

TYROSINE PHOSPHORYLATION OF AN INTERLEUKIN 2 RECEPTOR-LIKE PROTEIN IN CELLS TRANSFORMED BY HUMAN T CELL LEUKEMIA VIRUS TYPE I

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Tyrosine phosphorylation of key proteins appears to be involved in the regulation of cell growth (reviewed in reference 1), and changes in such phosphorylation have been implicated in the mechanism of cell transformation mediated by the oncogenes whose products have tyrosine kinase activity (reviewed in reference 2). By using antibodies against *o*-phosphotyrosine (Ptyr antibodies),¹ we have shown that *o*-phosphotyrosine-containing proteins (Ptyr proteins) with molecular masses of 310–23 kD become detectable or increase in various types of human leukemic cells (3, 4) and carcinomas (5), and that at least two of them are present on the outer surface of K562 human leukemic cells (3). We have also found that antibodies reactive with Ptyr are present in sera of patients with various types of malignant diseases (6). Its incidence was particularly high in patients with adult T cell leukemia (ATL), a disease caused by human T cell leukemia virus type I (HTLV-I), and also in healthy carriers of this virus (our unpublished data). Considering the possibility that malignancy-associated Ptyr proteins, particularly those present on the cell surface, are involved in the induction of such antibodies, we analyzed Ptyr proteins contained in various types of HTLV-I-transformed (or HTLV-I-bearing) cell lines, putting emphasis on those associated with cell surface. We show here evidence that at least two species of Ptyr-proteins are present on the surface of these cells, and that one with a molecular mass of 64 kD and pI 4.5 has the characteristics of the interleukin 2 (IL-2) receptor (IL-2-R). IL-2-R is constitutively expressed on the cell membrane of various HTLV-I-bearing human T and B cells, and of ATL cells (7, 8), and their impaired regulatory function has been suspected to be associated with the mechanism of transformation of HTLV-I-bearing T cells (9). The present finding that the IL-2-R-like protein present on the surface of these cells is phosphorylated at tyrosine may throw new light on this issue.

Materials and Methods

Cells and Antibodies. Human T cells used were three IL-2-independent cell lines. TL-Su, derived from an IL-2-dependent cell line; TCL-Ter, transformed in vitro with HTLV-

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¹ *Abbreviations used in this paper:* ATL, adult T cell leukemia; 2-D, two-dimensional; HTLV, human T cell leukemia virus; IF, immunofluorescence; Ptyr, *o*-phosphotyrosine.

I; and TL-Oml, established directly from an ATL patient (8). HPB-ALL (10), MOLT-4 (11), and CCRF-CEM (12) are human T cell lines unrelated to HTLV-I. Cells were grown in RPMI 1640 with 15% FCS (Gibco Laboratories, Grand Island, NY). Normal peripheral blood lymphocytes were obtained from healthy persons by the Ficoll-Paque method (Pharmacia Fine Chemicals, Uppsala, Sweden). Normal human T cells activated with phytohemagglutinin (PHA) were cultured in the presence of human IL-2 (13). Phosphate-free medium was composed of 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 mM glutamine, 5 mM glucose, and 10% FCS dialyzed against 0.9 M NaCl. Preparation, purification, and characterization of mouse mAb H1-D11, and rabbit polyclonal antibodies both reactive with P_{tyr} were described previously (14). mAb directed against different epitopes on IL-2-R molecules (anti-IL-2-R antibodies), mAb H-31 and HIEI, (13, 15) and that against HTLV-I p19, mAb GIN14, (16) have been described.

Immunofluorescent (IF) Staining. IF staining of acetone-fixed cells and intact cells with P_{tyr} antibody, mAb H1-D11, and fluorescein-conjugated anti-mouse IgG (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) was as described (14). Preimmune mouse IgG was used as a control. For blocking test, mAb H1-D11 was pretreated with 2 mM P_{tyr} (Sigma Chemical Co., St. Louis, MO) at 37°C for 1 h.

Isolation of P_{tyr} Proteins from Whole Cells. The procedure was essentially the same as described previously (3, 5). Briefly, 5×10^6 cells were homogenized in 1 ml of cold buffer A (10 mM Tris-HCl, pH 7.3, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 100 kallikrein units/ml aprotinin), and centrifuged at 15,000 g for 30 min. The supernatant was applied to a Sepharose 4 B column (0.5 ml) to which polyclonal rabbit P_{tyr} antibodies (5 mg protein) had been conjugated. The column was washed with buffer B (10 mM Tris-HCl, pH 7.3, and 50 mM NaCl), then P_{tyr} proteins were eluted with buffer C (10 mM Tris-HCl, pH 7.3, 10 mM NaCl, and 40 mM sodium phenylphosphate).

Isolation of Cell Surface P_{tyr} Proteins. The method was described previously (3). Briefly, intact cells (5×10^6) were exposed to polyclonal P_{tyr} antibodies in 0.3 ml PBS, pH 7.4, containing 0.02 M NaN₃ for 20 min, and then washed with the same buffer. Protein A-Sepharose CL-4 B (Pharmacia Fine Chemicals) was added to the cell suspension. After 30 min, the cells and Sepharose beads were collected by centrifugation at 100 g for 10 min, resuspended in 3 ml of buffer A, and the resulting mixture of cell lysate and beads was divided into two portions. One portion was quickly diluted with 3 ml of buffer A, layered on a discontinuous gradient composed of 15, 30, and 40% sucrose in buffer A, and centrifuged at 200 g for 10 min. Sepharose beads with adsorbed immune complexes sedimented into the 40% sucrose layer were collected and washed with buffer B, and then P_{tyr} proteins were dissociated from the beads/immune complexes with buffer C (rapid isolate). The remaining portion of cell lysate was kept as such for 1 h, then centrifuged through the sucrose gradient, and P_{tyr} proteins were obtained in the same manner as above (delayed isolate). All the procedure was performed in an ice bath.

Analysis of P_{tyr} Proteins by Labeling with ¹²⁵I. The method for labeling P_{tyr} as well as tyrosine residues with ¹²⁵I in the presence of chloramine T, and the analysis for labeled P_{tyr} proteins by SDS/PAGE followed by autoradiography were described previously, as was the analysis for ¹²⁵I-labeled P_{tyr} residues by partial acid hydrolysis of ¹²⁵I-labeled P_{tyr} proteins followed by two-dimensional (2-D) separation of iodinated P_{tyr} derivatives (3, 5). ¹²⁵I-labeled proteins were analyzed also by the 2-D method of O'Farrell (17).

Analysis of P_{tyr} Proteins by Immunoblotting. Proteins separated by SDS/PAGE as above were electroblotted onto Durapore filters (Millipore Co., Bedford, MA), and P_{tyr} proteins were visualized by treatments first with affinity-purified rabbit P_{tyr} antibodies and then with biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) and streptavidin bridge reagents (Amersham International Plc., Buckinghamshire, United Kingdom) according to the Amersham protocol.

Isolation of Proteins Reactive with Anti-IL-2-R Antibodies. TL-Su cells (5×10^6) were homogenized in 1 ml of cold buffer A and centrifuged at 15,000 g for 30 min. The supernatant was applied to a column of 0.5 ml Sepharose 4 B to which 2 mg protein of either mAb H-31 or HIEI had been conjugated, and the column was washed repeatedly with buffer B. Proteins bound to the column were then eluted with buffer B containing

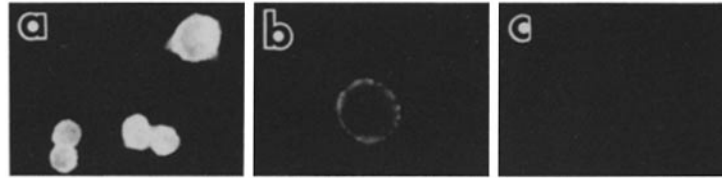


FIGURE 1. IF staining of HTLV-I-transformed cells with Ptyr mAb H1-D11. (a) TL-OmI cells fixed with acetone ($\times 1,200$); (b) membrane immunofluorescence of intact TL-OmI cells ($\times 1,200$); (c) the same as a except that Ptyr antibody was pretreated with 2 mM Ptyr ($\times 400$). Essentially the same results were obtained with TL-Su and TCL-Ter cells (not shown).

0.1% SDS, concentrated by lyophilization, and precipitated with 20% TCA. This will be referred to as IL-2-R preparation. In a control, the same cell extract was applied to a column containing mAb GIN14, which has no affinity to IL-2-R or Ptyr.

For further isolation of components phosphorylated at tyrosine, IL-2-R preparations precipitated with TCA were dissolved in buffer B and subjected to immunoaffinity chromatography, this time on columns to which polyclonal Ptyr antibodies were immobilized. Ptyr proteins were obtained in the same manner as described in the preceding section.

Dephosphorylation of IL-2-R-like Proteins. IL-2-R preparations obtained as above from TL-Su cells (10^6) were treated with 5 μ g bovine intestine alkaline phosphatase type VII-S (Sigma Chemical Co.) in 30 μ l of 50 mM Tris-HCl, pH 7.5, containing 1 mM DTT (Sigma Chemical Co.) at 30°C for 30 min. The product was heated at 100°C for 3 min in 2% SDS containing 5% 2-ME and subjected to immunoblotting analysis for Ptyr proteins.

Results

Detection of Ptyr Proteins by IF Staining. HTLV-I-transformed cells (TL-Su, TCL-Ter, and TL-OmI) fixed with acetone were IF stained with Ptyr antibody; >90% of cells showed strong cytoplasmic fluorescence and weak nucleolar fluorescence (Fig. 1A). When intact cells were stained, bright membrane fluorescence was seen in 20–30% of cells (Fig. 1B). The IF staining was blocked completely by pretreatment of Ptyr antibody with 2 mM Ptyr (Fig. 1C), but not with phosphoserine or phosphothreonine (not shown), suggesting that the staining is due to the specific recognition of Ptyr residues.

For comparative purposes, three T cell lines unrelated to HTLV-I (HPB-ALL, MOLT-4, and CCRF-CEM) and normal T cells activated with PHA were IF-stained in the same manner; cytoplasmic fluorescence was strongly positive in >90% of these cells when fixed with acetone, but membrane fluorescence on intact cells was practically negative. Other controls were negative in which Ptyr antibody was replaced by preimmune mouse IgG or lymphocytes from healthy persons were IF-stained (not shown). Thus, the membrane fluorescence with Ptyr antibody was characteristic of HTLV-I-transformed cells among human T cells examined.

Size Class Analysis of Total Ptyr Proteins. Ptyr proteins were isolated by affinity chromatography with polyclonal Ptyr antibodies from whole-cell extracts prepared from TL-Su, TCL-Ter, and TL-OmI cells. Proteins obtained were labeled with 125 I and separated by SDS-PAGE (Fig. 2, A–C). 10 size classes of proteins with molecular masses of 81–28 kD were detected in TCL-Ter and TL-OmI cells, and at least 7 classes were found in TL-Su cells. Among these, 70, 64, and 45 kD bands were commonly found in these HTLV-I-transformed cells. These

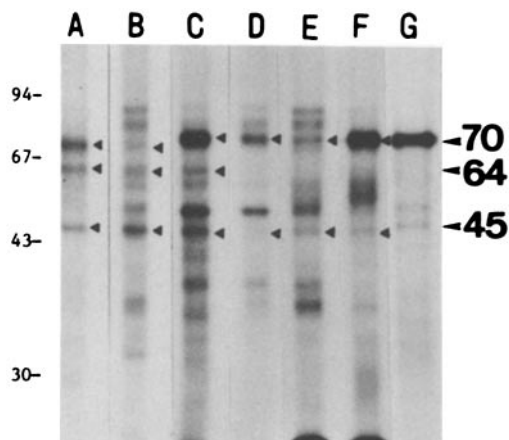


FIGURE 2. Ptyr proteins isolated from whole-cell extracts from human T cells. Total Ptyr proteins isolated by affinity chromatography with polyclonal Ptyr antibodies were labeled with ^{125}I and analyzed by SDS-PAGE. (A) TL-Su, (B) TCL-Ter, (C) TL-OmI, (D) HPB-ALL, (E) MOLT-4, (F) CCRF-CEM, and (G) normal human T cells activated with PHA. Marker proteins: phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), and carbonic anhydrase (30 kD).

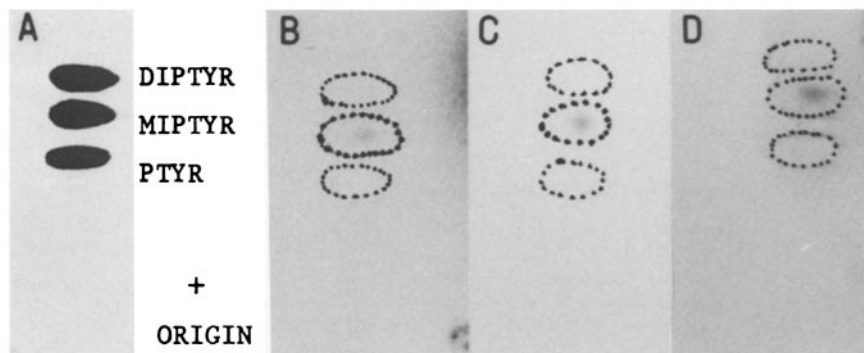


FIGURE 3. Identification of ^{125}I -labeled Ptyr in proteins. (A) Diagram showing the 2-D separation of authentic Ptyr, monoiodo-*o*-phosphotyrosine (MIPTYR), and diiodo-*o*-phosphotyrosine (DIPTYR) detected with ninhydrin and then by a color reaction for iodinated compounds (24). (B and C) Analysis of the 64 and 45 kD proteins, respectively, obtained from TL-OmI cells as shown in Fig. 2C. (D) Analysis of the 64 kD protein isolated by affinity chromatography with anti-IL-2-R followed by SDS/PAGE, as shown in Fig. 6B. The protein bands were cut from the gels, partially hydrolyzed, and then analyzed for ^{125}I -labeled Ptyr derivatives by 2-D separation. Authentic Ptyr, MIPTYR, and DIPTYR were included in the samples as markers. Iodinated Ptyr recovered was mostly MIPTYR under the conditions (i.e., relative amounts of total proteins vs. ^{125}I) used here. Results essentially the same as D were obtained for the 64 kD (pI 4.5) proteins obtained by sequential affinity isolation with anti-IL-2-R and then with Ptyr antibodies, as shown in Fig. 6, C and E (not shown).

three bands as well as those of 55, 50, 36, and 28 kD were cut from gels, and proteins eluted were partially hydrolyzed and analyzed for Ptyr residues; ^{125}I -iodinated Ptyr derivatives were detected in all of these proteins. The results for the proteins of 64 and 45 kD size classes from TL-OmI are shown in Fig. 3 as examples.

Ptyr proteins of similar size classes, including those of 70 and 45 kD were detected also in the other types of T cell lines unrelated to HTLV-I and activated normal T cells (Fig. 2, D-G), but the 64 kD band was not detected in any of these cells.

Cell Surface Ptyr Proteins. Ptyr proteins present on the outer surface of HTLV-I-transformed cells were analyzed by the method developed for this

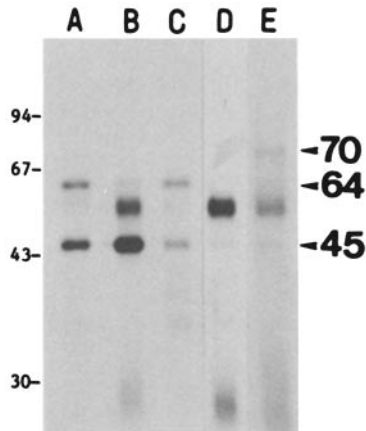


FIGURE 4. Cell surface Ptyr proteins isolated from human T cells. (A–D) Ptyr proteins in the rapid isolates from TL-Su, TCL-Ter, TL-Oml, and HPB-ALL cells, respectively. (E) Ptyr proteins in the delayed isolate from TCL-Ter cells.

purpose (3). To do this, intact cells were first exposed to Ptyr antibodies to allow antibody binding to Ptyr residues on the cell surface. Cells were then washed and lysed in the presence of protein A–Sepharose beads, and immune complexes were quickly isolated by being bound to the beads (rapid isolate). As seen in Fig. 4, A–C, Ptyr proteins of the size classes 64 and 45 kD were commonly detected in the three HTLV-I-transformed cell line cells. A band of ~55 kD was an artifact due to contaminating IgG H chains, and its appearance was erratic (Fig. 4, B, D, and E).

When the same cell lysates mixed with protein A–Sepharose were allowed to stand for 1 h and then immune complexes were isolated in the same manner as above (delayed isolate), the 64 and 45 kD bands diminished and a new 70 kD band appeared (Fig. 4E). As discussed previously (3), our interpretation of the result is that the surface Ptyr proteins primarily bound by Ptyr antibodies were displaced, during the 1-h standing, by the 70 kD Ptyr protein presumably having higher affinity to Ptyr antibody. In support of this, a pattern similar to that in Fig. 4E was also observed when the rapidly isolated beads/immune complexes were again mixed with a whole cell lysate for 1 h and the beads/immune complexes were reisolated by centrifugation for the analysis of Ptyr proteins (not shown). These results suggested that Ptyr residues of the 64 and 45 kD proteins are present on the outer cell surface and are responsible for the bright membrane immunofluorescence of intact HTLV-I-transformed cells.

The 64 kD band was again undetectable on the surface of the cells unrelated to HTLV-I or HPB-ALL, though a faint band was detected at the position of 45 kD (Fig. 4D).

Heterogeneity of the 64 kD Ptyr Protein. 2-D separation of total Ptyr proteins from TL-Su cells showed that the 64 kD of interest is heterogeneous in terms of pI (Fig. 5A); it gave an apparently heterogeneous major spot of pI 4.4–5.3 and two faint spots of pI 6.0 and 6.2. It was found in a separate experiment that the 64 kD Ptyr protein found on the cell surface (Fig. 4A) corresponds with this major component of 64 kD (pI 4.4–5.3) (not shown). This was then found to be composed of at least two distinct species of Ptyr proteins of 64 kD (pI 4.5 and 4.8–5.3), as shown in the next section.

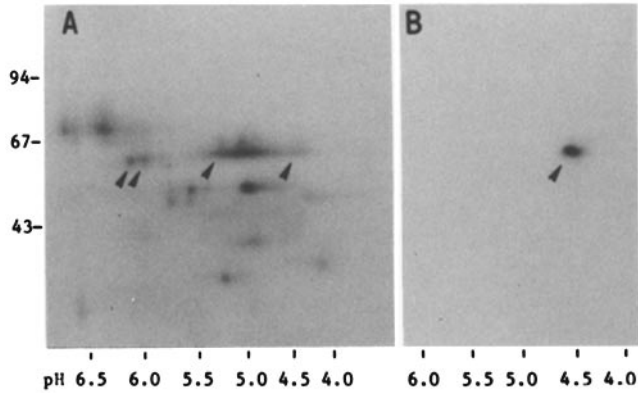


FIGURE 5. 2-D separation of P Tyr proteins. (A) Total P Tyr proteins isolated from TL-Su cells by affinity chromatography with P Tyr antibodies and labeled with ¹²⁵I. The arrowheads denote a heterogeneous major 64 kD component of pI 4.4-5.3 and two minor 64 kD components of pI 6.0 and 6.2. (B) The 64 kD band of Fig. 6C was cut from the gel and the protein eluted and analyzed. The arrowhead denotes a spot of 64 kD (pI 4.5) protein.

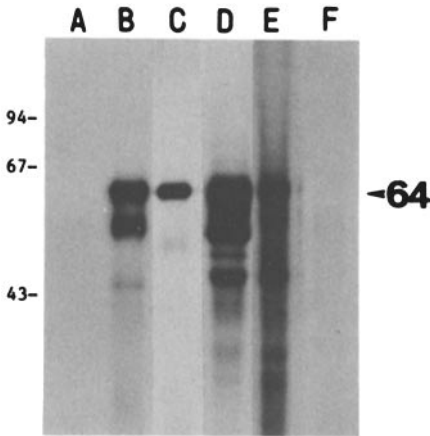


FIGURE 6. Proteins isolated by sequential affinity chromatography with anti-IL-2-R and P Tyr antibodies. (B and D) Proteins isolated from TL-Su cell extracts with mAb H-31 and HIEI, respectively, were labeled with ¹²⁵I and analyzed. (A) Control in which mAb GIN14 was used for affinity chromatography; other conditions were the same as B and D. (C and E) Proteins obtained by affinity isolation with mAb H-31 and HIEI, as in B and D, respectively, were again subjected, before iodination, to affinity chromatography with P Tyr antibodies, and P Tyr proteins obtained were analyzed as above. (F) An example of controls in which P Tyr antibody columns were pretreated with 2 mM P Tyr.

An IL-2-R-like Protein of 64 kD, Phosphorylated at Tyrosine. The physical properties of the acidic 64 kD P Tyr protein apparently corresponded to a certain species of IL-2-R previously found on the membrane of ATL-derived cells by Wano et al. (18). To examine this possibility, candidate proteins were isolated from TL-Su cells by immunoaffinity chromatography with two distinct types of mAb, H-31 and HIEI, directed against different antigenic structures on IL-2-R molecules. Each of these preparations was divided into two equal portions, and one portion was labeled with ¹²⁵I as such and analyzed by SDS/PAGE. Major bands were observed at the positions of ~64 and 50-55 kD, and a minor band was seen at ~45 kD in both the IL-2-R preparations (Fig. 6, B and D). An additional band of 70 kD was found in some preparations obtained with mAb HIEI that is not clearly seen here. These 64 and 45 kD proteins were found to have pI of ~4.5 and 4.3, respectively, in 2-D separation (not shown). No bands were detected in a control in which mAb GIN14 (unrelated to IL-2-R) was used for affinity chromatography (Fig. 6 A). The remaining halves of the IL-2-R preparations were sequentially subjected to immunoaffinity chromatography a second time with P Tyr antibody. Proteins obtained were labeled with ¹²⁵I and analyzed by SDS-PAGE (Fig. 6, C and E). ~60-90% of the 64 kD component contained in these IL-2-R preparations were bound to P Tyr antibody columns and eluted from them with phenylphosphate. The 64 kD P Tyr protein thus

obtained gave a single spot at \sim pI 4.5 in 2-D separation (Fig. 5B), this apparently corresponding with the most acidic component of the 64 kD class P_{tyr} proteins observed in Fig. 5A. The presence of ¹²⁵I-labeled P_{tyr} was confirmed in the 64 kD (pI 4.5) protein thus isolated (Fig. 3D).

The rest of the IL-2-R-like protein of 64 kD (pI 4.5) was recovered in the fraction unbound to P_{tyr} antibody columns, and it was not bound when applied again to fresh columns of P_{tyr} antibodies, indicating the lack of P_{tyr} residues in this fraction of the 64 kD (pI 4.5) protein. The ratio of the 64 kD IL-2-R-like proteins bound vs. unbound to P_{tyr} antibodies varied from cell sample to sample; the conditions that caused such variation were not clear.

The 45 kD protein in the IL-2-R preparation obtained with mAb H1E1 also behaved as a P_{tyr} protein (Fig. 6E), and its partial hydrolysate contained ¹²⁵I-labeled P_{tyr} (not shown). This component was not analyzed further here, because it was not clearly detectable in IL-2-R preparations obtained with mAb H31. No protein bands were detected in a control in which P_{tyr} antibodies immobilized on columns were blocked with P_{tyr} before application of IL-2-R preparations, suggesting that the isolation of proteins was due to the specific recognition by P_{tyr} antibodies (Fig. 6F).

In contrast to the protein of 64 kD (pI 4.5), the 64 kD P_{tyr}-protein of pI 4.8–5.3 found in the whole-cell extract of TL-Su (Fig. 5A) was not bound to the column of either anti-IL-2-R antibody and was clearly distinguishable, in this respect, from the IL-2-R-like P_{tyr} protein of 64 kD (pI 4.5).

Nonrecognition of Dephosphorylated IL-2-R-like Proteins by P_{tyr} Antibodies. IL-2-R molecules have been reported to be sulfated at unidentified sites (19). The following experiments show that phosphorylated groups, but not sulfated ones, are responsible for the recognition of IL-2-R-like proteins by P_{tyr} antibodies. IL-2-R preparations obtained with mAb H1E1 were analyzed for P_{tyr} proteins this time by immunoblotting using P_{tyr} antibodies. As shown in Fig. 7B, the 64 and 45 kD P_{tyr} proteins were clearly observed, in accordance with the analysis by ¹²⁵I-labeling shown in Fig. 6E. In this particular experiment, the 70 kD component was also seen as a major band. No bands were seen in a control (Fig. 7C) in which immunostaining with P_{tyr} antibodies was blocked with P_{tyr}, this supporting validity of the analytical method. The recognition by P_{tyr} antibodies was also abrogated when the same IL-2-R preparation was pretreated with alkaline phosphatase under the conditions in which P_{tyr} residues were preferentially dephosphorylated in a variety of P_{tyr} proteins (20) (Fig. 7A). In another series of controls, we confirmed that this enzyme treatment did not cause any detectable degradation of protein moieties of these P_{tyr} proteins (data not shown).

Discussion

IF staining of HTLV-I-transformed human T cells with P_{tyr} antibody showed the presence of P_{tyr} proteins on the outer cell membrane as well as in the cytoplasm and nucleoli. The membrane fluorescence was positive only on HTLV-I-transformed cells among various types of human T cells examined. This was substantiated by the identification of 7–10 size classes of P_{tyr} proteins in whole cell extracts from HTLV-I-transformed cells, and at least 2 classes of them, the

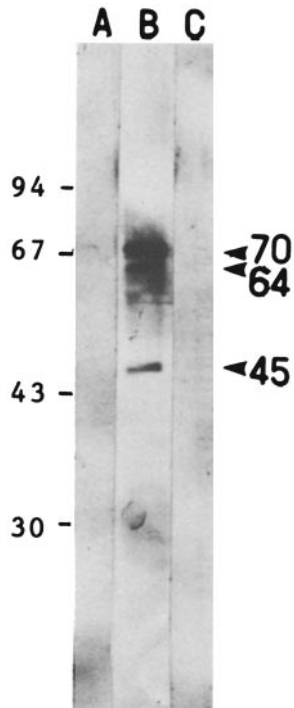


FIGURE 7. Nonrecognition of dephosphorylated IL-2-R-like proteins by P_{tyr} antibodies. (B) An IL-2-R preparation obtained from TL-Su cells with mAb H1E1 was analyzed for P_{tyr} proteins by immunoblotting using P_{tyr}-antibodies. (A) The same IL-2-R preparation as above was first treated with bovine alkaline phosphatase and then immunoblotted. (C) The same as B except that P_{tyr} antibodies pretreated with 2 mM P_{tyr} were used.

64 and 45 kD sizes, on the outer cell surface. P_{tyr} proteins of the 64 kD class seemed to be common to HTLV-I-transformed cells; they were not detected in other human T cell lines unrelated to HTLV-I or normal T cells treated with PHA.

P_{tyr} proteins of the 64 kD class found in HTLV-I-transformed cells were heterogeneous in pI; the class contained two major components, of pI 4.5 and 4.8–5.3. Of these, the P_{tyr} protein of 64 kD (pI 4.5) was found to have the characteristics of IL-2-R in that it was commonly recognized by two types of mAb directed against distinct epitopes on the IL-2-R molecule, and that its molecular mass and pI apparently corresponded with the figures, 60–65 kD (pI 4.2–4.5), reported for the mature form of IL-2-R present on the membrane of ATL-derived T cells (18). The P_{tyr} proteins of 64 kD (pI 4.5) appeared to make up only a fraction of the proteins isolated with anti-IL-2-R antibodies. This component most probably represents a particular molecular species of IL-2-R that underwent specific posttranslational modifications, including tyrosine phosphorylation, in HTLV-I-transformed cells, or it represents, if not IL-2-R itself, a protein closely related to it. This may account for the fact that the membrane fluorescence with P_{tyr} antibody is positive only on 20–30% of HTLV-I-transformed cells, while IL-2-R are expressed on the surface of most of these cells (8).

According to Shackelford et al. (21), IL-2-R can be phosphorylated at serine and threonine but not at tyrosine in HTLV-I-transformed human T cells labeled with ³²Pi (inorganic phosphate). Including this, no one has reported tyrosine phosphorylation of proteins having the characteristics of IL-2-R. We have recently found that there are two classes of P_{tyr} proteins in human cells: one major

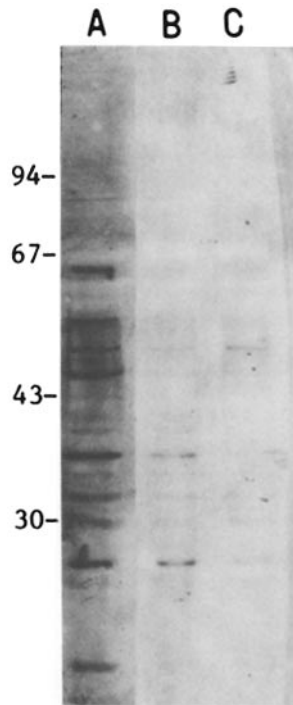


FIGURE 8. Disappearance of Ptyr proteins in cells cultured in phosphate-deficient medium. (A) Immunoblotting analysis of total Ptyr proteins extracted from growing TL-Su cells. (B and C) The same analysis after incubation of cells in phosphate-deficient medium for 3 and 6 h, respectively.

class can be detected by the conventional metabolic labeling with ^{32}P i followed by isolation with Ptyr antibodies; the other cannot be detected by this method but can be detected among the proteins that bind to Ptyr antibodies by in vitro labeling with ^{125}I . Presumably, proteins of the second class are present in relatively large quantities and have Ptyr residues at which the phosphate undergoes very slow turnover. We were puzzled, however, by the fact that such Ptyr proteins could not be detected even after prolonged labeling with ^{32}P i, and finally found that the amounts of either class of Ptyr proteins decrease dramatically when cells were cultured in phosphate-deficient medium, which has been generally used for the labeling (to be published elsewhere). The same phenomenon was observed also in human T cells used in the present study. As shown in Fig. 8, all the species of Ptyr proteins detected by immunoblotting in growing cells diminished or disappeared rapidly when cells were cultured in medium lacking Pi. This may well account for the failure of detection of the second class of Ptyr proteins even by long-term labeling with ^{32}P i. Such proteins may have escaped detection in much previous work, and the tyrosine-phosphorylated IL-2-R may belong to this class.

DNA sequencing of the IL-2-R gene (22, 23) showed that the IL-2-R molecule has eight tyrosine residues that are all located in the extracellular region of the molecule. The present finding that the Ptyr residues of the 64 kD IL-2-R-like protein seemed to be exposed to the outer cell surface is compatible with the DNA data. The mechanism by which proteins on the outer cell surface are phosphorylated at tyrosine is unknown. Presumably, the phosphorylation is an intracellular event that takes place before or during the transfer of newly formed

proteins to the cell surface. The IL-2-R molecules induced in HTLV-I-transformed cells have characteristic features (18); their relation to the present finding will be an important question to be answered in future studies.

None of the P_{tyr} proteins detected in the HTLV-I-transformed cells comigrated in SDS-PAGE with proteins immunoprecipitable with antibodies to HTLV-I-coded proteins (our unpublished observation). That these P_{tyr} proteins are not virus-encoded is also supported by the fact that they were detected in TL-OmI cells, which do not express any viral antigens (8).

Summary

Three interleukin 2 (IL-2)-independent human T cell lines transformed with human T cell leukemia virus type I were analyzed for *o*-phosphotyrosine-containing proteins (P_{tyr} proteins). Membrane and intracellular immunofluorescence was positive with antibody to P_{tyr} (P_{tyr} antibody). 10 size classes of P_{tyr} proteins with molecular masses of 81–28 kD were isolated with P_{tyr} antibodies. Among these, proteins of 64 kD (pI 4.5 and 4.8–5.3) and 45 kD (pI 4.3) were found on the outer cell surface. The P_{tyr} protein of 64 kD (pI 4.5) had the characteristics of the IL-2 receptor (IL-2-R) in that it was recognized by two monoclonal antibodies directed against different epitopes on the IL-2-R.

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References

1. Heldin, C.-H., and B. Westermark. 1984. Growth factors: mechanism of action and relation to oncogene. *Cell*. 37:9.
2. Krueger, J. G., E. A. Garber, and A. R. Goldberg. 1983. Subcellular localization of pp60^{src} in RSV-transformed cells. *Curr. Top. Microbiol. Immunol.* 107:51.
3. Ogawa, R., M. Ohtsuka, and Y. Watanabe. 1986. Complement-mediated lysis of K562 human leukemic cells by antibodies to phosphotyrosine and identification of cell surface proteins phosphorylated on tyrosine. *Cancer Res.* 46:2507.
4. Ogawa, R., M. Ohtsuka, Y. Watanabe, K. Noguchi, S. Arimori, and H. Sasadaira. 1985. Immunofluorescent staining of human leukemic cells with monoclonal antibody to phosphotyrosine. *Jpn. J. Cancer Res. (Gann)*. 76:567.
5. Ogawa, R., M. Ohtsuka, H. Sasadaira, M. Hirasa, H. Yabe, H. Uchida, and Y. Watanabe. 1985. Increase of phosphotyrosine-containing proteins in human carcinomas. *Jpn. J. Cancer Res. (Gann)*. 76:1049.
6. Ogawa, R., M. Ohtsuka, K. Noguchi, K. Iwamura, and Y. Watanabe. 1985. Anti-O-phosphotyrosine antibodies in human sera. *Microbiol. Immunol.* 29:759.
7. Hattori, T., T. Uchiyama, T. Toibana, K. Takatsuki, and H. Uchino. 1981. Surface phenotype of Japanese adult T-cell leukemia cells characterized by monoclonal antibodies. *Blood*. 58:645.
8. Sugamura, K., M. Fujii, M. Kannagi, M. Sakitani, M. Takeuchi, and Y. Hinuma. 1984. Cell surface phenotypes and expression of viral antigens of various human cell lines carrying human T-cell leukemia virus. *Int. J. Cancer*. 34:221.
9. Sugamura, K., S. Nakai, M. Fujii, and Y. Hinuma. 1985. Interleukin 2 inhibits in vitro growth of human T cell lines carrying retrovirus. *J. Exp. Med.* 161:1243.
10. Morikawa, S., E. Tatsumi, M. Baba, T. Harada, and K. Yasuhira. 1978. Two E-rosette-forming lymphoid cell lines. *Int. J. Cancer*. 21:166.
11. Srivastava, S., and J. Minowada. 1973. Terminal deoxynucleotidyl transferase activity in a cell line (MOLT-4) derived from the peripheral blood of a patient with acute lymphoblastic leukemia. *Biochem. Biophys. Res. Commun.* 51:529.

12. Foley, G. E., H. Lazarus, S. Farber, B. G. Uzman, B. A. Boone, and R. E. McCarthy. 1985. Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer*. 18:522.
13. Tanaka, Y., H. Tozawa, M. Hayami, K. Sugamura, and Y. Hinuma. 1985. Distinct reactivities of four monoclonal antibodies with human interleukin 2 receptor. *Microbiol. Immunol.* 29:959.
14. Ohtsuka, M., S. Ihara, R. Ogawa, T. Watanabe, and Y. Watanabe. 1984. Preparation and characterization of antibodies to O-phosphotyrosine and their use for identification of phosphotyrosine-containing proteins. *Int. J. Cancer*. 34:855.
15. Tanaka, Y., H. Tozawa, Y. Koyanagi, N. Yamamoto, and Y. Hinuma. 1984. A new monoclonal antibody recognizing an antigen of human lymphocytes similar or identical to Tac antigen. *Microbiol. Immunol.* 28:1041.
16. Tanaka, Y., Y. Koyanagi, T. Chosa, N. Yamamoto, and Y. Hinuma. 1983. Monoclonal antibody reactive with both p28 and p19 of adult T-cell leukemia virus-specific polypeptides. *Jpn. J. Cancer Res. (Gann)*. 74:327.
17. O'Farrell, J. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007.
18. Wano, Y., T. Uchiyama, K. Fukui, M. Maeda, H. Uchino, and J. Yodoi. 1984. Characterization of human interleukin 2 receptor (Tac antigen) in normal and leukemic T cells: co-expression of normal and aberrant receptors on Hut-102 cells. *J. Immunol.* 132:3005.
19. Leonard, W. J., J. M. Depper, M. Kronke, R. J. Robb, T. A. Waldmann, and W. C. Greene. 1985. The human receptor for T-cell growth factor. *J. Biol. Chem.* 260:1872.
20. Swarup, G., S. Cohen, and D. Garbers. 1981. Selective dephosphorylation of proteins containing phosphotyrosine by alkaline phosphatases. *J. Biol. Chem.* 256:8197.
21. Shackelford, D., and I. Trowbridge. 1984. Induction of expression and phosphorylation of the human interleukin 2 receptor by a phorbol diester. *J. Biol. Chem.* 259:11706.
22. Leonard, W. J., J. M. Depper, G. R. Crabtree, S. Rudikoff, J. Pumphrey, R. J. Robb, M. Kronke, P. B. Svetlik, N. J. Pfeffer, T. A. Waldmann, and W. C. Greene. 1984. Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. *Nature (Lond.)*. 311:626.
23. Nikaido, T., A. Shimizu, N. Ishida, H. Sabe, K. Teshigawara, M. Maeda, T. Uchiyama, J. Yodoi, and T. Honjo. 1984. Molecular cloning of cDNA encoding human interleukin-2 receptor. *Nature. (Lond.)*. 311:631.
24. Patterson, S. J., and R. L. Clements. 1964. The application of paper and thin-layer chromatography to the identification of thyroxine in a feeding-stuffs additive. *Analyst*. 89:328.