Complement-mediated Lysis of K562 Human Leukemic Cells by Antibodies to Phosphotyrosine and Identification of Cell Surface Proteins Phosphorylated on Tyrosine

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ABSTRACT

Intact K562 human leukemic cells showed bright membrane immunofluorescence after staining with monoclonal antibody to O-phosphotyrosine (PTyr). Up to 60% of the cells were lysed with mouse, rabbit, or human antibodies to PTyr by a complement-mediated mechanism. A new method has been developed for identifying proteins that have PTyr residues on the outside of cell membrane, and at least two species of PTyr-containing proteins with the molecular weights of 45,000 and 36,000 were identified as the most probable candidates of the antigens responsible for the membrane fluorescence and cell lysis.

INTRODUCTION

By means of IF staining with antibodies to PTyr, we have recently shown that human leukemic cells obtained from patients with various types of leukemia have elevated levels of PTyr-proteins and, in this respect, can be distinguished clearly from normal hematological cells including normal blast cells in the bone marrow. This finding suggested the possible use of antibodies to PTyr for immunodiagnostic purposes.

One typical example was the K562 human leukemic cell line which was established from a patient with chronic myelogenous leukemia and shown to have an altered c-abl product having tyrosine protein kinase activity. IF staining of acetone-fixed cells showed bright fluorescence in the cytoplasm and nucleolus-like structures in the nuclei. In the present paper, we show that at least two species of PTyr-proteins are present also on the outside of plasma membrane of K562 cells, and intact cells are recognized by PTyr-antibodies and killed in the presence of complement.

MATERIALS AND METHODS

Cells and Antibodies. K562 cells were cultured in RPMI 1640 medium supplemented with 15% FBS (Gibco Laboratories, New York, NY). Normal granulocytes were obtained from peripheral blood of three healthy persons by centrifugation of heparinized blood samples on a Ficoll-paque layer (Pharmacia Fine Chemicals, Uppsala, Sweden). Mouse mcAb (H1-D11) and rabbit polyclonal antibodies, both reactive with PTyr, were prepared as described (4). Human antibodies to PTyr were obtained from a patient with carcinoma of the esophagus as described (5). These will be referred to as PTyr-antibodies. Immunoglobulins were precipitated by half saturation with ammonium sulfate and dissolved in PBS to give the original volume of serum or ascites fluid.

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The abbreviations used are: IF, immunofluorescent; PTyr, O-phosphotyrosine; PTyr-proteins, O-phosphotyrosine-containing proteins; PTyr-antibodies, antibodies to O-phosphotyrosine; FBS, fetal bovine serum; mcAb, monoclonal antibody; PBS, phosphate-buffered saline; RPMI-FBS, RPMI 1640 medium with 5% fetal bovine serum; TCA, trichloroacetic acid; DlPTyr, diiodophosphotyrosine.

Membrane IF Staining. Intact K562 cells were allowed to react with mcAb H1-D11 at a dilution of 1:64 in PBS containing 0.02 M NaN3 (PBS-Na3) and in the cold, and then stained with fluorescein-conjugated anti-mouse IgG (Microbiol. & Biol. Laboratories, Nagoya, Japan) as described (6). Preimmune mouse IgG was used in a control. For antibody-blocking tests, mcAb H1-D11 was pretreated with 2 μM PTyr (Sigma Chemical Co., St. Louis MO) at 37°C for 1 h and then used for IF staining.

Microcytotoxicity Tests with PTyr-antibodies. The method described by North (7) was used. Ten μl of 2-fold serial dilutions of PTyr-antibodies, starting at an initial dilution of 1:5, in RPMI-FBS was added to 10 μl cell suspension in RPMI-FBS (5-7 x 10⁴ cells/ml, with ≥95% viability). The mixture then received 10 μl rabbit complement (LOW-TOX-H; Cedarlane Laboratories, Ontario, Canada; diluted 1:4 in RPMI-FBS). After incubation at 37°C for 45 min, percentages of dead cells were determined by staining with trypan blue. As controls, dead cells were determined without antibodies (complement controls, or c-cont.) or without complement (antibody controls). The percentage of specific cytotoxicity was calculated from the formula

\[
\frac{\text{% dead cells}_{\text{experimential}} - \text{% dead cells}_{\text{com}}}{\text{% dead cells}_{\text{com}}} \times 100
\]

100% - \% dead cells_{com}.

Blocking tests were conducted with PTyr-antibodies pretreated with 2 μM PTyr.

Isolation of PTyr-proteins from Whole K562 Cells. K562 cells (5 x 10⁶) were homogenized in 2 ml of ice-cold buffer A (1% Triton X-100, 10 mM Tris-HCl, pH 7.3, 150 mM NaCl, 5 mM EDTA and aprotinin, 100 kallikrein units/ml) and centrifuged at 15,000 x g for 30 min. The supernatant will be referred to as "whole cell extract." It was then subjected to immunoaffinity chromatography on a Sepharose 4B column (0.5 ml) to which rabbit PTyr-antibodies (5 mg globulins) had been conjugated (4). The loaded column was washed with buffer B (50 mM NaCl and 10 mM Tris-HCl, pH 7.3), and then PTyr-proteins were eluted with buffer C (10 mM NaCl, 10 mM Tris-HCl, pH 7.3, and 40 mM sodium phenylphosphate) and lyophilized.

Isolation of Cell Surface PTyr-proteins. All the procedures were performed on ice. Intact K562 cells (5 x 10⁶) were allowed to react with rabbit PTyr-antibodies in 0.3 ml of PBS-Na3 for 20 min, then washed three times with PBS-Na3 and resuspended in 0.3 ml of the same buffer. Protein A-Sepharose CL-4B (0.3 ml; Pharmacia Fine Chemicals, Uppsala, Sweden) was added to the antibody-treated cell suspension. After 30 min, cells and Sepharose beads were collected by centrifugation at 100 x g for 10 min and resuspended in 3 ml of buffer A to lyse cells. The cell lysate-Sepharose mixture was divided into two portions. One portion was diluted quickly with 3 ml of buffer A and layered on the top of a discontinuous gradient prepared with 1 ml each of 40, 30, and 15% sucrose in buffer A, and centrifuged at 200 x g for 10 min. Most of the cell components were distributed at the bottom of and above the 30% sucrose layer. Sepharose beads sedimented in the 40% sucrose layer were collected, washed once with buffer-A and three times with buffer B, and then PTyr-proteins bound to the beads (in a form of immune complex) were eluted with buffer C. The other portion was allowed to stand as such for 1 h, then immune complexes bound to Sepharose beads were collected by centrifugation, and PTyr-proteins were obtained in the same manner as above. PTyr-proteins thus obtained were lyophilized.

Labeling of PTyr-proteins with ¹²⁵I. Lyophilized PTyr-proteins were desalted by washing with 20% TCA followed by removal of TCA with acetone and ether. Desalted proteins were labeled with 0.5 mCi/sample.
of Na$^{25}$I (carrier free; Japan RI Associates, Tokyo, Japan) in the presence of chloramine-T by the method of Brown et al. (8). Samples were then washed extensively with cold 20% TCA and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography as described (4).

Identification of Iodinated PTyr in Proteins. $^{125}$I-labeled proteins were eluted from gels (9), partially hydrolyzed in 6 N HCl at 100°C for 100 min, and then analyzed for $^{125}$I-labeled DIPtyr by two-dimensional separation as described (10). Authentic moniodophosphotyrosine and DIPtyr added as markers were detected by a color reaction for iodinated compounds (11). Detailed procedures for the synthesis of the markers and the identification of iodinated PTyr in proteins will be published elsewhere.

RESULTS

Membrane Immunofluorescence. IF staining of intact K562 cells with mouse mcAb under conditions which prevent redistribution of antigen-antibody complexes on the cell surface showed bright membrane fluorescence in 60–70% of cells (Fig. 1A). This fluorescence pattern contrasted well with the cytoplasmic and nuclear fluorescence which was observed in acetone-fixed K562 cells IF stained with the same antibody (1), suggesting the presence of P Tyr-proteins on the exterior of K562 cells. The membrane fluorescence was negative when mcAb was pretreated with 2 mM P Tyr (Fig. 1B) or it was replaced by preimmune mouse IgG (not shown). Granulocytes obtained from healthy persons were totally IF negative as examined with either intact or acetone-fixed cells (not shown).

Complement-mediated Cell Lysis. K562 cells were found to be lysed with P Tyr-antibodies in the presence of complement (Fig. 2). Essentially the same results were obtained with mouse, rabbit, and human P Tyr-antibodies. The cell lysis was inhibited by pretreatment of P Tyr-antibodies with 2 mM P Tyr, suggesting dependency of the lysis on the specific recognition of P Tyr residues by antibodies on the surface of K562 cells. In contrast, no significant lysis was observed with normal granulocytes from healthy persons, this being consistent with their negative membrane fluorescence.

Identification of Cell Surface P Tyr Proteins. Selective labeling of cell surface proteins with $^{125}$I by the peroxidase method is not applicable in this study because P Tyr residues are readily iodinated under the conditions* and this would flaw the specific recognition by P Tyr-antibodies in the subsequent isolation of P Tyr-proteins. We therefore adopted a method in which P Tyr residues on the surface of intact cells were first allowed to react with P Tyr-antibodies, then cells were washed and lysed in the presence of protein A-Sepharose beads, and immune complexes were quickly isolated by being bound to the beads. There is a possibility that surface P Tyr-proteins primarily bound to antibodies are replaced during the manipulation by other species of P Tyr-proteins which are abundant in the cell lysate or have higher affinity for antibodies than that of originally bound antigens. To test this, the lysate was divided into two portions; one portion was immediately subjected to centrifugation in a discontinuous sucrose gradient to rapidly separate immune complexes from the rest of the cell lysate (referred to as “rapid isolate”), and the other portion was kept as such for 1 h before isolation of immune complexes (“delayed isolate”). P Tyr-proteins obtained by these two methods as well as total P Tyr-proteins isolated from whole cells were labeled with $^{125}$I and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3 and Table 1).

At least 11 species of P Tyr-proteins were detected in the whole cell extract; these included proteins of $M$, 55,000, 45,000, 43,000, 40,000, 33,000, and 28,000 (Fig. 3, Lane A; Table 1, Experiments 1 and 2). In contrast, major P Tyr-proteins detected in the rapid isolate were of $M$, 45,000 and 36,000 (Fig. 3, Lane C; Table 1, Experiments 3–6), and those detected in the delayed isolate were of $M$, 55,000 and 75,000 (Fig. 3, Lane E; Table 1, Experiments 7 and 8); the $M$, 45,000 and 36,000 bands were faint or undetectable in the latter. That these proteins contain P Tyr residues was confirmed by the following two types of tests: (a) $^{125}$I-labeled DIPtyr was identified in partial hydrolysates of all the proteins listed in Table 1 except the $M$, 81,000 protein which was not analyzed because of its low radioactivity. As examples, the analyses for DIPtyr in the $M$, 45,000 and 36,000 proteins from the rapid isolate are shown

![Fig. 1. Membrane immunofluorescence of K562 cells. A, intact K562 cells IF stained with mouse mcAb; B, The same as A except that P Tyr-antibody was pretreated with 2 mM P Tyr. × 600.](image-url)

![Fig. 2. Complement-mediated lysis of K562 cells. Cytotoxicity tests with mouse mcAb (A), rabbit P Tyr-antibodies (B), and human P Tyr-antibodies (C).](image-url)
PHOSPHOTYROSYL PROTEINS ON K562 CELL SURFACE

Table 1. PTyr-proteins isolated from K562 cells

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Experiments 1 and 2. PTyr-proteins found in the whole cell extract; Experiments 3-6, those in the rapid isolate; Experiments 7 and 8, those in the delayed isolate. Experiments 3 vs. 7 and 4 vs. 8 are the results of two series of experiments, each conducted with the same cell sample. The autoradiograms for Experiments 1, 3, and 7 are shown in Fig. 2. Lanes A, C, and E, respectively, and that for Experiment 6 is shown in Fig. 4. Lane A. ++ represent strong bands.

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of [32P]labeled PTyr-proteins isolated from K562 cells. Lane A. PTyr-proteins isolated from the whole cell extract by affinity chromatography on a Sepharose column to which rabbit PTyr-antibodies had been conjugated. Lane B, the same as Lane A except that the affinity column had been saturated with sodium phenylphosphate before application of the sample. Lanes C and E. PTyr-proteins obtained from the rapid and delayed isolate, respectively. Lanes D and F, the same as Lanes C and E, respectively, except that intact K562 cells were treated phenylphosphate before application of the sample. Lanes C and E, PTyr-proteins isolated from K562 cells. Lane A, PTyr-proteins isolated from K562 cells. Lane A. ++ represent strong bands.

DISCUSSION

We reported previously that human leukemic cells, including the K562 cell line, can be distinguished from normal hematological cells by their increased content of PTyr-proteins in the cytoplasm and nucleolus-like structures (1). Here we present evidence that a certain species of PTyr-proteins are present on the exterior of cell membrane of K562 cells. This was shown first by membrane immunofluorescence and cell lysis with PTyr-antibodies and then substantiated by the identification of at least two species of surface PTyr-proteins.

A new method has been developed in this study for analyzing proteins having PTyr residues outside the cell membrane, and the M, 45,000 and 36,000 components were identified as the most probable candidates for PTyr-proteins that are responsible for membrane fluorescence and cell lysis. For comparative purposes, membrane proteins were also analyzed by the conventional procedure in which amino acid residues susceptible to iodination on the K562 cell membrane were labeled with [125I] by the peroxidase method (12) and then PTyr-proteins labeled were analyzed after their specific isolation by affinity chromatography with PTyr-antibodies. PTyr-proteins of M, 100,000, 81,000, 75,000, and 55,000 were detected as the major components together with several minor ones, while the M, 45,000 and 36,000 bands were very faint. We tentatively interpret these contradictory results to mean that the PTyr-antibodies used in this study do not appreciably cross-react with iodinated PTyr residues, and that the membrane proteins detected in this analysis represent mostly transmembrane proteins which have PTyr residues in a form protected from the surface iodination, e.g., those projecting to the inside of cell membrane. We have been unable to determine the extent of cross-reaction of PTyr-antibodies with DITyr because the amount of DITyr that could be synthesized by our method was insufficient for these purposes.

A PTyr-protein of M, 36,000 has been found in avian and mammalian cells transformed with retroviruses whose v-onc products possess tyrosyl protein kinase activity. The M, 36,000 protein in avian cells has been shown to be associated with cytoplasmic sites of the plasma membrane of transformed cells and its biological significance in cell transformation has been studied extensively [reviewed by Krueger et al. (13)]. A homologous or essentially the same M, 36,000 PTyr-protein has been studied also in A431 human epidermoid carcinoma cells treated with epidermal growth factor, suggesting that it is a highly conserved protein and possibly involved in the regulation of cell growth (14, 15), although no definite function has been assigned to these M, 36,000 proteins so far. Whether these M, 36,000 proteins are the same as that found in the present study is an important question to be asked in future studies.

The cytotoxicity test with PTyr-antibodies, about 60% of

fractionated. This was verified by a model experiment in which a portion of the rapid isolate was again mixed with the whole cell lysate for 30 min and then immune complexes were reisolated; the replacement of PTyr-proteins was observed as expected (Fig. 5). Thus the M, 45,000 and 36,000 proteins are most probably the major components responsible for the membrane fluorescence and cell lysis with PTyr-antibodies. The M, 55,000 protein was also observed in the rapid isolates, although its amount relative to the others varied from experiment to experiment. Therefore, the possibility that a fraction of the M, 55,000 protein also resides on the cell membrane cannot be excluded.

in Fig. 4. (b) Control experiments in which immune reactions with PTyr-antibodies were blocked with PTyr or phenylphosphate showed that the isolation of these proteins is based on the specific recognition by PTyr-antibodies (Fig. 3, Lanes B, D and F).

A simple interpretation of these results is that the M, 45,000 and 36,000 proteins have PTyr residues on the outside of cell membrane and are primarily bound by PTyr-antibodies, and that these proteins are replaced later by the M, 55,000 and 75,000 proteins when the cell lysate is kept without being

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Fig. 4. Identification of 125I-labeled DIP-Tyr. A. An illustration of authentic PTyr, monooiodophosphotyrosine (MIPTYR), and DIP-Tyr spots separated on a thin-layer plate and detected with ninhydrin or by a color reaction for iodinated compounds. Ninhydrin failed to locate DIP-Tyr precisely due to interference by iodine. B and C, identification of DIP-Tyr in the M, 45,000 and 36,000 proteins shown in Fig. 3, Lane C, respectively. The radioactive spots coincide with the marker DIP-Tyr.

![Diagram of DIP-Tyr, MIPTYR, and PTyr spots](image)

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Fig. 5. Demonstration of the replacement of antibody-bound PTyr-proteins. The rapid isolate (complexes composed of protein A-Sepharose, PTyr-antibodies, and surface PTyr-proteins) was divided into two portions. One portion was analyzed as such for PTyr-proteins (Lane A). The other portion was mixed with the whole cell extract (obtained from the same amount of cells as that used for preparing the rapid isolate) and kept for 30 min on ice, and then analyzed for the PTyr-proteins bound to the complex (Lane B). MW, molecular wt; K, thousands.

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the cells were lysed at the maximum. The fact that membrane fluorescence was positive in 60–70% of the cell population and that the rest was very faint or negative suggests a possibility that cells having below-threshold amounts of PTyr residues on the surface, at any given time, are insusceptible to antibody-dependent cell lysis. Alternatively, this could be an inherent limitation of this assay method, e.g., due to inevitable limitation of the amount of complement that can be added to the assay system. Whichever it may be, the specific recognition of the cell surface of leukemic cells with PTyr-antibodies suggests its possible use in "targeting" for either immunodiagnostic or therapeutic purposes.
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