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IL-2-dependent in vivo and in vitro tyrosine phosphorylation of IL-2 receptor γ chain

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We previously reported a molecule, p64, which was tentatively named the γ chain, coprecipitable with the β chain of human interleukin-2 receptor (IL-2R). The present study demonstrated that the γ chain, as well as the β chain expressed on IL-2-responsive cells, is phosphorylated on tyrosine residues in an IL-2-dependent manner in vivo and in vitro. The in vivo tyrosine phosphorylation of both chains was similarly induced within 1 min after IL-2 stimulation, and their in vitro tyrosine phosphorylation with the anti-IL-2Rβ antibody-directed immunocomplex was also increased by treatment of cells with IL-2. These results suggest that a tyrosine kinase is associated with the βγ subunit complex, of which activation by IL-2 may result in transduction of intracellular signals.

Interleukin-2 receptor; Tyrosine kinase

1. INTRODUCTION

Interleukin 2 (IL-2) is a well-characterized lymphokine that influences various types of cells, including T, B and NK cells, macrophages and glioma cells, through interaction with its specific receptor, IL-2 receptor (IL-2R), on their surfaces [1]. IL-2R is composed of at least two subunits, the α (p55) and β (p75) chains. IL-2Rβ but not IL-2Rα has been shown to be a molecule responsible for transduction of growth signals when expressed on lymphoid and oligodendroglioma cells [2,3]. Recent studies using IL-2Rα and -β cDNA-transfected fibroblast cell lines have shown that the α and β subunits are not enough to form the high affinity functional IL-2 receptor [4]. Therefore, we anticipated the presence of the other component involved in the IL-2R complex. We have recently identified a novel molecule, p64, designated as the γ chain of IL-2R, associated with IL-2Rβ by immunoprecipitation of IL-2-treated cell lysates with an anti-IL-2Rβ monoclonal antibody [5]. Transfection experiments with IL-2Rβ cDNA demonstrated that the association of IL-2Rγ with IL-2Rβ correlates with the formation of functional IL-2Rβ [6].

IL-2 has been shown to induce tyrosine phosphorylation of cellular proteins [7], and we and others have demonstrated that IL-2Rβ was phosphorylated at tyrosine residues in an IL-2-dependent manner [8–10]. In addition, recent reports showed that $p56^{lck}$, a cytoplasmic tyrosine kinase, is physically associated with IL-2Rβ [11] and activated by IL-2 stimulation [12]. We have, however, detected IL-2-induced tyrosine phosphorylation of IL-2Rβ in some $p56^{lck}$-negative cell lines (Kumaki et al., manuscript submitted for publication). Collectively it was of interest to see whether the γ chain is phosphorylated in response to IL-2 in vivo and in vitro. In the present study we found that IL-2 induced tyrosine phosphorylation of IL-2Rγ in vivo, and that a tyrosine kinase activated by IL-2 treatment was present in an anti-IL-2Rβ antibody-directed immunocomplex from cell lines that contained or lacked $p56^{lck}$.

Abbreviations: IL-2, interleukin-2; IL-2R, interleukin-2 receptor; FCS, fetal calf serum; mAb, monoclonal antibody; 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; PHA, phytohemagglutinin; PBL, peripheral blood leukocyte.

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2. MATERIALS AND METHODS

2.1. *Cells and monoclonal antibody*

MOLTβ-12 is a subline of MOLT-4, a human T-cell line, which was generated by stable transfection with IL-2Rβ cDNA and expresses exogenous IL-2Rβ and endogenous IL-2Rγ molecules, resulting in expression of 6,100 sites/cell of intermediate-affinity IL-2R (K_d = 1.7 nM) [6]. MT-2 is an HTLV-I-transformed human T-cell line. These cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS. TU11 mAb (IgG 1) is specific for human IL-2Rβ and does not interfere with IL-2 binding to IL-2Rβ [13]. PY20 mAb (IgG 2b, ICN) is specific for phosphotyrosine [14].

2.2. *Synthesized oligopeptides*

Two kinds of oligopeptides, X-67 (RRVYFAYDPYGEE) and X-68 (RRLPLNADAYLGL), were synthesized according to the published human IL-2Rβ amino acid sequence [2]. X-67 consists of amino acids from position 354 to 364 and X-68 from 502 and 513 with some modifications, namely that serine or threonine residues were exchanged for glycine or alanine, respectively, to remove the residues acting as acceptor molecules of serine/threonine kinase reaction. One or two arginine residues were added at the N-terminal ends of these oligopeptides as shown with the R-R-SRC peptide [15].

2.3. *In vivo phosphorylation and immunoprecipitation*

MOLTβ-12 cells (1×10^8 cells) were labeled with [^{32}P]orthophosphate (10 mCi/ml, Amersham), treated with IL-2 and immunoprecipitated with TU11 mAb and Protein A–Sepharose CL-4B (Pharmacia) pretreated with rabbit anti-mouse IgG antibody (Zymed), as described previously [16]. The Sepharose beads were washed and subjected to 2D PAGE.

2.4. *Phosphoamino acid analysis*

Gel pieces containing molecules of interest were excised from the 2D gels and subjected to phosphoamino acid analysis as described previously [8]. Phosphoamino acids were quantitated with a Bio-Image analyzer BAS2000 (Fuji Film) [17]. Phosphoamino acid markers were detected by ninhydrin staining.

2.5. *In vitro kinase assay*

MOLTβ-12 cells or MT-2 cells were starved of fetal bovine serum for 18 or 12 h, respectively. Cells (5×10^7 cells/sample) were then stimulated with IL-2 for 10 min at 37°C. Membrane fraction of cells was prepared, lysed with the cell extraction buffer (50 mM TRIS-HCl, pH 7.5, 1% Nonidet P-40, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride and 1% aprotinin) and immunoprecipitated with TU11 mAb. Immunoprecipitates were resuspended in 10 μl of kinase reaction buffer (50 mM HEPES, 10 mM $MnCl_2$, 2 mM DTT, 0.3 mM Na_3VO_4, pH 7.3) with or without exogenous substrates (X-67 or X-68). The kinase reaction was started by addition of 20 μCi of [γ-^{32}P]ATP (> 5,000 Ci/mmol, Amersham) and continued for 5 min at 37°C. The reaction mixture without exogenous substrates was analyzed on 2D PAGE. 1D PAGE of 20% gel was used for the reaction mixture with exogenous substrates.

3. RESULTS AND DISCUSSION

We determined whether phosphorylation of IL-2Rγ is induced by IL-2 in vivo. MOLTβ-12 cells, which have been shown to respond to IL-2 in growth signal and in tyrosine phosphorylation of IL-2Rβ under the FCS starved conditions (Kumaki et al., manuscript submitted for publication), were used for this analysis. Cells were labeled with [^{32}P]orthophosphate for 2 h and then treated with 30 nM IL-2 for the indicated times. Cell lysates were immunoprecipitated with TU11 mAb specific for IL-2Rβ and analyzed by 2D PAGE. Without IL-2 treatment (indicated as 0′ in Fig. 1) TU11 mAb precipitated substantially a single molecule corresponding to the β chain (pI 4.3–4.7; mol. wt. 75 kDa), whereas 1 min after IL-2 stimulation not only the β chain but also an additional molecule (pI 4.1–4.4; mol. wt. 64 kDa), corresponding to the γ chain, were precipitated (Fig. 1, left panels). The densities of both spots increased up to 15 min after stimulation and then reached a plateau.

Phosphorylated IL-2Rβ and IL-2Rγ molecules were excised from the gel and analyzed for phosphoamino acids (Fig. 1, middle and right panels). The IL-2Rβ molecule from the IL-2-untreated cells contained mainly phosphoserine and phosphothreonine, but little, if any, phosphotyrosine. After IL-2 treatment, in addition to phosphoserine and phosphothreonine, phosphotyrosine was clearly seen in phosphorylated IL-2Rβ, as shown with the IL-2-dependent human T-cell line, ILT-Mat [8]. Similarly, phosphoserine and phosphothreonine of IL-2Rβ increased as the incubation period with IL-2 increased up to 30 min. The maximal level of tyrosine phosphorylation of IL-2Rγ was seen 15 min after stimulation, like IL-2Rβ. Similar results were obtained with PHA-stimulated normal human PBL (data not shown).

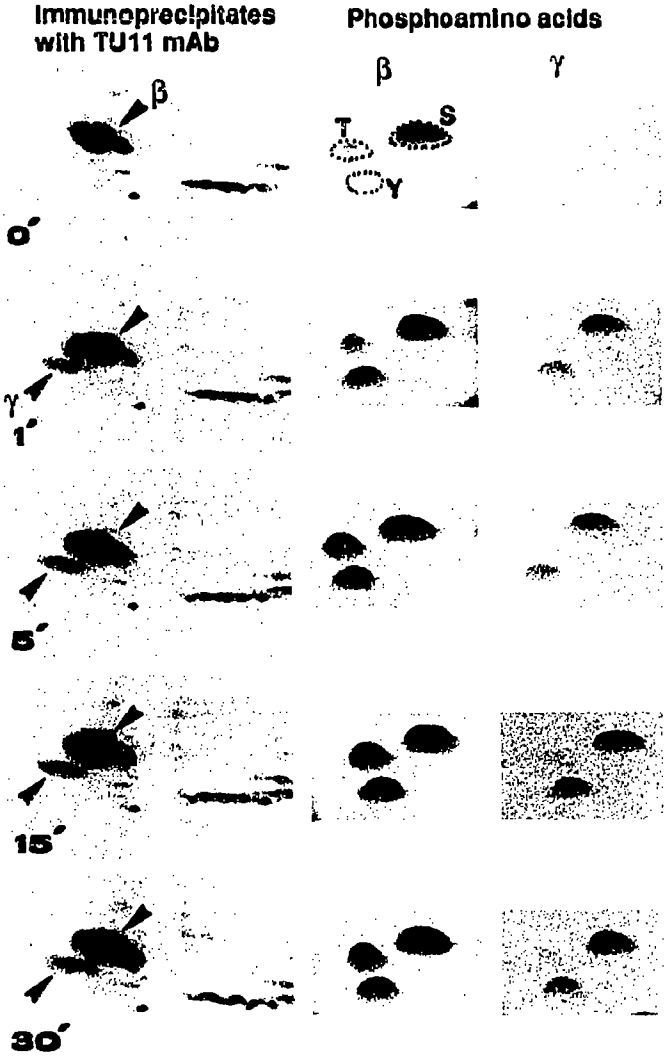

Fig. 1. Kinetics of in vivo phosphorylation of IL-2Rβ and -γ. MOLTβ-12 cells were labeled with [^{32}P]orthophosphate for 2 h and then stimulated with 30 nM IL-2 for the indicated times. Cell lysates were immunoprecipitated with TU11 mAb and subjected to 2D PAGE (left panel). IL-2Rβ and -γ are indicated as arrowheads β and γ, respectively. Spots corresponding to IL-2Rβ and -γ were excised from the gel and analyzed for phosphoamino acids (middle or right panel). The position of phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) are indicated.

It was still unclear, however, whether tyrosine phosphorylation of IL-2Rγ is induced by IL-2 stimulation, because IL-2Rγ is detectable in the co-precipitate with IL-2Rβ only after treatment of cells with IL-2. To ad-

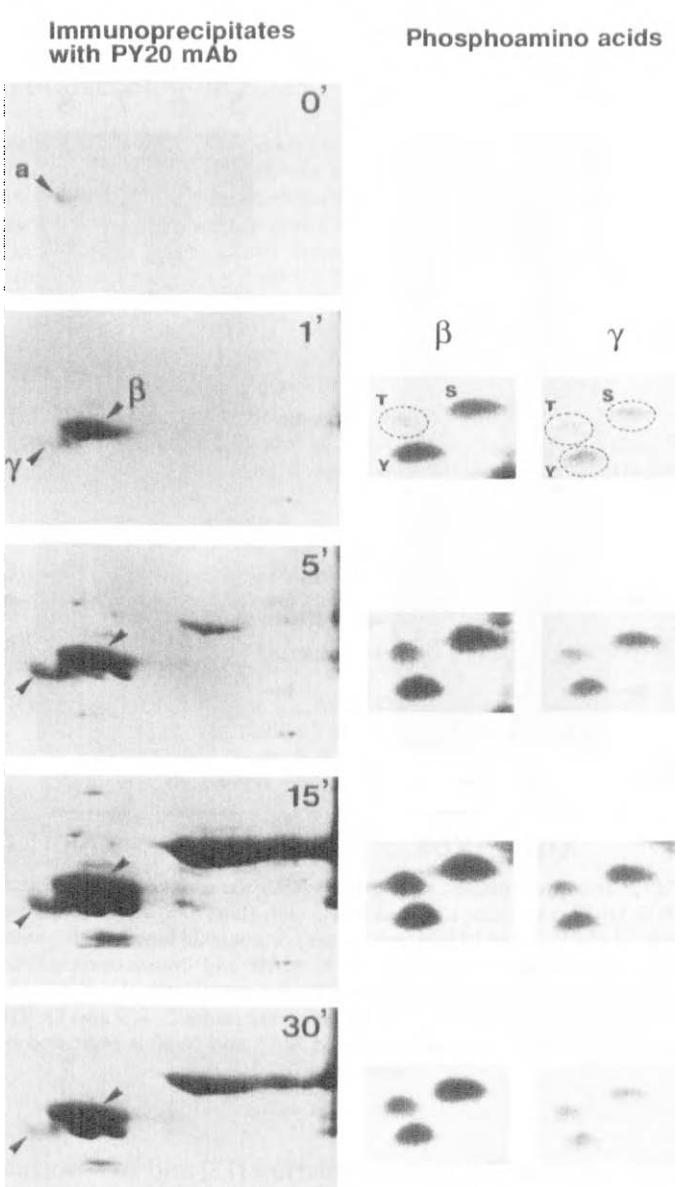

Fig. 2. Kinetics of in vivo tyrosine phosphorylation of cellular proteins. MOLTβ-12 cells were labeled and stimulated with IL-2 as described in the legend to Fig. 1. Cell lysates were immunoprecipitated with PY20 mAb and subjected to 2D PAGE (left panel). Phosphoamino acid analyses of IL-2Rβ and -γ are shown in the middle and right panels, respectively.

dress this question we attempted to detect IL-2Rγ directly with anti-phosphotyrosine antibody, PY20 mAb. Immunoprecipitation with PY20 mAb of the [^{32}P]orthophosphate-labeled, IL-2 stimulated MOLTβ-12 cell lysate detected at least six tyrosine phosphorylated molecules. Among them two molecules were confirmed to be identical with IL-2Rβ and γ, as judged by identity of (i) molecular weights and isoelectric points and (ii) peptide mapping patterns, and the fact that (iii) these two molecules, which precipitated with PY20 mAb, were completely pre-cleared with TU11 mAb (data not shown). The kinetics of the tyrosine phosphorylation of IL-2Rγ

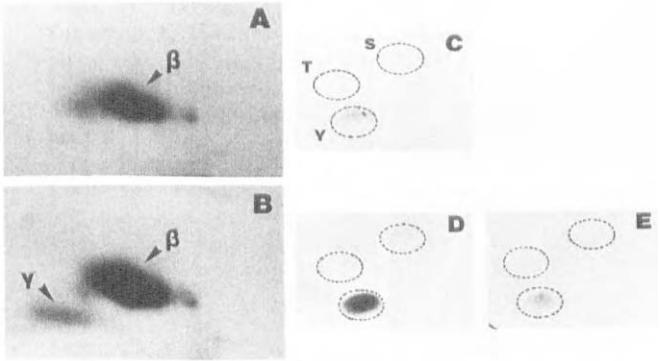

Fig. 3. In vitro phosphorylation of IL-2Rβ and -γ in MOLTβ-12 cells. MOLTβ-12 cells were stimulated with 30 nM IL-2 for 10 min(B) or not (A). Cell lysates were immunoprecipitated with TU11 mAb and the immunoprecipitates were subjected to immunocomplex kinase assay and then analyzed by 2D PAGE. IL-2Rβ and -γ are indicated as arrowheads β and γ, respectively. IL-2Rβ and -γ were excised from the gel and analyzed for phosphoamino acids. C, D and E correspond to β from IL-2-unstimulated cells, β and γ from IL-2-stimulated cells. The position of phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) are indicated.

was examined by using PY20 mAb. ^{32}P-Labeled MOLTβ-12 cells were stimulated by 30 nM IL-2 for the indicated times, and then their lysates were precipitated with PY20 mAb. Before IL-2 stimulation only one spot, which is not identical with either IL-2Rβ or γ, was detected (Fig. 2, arrowhead a). The IL-2Rβ and γ spots became detectable 1 min after stimulation and increased up to 15 min after stimulation (Fig. 2, left panels). The maximal level of tyrosine phosphorylation of IL-2Rγ was seen within 15 min after stimulation, demonstrating that tyrosine phosphorylation of IL-2Rγ was induced by IL-2 treatment, like IL-2Rβ (Fig. 2, middle and right panels). This result was consistent with that obtained with TU11 mAb. The IL-2-induced tyrosine phosphorylation of the other four molecules was detectable within 5 min after stimulation and reached maximal levels at 15 min after stimulation, which appeared later than IL-2Rβ and γ. These results indicated that the IL-2Rγ chain, as well as the β chain, is a substrate in tyrosine phosphorylation which is induced by IL-2 treatment, and suggest that tyrosine and serine/threonine kinases are present in the IL-2/IL-2R complex containing IL-2Rβ and IL-2Rγ.

The tyrosine or serine/threonine kinase activity apparently associated with the IL-2Rβ and γ immunocomplex was analyzed in vitro. Lysates of MOLTβ-12 cells treated or untreated with IL-2 were immunoprecipitated with TU11 mAb. Immunoprecipitates were subjected to a kinase reaction with [γ-^{32}P]ATP and analyzed by 2D PAGE. With the lysate from IL-2-untreated MOLTβ-12 cells, IL-2Rβ but not IL-2Rγ was detected as a phosphorylated protein (Fig. 3A). Treatment with IL-2 not only increased phosphorylation of IL-2Rβ but also made it possible to detect phosphorylation of IL-2Rγ (Fig. 3B). These phosphorylated spots

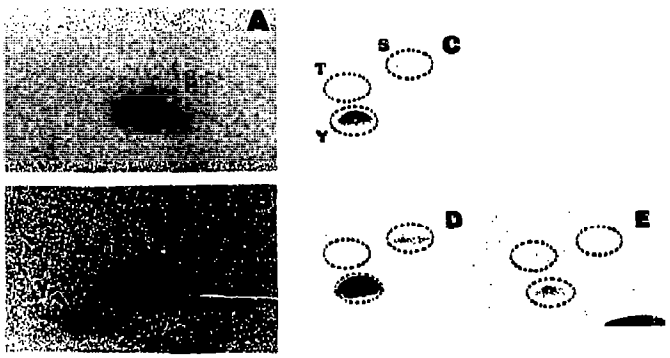

Fig. 4. In vitro phosphorylation of IL-2Rβ and -γ in MT-2 cells. MT-2 cells were used and analyzed as described in the legend to Fig. 3.

of IL-2Rβ and γ were excised from the gels and analyzed for phosphoamino acids. Phosphotyrosine was seen dominantly in both IL-2Rβ and IL-2Rγ in the IL-2-treated cells, and phosphoserine and phosphothreonine were rarely seen (Fig. 3C–E). These results raise the question of whether IL-2 induces activation of the tyrosine kinase or an increase in the amount of the tyrosine kinase physically associated with IL-2R. At present we do not have enough data to answer this question. Very similar results were seen in an in vitro kinase experiment with the cell lysate from MT-2 cells expressing IL-2Rβ endogenously (Fig. 4).

Besides tyrosine phosphorylation of IL-2Rβ and IL-2Rγ, IL-2 also induced serine and threonine phosphorylation of IL-2Rβ in vivo. However, we previously reported that the kinetics of the serine and threonine phosphorylation appeared considerably slower than that of the tyrosine phosphorylation of IL-2Rβ [8]. Taken together with results from our in vitro kinase assay a tyrosine kinase may be tightly associated with IL-2R, and its activation may be a primary effect of IL-2 stimulation followed by activation of serine/threonine kinase(s). Very recently, Fung et al. reported that IL-2Rβ immunocomplex has certain serine/threonine kinase activity in addition to tyrosine kinase activity [18].

IL-2-induced activation of tyrosine kinase in the IL-2Rβ immunocomplex was further examined with exogenous substrates. The kinase reaction was carried out with the immunoprecipitates plus the exogenous oligopeptide substrates which were synthesized according to the human IL-2Rβ amino acid sequence. With the TU11 mAb-directed immunocomplex from IL-2-treated MOLTβ-12 and MT-2 cells significantly elevated tyrosine phosphorylation of the exogenous substrates was detected, whereas little if any phosphorylation was seen with the immunocomplex from IL-2-untreated cells (Fig. 5). Taken together an association with IL-2Rβ of a tyrosine kinase that is activated by interaction of IL-2 with the IL-2R complex seems likely.

A tyrosine kinase molecule, $p56^{lck}$, has been reported

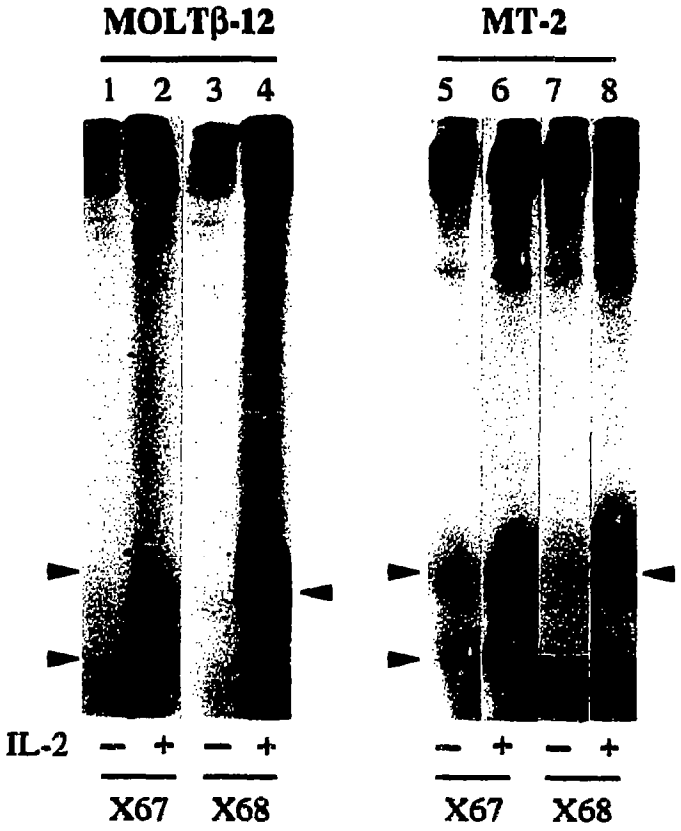

Fig. 5. Immunocomplex kinase assay with the exogenous substrates. MOLTβ-12 cells (lane 1–4) and MT-2 cells (lane 5–8) were stimulated with 30 nM IL-2 for 10 min (even lanes) or not (odd lanes). Cell lysates were immunoprecipitated with TU11 mAb, and immunoprecipitates were subjected to immunocomplex kinase assay with the X-67 substrate (lanes 1, 2, 5 and 6) or X-68 substrate (lanes 3, 4, 7 and 8). The apparent position of phosphorylated X-67 and X-68 is indicated by arrowheads.

to be activated by IL-2 stimulation [12] and to associate with IL-2Rβ [11], suggesting the possibility that $p56^{lck}$ participates in the tyrosine phosphorylation of IL-2Rβ and IL-2Rγ. We found, however, that MT-2 and MT-1 cells, which are negative for $p56^{lck}$ [19], phosphorylate tyrosine residues of IL-2Rβ and IL-2Rγ in response to IL-2 in vivo (our unpublished data). Moreover, IL-2-induced activation of the tyrosine kinase possibly associated with IL-2Rβ was also demonstrated with the MT-2 cell lysates. These results indicate that a tyrosine kinase(s) other than $p56^{lck}$ also participate(s) in phosphorylation of IL-2Rβ and IL-2Rγ, at least in MT-2 cells.

In the present study, we clearly demonstrate that IL-2 induces tyrosine phosphorylation of the IL-2Rβ and γ subunits through activation of the tyrosine kinase which is included in the IL-2 receptor complex. Similar tyrosine kinase activities were reported in the immunocomplex obtained by anti-IL-2Rβ or anti-IL-2 antibodies [18,20]. Our preliminary study suggests that IL-2Rγ itself is not a tyrosine kinase. Therefore, molecular and functional characterization of the tyrosine kinase which

is involved in IL-2-induced signal transduction is essential for better understanding the mechanism of signal transduction from IL-2R.

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