INTERLEUKIN 2 (IL-2)-INDUCED TYROSINE PHOSPHORYLATION OF IL-2 RECEPTOR p75

By HIRONOBU ASAO, TOSHIKAZU TAKESHITA, MASATAKA NAKAMURA, KINYA NAGATA, and KAZUO SUGAMURA

From the Department of Microbiology, Tohoku University School of Medicine, Sendai 980, Japan

Expansion of T lymphocytes after exposure to antigens results from clonal growth of the T cells specific for the antigens. Proliferation of these T cells is initiated by the interaction of IL-2 and its receptor (IL-2R), which are transiently secreted as a growth factor lymphokine and expressed on the cell surface as an acceptor of the growth factor, respectively. There exist at least two classes of IL-2R, which differ in their affinity to IL-2. The high affinity receptor is responsible for conferring signals mediating various functions in T, B, and non-T non-B cells such as internalization of IL-2R, activation of gene expression and culminating in cell growth (reviewed in reference 1). The chemical crosslinking analysis with ^{125}I-labeled IL-2 revealed that the high affinity IL-2R is a complex of at least two distinct subunits, p55 and p75 (2–6). The p55 molecule alone having failed to mediate growth signals (7, 8), much attention focuses on the structure and function of p75 that is thought to be involved in transducing signals.

Several of the growth factor receptors are known to possess tyrosine kinase domains, and the tyrosine phosphorylation of receptor molecules has been considered to be involved in the pathway of growth signals as one of the earliest detectable responses to ligand bindings (reviewed in reference 9). We previously detected the phosphotyrosine proteins in the immunocomplex purified with anti-IL-2Rp55 (10), and a recent report also demonstrated IL-2-induced tyrosine phosphorylation of several proteins with antiphosphotyrosine antibody (11), although the molecular nature of these phosphotyrosine proteins has not been clarified. We have recently established two independent mAbs specific for human IL-2Rp75, which enabled us to characterize the IL-2Rp75 molecule (12, 13). The present study uses one of these mAbs to demonstrate the IL-2-induced tyrosine phosphorylation of IL-2Rp75 and association of IL-2Rp75 with tyrosine kinase activity.

Materials and Methods

Cells.   The cell line used here was an IL-2-dependent human T cell line, ILT-Mat, carrying human T cell leukemia virus type I (HTLV-I) and expressing both high affinity and low affinity IL-2R (12). ILT-Mat cell line was maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, antibiotics, and 0.9 nM recombinant human IL-2 (Shionogi Pharmaceutical Co., Japan).

Monoclonal Antibodies.   TU11 mAb (IgG1) is specific for human IL-2Rp75 and does not interfere with IL-2 binding to IL-2R (13). X63 mAb (IgG1) produced by the P3X63-Ag8 cell line is a control mAb.
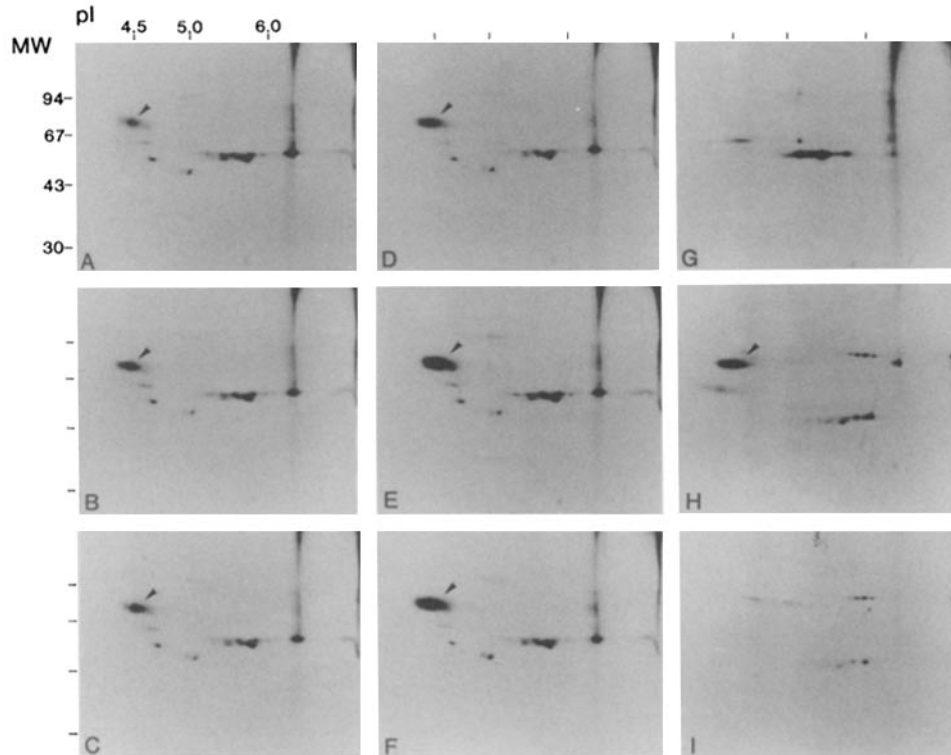
In Vivo Phosphorylation.   ILT-Mat cells and activated peripheral blood leukocytes were labeled with [^{32}P]orthophosphate as described previously (14). In brief, cells were preincubated in the absence of IL-2 for 10–14 h, and resuspended in phosphate-free Eagle's MEM supplemented with 20 mM Hepes and 10% dialyzed FCS. [^{32}P]Orthophosphate (Amersham Corp., Arlington Heights, IL) was added into the culture at a concentration of 1 mCi/ml and cells were cultured for another 2 h. Then rIL-2 was added and the cells were further cultured for the indicated times. The cells were then lysed in the cell extraction buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 10 mM EDTA, 10 mM Na$_4$P$_2$O$_7$, 10 mM Na$_2$MoO$_4$, 100 mM NaF, 2 mM Na$_3$VO$_4$, 0.1% NaN$_3$, 2 mM PMSF, and 1% aprotinin). The lysate was centrifuged at 10,000 *g* for 20 min and the resultant supernatant was used for immunoprecipitation analysis.

Radioimmunoprecipitation.   Radioimmunoprecipitation analyses were done by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) as described previously (12, 14). Briefly, ^{32}P-labeled cell lysates were preabsorbed with protein A–Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ), and then incubated with TU11 mAb or X63 mAb and protein A–Sepharose CL-4B pretreated with rabbit polyclonal anti-mouse IgG antibody (Zymed Laboratories, San Francisco, CA) for over 4 h at 4°C. The Sepharose beads were washed and suspended in the lysis buffer for isoelectric focusing (IEF). Then the eluates were analyzed by 2-D PAGE. As a control, ^{125}I surface-labeled ILT-Mat cell lysates were analyzed under the same procedure as mentioned above.

Phosphoamino Acid Analysis.   Gel pieces corresponding to ^{32}P-labeled IL-2Rp75 spots were excised from the 2-D gels, washed in 10% methanol, and lyophilized to dryness. The gel slices were incubated for 12 h at 37°C in 0.5 ml of 50 mM ammonium bicarbonate containing 25 µg of self-digested proteinase K (Merck & Co., Rahway, NJ). The gel slices were then removed, and the supernatants were lyophilized to dryness. Phosphoamino acid analysis was performed according to the method of Cooper et al. (15). The dried materials were hydrolyzed in 5.7 N HCl for 1 h and the phosphoamino acids were recovered on AG1-X8 resin (Cl⁻ form; Bio-Rad Laboratories, Richmond, CA), and the separation of phosphoamino acids was achieved by high voltage electrophoresis in two dimensions on a thin-layer cellulose plate. The dried plate was exposed to x-ray film for autoradiography. Phosphoamino acid markers were detected by ninhydrin staining.

Results

The phosphorylation of IL-2Rp75 was first examined in IL-2-dependent ILT-Mat cells. After incubation without IL-2 for 14 h, ILT-Mat cells were cultured with [^{32}P]orthophosphate for 2 h, and then stimulated with 2 nM IL-2 for the indicated times. The radiolabeled cell lysates were immunoprecipitated with TU11 mAb specific for the p75 subunit of IL-2R. The precipitated immunocomplexes were analyzed by 2-D PAGE (Fig. 1). The p75 phosphorylation specifically increased during IL-2 stimulation for 5 min, and the increase tended to continue for at least 30 min. The location (mol wt: 71,000–81,000; pI 4.3–4.7) of the phosphorylated p75 spot in Fig. 1, *A–E* was confirmed to be identical to that of the ^{125}I surface-labeled p75 spot in Fig. 1 *G*. The phosphorylated p75 molecule was then examined for phosphoamino acids. The phosphorylated p75 polypeptides were extracted from 2-D gels depicted in Fig. 1 and subjected to the phosphoamino acid analysis (Fig. 2). Phosphoserines

FIGURE 1. Kinetic study of in vivo phosphorylation of the IL-2R p75 in an IL-2-dependent human T cell line, ILT-Mat. ILT-Mat cells were cultured without IL-2 for 14 h, and then resuspended in the phosphate-free medium. [^{32}P]Orthophosphate was added into the culture and the cells were cultured for another 2 h. Subsequently, 2 nM recombinant human IL-2 was added and the cells were further cultured for 0 (A), 1 (B), 5 (C), 15 (D), and 30 min (E and F), and then lysed. The cell lysates were immunoprecipitated with TU11 mAb (A-E) and X63 mAb (F). ^{125}I surface-labeled ILT-Mat cells were also used. Their lysates were immunoprecipitated with TU11 mAb (G) and X63 mAb (H). The immunoprecipitates were analyzed by 2-D PAGE. Arrows show spots for IL-2R p75. Molecular sizes (mol wt $\times$ 10^{-3}) and isoelectric points (pI) of standards are indicated at the left and the top, respectively.

and phosphothreonines appeared as dominant and minor spots, respectively, but phosphotyrosines were not appreciably detected in the p75 derived from cells unstimulated by IL-2. However, the tyrosine phosphorylation was induced as early as 1 min after IL-2 stimulation and rapidly increased within 5 min of IL-2 stimulation, and the increase tended to continue for at least 15 min. On the other hand, the amounts of phosphoserine and phosphothreonine were slightly increased during the first 5 min of IL-2 stimulation. Subsequently, the threonine phosphorylation increased significantly and reached the maximum level after 15 min of IL-2 stimulation, whereas the serine phosphorylation was marginally increased during at least 30 min of IL-2 stimulation. These results suggest that the tyrosine phosphorylation of the IL-2Rp75 is one of the earliest events induced by the interaction of the receptor with IL-2. Similar IL-2-dependent phosphorylation of tyrosine, serine, and threonine residues of IL-2Rp75 was observed with normal peripheral blood leukocytes stimulated with phytohemagglutinin and IL-2 (data not shown).
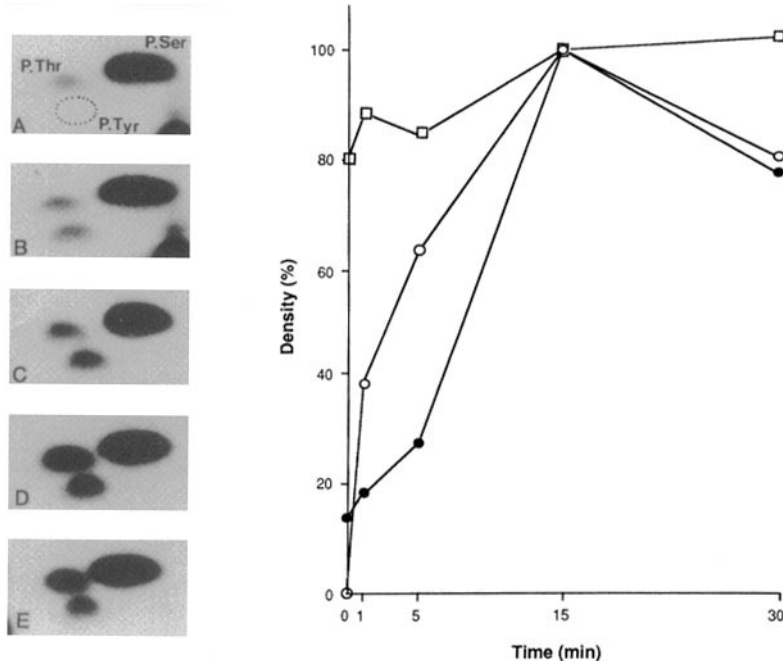
FIGURE 2. Phosphoamino acid analysis of the in vivo phosphorylated IL-2R p75. The spots of the IL-2R p75 in Fig. 1, A-E were excised, and analyzed for phosphoamino acids. A-E of the left-hand panel correspond to A-E of Fig. 1, respectively. The positions of phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) are as indicated. The amount of phosphotyrosine (O), phosphoserine (□), and phosphothreonine (●) was quantitated with a densitometer (Video densitometer, Bio-Rad Laboratories) and is shown in the right-hand panel. 100% indicates the densities of the spots at 15 min after IL-2 stimulation.

Next, the possibility that the IL-2-induced tyrosine phosphorylation of IL-2Rp75 is mediated by the high affinity IL-2R was tested by experiments using various concentrations of IL-2. ILT-Mat cells incubated without IL-2 for 14 h were transferred to the culture medium containing [^{32}P]orthophosphate. After incubation for 2 h, IL-2 at indicated concentrations was added to the culture. After another 5-min incubation, cell lysates were immunoprecipitated with TU11 mAb and analyzed by 2-D PAGE, and the phosphorylated p75 polypeptides were examined for phosphoamino acids (Fig. 3). An increase in the tyrosine phosphorylation of p75 was observed with IL-2 at a concentration of 15 pM, and it reached a plateau level at 250 pM and 4 nM IL-2, whereas the serine and threonine phosphorylation was nearly independent of IL-2 concentration. 15 pM IL-2 that commenced the enhancement of the tyrosine phosphorylation of p75 corresponds to the dissociation constant (K_d = 5–50 pM) of the high affinity receptor to IL-2, and the enhancement appears to be proportional to the amount of the high affinity receptors occupied by IL-2. Furthermore, the IL-2 dependency of the tyrosine phosphorylation exactly correlated with the IL-2-induced [^{3}H]TdR incorporation of cells (Fig. 4). These observations indicate that IL-2 specifically induces the tyrosine phosphorylation of p75 through the high affinity IL-2R as well as signals for the cell growth.
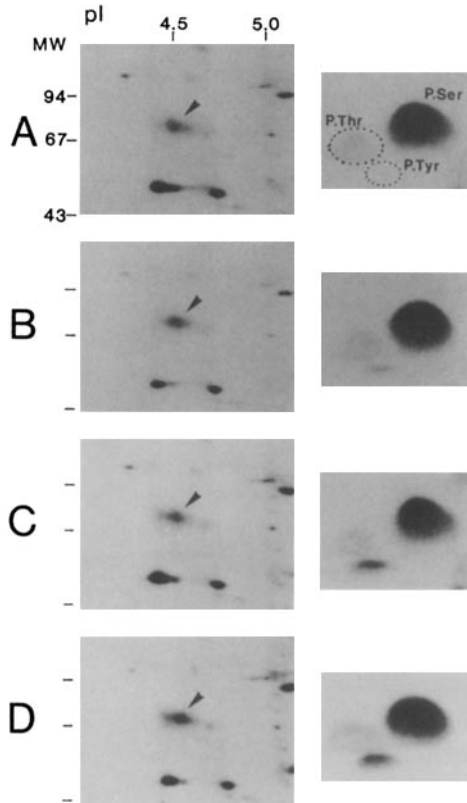
FIGURE 3. IL-2 dose-responses of in vivo phosphorylation of IL-2Rp75. ILT-Mat cells cultured without IL-2 for 14 h were labeled with [^{32}P]-orthophosphate, whereupon IL-2 was added directly to the medium at concentrations of 0 M (*A*), 15 pM (*B*), 250 pM (*C*), and 4 nM (*D*), and then the cells were incubated for 5 min and lysed. Cell lysates were immunoprecipitated with TU11 mAb, and resolved on 2-D PAGE (*left-hand panels*). Arrows show spots for IL-2Rp75. The spots indicated by arrows were excised and analyzed for phosphoamino acids (*right-hand panels*). The positions of the phosphoamino acid marker are indicated.
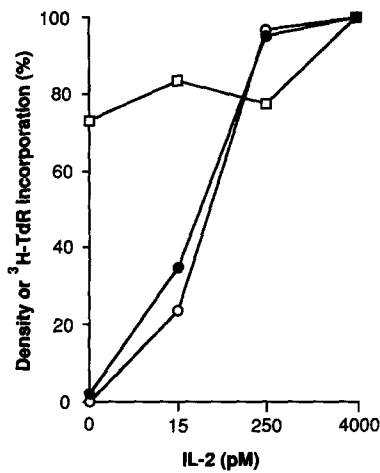
FIGURE 4. Correlation between the IL-2-induced tyrosine phosphorylation of IL-2Rp75 and [^{3}H]TdR incorporation of ILT-Mat cells. The spots of phosphotyrosine (O) and phosphoserine (□) in Fig. 3 were quantitated with a densitometer. The [^{3}H]TdR incorporation assay with ILT-Mat cells was performed in the presence of IL-2 at the indicated concentrations as described previously (14). The incorporated [^{3}H]TdR was measured and represented in percentages (●). 100% indicates the maximum densities of the spots and the maximum level of the [^{3}H]TdR incorporation, both of which were observed at 4 nM IL-2.

Discussion

The present study clearly demonstrated that IL-2 rapidly stimulates the phosphorylation of IL-2Rp75 on tyrosine residues in IL-2-dependent T cells. We also found that the IL-2-induced tyrosine phosphorylation of p75 was mediated by the high affinity IL-2R, correlating with the IL-2-induced cell growth. Therefore, the present direct demonstration of tyrosine phosphorylation of the IL-2Rp75 is of great importance in the understanding of IL-2-induced signal transduction.

The correlation between tyrosine phosphorylation of the IL-2Rp75 and cell growth signaling was reminiscent of tyrosine phosphorylation of other growth factor receptors such as the epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, insulin receptor, insulin-like growth factor 1 (IGF-1) receptor, and colony-stimulating factor 1 (CSF-1) receptor (reviewed in reference 9). In these cases, the cytoplasmic portion of the receptor molecules has a tyrosine kinase domain, and their tyrosine phosphorylation, which is associated with cell growth, was directed by the receptors themselves. We have previously detected at least three classes of phosphotyrosine proteins with molecular masses of 70, 64, and 45 kD that were detectable with antiphosphotyrosine antibody and anti-IL-2Rp55 mAb, suggesting that these phosphoproteins could associate with IL-2Rp55, although the relationship between the proteins and IL-2Rp75 has not been clarified yet (10). A recent report using antiphosphotyrosine antibody further suggested that IL-2 induces tyrosine phosphorylation of some cellular proteins; however, whether they include IL-2R components is unknown (11). In the present study, the tyrosine phosphorylation of IL-2Rp75 was induced as early as 1 min after IL-2 stimulation, suggesting that IL-2R associates with tyrosine kinase activity that can be stimulated by the interaction between IL-2 and its receptor.

The IL-2Rp55 is known to have no kinase domain in its cytoplasmic portion (16, 17), and very recently the complete nucleotide sequence of human IL-2Rp75 was reported, which indicated that the IL-2Rp75 does not contain typical consensus residues (Gly X Gly X X Gly) of protein kinases, but contains intracytoplasmic six tyrosine residues and triplet residues (Ala-Pro-Glu) that have been implicated in the consensus motif for a catalytic domain of protein kinases including tyrosine kinases (18). Accordingly, it seems too early to rule out the possibility that the tyrosine phosphorylation of p75 is directed by p75 itself. Alternatively, a molecule other than p55 and p75, associated with the high affinity IL-2R, may catalyze tyrosine phosphorylation of the IL-2Rp75. We have also detected the IL-2-induced phosphorylation on several cellular proteins, and only phosphoserines were observed in these phosphoproteins (14). Here, the phosphorylation on serine/threonine residues of IL-2Rp75 was more abundantly detected than that on tyrosine residues; however, the rapid response to IL-2 stimulation was observed only in the tyrosine phosphorylation of IL-2Rp75. These observations indicate that certain tyrosine kinases should be activated by IL-2 stimulation more directly than certain serine/threonine kinases.

The ^{125}I-IL-2 chemical crosslinking study has shown that the IL-2Rp75 is detected as two distinct molecules bound with ^{125}I-IL-2, which appeared to be p75 and p70, in cells expressing the high affinity IL-2R or the intermediate affinity IL-2R (4, 5, 19). We have also confirmed the doublet form of IL-2Rp75 by using TU11 mAb (13). It is possible that an associated molecule, for example p70, with the high affinity IL-2R catalyzes the tyrosine phosphorylation of IL-2Rp75. On the other

hand, since T cell–specific molecules CD4 and CD8 are known to associate with p56[lck], a membrane-associated tyrosine kinase protein (reviewed in reference 20), it may be well that the high affinity IL-2R is associated with such a tyrosine kinase molecule in the cytoplasmic membrane. Our present results provide the first demonstration of an association of IL-2Rp75 with tyrosine kinase activity. The nature of the tyrosine kinase molecule is presently under investigation.

Summary

We have recently established a mAb named TU11 mAb specific for the p75 subunit of human IL-2 receptor (IL-2R). The present study using TU11 mAb demonstrates the IL-2-induced phosphorylation of IL-2Rp75 on tyrosine residues in IL-2-dependent T cells. The tyrosine phosphorylation is mediated by the high affinity IL-2R, correlates with the IL-2-induced cell growth, and rapidly increases during the first 5 min of IL-2 stimulation. Phosphorylation of serine and threonine residues of IL-2Rp75 is also detected, but its IL-2 dependency is not significant during at least the first 5 min. These results suggest some roles of a tyrosine kinase associated with IL-2Rp75 in the IL-2-induced signal-transducing pathway.

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