Interleukin-4 (IL-4) Induces Protein Tyrosine Phosphorylation of the IL-4 Receptor and Association of Phosphatidylinositol 3-Kinase to the IL-4 Receptor in a Mouse T Cell Line, HT2*

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To study the signal transduction mechanism of interleukin-4 (IL-4), we have examined the effects of IL-4 on protein tyrosine phosphorylation in a mouse IL-2-dependent T cell line, HT2. Mouse IL-4 induces HT2 proliferation in a dose-dependent manner. Western blotting analyses using anti-phosphotyrosine antibody showed that IL-4 induces tyrosine phosphorylation of four proteins (140, 110, 100, and 92 kDa) in a dose-dependent manner. Protein tyrosine phosphorylation was detected within 1 min and reached a plateau approximately at 10 min after IL-4 stimulation.

Immunoprecipitation using anti-IL-4 receptor antibody revealed that the 140-kDa tyrosine-phosphorylated protein is the IL-4 receptor (IL-4R) itself. Furthermore, we demonstrate that phosphatidylinositol 3-kinase (PI 3-kinase) activity in immunoprecipitates with anti-IL-4R antibody increases after IL-4 stimulation. These data indicate that IL-4 induces activation of tyrosine kinase and also induces association between IL-4R and PI 3-kinase.

Interleukin-4 (IL-4) is a T cell-derived lymphokine which was originally designated as the B cell co-stimulatory factor with anti-IgM antibody (1). A large number of studies have revealed that IL-4 exerts the variety of biological effects not only on B cells but also on many hematopoietic cells, including T cells, mast cells, macrophages, and hematopoietic progenitors (for review, see Refs. 2 and 3). It has been demonstrated that IL-4 also acts on nonhematopoietic cells (4, 5). IL-4 exerts its biological effects through a specific high-affinity IL-4 receptor (IL-4R) on the cell surface (6-11). Recently, cDNA clones encoding human and mouse IL-4Rs have been isolated (12-15), and both human and mouse IL-4Rs were found to be members of the cytokine receptor family. The molecularly cloned human IL-4R was able to transduce the growth signal when human IL-4Rs were expressed on mouse T cells (15), indicating that the molecularly cloned human IL-4R is functionally active. IL-4Rs have a long cytoplasmic domain which is essential to transduce the IL-4 signal. However, there are no known catalytic motifs in the cytoplasmic domain of IL-4R or in other cytokine receptors. We have further identified the critical region for signal transduction in the cytoplasmic domain of human IL-4R (16), confirming that the cytoplasmic domain of IL-4R is essential for IL-4 signal transduction.

However, in spite of these structural studies, the details of the signal transduction mechanism of IL-4 remain unknown. Although biochemical mechanisms such as involvement of protein kinase C or Ca2+ influx in signal transduction of IL-4 have been reported by several groups, results have not been consistent, but have depended on materials used in each experiment (17-20).

The initial response of polypeptide growth factor receptors such as epidermal growth factor receptor (EGF-R) or platelet-derived growth factor receptors (PDGF-R) is activation of an intrinsic tyrosine kinase in their receptors. This activation is the essential reaction to transduce the growth signal through these receptors (for review, see Ref. 21). Like these growth factors, many cytokines such as IL-2, IL-3, IL-5, IL-6, IL-7, erythropoietin, G-CSF, and GM-CSF are known to induce protein tyrosine phosphorylation upon ligand stimulation (for review, see Ref. 22), although their receptors have no tyrosine kinase domain in receptor molecules. In particular, it has been demonstrated that IL-2 induces physical association between IL-2 receptor (IL-2R) β-chains and the cytokine kinases, lck (23, 24) and lyn (25), and activates these tyrosine kinases. Furthermore, IL-2 has been shown to induce association of phosphatidylinositol 3 kinase (PI 3-kinase) to IL-2R β-chains (26, 27) and dissociation of active ras-1 serine/threonine kinase from IL-2R β-chains (28). Thus, it is likely that tyrosine kinases may play an important role in signal transduction in the cytokine receptor system.

In the case of IL-4, it has been demonstrated that IL-4 induces protein phosphorylation in 44-42-kDa protein in the cell membrane fraction prepared from mouse B cells (17, 29), although phosphorylated amino acids have not been determined in those reports. IL-4 has also been shown to induce protein tyrosine phosphorylation in several proteins (30-32). However, these tyrosine phosphorylated proteins have not been characterized yet. In the present study, we have investigated the protein tyrosine phosphorylation induced by mouse IL-4 in an IL-4-responsive mouse T cell line to characterize tyrosine-phosphorylated proteins. We demonstrate here that IL-4 induces tyrosine phosphorylation in IL-4R itself and increases association of PI 3-kinase to IL-4R.

MATERIALS AND METHODS

Antibodies—Monoclonal anti-phosphotyrosine antibodies PY20 and PY99 were obtained from ICN. Monoclonal anti-phosphotyrosine antibody 4G10 was obtained from UBI. Polyclonal rabbit anti-phos-
phototyrosine antibody was purchased from Zymed. Monoclonal mouse IL-4R antibody was supplied from Genzyme.

Cell Culture—Mouse IL-2-dependent cell line HT2 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml of streptomycin, 100 units/ml of penicillin, 5 × 10⁻⁵ M 2-mercaptoethanol, and recombinant mouse IL-2 (provided by Dr. Gerard Zuwari of DNAX Research Institute) as described previously (33).

Cell Proliferation Assay—HT2 cells were washed twice with factor-depleted medium and were incubated with appropriate amounts of IL-4 for 32 h. Tritium thymidine (1 μCi/well) was added, and cells were further incubated for 4 h. Thymidine incorporation was measured with a Beckman liquid scintillation counter.

Western Blotting By Anti-phototyrosine Antibody—Cells were washed with RPMI 1640 medium containing 0.075% bovine serum albumin and 5 × 10⁻⁵ M 2-mercaptoethanol (starvation medium), suspended in starvation medium at 1 × 10⁶ cells/ml, and incubated for 6 h at 37°C. After incubation, cells were collected, resuspended in starvation medium containing 50 μM sodium orthovanadate at 4 × 10⁶ cells/ml, and further incubated for 30 min at 37°C. Aliquots of 2.5 × 10⁶ cells were then stimulated with either mouse IL-4 or mouse IL-2 for an appropriate time at 37°C, followed by centrifugation for 5,000 × rpm for 2 min with a microcentrifuge. Cells were lysed for 30 min on ice by adding 25 μl of lysis buffer containing 50 mM HEPES buffer (pH 7.4), 1% Triton X-100, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, and 0.1% aprotinin. Cell lysates were cleared by centrifugation for 15 min at 15,000 × rpm with a microcentrifuge and subjected to SDS-PAGE (4-12% linear gradient gel, NOVEX). After proteins were electrophoretically transferred to nitrocellulose filters (Hybond-ECL, Amersham Corp.), the filters were blocked with 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 3% BSA, and 0.05% NaN₃ overnight at room temperature. Tyrosine-phosphorylated proteins were probed with polyclonal rabbit anti-phototyrosine antibody (1:1000) (Zymed) or several different monoclonal mouse anti-phototyrosine antibodies and visualized by an ECL system (Amersham Corp.).

Immunoprecipitation—Cell lysates were prepared as described above. The proteins were immunoprecipitated from clarified cell lysates by addition of 30 μl of protein G-Sepharose which was pre-conjugated with 5 μg of monoclonal anti-mouse IL-4R antibody and incubated overnight at 4°C. Protein G-Sepharose was washed four times with 1 ml of lysis buffer, once with 1 ml of lysis buffer without 1% Triton X-100, and boiled for 10 min with SDS-PAGE sample buffer. Eluted materials were applied to SDS-PAGE and transferred electrically to nitrocellulose filters (Hybond-ECL, Amersham Corp.). the filters were blocked with 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 3% BSA, and 0.05% NaN₃ overnight at room temperature. Tyrosine-phosphorylated proteins were probed with polyclonal rabbit anti-phototyrosine antibody (1:1000) (Zymed) or several different monoclonal mouse anti-phototyrosine antibodies and visualized by an ECL system (Amersham Corp.).

P1 3-Kinase Assay in Immunoprecipitates with Anti-IL-4R Antibody—Cells were lysed before or after IL-4 stimulation by the lysis buffer described above, in which 0.5% digitonin was substituted for 1% Triton X-100 and 20 mM MgCl₂ was added. In this immunoprecipitation experiment, protein G-Sepharose was pre-conjugated with 5 μg of anti-IL-4R antibody in the presence of 10 μg of rabbit anti- rat IgG+M(H+L) (Zymed) antibody to enhance the capacity of protein G-Sepharose for immunoprecipitation. Immunoprecipitates with anti-mouse IL-4R antibody on protein G-Sepharose were washed twice with lysis buffer, washed twice with 10 mM Tris-HCl (pH 7.5) containing 20 mM MgCl₂ and 0.5 M LiCl, and finally washed twice with 100 mM LiCl (pH 7.5) containing 20 mM MgCl₂ and 100 mM NaCl. P1 3-kinase activity in immunoprecipitates with anti-IL-4R antibody was assayed as described previously (34), with slight modifications. In brief, 10 μg of sonicated P1 (1 mg/ml in 20 mM HEPES buffer (pH 7.5)) was added to immunoprecipitates, and the mixture was incubated for 10 min at 4°C. The reaction was started by adding 40 μl of reaction mixture containing 20 mM HEPES buffer (pH 7.5), 30 mM MgCl₂, 200 μM ATP, 200 μM adenosine, 100 μCi of [γ-³²P] ATP. The reaction was carried out at 25°C for 15 min and terminated by adding 100 μl of 1 M HCl. The reaction mixture was then extracted with 200 μl of chloroform/methanol (1:1) mixture. Extracts were applied to a thin-layer chromatography (TLC) plate (Merck no. 5735, E. 511 D; E) which were presoaked in 1% oxalic acid for 2 min, dried, and heat-treated at 85°C for 30 min. Products of P1 kinase were developed with CHCl₃/methanol/4 M NH₄OH (9:7:2), and each product was visualized by radioautography.

Results

Protein Tyrosine Phosphorylation Induced by IL-4—To examine IL-4-dependent tyrosine phosphorylation, we have used the IL-2-dependent murine T cell line HT2. As reported previously (33), mouse IL-4 induced the proliferation of HT2 cells in a dose-dependent manner (Fig. 1).

We have investigated protein tyrosine phosphorylation in this cell line upon stimulation of IL-4. In order to demonstrate protein tyrosine phosphorylation induced by IL-4, cells were deprived of IL-2, and after stimulation with IL-4, cells were lysed and cell lysates were subjected to Western blotting using several different anti-phototyrosine antibodies, including PY20, PY69, 4G10, and polyclonal rabbit anti-phototyrosine antibody. Among monoclonal antibodies, 4G10 gave the most significant signals. However, polyclonal anti-phototyrosine antibody gave the more significant signals than 4G10 in Western blotting. As shown in Fig. 2A, polyclonal anti-phototyrosine antibody recognized tyrosine phosphorylation of four proteins of 140, 110, 100, and 92 kDa after IL-4 stimulation, whereas 4G10 did not recognize tyrosine phosphorylation of 100 kDa. Although tyrosine phosphorylation of the 92-kDa protein was very weak compared with other proteins, it was detected reproducibly in many experiments.

In some experiments, we detected tyrosine phosphorylation of 125-kDa protein as shown in Fig. 2A. However, we were not able to detect tyrosine phosphorylation of 125-kDa protein reproducibly. This tyrosine-phosphorylated protein may be very sensitive to phosphatase or proteinase. Tyrosine phosphorylation of these proteins induced by IL-4 was observed in a dose-dependent manner (Fig. 2B). Weak tyrosine phosphorylation in these proteins was detected at an IL-4 concentration of 0.1 ng/ml of IL-4 and reached a plateau at 10 ng/ml IL-4. The dose response of protein tyrosine phosphorylation by IL-4 was similar to that of proliferation by IL-4, suggesting that the tyrosine phosphorylation event may correlate with IL-4-dependent growth signal transduction.

In contrast to tyrosine phosphorylation by IL-4, IL-2 induced a different pattern of tyrosine phosphorylation in HT2 cells from that induced by IL-4 (Fig. 2B). Four tyrosine-phosphorylated proteins (115, 95, 85, and 54 kDa) were detected upon stimulation of IL-2. This result suggested that signal transduction pathway of IL-4 is different from that of IL-2 in HT2 cells.

Fig. 1. Proliferation of HT-2 by IL-4. HT2 cells were washed twice with factor-depleted medium, and cells (3,500 cells/well) were stimulated with the indicated concentrations of IL-4. [3H]Thymidine was added during the last 4 h of a 36-h culture. One representative experiment out of three.
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**FIG. 2.** Protein tyrosine phosphorylation induced by IL-4 and IL-2 in HT2 cells. **A,** factor-deprived cells ($2 \times 10^6$ cells) were stimulated with 10 ng/ml of IL-4 for 10 min. Cells were then lysed, and lysates were resolved by SDS-PAGE. Proteins were electrically transferred to nitrocellulose filters and probed with either polyclonal rabbit anti-phosphotyrosine antibody or 4G10 (1:1000 dilution). Tyrosine-phosphorylated proteins were visualized with the Amersham ECL system as described under “Materials and Methods.” **B,** factor-deprived cells ($2 \times 10^6$ cells) were stimulated with various concentration of IL-4 for 10 min, or factor-deprived cells ($1 \times 10^6$ cells) were stimulated with 10 ng/ml of IL-2 for 10 min. Cell lysates were prepared, and Western blotting analyses were carried out as described under “Materials and Methods.” The mobilities of molecular markers are indicated by **arrows** on the right. The migration positions of tyrosine phosphorylated proteins are indicated by **bars.**

**FIG. 3.** Kinetics of tyrosine phosphorylation induced by IL-4. Factor-deprived cells ($2 \times 10^6$ cells) were stimulated with 10 ng/ml IL-4 for indicated times. Cell lysates were prepared, and Western blotting analyses were carried out as described under “Materials and Methods.” The mobilities of molecular markers are indicated by **arrows.**

Tyrosine phosphorylation of four proteins was detected within 1 min and reached a plateau approximately at 10 min after IL-4 stimulation and gradually decreased thereafter (Fig. 3A). Addition of the excess amount of phosphotyrosine during Western blotting completely blocked detection of any inducible tyrosine phosphorylated proteins (Fig. 3B). However, addition of the excess amount of phosphoserine or phosphothreonine did not block detection of tyrosine phosphorylation by polyclonal anti-phosphotyrosine antibody (data not shown). These results clearly demonstrated that phosphorylated proteins induced by IL-4 are phosphorylated in tyrosine residues.

Protein tyrosine phosphorylation induced by IL-4 was inhibited by addition of monoclonal anti-IL-4 antibody 11B11 which competes with IL-4R for IL-4 binding (35), indicating that ligand binding was required for tyrosine phosphorylation (data not shown).

**Immunoprecipitation of the IL-4 Receptor**—It has been demonstrated that IL-2R ß-chain and erythropoietin receptor are tyrosine phosphorylated upon ligand stimulation (36–39). These reports indicated that ligand activates tyrosine kinase, and the activated tyrosine kinase phosphorylates receptors at tyrosine residues. Therefore, we next investigated whether IL-4R is tyrosine-phosphorylated upon stimulation of IL-4. In order to test this possibility, we immunoprecipitated IL-4R with anti-mouse IL-4R antibody after IL-4 stimulation and analyzed the immunoprecipitates with Western blotting using either anti-IL-4R antibody or anti-phosphotyrosine antibody. As can be seen in Fig. 4, IL-4R was immunoprecipitated with anti-IL-4R antibody from cell lysate and clearly phosphorylated at tyrosine residues upon stimulation of IL-4.

The amount of IL-4R in immunoprecipitates appeared to be constant during 15 min of incubation, and it decreased after a 60-min incubation probably due to internalization and degradation of IL-4R. Tyrosine phosphorylation of IL-4R reached a plateau within 5 min and gradually declined thereafter. Decline of tyrosine phosphorylation of IL-4R may be partly due to dephosphorylation of IL-4R in addition to internalization and degradation of IL-4R, since decline of tyrosine phosphorylation of IL-4R at 60 min was more pronounced than decrease of the amount of IL-4R in several experiments.
activity at 5 min after IL-4 stimulation, as measured by a PhosphorImager (Molecular Dynamics), was approximately 2-fold compared with that at 0 min in this particular experiment. We sometimes detected the maximum level of PI 3-kinase activity in immunoprecipitates with anti-IL-4R antibody at 1 min after IL-4 stimulation in separate experiments. These results suggest that the association of PI 3-kinase to IL-4R is rapid and rather transient.

Without anti-IL-4R antibody during immunoprecipitation, PI 3-kinase activity was not detected, indicating that PI 3-kinase activities detected in immunoprecipitates were not nonspecific binding of PI 3-kinase to protein G-Sepharose. We detected IL-4R-associating PI 3-kinase activity in nonstimulated cells. This result suggested that a small fraction of PI 3-kinase may constitutively associate with IL-4R or that PI 3-kinase may associate with IL-4R nonspecifically under this experimental condition. PI 3-kinase activity in these immunoprecipitates was completely inhibited in the presence of 0.5% Nonidet P-40, demonstrating that PI kinase activity in this assay system is specific for PI 3-kinase as described previously (40) (data not shown). When immunoprecipitates with anti-PI 3-kinase antibody were probed by anti-IL-4R antibody in Western blotting, no significant signal was detected, suggesting that only small fraction of PI 3-kinase associates with IL-4R (data not shown).

**DISCUSSION**

The signal transduction mechanism of IL-4 has not been clearly elucidated. IL-4R belongs to the cytokine receptor family and does not have any known catalytic domain in the receptor molecule. However, in spite of noncatalytic receptors, many cytokines have been demonstrated to induce tyrosine phosphorylation, which is thought to be important for signal transduction (for review, see Ref. 22), suggesting that a tyrosine kinase may be involved in the signal transduction pathway of cytokines. With regard to signal transduction of IL-4, several reports have also indicated that IL-4 induces tyrosine phosphorylation of certain proteins (30–32). However, these tyrosine-phosphorylated proteins have not been characterized. Therefore, we investigated protein tyrosine phosphorylation induced by IL-4 in a IL-4-responsive mouse T cell line, HT2 cells, to clarify the characters of tyrosine-phosphorylated proteins.

By Western blotting using anti-phosphotyrosine antibody, we demonstrated that IL-4 rapidly induces tyrosine phosphorylation of four different proteins. We further demon-
It has been demonstrated that IL-4 induces dephosphorylation of an 80-kDa phosphoprotein from tyrosine residues in human leukemic cell lines (46). We have not detected any significant dephosphorylation in HT2 cells with using Western blotting method. Although we cannot exclude the possibility that Western blotting method may not be sensitive enough to detect dephosphorylation or that the anti-phosphotyrosine antibody we used in this study cannot recognize 80-kDa phosphoprotein. Alternatively, this discrepancy may be explained by the difference in signal transduction pathways between human and mouse, as suggested previously (20).

We have also found that IL-4 induces association of PI 3-kinase to IL-4R. PI 3-kinase is known to be a cytoplasmic signaling protein and physically associates with activated receptors of PDGF, EGF, CSF-1, and insulin (34, 47-49). Although the physiological function of PI 3-kinase is still unknown, several reports using mutants of polyoma middle T antigen (50), the v-src gene (61), and PDGF-R (52) support the importance of PI 3-kinase for signal pathway of proliferation. PI 3-kinase is composed of two subunits, p85, the adapter unit between the catalytic unit and phosphorylated proteins, and p110, the catalytic subunit (53-55). The binding of p85 to PDGF-R is mediated through the SH2 region (SH: Src homology domain) of p85 and specific motifs (Tyr-X-X-Met; YXXM) including phosphorylated tyrosine residue in the cytoplasmic domain of PDGF-R, the polyoma middle T antigen, and related molecules (for review see Ref. 56).

Although IL-4 has no YXXM motif which allow PI 3-kinase to bind IL-4R, the fact that immunoprecipitates with anti-IL-4R antibody contain PI 3-kinase activity indicates that PI 3-kinase physically associates with IL-4R, suggesting the possibilities that PI 3-kinase may associate with IL-4R indirectly, probably through other tyrosine-phosphorylated proteins which have YXXM motifs or that PI 3-kinase may associate with IL-4R directly through alternate binding sites. PI 3-kinase activity in the immunoprecipitates with anti-p85 antibody was constant before and after the IL-4 stimulation, indicating that IL-4 does not cause activation of PI 3-kinase, but causes translocation of PI 3-kinase from cytosol to plasma membrane fraction, where substrates are available.

Our findings raise the possibility that PI 3-kinase may participate in the IL-4-mediated signal transduction pathway in mouse T cells. Currently, characterization of tyrosine kinase activated by IL-4 is in progress.

During preparation of this manuscript, Wang et al. (57) have published a paper in which they demonstrated that IL-4 induces tyrosine phosphorylation of 170-, 135-140-, 100-120-, and 97-kDa proteins in a 3-dependent myeloid pro-genitor cell line FDCP-2. They also demonstrated that the tyrosine-phosphorylated 135-140-kDa protein is IL-4R and that PI 3-kinase associates with both tyrosine-phosphorylated 170-kDa protein and IL-4R (57). With regard to tyrosine phosphorylation of IL-4R and association of PI 3-kinase to IL-4R, their results are consistent with ours. However, we did not detect tyrosine phosphorylation of the 170-kDa protein in HT2 even by using 4G10 which they used in their experiments. This discrepancy suggests that signal transduction pathway of IL-4 may be different in different cell types.

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