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TYROSINE PHOSPHORYLATION OF INTERLEUKIN 2 RECEPTOR-RELATED PROTEINS IN PHYTOHEMAGGLUTININ-ACTIVATED HUMAN LYMPHOCYTES

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Three classes of proteins (mol wts 70k, 64k and 45k) having the characteristics of interleukin 2 receptor were detected in phytohemagglutinin-activated human lymphocytes using two monoclonal antibodies which recognize distinct epitopes on the receptor. It was shown that at least portions of these proteins were phosphorylated on tyrosine by analyses for phosphotyrosine by immunoblotting and by immunoaffinity chromatography with antibodies to phosphotyrosine. In addition an iodinated phosphotyrosine derivative was identified in partial hydrolysates of these proteins iodinated in vitro.

Tyrosine phosphorylation of key proteins has been implicated in the regulation of cell growth mediated by certain growth factors as well as in the mechanism of cell transformation caused by src-related oncogenes (reviewed in refs. 1 and 2). We have recently found that there is a class of PTyr-proteins which cannot be detected by the conventional method of metabolic labeling with [^{32}P]Pi but can be detected among proteins isolated with anti-PTyr antibodies by in vitro iodination with ^{125}I (3). Presumably proteins of this class have PTyr residues at which the phosphate undergoes slow turnover. Even long-term labeling with ^{32}P did not enable the detection of these proteins because they disappeared rapidly when cells were cultured in Pi-minus medium generally used for ^{32}P labeling.

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Abbreviations. PTyr, phosphotyrosine; IL-2-R, interleukin 2 receptor; PHA, phytohemagglutinin; HTLV-I, human T cell leukemia virus type I; SDS/PAGE, sodium dodecylsulfate/polyacrylamide gel electrophoresis.

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By using our ^{125}I method, we have been able to show that PTyr-proteins indeed increase in a wide variety of human carcinomas and leukemic cells (4,5,6).

Our method also could detect tyrosine phosphorylation of IL-2-R or proteins related to it, which were induced on the surface of human lymphoid cells transformed with HTLV-I (7). The receptors were found to be induced by a function of the virus genome (8) and this induction has been implicated in the mechanism of transformation of infected cells (9). To determine whether the tyrosine phosphorylation of IL-2-R is unique to the cells transformed with the virus, we analyzed IL-2-R induced in PHA-activated normal human lymphocytes.

MATERIALS AND METHODS

Lymphocytes and antibodies. Normal human lymphocytes from peripheral blood were treated with 1% PHA and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and human interleukin 2 for 7 days (10). Two mouse monoclonal antibodies to IL-2-R, mAb H-31 and HIEI, used here recognize different epitopes on the IL-2-R molecule (10,11). Rabbit polyclonal antibodies to PTyr were prepared and affinity-purified as described (12). Mouse monoclonal antibodies with no affinity to IL-2-R or PTyr, mAb GIN-14 and P3/X63-Ag8 (10,11), were used as controls.

Isolation and ^{125}I-labeling of IL-2-R. PHA-activated cells (1×10^7) were homogenized in 1ml cold buffer A (10mM Tris-Cl, pH 7.3, 150mM NaCl, 5 mM EDTA, 1% Triton X-100, and 100 Kallikrein units/ml of aprotinin) and centrifuged at 15,000g for 30 min (6). The supernatant was applied to a 0.5ml Sepharose 4B column to which mAb H-31 or HIEI had been immobilized. The column was washed extensively with buffer B (50mM NaCl and 10mM Tris-Cl, pH 7.4), and proteins bound to the column were eluted with buffer B containing 0.1% SDS. Proteins eluted (this will be referred to as IL-2-R preparation) were precipitated with 20% trichloroacetic acid, washed with cold acetone, and then labeled with ^{125}I as described (4). SDS/PAGE analysis of labeled proteins and autoradiography were as described previously (4,6).

Immunoblotting. Proteins separated by SDS/PAGE were electroblotted to Durapore filters (Millipore Co., Bedford, Mass). IL-2-R or PTyr-proteins were visualized by immunostaining with mAb HIEI or affinity-purified anti-PTyr antibodies, respectively, with the aid of biotinylated anti-mouse or rabbit antibody (Vector Lab. Inc., Burlingame, Calif) and streptavidin bridge reagents (Amersham Int. Plc, Buckinghamshire, UK) according to the manufacturer's protocol.

Isolation of tyrosine-phosphorylated IL-2-R. IL-2-R preparations obtained from 1×10^7 cells as above were subjected to immunoaffinity chromatography on 0.5ml Sepharose columns to which anti-PTyr antibodies (5mg IgG) had been immobilized. The procedure was the same as described for the isolation of IL-2-R except that PTyr-proteins were eluted from the columns with 40mM phenylphosphate in buffer B.

Dephosphorylation of IL-2-R. IL-2-R preparations from 1×10^7 cells were incubated in 30µl of 50mM Tris-Cl, pH 7.5, containing 1mM dithiothreitol and 5µg bovine-intestine alkaline phosphatase type VII-S (Sigma Chem. Cor.,St. Louis, Mo) at 30°C for 30 min (13), and then heated at 100°C for 3 min in the presence of 2% SDS and 5% mercaptoethanol.

Identification of iodinated PTyr derivatives. The procedure has been described (3). Briefly, ^{125}I-labeled proteins were eluted from gels, partially hydrolyzed with 6N HCl at 100°C for 100 min, then analyzed for ^{125}I-labeled PTyr derivatives by two-dimensional separation followed by autoradiography. Authentic monoiodo-

and diiodo-phosphotyrosine, and PTyr included in the analysis were located with ninhydrin and by a color reaction for iodinated compounds (14).

RESULTS AND DISCUSSION

Proteins having the characteristics of IL-2-R were isolated by immunoaffinity chromatography from the lysates of PHA-activated human lymphocytes, then labeled with ^{125}I, and analyzed by SDS/PAGE followed by autoradiography. As shown in Fig. 1, D and F, proteins of apparent mol wts of 70k, 64k and 45k were commonly detected with either anti-IL-2-R antibody, mAb H-31 or HIEI. Several faint bands of mol wts smaller than 45k were consistently seen in IL-2-R preparations obtained with mAb HIEI, but they were not detected in samples obtained with mAb H-31 and therefore their identity is not clear. No bands were seen in controls in which antibodies to IL-2-R were replaced by control antibodies (Fig. 1, C & E).

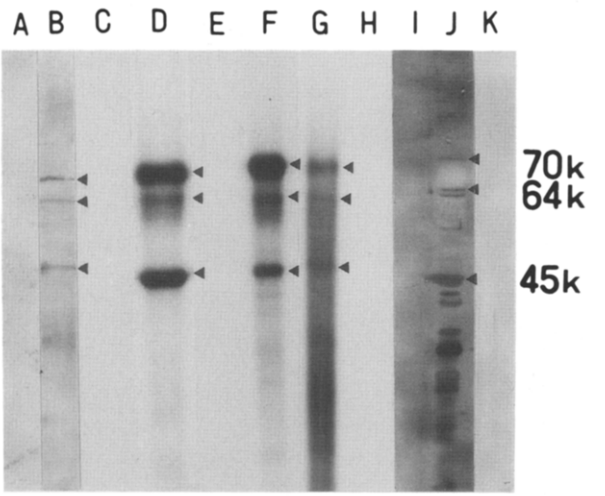

Fig. 1. Lane A: immunoblotting analysis of IL-2-R stained with a control antibody, GIN-14; and lane B: stained with mAb HIEI. Lanes D & F: SDS/PAGE of ^{125}I-labeled IL-2-R proteins isolated with mAb H-31 and HIEI, respectively; and lanes C & E: controls in which H-31 and HIEI were replaced by mAb GIN-14 and P3/X63-Ag8, respectively. Lane G: PTyr-proteins isolated by sequential immunoaffinity chromatography first with mAb HIEI and second with anti-PTyr antibodies, iodinated, and analyzed by SDS/PAGE; and lane H: a control in which the anti-PTyr column was pretreated with PTyr, other conditions were the same as in lane G. Lane J: immunoblotting analysis for PTyr of an IL-2-R preparation obtained with mAb HIEI; lane K: the same as lane J except that immunostaining was blocked with 2mM PTyr; and lane I: a dephosphorylated IL-2-R preparation was immunoblotted in the same manner as in lane J. Mol wt markers (not shown) were: phosphorylase b (94k), bovine serum albumin (67k), ovalbumin (43k) and carbonic anhydrase (30k).

The possibility that the 70k protein represents a complex of an interleukin 2 molecule and its receptor protein (15) may be excluded, because, when the 70k protein extracted from gels was heated in 8M urea under reducing conditions and the product was again analyzed by SDS/PAGE, no changes were observed in its mobility on gels (not shown). We assume that these three size classes of proteins represent IL-2-R itself or proteins closely related to it, because they share two distinct epitopes characteristic of IL-2-R (Fig. 1, D & F) and no such proteins were found in PHA untreated peripheral lymphocytes (data not shown).

Similar protein bands of mol wts 70k, 64k and 45k were also observed when cell extracts were directly subjected to SDS/PAGE, blotted onto membranes, and immunostained with mAb HIEI (Fig. 1, B). Again, no bands were seen in the control, stained with the control antibody (Fig. 1, A).

The mol wts of IL-2-R proteins determined here somewhat differ from those reported previously for IL-2-R and its precursors: 60k-65k and 40k respectively in ref. 16, 55k and 33k-37k respectively in ref. 17, and 60k in ref. 11. Such discrepancy could have partly resulted from difference in the methods by which these proteins were labeled besides cell differences. Biosynthetic labeling employed by these groups may have preferentially detected de novo synthesized intermediates of the processing of IL-2-R as well as its mature forms. By contrast, the two methods used here are expected to detect IL-2-R proteins which are present in relatively large quantities regardless of their metabolism.

To examine whether these proteins isolated with anti-IL-2-R antibodies are phosphorylated on tyrosine, they were subjected, before iodination, to immunoaffinity chromatography this second time on columns to which anti-PTyr antibodies were immobilized. At least portions of the 70k, 64k and 45k proteins were bound to the column and eluted with a ligand, phenylphosphate (Fig. 1, G). No bands were detected when the affinity columns were blocked with PTyr before the application of samples (Fig. 1, H). The band intensities in lanes F and G cannot be directly compared, because the total amount of proteins subjected to iodination was much less in lane G. This higher ^{125}I/protein ratio presumably caused more labeling of proteins in lane G than in lane F. For presumably the same reason,

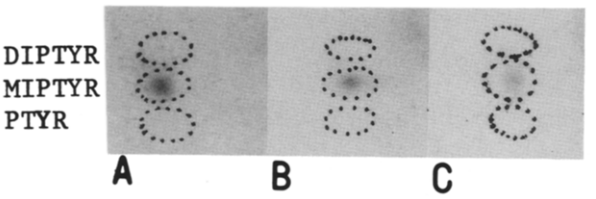

Fig. 2. Two-dimensional analysis of ^{125}I-labeled PTyr derivatives in partial hydrolysates of the 70k (A), 64k (B), and 45k (C) proteins extracted from the gel shown in Fig. 1, lane F. The right bottom corner of each diagram denotes the origin. MIPTYR: monoiodophosphotyrosine; and DIPTYR: diiodophosphotyrosine.

nonspecific background ^{125}I was high in lane G, and tended to obscure the bands that migrated into the lower half of the gel.

Immunoblotting analysis also showed that the 70k, 64k and 45k proteins are phosphorylated on tyrosine. In this test, proteins isolated with mAb HIEI were subjected to SDS/PAGE, electroblotted onto membranes, and immunostained with anti-PTyr antibodies. No definite bands were detected in controls in which immunostaining was blocked with 2mM PTyr (Fig. 1, K). Also, the same IL-2-R preparation was first treated with phosphatase under the conditions which facilitated dephosphorylation of PTyr (13) and then subjected to immunoblotting (Fig. 1, I). In the latter control, we confirmed that the phosphatase treatment did not cause detectable degradation of the protein moieties (data not shown). In the analysis shown in Fig. 1, J, in addition to the bands of 70k, 64k and 45k a number of new bands were seen; these included several clear bands of mol wts smaller than 45k. Since these bands were undetectable, or very faint if any, in the analyses shown in Fig. 1, B and F, the results suggest that these proteins were highly posphorylated on tyrosine, compared to the major 70k, 64k and 45k proteins. The nature of these proteins is as yet not clear.

To further ascertain the existence of PTyr in the 70k, 64k and 45k proteins, each protein was extracted from gels, partially hydrolyzed, and analyzed for iodinated PTyr derivatives by two-dimensional separation; ^{125}I-labeled monoiodophosphotyrosine was detected in all of these proteins (Fig. 2)

The above results were essentially the same as those obtained for human T cells transformed with HTLV-I (7) with respect to: (i) the apparent mol wts of

the major classes of proteins recognized by anti-IL-2-R antibodies and (ii) their partial phosphorylation on tyrosine. Therefore such phosphorylation is not an event unique to virus-transformed cells. It has been shown that two distinct types of IL-2-R with high and low affinities for interleukin 2 are induced in lectin-activated lymphocytes (15), and that IL-2-R expressed on HTLV-I-transformed cells is mostly of the low affinity type (9). Whether or not the phosphorylation of tyrosine residues is the molecular bases for the difference between the high and low affinity receptors will be an important issue in future studies.

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