocyte extracts previously incubated with immobilized antipeptide

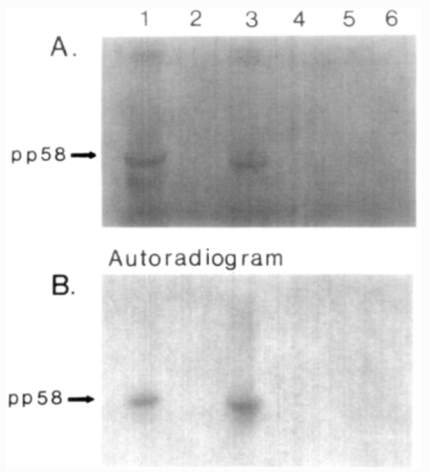

Fig. 2. Detection of pp58 by immunoblotting and evidence for its autophosphorylation. Experimental procedures are as described in Table 1 and in the text.
A) Detection of pp58 by immunoblotting technique.
Lane 1; normal T-lymphocyte extract immunoprecipitated with antipeptide antibodies
Lane 2; normal T-lymphocyte extract immunoprecipitated with control rabbit IgG
Lane 3; JURKAT extract immunoprecipitated with antipeptide antibodies
Lane 4; JURKAT extract immunoprecipitated with control rabbit IgG
Lane 5; human platelet extract immunoprecipated with antipeptide antibodies
Lane 6; human platelet extract immunoprecipitated with control rabbit IgG
B) Autoradiogram of immunoblot shown in A. Lanes are as described in A.

antibodies, but not with an irrelevant rabbit IgG-Sepharose (Fig. 2a). The autoradiogram of the immunoblot detected the incorporation, of ^{32}P into pp58, demonstrating that pp58 is capable of autophosphorylation (Fig. 2b). Cellular $pp60^{c-src}$ expressed in detergent extracts of human platelets (13) was not detected either by the immunoblotting procedure or by autoradiography (Fig. 2a and 2b).

Summary

The peptide specific antibodies described in this communication specifically immunoprecipitate a ^{32}P-labeled pp58 membrane-associated protein from human JURKAT T-leukemia cells and normal human T-lymphocytes (Fig. 2). They also detected this protein in immunoblots (Fig. 2). The evidence suggests that pp58 is a human T-lymphocyte PTK, because immunoprecipitation of pp58 from detergent extracts correlates with a loss of PTK activity in the extracts and also because the pp58 in immune complex exhibits significant PTK activity against exogenous substrates (see Table 1). Furthermore pp58 is capable of apparent autophosphorylation (Fig. 2) which is characteristic of several PTKs.

The peptide specific antibodies crossreacted with murine $pp56^{T-cell}$ PTK but did not react with $pp60^{c-src}$ expressed in human platelets (Table 1). Recently antibodies raised against a synthetic peptide representing the autophosphorylation site of $pp60^{v-src}$ have been shown to also react with the murine $pp56^{T-cell}$ and to inhibit the PTK activity of $pp56^{T-cell}$ and $pp60^{c-src}$ (7,18). The peptide specific antibodies described in this communication have the advantage of specificity since they do not crossreact with an epitope shared by $pp60^{c-src}$ and furthermore they do not block PTK activity (Table 1). Consequently these specific antibodies should be useful for further in vivo and in vitro studies of the regulation and cellular function of the pp58 PTK expressed in human T-lymphocytes.

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