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Ligand-stimulated tyrosine phosphorylation of the IL-2 receptor β chain and receptor-associated proteins

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Interleukin-2 (IL-2) stimulates the rapid phosphorylation on tyrosine of several specific cellular proteins. However, the high-affinity human IL-2 receptor, composed of an α (p55) and β (p70/75) subunit, does not contain a cytoplasmic tyrosine kinase domain. In this study, we investigated the identities of the proteins phosphorylated on tyrosine in response to IL-2 stimulation to examine possible pathways of signal transduction. By the use of immunoblotting with anti-phosphotyrosine antibodies, we demonstrate that IL-2 augments tyrosine phosphorylation of the IL-2 receptor β chain in human cell lines expressing either high-affinity (α/β) receptors or only the β chain. In IL-2-dependent mouse T cell lines, a 100 000-Da protein was phosphorylated on tyrosine in response to IL-2 and is proposed to be the mouse IL-2 receptor β chain. Two other cellular proteins, pp55 and pp105 in human or pp55 and pp115 in mouse cell lines, were phosphorylated on tyrosine in response to IL-2 and coimmunoprecipitated with the high-affinity IL-2 receptor after chemical crosslinking of IL-2-stimulated cells. Thus, the IL-2 receptor may associate with additional subunits or with cellular proteins involved in signal transduction.

Introduction

Activation of T lymphocytes by antigen or mitogen induces de novo transcription of the protooncogenes c-*fos* and c-*myc* and the genes for the transferrin receptor, the growth factor interleukin-2 (IL-2),[1] and the IL-2 receptor (reviewed in Crabtree, 1989). The interaction of IL-2 with its receptor drives the clonal expansion of antigen-reactive T cells and, consequently, determines the magnitude and duration of a T cell response. Three classes of IL-2 receptor, differing in their affinity for binding ligand, have been defined (Robb *et al.*, 1984; Lowenthal and Greene, 1987; Wang and Smith, 1987). The high-affinity receptor ($K_d \sim 10^{-11}$ M) is composed of at least two integral membrane proteins, α (CD25, p55, Tac) and β (p70/75) (Sharon *et al.*, 1986; Tsudo *et al.*, 1986; Dukovich *et al.*, 1987; Teshigawara *et al.*, 1987). Expressed independently, the α and β chains bind IL-2 with low ($K_d \sim 10^{-8}$ M) and intermediate ($K_d \sim 10^{-9}$ M) affinity, respectively. In most proliferating T cells, the high-affinity receptor mediates growth signal transmission, although ~10-fold more α than β subunits are expressed on the cell surface. The binding of IL-2 to the β chain alone, however, is sufficient to initiate receptor internalization (Robb and Greene, 1987) and signal transduction. For example, large granular lymphocytes expressing only β chain respond to IL-2 by increased natural killer activity and proliferation, but at higher concentrations of IL-2 than required for normal T lymphoblast proliferation (Le Thi Bich-Thuy *et al.*, 1987; Siegel *et al.*, 1987; Tsudo *et al.*, 1987a; Hori *et al.*, 1988).

Events leading to signal transduction subsequent to IL-2 binding to the high-affinity receptor complex have not been well defined. Activation of phospholipase C-mediated phosphatidylinositol hydrolysis has been observed in some cell lines (Bonvini *et al.*, 1987) but not in others (Mills *et al.*, 1986; Kozumbo *et al.*, 1987). Calcium mobilization and activation of protein kinase C (PKC), both consequences of phosphatidylinositol turnover, do not appear to be required for IL-2-driven proliferation (Mills *et al.*, 1985a, 1988; Valge *et al.*, 1988). Like other growth factors, IL-2 rapidly induces cytosolic alkalinization by activation of a Na^+/H^+ antiport, but inhibition of these events does not prevent IL-2-dependent proliferation (Mills *et al.*, 1985b).

IL-2 rapidly induces an increase in serine phosphorylation of several cytosolic and mem-

[1] Abbreviations: CSF-1, colony stimulating factor-1; DCS, defined calf serum; DMEM, Dulbecco's minimum essential medium; DSP, dithiobis (succinimidylpropionate); EGF, epidermal growth factor; FBS, fetal bovine serum; GAP, GTPase-activating protein; IL-2, interleukin-2; mAb, monoclonal antibody; 2-ME, 2-mercaptoethanol; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMA, phorbol myristate acetate; Tris, tris(hydroxymethyl)aminomethane.

brane proteins (Ishii *et al.,* 1988) and also leads to tyrosine phosphorylation of a number of proteins (Morla *et al.,* 1988; Saltzman *et al.,* 1988, 1989; Farrar and Ferris, 1989). Thus, IL-2 binding appears to result in the activation of a number of serine/threonine and tyrosine kinases and/or inhibition of phosphatases. Neither the 13-amino acid nor the 286-amino acid cytoplasmic domain of the IL-2 receptor α or β chains, respectively, has sequence similarity to protein tyrosine kinases or other known kinases (Taniguchi *et al.,* 1983; Leonard *et al.,* 1984; Hatakeyama *et al.,* 1989).

In this report, we sought to identify the substrates for IL-2-stimulated tyrosine phosphorylation in mouse and human cell lines and to determine whether any phosphotyrosyl proteins were physically associated with the IL-2 receptor complex. We demonstrate that IL-2 treatment induces the tyrosine phosphorylation of the IL-2 receptor β chain in cell lines expressing either high-affinity receptors or β chain alone and that two other proteins phosphorylated on tyrosine in response to IL-2 are coimmunoprecipitated with the IL-2 receptor.

Results

IL-2 increases tyrosine phosphorylation of cellular proteins

In initial studies, the proteins phosphorylated on tyrosine in response to IL-2 were compared in mouse and human T cell lines. Three cytotoxic mouse T cell clones (CTLL-2, L3AI, and CI 36) and one mouse helper clone (HT2b) were incubated for 15 min with rIL-2, and the total detergent-soluble cell proteins were subjected to electrophoresis and immunoblotted with affinity-purified rabbit anti-phosphotyrosine antibodies. All four mouse cell lines showed an increase in anti-phosphotyrosine staining of proteins of M_r = 55 000, 115 000, and 92 000, and 96 000, which variably appeared as a doublet or as a single band (Figure 1). In the human T cell line HUT 102B2, phosphorylation of proteins of M_r = 105 000, 55 000, and a doublet of 96 000 and 92 000 was augmented. Additional proteins of M_r = 165 000 and 40 000 showed a much smaller but reproducible increase in tyrosine phosphorylation in response to IL-2. The increase in tyrosine phosphorylation stimulated by IL-2 in all cell lines was detected within 2 min, increased up to 30 min, and decreased after 60 min (data not shown). The 55 000-Da phosphoprotein did not comigrate with the α chain of the IL-2 receptor, which migrates as a broad band of $M_r \sim$ 50 000–60 000 and varies in molecular weight between cell lines (Shackelford and Trowbridge, 1984). Furthermore, the α chain migrates faster under nonreducing conditions because of the presence of intramolecular disulfide bonds, whereas the 55 000-Da phosphotyrosyl protein did not (data not shown).

Stimulation of protein tyrosine phosphorylation by IL-2 was inhibited in mouse cell lines by the anti-mouse IL-2 receptor α chain monoclonal antibody (mAb) PC61, which blocks ligand binding (Figure 2, lanes 10–12), but not by the mAb 7D4, which binds to the α chain and does not prevent IL-2 binding (lane 13). Neither antibody acted as an agonist inducing phosphorylation in the absence of IL-2 (lanes 14 and 15). Likewise, the anti-human IL-2 receptor α chain mAb anti-Tac, which blocks the binding of IL-2 to the receptor, inhibited the increase in tyrosine phosphorylation in HUT 102B2 cells, whereas the mAb 7G7/B6, which binds to the α chain but does not prevent ligand binding, did not inhibit tyrosine phosphorylation in response to IL-2 (data not shown). In addition, the anti-human IL-2 receptor β chain mAb Mik-β3 did not inhibit the increase in phosphorylation, consistent with its inability to block IL-2 binding. Preincubation of CTLL-2 cells with 10 mM 2-deoxy-D-glucose and 15 mM Na azide, conditions that inhibit internalization of clustered IgE receptors on rat basophilic leukemia cells (Menon *et al.,* 1986), inhibited the IL-2-induced increase in tyrosine phosphorylation (lanes 4–6). Sodium azide alone did not inhibit the increase in phosphorylation (lane 16).

IL-2 stimulates tyrosine phosphorylation of the human IL-2 receptor β chain

To determine whether any of the proteins phosphorylated on tyrosine in response to IL-2 were directly associated with the human IL-2 receptor, immunoprecipitates were prepared, using mAbs to the α or β subunit, and immunoblotted with anti-phosphotyrosine antibodies. The anti-α chain mAb 7G7/B6 precipitated a 75 000-Da protein from HUT 102B2 cells that showed a sevenfold increase in anti-phosphotyrosine staining in response to IL-2 stimulation (Figure 3). The molecular mass of the 75 000-Da phosphoprotein suggested that it was the β chain of the IL-2 receptor. This was confirmed by its reactivity with the anti-β chain mAbs Mik-β3 (Figure 3) and TU27 (data not shown). The β chain immunoprecipitated by Mik-β3 was phosphorylated on tyrosine in unstimulated HUT

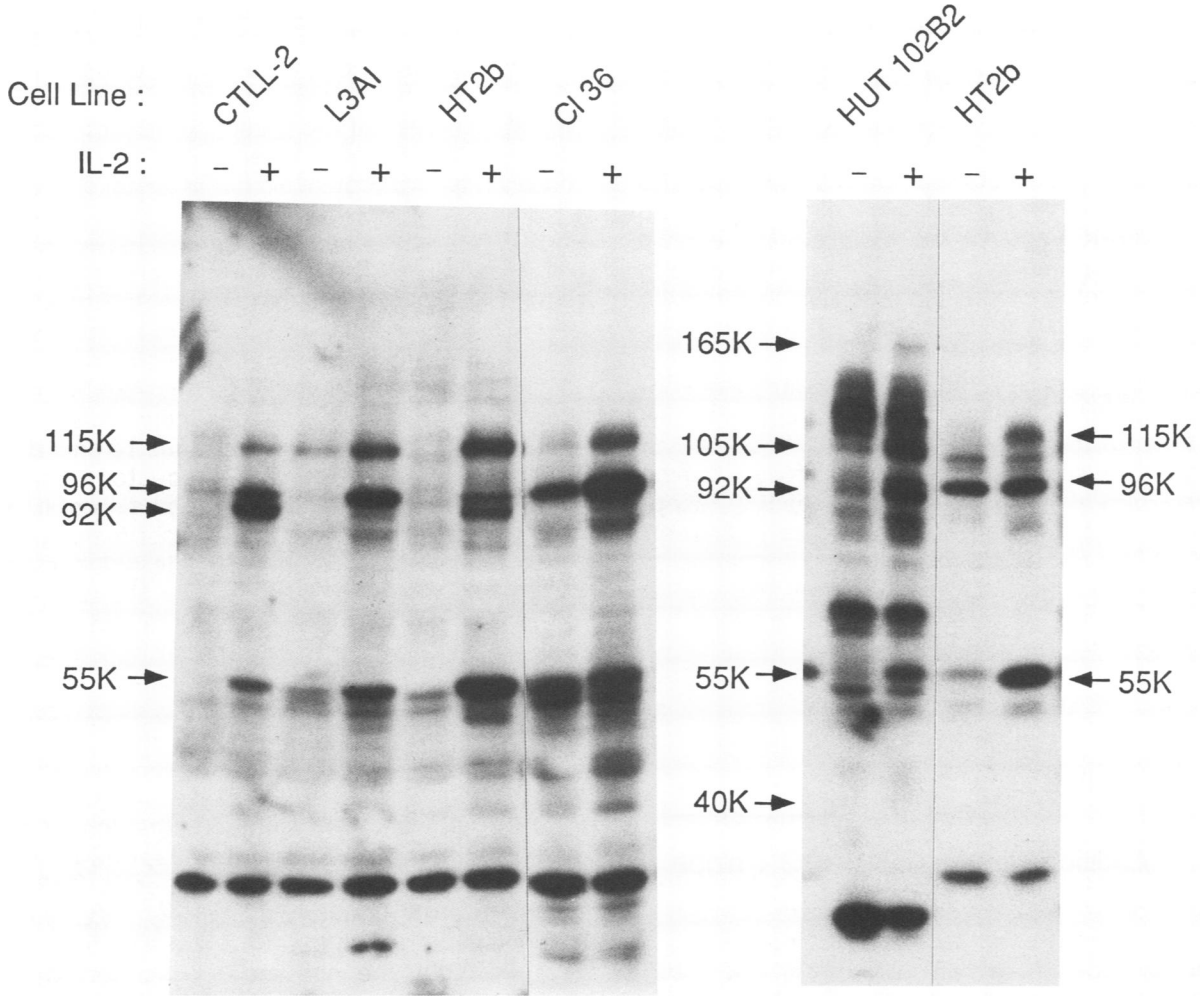

Figure 1. **IL-2 stimulates tyrosine phosphorylation in mouse (CTLL-2, L3AI, HT2b, and Cl 36) and human (HUT 102B2) cell lines.** Cells were incubated with (+) or without (−) 1000 U/ml rIL-2 for 15 min at 37°C, washed, and lysed as described in Methods. The samples (~6 × 10^5 cell equivalents each) were processed for immunoblots and stained with anti-phosphotyrosine antibodies. The arrows indicate the major proteins showing an increase in anti-phosphotyrosine staining in response to IL-2.

102B2 cells, but incubation with IL-2 increased the anti-phosphotyrosine staining two- to threefold. The increase in tyrosine phosphorylation of the β chain was 67% maximal after incubation with 10 U/ml rIL-2 (~35 pM rIL-2) (Figure 4). At this concentration, high-affinity, but not low-affinity, IL-2 receptors should be occupied. The IL-2-induced increase in phosphorylation of the β chain was demonstrated to be a general phenomenon. Increases in tyrosine phosphorylation of two- to sevenfold were observed in three other cell lines that bind IL-2 (Figure 3). The human T cell line MT-2 expresses high-affinity receptors composed of α and β, whereas HUT 78 expresses the β chain and undetectable amounts of α chain (Tsudo *et al.*, 1987b; Saltzman *et al.*, 1989; Takeshita *et al.*, 1989). The gibbon ape cell line MLA-144 produces only the IL-2 receptor β chain (Lowenthal and Greene, 1987; Tsudo *et al.*, 1986). A 55 000-Da phosphotyrosyl protein reproducibly and specifically co-precipitated with the β chains from MLA-144; HUT 78; and, to a lesser extent, HUT 102B2 cells. This protein is distinct from the IL-2 receptor α subunit, because it is found in MLA-144 and HUT 78 cells that do not express α and it did not comigrate with the IL-2 receptor α chain in HUT 102B2 cells. Other phosphotyrosyl proteins observed in the anti-α and anti-β chain immunoprecipitates from HUT

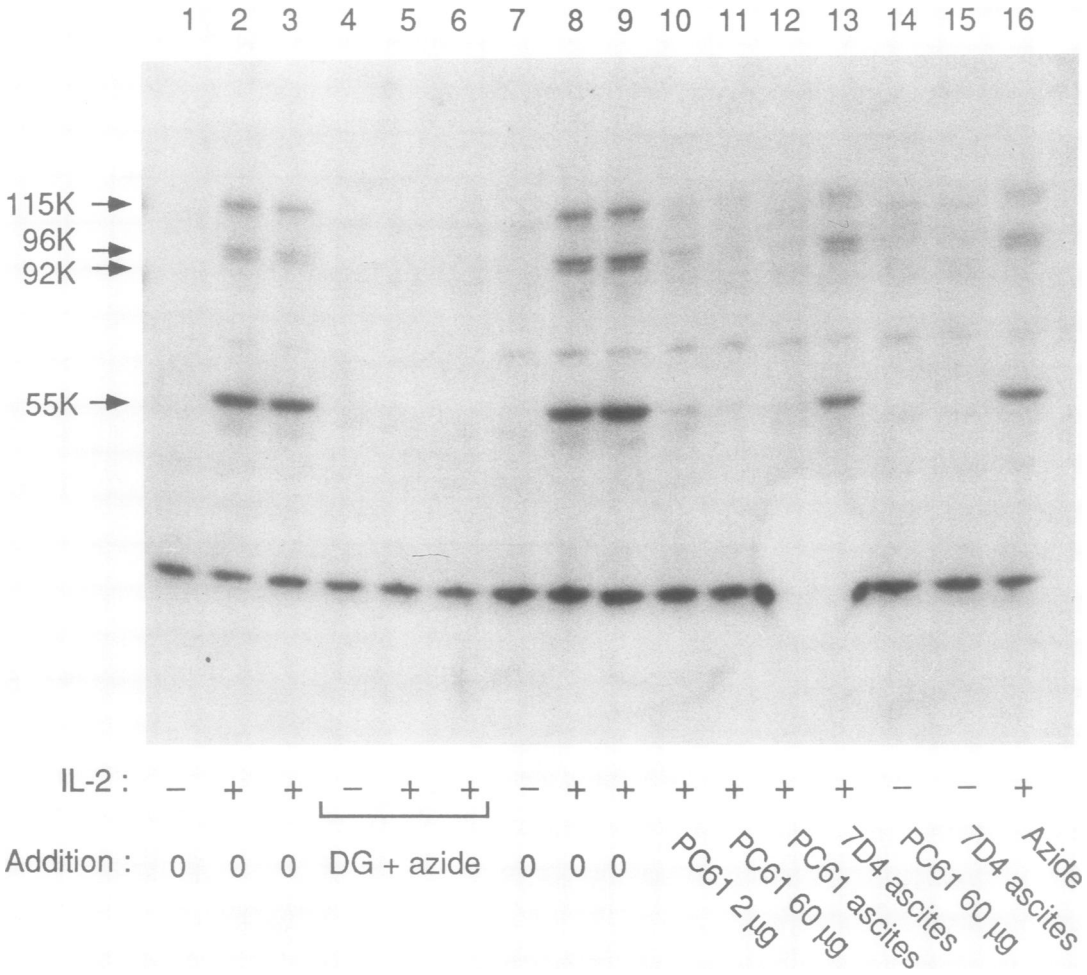

Figure 2. **Inhibition of tyrosine phosphorylation in mouse HT2b cells by anti-IL-2 receptor antibodies.** HT2b cells were preincubated at 37°C with antibody or other addition as indicated in the figure. After 20 min, no rIL-2 (lanes 1, 4, 7, 14, and 15), 10 U/ml (lanes 2, 5, 8, 10–13, and 16), or 100 U/ml (lanes 3, 6, and 9) of rIL-2 was added; the samples were incubated for an additional 15 min at 37°C, centrifuged, and lysed. The samples shown in lanes 1–6 were incubated in DMEM medium lacking glucose to assay the effects of 2-deoxy-D-glucose, whereas those in lanes 7–16 were incubated in RPMI medium. During the 20-min preincubation, the samples in lanes 4–6 received 10 mM 2-deoxy-D-glucose and 15 mM Na azide (DG + azide). The additions to the other samples were 2 µg/ml PC61 (lane 10), 60 µg/ml PC61 (lanes 11 and 14), 1:200 dilution of PC61 ascites (lane 12), 1:200 dilution of 7D4 ascites (lanes 13 and 15), or 15 mM Na azide (lane 16). The samples (~6 × 10^5 cell equivalents each) were processed for immunoblots and stained with anti-phosphotyrosine antibodies.

102B2 cells were not reproducibly observed and precipitated nonspecifically with all antibodies. These included a protein of M_r = 58 000 and the protein indicated by the open arrowhead (compare Figures 3 and 4). The HTLV-1-infected cell line MT-2 had an elevated content of phosphotyrosine-containing proteins compared with the other cell lines. Incubation with IL-2 led to increased phosphorylation of a 92 000-Da protein and a minor increase in phosphorylation of a 105 000-Da protein (Figure 3, lysate). Two of the major constitutively phosphorylated proteins, M_r = 58 000 and 125 000, co-precipitated nonspecifically with both the anti-β chain (Mik-β3) and the control rabbit anti-mouse Ig (RαM) immunoprecipitates.

Identification of two novel phosphotyrosyl proteins crosslinked to the IL-2 receptor

To investigate whether additional proteins might be noncovalently associated with the IL-2 receptor and dissociate after cell lysis in NP-40, cells incubated were with or without IL-2 and then subjected to chemical crosslinking with the cleavable crosslinker dithiobis(succinimidyl-propionate) (DSP). Under these conditions, Mik-

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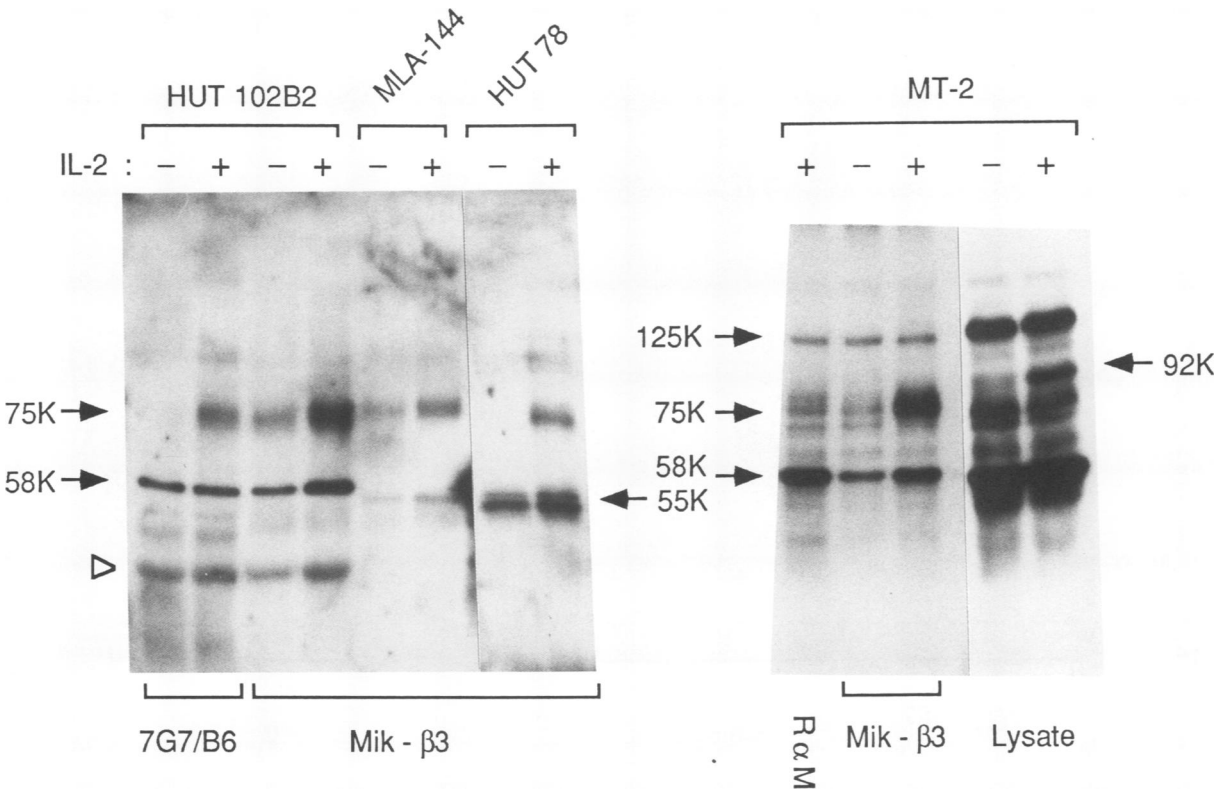

Figure 3. **IL-2 stimulates tyrosine phosphorylation of the IL-2 receptor β chain in different human (HUT102 B2, HUT 78, and MT-2) or ape (MLA-144) cell lines.** Cells were stimulated without (−) or with (+) rIL-2 (1000 U/ml) for 15 min at 37°C, washed, lysed, and immunoprecipitated. The lysates (~1 × 10^7 cell equivalents) were immunoprecipitated with 7G7/B6 adsorbed to protein A-Sepharose or Mik-β3 adsorbed to rabbit anti-mouse Ig coupled to Sepharose or with rabbit anti-mouse IgG coupled to Sepharose alone (RαM) as indicated. Aliquots (6 × 10^5 cell equivalents) of the detergent lysates of MT-2 cells were analyzed directly in the last two lanes. Samples were analyzed by immunoblotting with anti-phosphotyrosine antibodies.

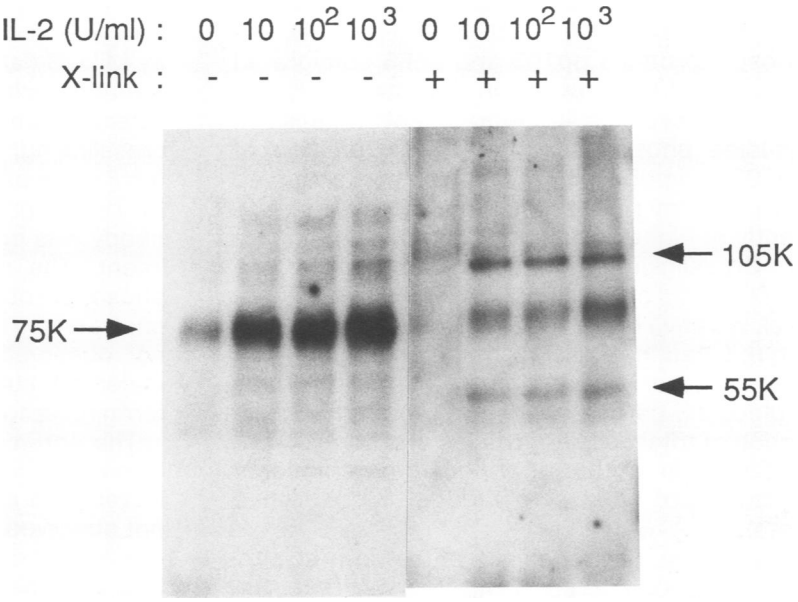

Figure 4. **IL-2 dependence of tyrosine phosphorylation of the IL-2 receptor β chain and associated proteins.** HUT 102B2 cells were incubated for 15 min with the indicated concentration of rIL-2. One-half of each sample was then treated with 0.8 mM DSP for 15 min on ice (X-link). After lysis, each sample (~1 × 10^7 cell equivalents) was immunoprecipitated with Mik-β3 adsorbed to rabbit anti-mouse IgG coupled to Sepharose. Samples were processed for immunoblots and stained with anti-phosphotyrosine antibodies. The autoradiograph of the samples subjected to crosslinking was exposed twice as long as that of the untreated samples.

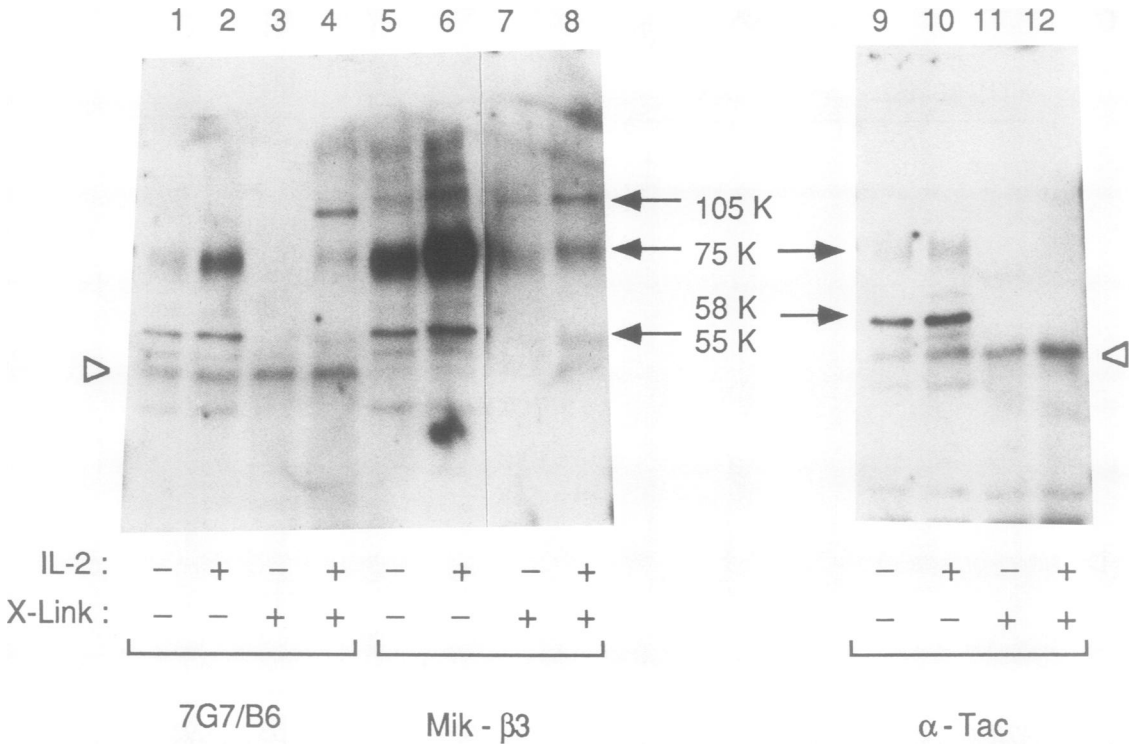

Figure 5. **Anti-phosphotyrosine immunoblot of anti-human IL-2 receptor immunoprecipitates.** HUT 102B2 cells were incubated with (+) or without (−) rIL-2 (1000 U/ml) for 15 min at 37°C. One-half of the samples (lanes 3, 4, 7, 8, 11, and 12) were then incubated with 0.8 mM DSP for 15 min on ice. All samples were lysed and immunoprecipitated from $\sim 1 \times 10^7$ cell equivalents each with the mAb indicated. The major proteins stained with anti-phosphotyrosine are indicated by arrows. The protein indicated by the open arrowhead is due to anti-phosphotyrosine staining of the mAb immunoadsorbent and is observed in the absence of incubation with cell lysate.

β3 immunoprecipitated anti-phosphotyrosine staining proteins of M_r = 105 000 (pp105), 75 000 (pp75, β chain), and 55 000 (pp55) from IL-2-stimulated HUT 102B2 cells (Figure 4). The phosphoproteins pp105 and pp55 comigrated with two of the proteins in the total cell lysate that displayed an IL-2-dependent increase in tyrosine phosphorylation. Co-precipitation of pp105 and pp55 with the β chain was observed with 10 U/ml rIL-2 and did not increase significantly at higher concentrations of rIL-2 (Figure 4). Crosslinking reduced the amount of the 75 000-Da β chain immunoprecipitated. In general, we have observed that chemical crosslinking reduces the ability to immunoprecipitate the IL-2 receptor β chain and other cell surface molecules, partly because of reduced solubilization of total cellular proteins and possibly because of disruption of antigenic determinants (Shackelford and Trowbridge, unpublished data).

To assess the specificity of the co-precipitation of pp105 and pp55 with the IL-2 receptor, immunoprecipitates using anti-α or anti-β chain mAbs were compared. Figure 5 confirms that IL-2 induces an increase in the tyrosine phosphorylation of the β chain immunoprecipitated by Mik-β3 (lanes 5 and 6) or co-precipitated by the anti-α chain mAb 7G7/B6 (lanes 1 and 2). After stimulation of cells with IL-2 and chemical crosslinking, pp105 was co-precipitated with the β chain using 7G7/B6 or Mik-β3 (lanes 4 and 8). The co-precipitation of pp55 with either antibody was only weakly observed in this experiment. The specificity of the crosslinking of phosphoproteins to the IL-2 receptor was demonstrated by the fact that pp105 and pp55 were not observed in immunoprecipitates of other major cell-surface proteins, such as the transferrin receptor or CD45 (T200) (data not shown).

The anti-α chain mAb anti-Tac also precipitated the 75 000-Da β chain, but a significant increase in anti-phosphotyrosine staining was not observed after IL-2 stimulation (lanes 9 and 10). Anti-Tac also did not immunoprecipitate the two tyrosine phosphorylated proteins, pp105

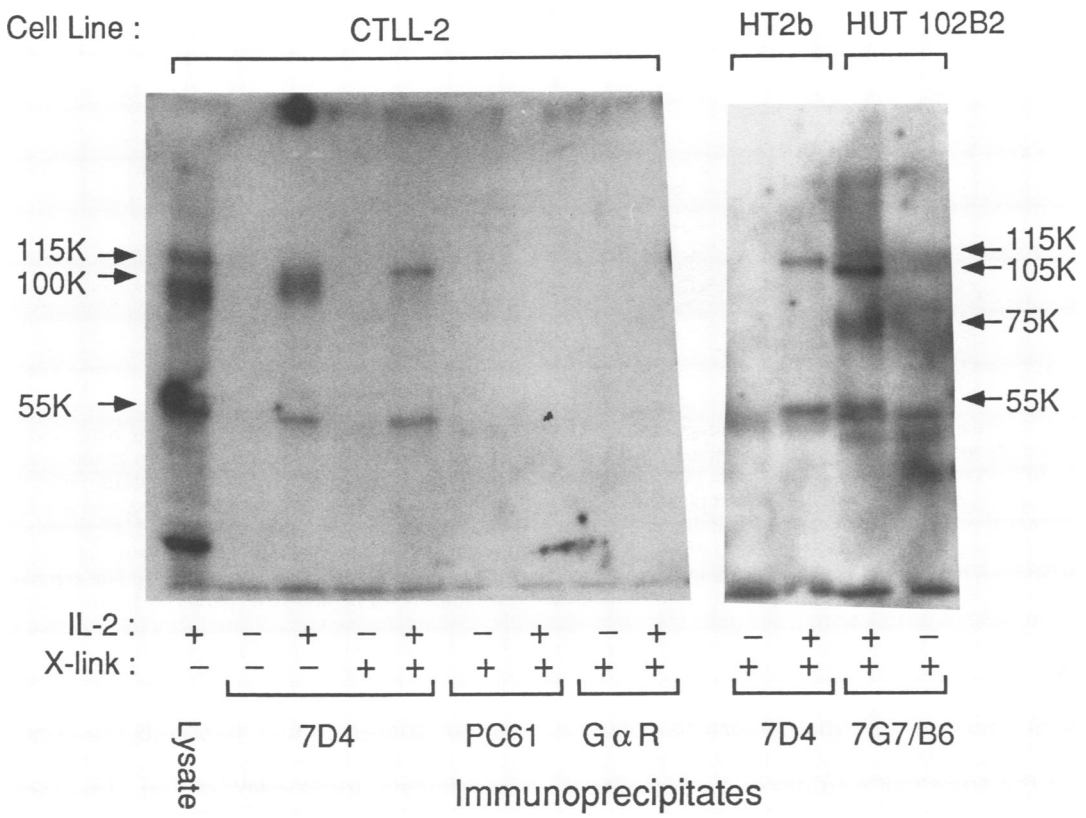

Figure 6. **Tyrosine phosphorylation of proteins associated with the mouse IL-2 receptor.** CTLL-2 cells were incubated with (+) or without (−) rIL-2 (1000 U/ml) for 15 min at 37°C. The samples indicated were subjected to crosslinking (X-link) with 0.8 mM DSP for 15 min on ice. An aliquot (6×10^5 cell equivalents) of the detergent lysate of CTLL-2 cells was analyzed directly in the first lane. Otherwise, the lysates ($\sim 1 \times 10^7$ cell equivalents) were immunoprecipitated with 7D4 or PC61 adsorbed to goat anti-rat Ig coupled to Sepharose or with goat anti-rat IgG coupled to Sepharose alone (GαR). The last four lanes show a direct comparison of the proteins co-precipitated with the IL-2 receptor α chain mAbs 7D4 or 7G7/B6 from mouse (HT2b) or human (HUT 102B2) cell lines, respectively, after stimulation with IL-2 and chemical crosslinking.

and pp55, after IL-2 stimulation and crosslinking (lanes 11 and 12). Anti-Tac competes with IL-2 for the binding site and thus cannot immunoprecipitate efficiently occupied receptors, as demonstrated by the inability of anti-Tac to immunoprecipitate ^{125}I-labeled IL-2 crosslinked to the receptor (Shackelford and Trowbridge, 1986). However, anti-Tac and 7G7/B6 immunoprecipitate indistinguishable amounts of IL-2 receptor α chain from cell-surface-iodinated HUT 102B2 cells even in the presence of IL-2 (data not shown), because there is a large excess of free α chain that binds and dissociates IL-2 rapidly (Robb *et al.,* 1984; Lowenthal and Greene, 1987; Wang and Smith, 1987). Therefore, the increase in anti-phosphotyrosine-staining of pp75 and the co-precipitation of pp105 and pp55 are only observed by the use of an anti-IL-2 receptor antibody that does not block the binding site and that thus can immunoprecipitate occupied receptors.

Comparison of IL-2 receptor-associated proteins in mouse and human cell lines

No mAbs to the presumptive β chain of the mouse IL-2 receptor are currently available. The β chain of the human IL-2 receptor, however, can be co-precipitated with anti-α chain mAbs, as demonstrated in Figures 3 and 5. Therefore, anti-mouse α chain mAbs were used to determine whether IL-2 induced tyrosine phosphorylation of components of the mouse IL-2 receptor in IL-2-dependent T cell lines. The anti-mouse α chain mAb 7D4 co-precipitated two anti-phosphotyrosine-staining proteins of M_r = 100 000 (pp100) and 55 000 (pp55) from IL-2-stimulated CTLL-2 cells (Figure 6). Similar results were obtained with the cell line HT2b (data

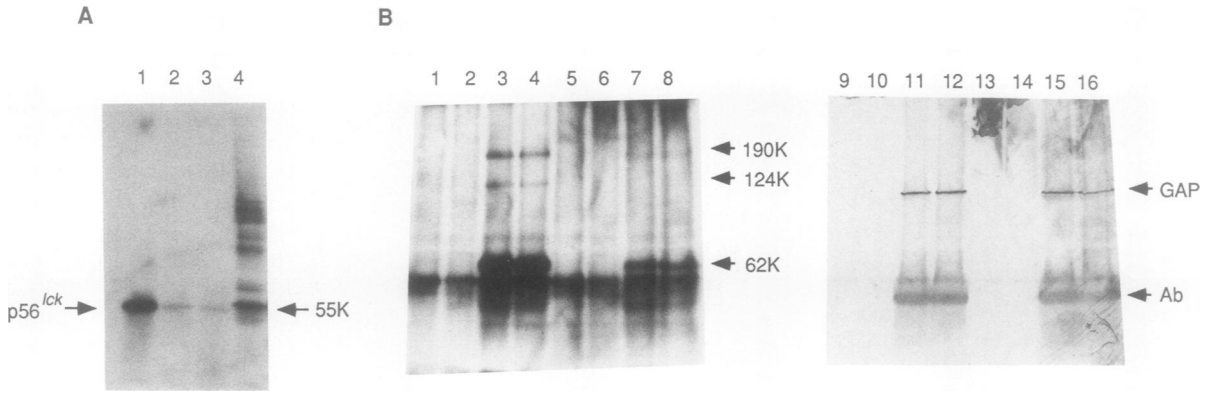

Figure 7. **IL-2 does not induce tyrosine phosphorylation of p56^{lck} or GAP.** (A) HUT 102B2 cells were incubated without (lane 2) or with (lanes 3 and 4) rIL-2. The samples were immunoprecipitated with rabbit anti-p56^{lck} serum (lanes 2 and 3). As a control, p56^{lck} was immunoprecipitated from the human T cell line HPB-MLT (lane 1). An aliquot of the detergent cell lysate from HUT 102B2 is shown in lane 4. The samples were analyzed by immunoblotting with anti-phosphotyrosine antibodies. (B) MT-2 (lanes 1–4 and 9–12) or HUT 102B2 (lanes 5–8 and 13–16) were stimulated with (lanes 2, 4, 6, 8, 10, 12, 14, and 16) or without (lanes 1, 3, 5, 7, 9, 11, 13, and 15) rIL-2. Cells were lysed and immunoprecipitated with anti-GAP serum and Pansorbin (lanes 3, 4, 7, 8, 11, 12, 15, and 16) or with Pansorbin alone (lanes 1, 2, 5, 6, 9, 10, 13, and 14). The samples were processed for immunoblots and stained with anti-phosphotyrosine (lanes 1–8) or anti-GAP (lanes 9–16) as described in Methods. The proteins pp190, pp124, and pp62 that stain with anti-phosphotyrosine are indicated. The GAP protein stained with anti-GAP serum is also indicated. The antibody heavy chain (Ab) was also visualized by this procedure.

not shown). Thus pp100, which migrates above the 92 000–96 000-Da phosphoproteins observed in the total cell lysates, is a candidate for a phosphorylated form of the mouse IL-2 receptor β subunit. The IL-2 receptor α chain immunoprecipitated from ^{125}I- or ^{35}S-methionine-labeled CTLL-2 cells migrates as a broad band of M_r = 55 000–70 000 and did not co-migrate with the phosphoprotein pp55 (data not shown). CTLL-2 cells were also subjected to chemical crosslinking with DSP after incubation with or without IL-2. Under these conditions, phosphoproteins of M_r = 115 000 (pp115) and 55 000 (pp55) were co-precipitated by 7D4 from IL-2-stimulated cells, and the yield of pp100 was reduced. In contrast, no phosphotyrosyl proteins were immunoprecipitated with the anti-mouse α chain mAb PC61 or the second antibody alone (goat anti-rat Ig, GαR) from cells stimulated with IL-2 and chemically crosslinked. Thus, analogous results were obtained with mouse and human cell lines with mAbs that do (PC61 and anti-Tac) or do not (7D4 and 7G7/B6) block binding of IL-2 to the α chain.

Comparison of the four mouse cell lines—CTLL-2, HT2b, L3AI, and CI 36—demonstrated that the mAb 7D4 immunoprecipitated pp115 and pp55 from each cell line after stimulation with IL-2 and crosslinking with DSP (data not shown). Similarly, mAb 7G7/B6 or Mik-β3 immunoprecipitated pp105 and, to a lesser extent, pp55 from MT-2, MLA-144, and lectin-activated human thoracic duct lymphocytes that were stimulated with IL-2 and subjected to chemical crosslinking (data not shown). The last four lanes of Figure 6 show a direct comparison of the proteins precipitated by 7D4 or 7G7/B6 from a mouse (HT2b) or human (HUT 102B2) T cell line, respectively. The difference in molecular weight between pp105 and pp115 correlated with differences observed in the total detergent cell lysates of mouse versus human T cell lines.

Proteins p56^{lck} and GAP are not tyrosine phosphorylated in response to IL-2

It was investigated whether the phosphoprotein pp55 was identical to the T cell protein tyrosine kinase, p56^{lck}. HUT 102B2 and other HTLV-1-infected cell lines were reported to contain undetectable amounts of p56^{lck} (Koga *et al.*, 1989). However, by the use of rabbit anti-p56^{lck} antiserum, we immunoprecipitated a small amount of p56^{lck} that possessed tyrosine kinase activity from the subline of HUT 102B2 used. This protein showed no change in tyrosine phosphorylation in response to IL-2 and migrated slightly faster than the IL-2-responsive 55 000-Da phosphoprotein (Figure 7A).

Recently, the GTPase-activating protein (GAP) was demonstrated to be phosphorylated on tyrosine in response to epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) and co-precipitated with the EGF or

PDGF receptor after ligand binding (Molloy *et al.,* 1989; Ellis *et al.,* 1990; Kazlauskas *et al.,* 1990; Kaplan *et al.,* 1990). The GAP protein of M_r = 124 000 is not phosphorylated in normal fibroblasts, but, in virus-transformed cells, it is constitutively phosphorylated on tyrosine and co-precipitates with two additional phosphotyrosyl proteins of M_r = 190 000 and 62 000 (Ellis *et al.,* 1990). It was investigated whether GAP was phosphorylated in response to IL-2 and associated with the IL-2 receptor. No anti-phosphotyrosine staining of GAP immunoprecipitated from human HUT 78 cells incubated with or without IL-2 was detected (data not shown). The GAP protein was confirmed to be present in the immunoprecipitate and in the total cell lysate by immunoblotting with anti-GAP antiserum. The GAP protein was phosphorylated on tyrosine and associated with the phosphotyrosyl proteins pp190 and pp62 in the virally infected MT-2 cells and, to a lesser extent, in HUT 102B2 cells, but no change in tyrosine phosphorylation of these proteins was detected in response to IL-2 (Figure 7B). In addition, anti-IL-2 receptor α chain immunoprecipitates from human (HUT 102B2) or mouse (HT2b) cells that were stimulated with IL-2 and subjected to chemical crosslinking or left untreated were immunoblotted with anti-GAP antiserum. No GAP protein was found to be co-precipitated with the IL-2 receptor in the presence or absence of IL-2 or crosslinking.

Discussion

One of the earliest metabolic changes detected after treatment with many peptide growth factors is an increase in protein tyrosine phosphorylation. Several growth-factor receptors—including those for EGF, PDGF, colony stimulating factor-1 (CSF-1), and insulin—contain a cytoplasmic protein tyrosine kinase catalytic domain that is activated upon ligand binding, resulting in autophosphorylation of the receptor and other proteins (reviewed in Ullrich and Schlessinger, 1990). Although the α and β chains of the IL-2 receptor do not contain a tyrosine kinase domain, IL-2 induces a rapid increase in tyrosine phosphorylation of several specific cellular proteins.

Our studies demonstrate that the β chain of the IL-2 receptor is one of the substrates for IL-2-stimulated tyrosine phosphorylation. The β chain was not a major anti-phosphotyrosine staining protein in immunoblots of total detergent cell lysates but was immunoprecipitated with antibodies to the β chain or α chain of the IL-2 receptor. After the current study was completed, Asao and co-workers (1990) also reported that IL-2 induces an increase in tyrosine phosphorylation of the IL-2 receptor β chain immunoprecipitated from one ^{32}P-labeled IL-2-dependent human T cell line. Our results provide independent confirmation, using different methodology to assess phosphorylation of the β chain; extend the results to a variety of human cell lines; and identify a 100 000-Da phosphotyrosyl protein as the putative mouse β chain. In the present study, human cells that express high-affinity IL-2 receptors (composed of α and β subunits) or only β chain exhibited an IL-2-stimulated increase in tyrosine phosphorylation of the β chain. This is consistent with previous reports demonstrating that cells expressing only β chain can transmit a proliferative signal (Le Thi Bich-Thuy *et al.,* 1987; Siegel *et al.,* 1987; Tsudo *et al.,* 1987a; Hori *et al.,* 1988). Other recent reports have provided evidence that the IL-2 receptor β chain is phosphorylated on tyrosine when IL-2 is chemically crosslinked to the receptor, but have not shown that IL-2 induces changes in β chain phosphorylation (Sharon *et al.,* 1989; Mills *et al.,* 1990). Ferris *et al.* (1989) did not detect any IL-2-induced ^{32}P-labeled proteins associated with anti-Tac immunoprecipitates from human T lymphocytes. Our results, however, suggest that use of the mAb anti-Tac, which competes for the IL-2 binding site, may preclude detection of IL-2-stimulated changes in phosphorylation.

Two anti-phosphotyrosine staining proteins in addition to the β chain were specifically co-precipitated with the IL-2 receptor from cells stimulated with IL-2 and subjected to chemical crosslinking. The proteins, pp55 and pp105 in human cells and pp55 and pp115 in mouse cells, comigrated with proteins in the total cell lysate that showed IL-2 enhanced anti-phosphotyrosine staining. (To simplify discussion, the 55 000-Da protein is referred to as pp55 in both species, although its identity has not been demonstrated.) It could not be determined whether pp55 and pp105 or pp115 were constitutively associated with the IL-2 receptor and were phosphorylated in response to IL-2 or whether the association was dependent on ligand binding. The data suggest that only occupied high (α/β)- or intermediate (β)-affinity IL-2 receptors are associated with tyrosine-phosphorylated pp55 and pp105 or pp115. These phosphoproteins were co-precipitated with the IL-2 receptor at concentrations of

rIL-2 (35 or 350 pM) at which high-, but not low-, affinity receptors would be occupied. Furthermore, anti-Tac or PC61, which block the binding of IL-2 to the α chain, and therefore cannot immunoprecipitate occupied receptors, did not co-precipitate pp55 and pp105 or pp115 from IL-2-stimulated and chemically crosslinked cells. The crosslinking agent DSP can penetrate the membrane and crosslink cytosolic proteins to membrane proteins (Wang and Richards, 1975). The proteins pp55, pp105, and pp115 may be cytosolic proteins because, using the Triton X-114 phase separation method of Bordier (1981) to separate proteins that bind detergent from those that do not, the phosphoproteins partitioned into the phase depleted of detergent (unpublished data).

The proteins pp55 and pp105 or pp115 differ in M_r from the cellular proteins—phosphatidylinositol-3 kinase (85 kDa), phospholipase Cγ (145 kDa), the serine/threonine kinase, Raf-1 (74 kDa), and GAP (124 kDa)—that are phosphorylated on tyrosine in cells stimulated with PDGF or EGF and are associated with the PDGF receptor from growth factor-treated cells (Molloy *et al.,* 1989; Ellis *et al.,* 1990; Kaplan *et al.,* 1990; Kazlauskas *et al.,* 1990; Ullrich and Schlessinger, 1990). We were not able to detect GAP in immunoprecipitates of the IL-2 receptor from IL-2-stimulated cells, nor did IL-2 treatment enhance the phosphorylation of GAP. However, all growth factor receptors that contain a cytoplasmic tyrosine kinase domain do not phosphorylate the same spectrum of cellular proteins. For example, phospholipase Cγ is not a substrate for CSF-1 or insulin-stimulated tyrosine phosphorylation (Ullrich and Schlessinger, 1990), which correlates with the observation that CSF-1 or insulin does not stimulate membrane phosphatidylinositol turnover, in contrast to EGF and PDGF (Whetton *et al.,* 1986; Whitman and Cantley, 1988).

To date, attempts to demonstrate tyrosine kinase activity in vitro in anti-IL-2 receptor immunoprecipitates have not yielded consistent results. Benedict and co-workers (1987) and Merida and Gaulton (1989) found tyrosine kinase activity to be associated with IL-2 receptor immunoprecipitates, but the kinase was not identified. One candidate for an IL-2-stimulated protein tyrosine kinase is p56^{lck}, a member of the *src* family of tyrosine kinases that is expressed in essentially all T cells. We were not able to detect any IL-2-stimulated increase in tyrosine phosphorylation or kinase activity of p56^{lck}, or co-precipitation with the IL-2 receptor in human cells or in functional IL-2-dependent mouse T cell lines. Moreover, overexpression of p56^{lck} in the IL-2-dependent mouse cell line CTLL-2 did not augment IL-2-induced protein tyrosine phosphorylation (R. Hershberg, K. Luo, and B. Sefton, personal communication). Therefore, the level of expression of p56^{lck} does not correlate with the amount of IL-2-stimulated protein tyrosine phosphorylation, suggesting that other kinases may be involved.

Recently, many investigations have focused on the mechanisms of signal transduction through membrane receptors, including the T cell antigen receptor complex and surface IgM and IgD, that do not contain a tyrosine kinase domain yet mediate a rapid stimulation of protein tyrosine phosphorylation. By sequence similarities, the IL-2 receptor β chain belongs to a family of membrane receptors, including the IL-3, IL-4, and erythropoietin receptors, the members of which do not contain intrinsic tyrosine kinase activity. Nevertheless, IL-2, IL-3, and IL-4 all stimulate tyrosine phosphorylation but of different cellular substrates (Morla *et al.,* 1988). The growth factors IL-2, IL-3, and IL-4 also do not increase phosphatidylinositol turnover (Justement *et al.,* 1986; Mills *et al.,* 1986; Mizuguchi *et al.,* 1986; Whetton *et al.,* 1986; Kozumbo *et al.,* 1987). Therefore, the pathway of signal transmission utilized by the IL-2 receptor and other growth-factor receptors may differ substantially from that activated by the PDGF or EGF receptor. This may be reflected by differences in the cellular proteins found associated with each receptor. We have demonstrated that the β subunit of the receptor is phosphorylated on tyrosine, which may mediate its interaction with other cellular proteins. Two novel phosphotyrosyl proteins were co-precipitated with the high-affinity IL-2 receptor. These represent candidates for additional receptor subunits or cellular proteins necessary for signal transmission.

Methods

Cells

The human T leukemic cell lines HUT 102B2 (Robb *et al.,* 1984), HUT78 (Tsudo *et al.,* 1987b) and MT-2 (Takeshita *et al.,* 1989), and the gibbon ape T cell line MLA-144 (Tsudo *et al.,* 1986), were maintained in RPMI 1640 medium supplemented with 8% fetal bovine serum (FBS). The mouse T lymphocyte cell lines HT2b (Malek *et al.,* 1983) and Cl 36 (Kane *et al.,* 1989) were maintained in RPMI medium supplemented with 5×10^{-5} M 2-mercaptoethanol (2-ME) and 8% or 16%, respectively, defined calf serum (DCS, Hyclone, Logan, UT). The mouse T cell lines CTLL-2 (Malek *et al.,* 1983) and L3AI (Glasebrook *et al.,* 1981) were maintained

in Dulbecco's minimum essential medium (DMEM) medium supplemented with 5×10^{-5} M 2-ME and 4 or 8% DCS, respectively. All mouse T cell lines were IL-2 dependent and were supplemented with 5% supernatant of phorbol myristate acetate (PMA)-stimulated EL-4 cells as a source of IL-2.

IL-2 stimulation and crosslinking

The cell lines were harvested, washed once with medium, and cultured in RPMI 1640 or DMEM medium supplemented with 1% FBS or DCS 18–24 h before assay. The amount of PMA-stimulated EL-4 supernatant was reduced to 0.5% for the mouse cell lines during this culture period. After this incubation, the cells were stripped of bound ligand by a 10-s wash with 10 mM sodium citrate, pH 4, containing 0.14 M NaCl, followed by 10-fold dilution with RPMI medium. Cells (2×10^6 cells/ml) were resuspended in RPMI medium supplemented with 4% FBS. Recombinant IL-2 (1–1,000 U/ml), generously provided by Hoffmann-La Roche (Nutley, NJ), was added to the appropriate samples, and the cells were incubated at 37°C for 15 min. The cells then were centrifuged and washed once with phosphate-buffered saline (PBS, 0.14 M NaCl, 10 mM Na phosphate, pH 7.2) containing 1 mM $MgCl_2$. Cells to be subjected to chemical crosslinking were resuspended at 2×10^6 cells/ml in PBS containing 1 mM $MgCl_2$ and 0.8 mM DSP and incubated on ice for 15 min. The reaction was quenched by adding 1/100 volume of 1 M tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5. All cell samples were lysed at 4×10^7 cells/ml in 1% NP-40 containing 20 mM Tris-HCl, pH 8, 0.15 M NaCl, 2.5 mM EDTA, 0.5 mM Na_3VO_4, and 0.5 mM phenylmethylsulfonylfluoride. Aliquots of the cell lysates to be analyzed for total cell protein were diluted with an equal volume of 2× Laemmli gel (Laemmli, 1970) sample buffer and boiled for 5 min.

Immunoprecipitations

The anti-human IL-2 receptor α chain mAb anti-Tac (Leonard *et al.,* 1982) was provided by Dr. T. Waldmann (National Institutes of Health) and Dr. T. Uchiyama (Kyoto University, Kyoto, Japan); and 7G7/B6 (Rubin *et al.,* 1985), which also recognizes the α chain, was from Dr. D. Nelson (National Institutes of Health). The anti-IL-2 receptor β chain mAbs Mik-β3 and TU27 (Takeshita *et al.,* 1989; Tsudo *et al.,* 1989) were gifts from Dr. M. Tsudo (the Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and Dr. K. Sugamura (Tohoku University School of Medicine, Sendai, Japan), respectively. The anti-mouse IL-2 receptor α chain mAbs 7D4 (Malek *et al.,* 1983) and PC61 (Ceredig *et al.,* 1985) were obtained from Dr. T. Malek (University of Miami, Florida) and Dr. R. MacDonald (Ludwig Institute, Lausanne, Switzerland), respectively. Rabbit anti-p56^{lck} antiserum was provided by T. Hurley and Dr. B. Sefton (Salk Institute, La Jolla, CA). The rabbit anti-GAP antiserum, made against a fusion protein containing residues 171–448 (Ellis *et al.,* 1990), was kindly provided by Dr. T. Pawson (Mount Sinai Hospital Research Institute, Toronto, Canada).

To immunoprecipitate the human IL-2 receptor α chain, aliquots of the cell lysate were incubated with anti-Tac or 7G7/B6 that had been preadsorbed to Protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ). The human IL-2 receptor β chain mAb Mik-β3 or TU27 was preadsorbed to rabbit anti-mouse IgG covalently coupled to Sepharose. The anti-mouse α chain mAb 7D4 or PC61 was preadsorbed to goat anti-rat IgG coupled to Sepharose. As a control, aliquots of the lysates were incubated with Protein A-Sepharose, rabbit anti-mouse IgG-Sepharose, or goat anti-rat IgG-Sepharose alone. The proteins p56^{lck} or GAP were immunoprecipitated with 1–4 μl of the appropriate antiserum and 50 μl of a 10% solution of Pansorbin (Calbiochem, La Jolla, CA). Immunoprecipitates were washed once with 0.5% NP-40 containing 0.5 M NaCl, 50 mM Tris-HCl, pH 8, and 5mM EDTA and twice with 1% NP-40 containing 0.15 M NaCl, 20 mM Tris-HCl, pH 8, and 2.5 mM EDTA. Proteins were eluted by boiling in Laemmli gel sample buffer.

Immunoblotting

Antibodies to phosphotyrosine were prepared according to Kamps and Sefton (1988) using a mixture of phosphotyrosine, alanine, and glycine coupled to keyhole limpet hemocyanin as the immunogen. The staining of immunoblots with the affinity-purified rabbit anti-phosphotyrosine antibodies was blocked by phosphotyrosine but not by phosphoserine.

Immunoblotting was performed essentially as described by Towbin *et al.* (1979) with some modifications. Proteins were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 8.5% acrylamide/0.15% bisacrylamide gels according to Laemmli (1970). The separated proteins were transferred to Immobilon-P (Millipore, Bedford, MA) using a Novex Western Transfer apparatus (Novex, Encinitas, CA) or the Bio-Rad Trans-Blot Cell (Bio-Rad, Richmond, CA) in transfer buffer (192 mM glycine, 25 mM Tris base, and 20% [v/v] methanol) for 1–2 h at 120 mA. The Immobilon filters were incubated for 18 h at room temperature in blocking buffer (5% bovine serum albumin in 0.15 M NaCl, 10 mM Tris-HCl, pH 7.5, and 0.01% NaN_3) to prevent nonspecific antibody binding. The filters were incubated subsequently with affinity-purified rabbit anti-phosphotyrosine antibodies (2 μg/ml) in blocking buffer for 1–3 h at room temperature. After two washes in rinse buffer (0.15 M NaCl, 10 mM Tris-HCl, pH 7.5, and 0.01% NaN_3), the filters were incubated for 1–2 h with 10 μCi [^{125}I] Protein A (ICN Biochemicals, Costa Mesa, CA; 30–70 μCi/μg) in blocking buffer. The filters were washed twice with rinse buffer, once with rinse buffer containing 0.05% NP-40 and 0.05% Tween 20, and twice more with rinse buffer. Filters were exposed to Kodak (Rochester, NY) XAR film with an intensifying screen. Prestained SDS electrophoresis molecular weight markers were purchased from Sigma (St. Louis, MO). SDS-PAGE molecular weight standards were also obtained from Bio-Rad. Autoradiographs were scanned using an LKB (Piscataway, NJ) laser densitometer.

To detect the GAP protein, Immobilon filters were incubated with rabbit anti-GAP serum diluted 1:200 in blocking buffer. The bound antibody was visualized by the use of a horseradish peroxidase-conjugated goat anti-rabbit Ig serum followed by incubation with 4-chloro-1-naphthol.

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