

Interleukins 2, 4, 7, and 15 Stimulate Tyrosine Phosphorylation of Insulin Receptor Substrates 1 and 2 in T Cells

POTENTIAL ROLE OF JAK KINASES*

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The signaling molecules insulin receptor substrate (IRS)-1 and the newly described IRS-2 (4PS) molecule are major insulin and interleukin 4 (IL-4)-dependent phosphoproteins. We report here that IL-2, IL-7, and IL-15, as well as IL-4, rapidly stimulate the tyrosine phosphorylation of IRS-1 and IRS-2 in human peripheral blood T cells, NK cells, and in lymphoid cell lines. In addition, we show that the Janus kinases, JAK1 and JAK3, associate with IRS-1 and IRS-2 in T cells. Coexpression studies demonstrate that these kinases can tyrosine-phosphorylate IRS-2, suggesting a possible mechanism by which cytokine receptors may induce the tyrosine phosphorylation of IRS-1 and IRS-2. We further demonstrate that the p85 subunit of phosphoinositol 3-kinase associates with IRS-1 in response to IL-2 and IL-4 in T cells. Therefore, these data indicate that IRS-1 and IRS-2 may have important roles in T lymphocyte activation not only in response to IL-4, but also in response to IL-2, IL-7, and IL-15.

Interleukin 2 (IL-2)¹ and IL-4 are lymphokines that have potent mitogenic effects on peripheral blood lymphocytes (1, 2). These cytokines also have a number of overlapping biochemical effects. They bind a common hematopoietin receptor subunit termed γc (3, 4) and activate Janus family protein-tyrosine kinases JAK1 and JAK3 (5, 6). After IL-2-induced activation of

these protein-tyrosine kinases, tyrosine phosphorylation of many signaling components occurs, including the receptor proteins themselves (7), the adaptor molecule Shc (8), STAT (signal transducers and activators of transcription) proteins (9–12), and phosphatidylinositol 3-kinase (PI 3-kinase) (13).

Binding of IL-2 to its receptor results in rapid tyrosine phosphorylation of the receptor cytoplasmic domain (7). A number of SH2-containing signaling molecules such as the adaptor molecule Shc and the STATs are thought to bind directly to tyrosine residues on the intracellular regions of the receptor (10, 14). IL-2 also activates PI 3-kinase, but it is not clear whether this substrate binds to the IL-2 receptor. In contrast, it is clear that the IL-4 receptor can recruit PI 3-kinase via the tyrosine phosphorylation of the cytosolic docking protein IRS-1 or the newly cloned IRS-2 (4PS) molecule (15–17). These large cytosolic docking molecules have many phosphotyrosine residues which provide binding sites for important SH2-containing molecules such as Crk, Grb-2, Nck, PI 3-kinase, and SH-PTP2 to the receptor complex (18–21).

The phosphorylation of IRS-1 is thought to be important for glucose transport (22) and for both insulin- and IL-4-induced proliferation (23). Indeed, 32D cells expressing an IL-4 receptor mutant that does not phosphorylate IRS-1 will not proliferate in response to IL-4 (24). Collectively, these data suggest that IRS-1 and perhaps IRS-2 play an important role in the signaling response to growth factors such as insulin and IL-4. More recently, other cytokines and growth factors such as IFN α , IFN γ , IL-9, and leukocyte inhibitory factor have also been shown to induce the tyrosine phosphorylation of IRS-1 (25–29). These reports suggest that IRS-1 may be an important signaling component utilized by many growth factors.

Although IL-2 signal transduction has been investigated extensively, IL-2 has not been reported to induce phosphorylation of IRS-1 or IRS-2. Therefore, it was important to assess whether IL-2 could induce phosphorylation of IRS-1 and IRS-2 and thus provide a means of explaining PI 3-kinase recruitment in response to IL-2. We report here that IL-2, IL-7, and IL-15, as well as IL-4, rapidly stimulate the tyrosine phosphorylation of IRS-1 and IRS-2 in human T cells. In addition, we show that the Janus kinases JAK1 and JAK3 can associate with and tyrosine-phosphorylate IRS-1 and IRS-2. Moreover, we demonstrate a rapid IL-2-induced association of PI 3-kinase with IRS-1. These results suggest that IRS-1 and IRS-2 may have significant roles in T lymphocyte activation in response to not only IL-4 but also IL-2, IL-7, and IL-15.

MATERIALS AND METHODS

Cells and Reagents—Human T lymphocytes were obtained by Percoll gradient centrifugation from normal donors, following informed consent. The purity of the T cell population was 93–96%. Peripheral blood T lymphocytes were cultured in RPMI 1640 (Advanced Biotechnology, Inc., Columbia, MD) containing 100 μ g/ml gentamycin, 2 mM L-glutamine (Life Technologies, Inc.), and 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT). T cells were activated in RPMI 10% serum and phytohemagglutinin (PHA) (1 μ g/ml) for 72 h, washed in CO₂-acidified medium, and rested in 0.5% human serum for 4 h in order to optimize IL-2, IL-7, and IL-15 signaling.

Recombinant human IL-2 was kindly provided by Cetus Oncology Corp. IL-4 and IL-7 were obtained from PeproTech (Rocky Hill, NJ). IL-12 was received from Dr. Stanley Wolf (Genetics Institute, Cambridge MA). IL-15 was obtained from Immunex (Seattle, WA) and R & D Systems (Minneapolis, MN). IFN α was obtained from Hoffman-La Roche Inc. Monoclonal anti-JAK1 was obtained from Transduction Lab-

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¹The abbreviations used are: IL, interleukin; PI 3-kinase, phosphatidylinositol 3-kinase; STAT, signal transducers and activators of transcription; IRS, insulin receptor substrate; IFN, interferon; PHA, phytohemagglutinin; PAGE, polyacrylamide gel electrophoresis.

oratories (Lexington, KY). Rabbit polyclonal anti-JAK1, JAK2 antibodies, and monoclonal antiphosphotyrosine, 4G10, were purchased from Upstate Biotechnology Industries (Lake Placid, NY). Polyclonal rabbit anti-JAK3 was made in our laboratory (30), rabbit anti-IRS-1 and anti-IRS-2 sera were made against recombinant baculovirus-purified IRS-1 protein and against a unique COOH-terminal murine IRS-2 (23).

Immunoprecipitation and Immunoblotting—After stimulation with the indicated cytokines, T lymphocytes were washed once in ice-cold phosphate-buffered saline and lysed in buffer containing 0.5% Brij 96, 300 mM NaCl, 50 mM Tris, pH 7.5, with 2.5 M *p*-nitrophenyl guanidino-benzoate, 200 μ M sodium orthovanadate, and 10 μ g/ml both leupeptin and aprotinin (Sigma). The lysates were centrifuged at 12,000 \times *g*, and the postnuclear supernatants were immunoprecipitated with anti-IRS-1, anti-IRS-2, anti-JAK1 (UBI), anti-JAK3 (as above), or antiphosphotyrosine (4G10) from Upstate Biotechnology Industries. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis on 8% gels. Proteins were transferred to Immobilon membranes (Millipore Corp.) at 100 V at 4 °C for 3 h in 25 mM Tris, 190 mM glycine, 20% methanol, and 0.01% SDS. For immunoblotting with antiphosphotyrosine, the filters were first blocked with 1% gelatin and sequentially incubated with antiphosphotyrosine (1:2000), biotinylated goat anti-mouse IgG (Oncogene Science, Cambridge, MA), and horseradish peroxidase-conjugated streptavidin (Oncogene Science). Signal was detected using enhanced chemiluminescence ECL (Amersham). Filters that were subsequently reprobbed were stripped with 2% SDS, 60 mM Tris, pH 6.8, and 100 mM 2-mercaptoethanol at 70 °C for 30 min.

Transient Transfection—Cos-7 cells were maintained in RPMI 1640 with 5% fetal calf serum. These cells were transfected at 50–60% confluence using the DEAE-dextran method (31). In co-transfection experiments, expression of a total of 4 μ g of cDNA for IRS-2 and IL-2 receptor γ chain (γ c), JAK3 or JAK1 were transfected into Cos-7 cells.

RESULTS

IL-2 and IL-4 Induce Tyrosine Phosphorylation of IRS-1 and IRS-2—Whereas IL-4 stimulation induces rapid tyrosine phosphorylation of IRS-1, IL-2 has not been shown to induce phosphorylation of IRS-1 or the newly described IRS-2 protein. To determine whether IRS-1 became tyrosine-phosphorylated in response to both IL-2 and IL-4, human peripheral blood T cells were activated with PHA for 3 days, acid-washed, and incubated for 4 h in serum-deprived conditions. The cells were then washed and stimulated with IL-2 (1000 units/ml), IL-4 (100 units/ml), or IL-12 (100 units/ml) for 15 min, lysed, and immunoprecipitated with anti-IRS-1. The immunoprecipitates were separated by SDS-PAGE, and immunoblotted with antiphosphotyrosine. There was no basal tyrosine phosphorylation under conditions of factor deprivation (Fig. 1A, lane 1), but, as expected, IL-4-induced tyrosine phosphorylation of IRS-1 was detected after a 15-min stimulation (Fig. 1A, lane 4). However, tyrosine phosphorylation of IRS-1 was also observed in T cells after 5- and 15-min stimulation with IL-2 (Fig. 1A). Of note, the levels of IRS-1 tyrosine phosphorylation detected in response to IL-2 in peripheral blood T cells were not as high as those observed with IL-4. The tyrosine phosphorylation of IRS-1 was transient with maximum stimulation in response to IL-2 being observed at 15 min and declining thereafter (results not shown). We did not detect tyrosine phosphorylation of IRS-1 in response to IL-12 in T cells (Fig. 1A, lane 5). This is of interest since IL-2 and IL-12 activate distinct Janus kinases (32). These data suggest that tyrosine phosphorylation of IRS-1 occurs in response to IL-2 treatment of activated human T cells.

Recently, the IRS-1 homologue, IRS-2 (4PS), has been cloned from myeloid cells (17). IRS-1 and IRS-2 exhibit considerable overall sequence identity and can be used interchangeably by both insulin and IL-4 to mediate mitogenesis in the 32D cell system (17, 23). We therefore examined whether IL-2 could also induce the tyrosine phosphorylation of IRS-2. Fig. 1B shows that tyrosine phosphorylation of both IRS-1 and IRS-2 was detected in response to IL-2 and IL-4 in human T cells. The antibodies used to identify IRS-1 and IRS-2 are specific for each of the molecules; this is confirmed since these two proteins can

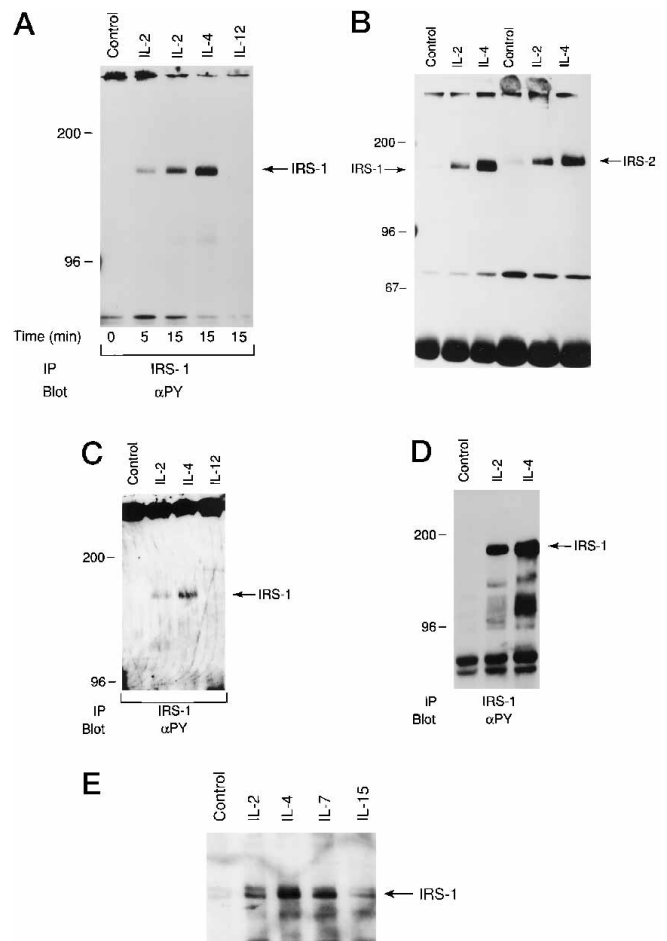


FIG. 1. Tyrosine phosphorylation of IRS-1 and IRS-2 by IL-2, IL-4, IL-7, and IL-15. A, IL-2 and IL-4 induce tyrosine phosphorylation of IRS-1 in T cells. PHA-activated T cells were treated with IL-2, IL-4, or IL-12 for 5 or 15 min, lysed, and immunoprecipitated with anti-IRS-1 and immunoblotted with antiphosphotyrosine. B, IL-2 and IL-4 induce IRS-1 and IRS-2 tyrosine phosphorylation in T cells. T cells either unstimulated or treated with IL-2 or IL-4 for 15 min, immunoprecipitated with anti-IRS-1 or anti-IRS-2 as indicated, and immunoblotted with antiphosphotyrosine. C, IL-2 and IL-4 phosphorylate IRS-1 Natural Killer (NK) cells; D, the NK 3.3 cell line. NK and NK 3.3 cells were untreated or treated with IL-2, IL-4, or IL-12 for 15 min, were lysed and immunoprecipitated with anti-IRS-1 antisera. E, T cells were treated with IL-2, IL-4, IL-7, and IL-15, immunoprecipitated with anti-IRS-1, and blotted with antiphosphotyrosine.

be distinguished by their migration on SDS-PAGE. As shown in Fig. 1B, consistent with the predicted molecular masses, IRS-2 (180 kDa) migrates slower than IRS-1 (165 kDa) (17). To confirm that equivalent levels of IRS-1 and IRS-2 were immunoprecipitated, the filter was reblotted with the respective antibodies, and the levels were identical in each lane (results not shown).

IL-2 is an important regulator of Natural Killer (NK) cell and B cell function. We therefore examined whether IL-2-dependent IRS-1 tyrosine phosphorylation could be detected in human peripheral blood NK cells or B cells. Increased IRS-1 phosphorylation was readily observed in response to IL-2 and IL-4 in NK cells, but again IL-12 did not induce tyrosine phosphorylation of IRS-1 in these cells (Fig. 1C). Marked increases in the levels of IRS-1 phosphotyrosine were also observed in the NK cell line, NK 3.3, in response to IL-2 and IL-4 treatment (Fig. 1D). IL-2-induced IRS-1 phosphorylation was also observed in an Epstein Barr virus-transformed B cell line from a normal donor and, in the T cell line, Kit 225 (results not shown). These data further suggest that IL-2-induced phosphorylation of IRS-1 may be an important signaling event in all human lym-

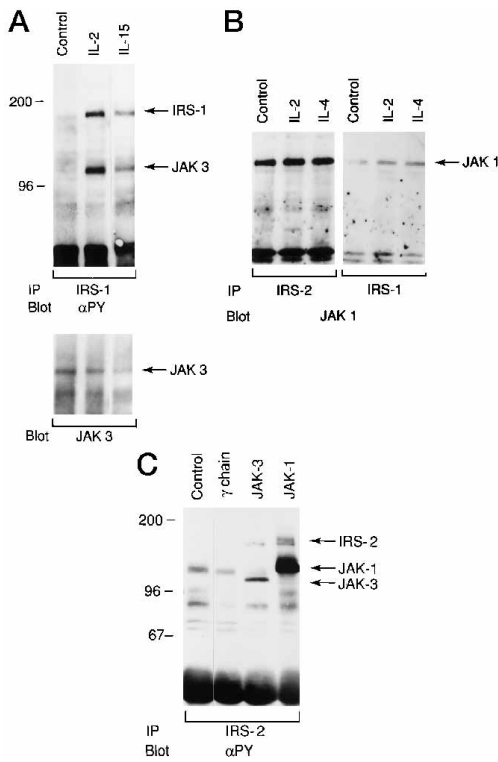


FIG. 2. IRS-1 and IRS-2 associate with JAK1 and JAK3. A, PHA-activated T cells were untreated or treated with IL-2 (1000 units/ml) or IL-15 (5000 units/ml) for 15 min, lysed, and immunoprecipitated with anti-IRS-1, and blotted with antiphosphotyrosine (upper panel) and anti-JAK3 (lower panel). B, T cells were untreated or treated with IL-2 (1000 units/ml) or IL-4 (100 units/ml) for 15 min, lysed, and immunoprecipitated with anti-IRS-1 or anti-IRS-2 and blotted with anti-JAK1. C, IRS-2 alone or IRS-2 with γ c, JAK3, or JAK1 was expressed in Cos cells and lysates from these cells were immunoprecipitated with IRS-2, resolved on SDS-PAGE, and blotted for phosphotyrosine.

phocyte subpopulations.

IL-7 and IL-15 also bind the IL-2 receptor γ c subunit and activate JAK1 and JAK3 in T cells (12, 33). We therefore examined whether tyrosine phosphorylation of IRS-1 would occur in response to IL-7 and IL-15. T cells were treated with IL-2, IL-4, IL-7 (100 units/ml), and IL-15 (5000 units/ml), lysed, and immunoprecipitated as above. As with IL-2 and IL-4, tyrosine phosphorylation of IRS-1 was detected in response to IL-7 and IL-15 in T cells (Fig. 1E). These findings establish that tyrosine phosphorylation IRS-1 occurs in T cells in response not only to IL-4 but also to IL-2, IL-7, and IL-15.

IRS-1 and IRS-2 Associate with the Janus Kinases JAK1 and JAK3—Tyrosine phosphorylation of IRS-1 in response to insulin is thought to result from the tyrosine kinase activity of the insulin receptor. Unlike the insulin receptor, the IL-2 and IL-4 receptors do not have intrinsic tyrosine kinase activity but have been demonstrated to be coupled to the Janus protein-tyrosine kinases, JAK1 and JAK3 (5, 6). We speculated that the IL-2- and IL-4-induced tyrosine phosphorylation of IRS-1 and IRS-2 might result from Janus kinase activity. To investigate this possibility, we examined whether these Janus kinases could associate with IRS-1. Following IL-2 stimulation of T cells, an additional tyrosyl phosphoprotein was detected in the anti-IRS-1 immunoprecipitates (Fig. 2A, upper panel). This protein migrated with a molecular mass of ~125 kDa, identical with that of JAK3. We therefore speculated that this protein might be JAK3 and confirmed this by reprobating the filter with JAK3 antiserum (Fig. 2A, lower panel). We also detected JAK1 protein-tyrosine kinase in both IRS-1 and IRS-2 immunoprecipitates from T cell lysates (Fig. 2B). These results strongly

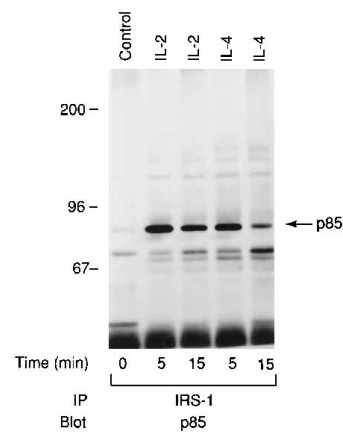


FIG. 3. IL-2 and IL-4 induce association of p85 with IRS-1. T cells were unstimulated or stimulated with IL-2 or IL-4 for 5 or 15 min, lysed, and immunoprecipitated with anti-p85.

suggest that the Janus kinases JAK1 and JAK3 associate with IRS-1 and IRS-2 in T cells.

To determine if JAK1 or JAK3 could interact directly with the IRS molecules and to examine whether these Janus kinases could induce tyrosine phosphorylation of the IRS proteins, we co-expressed IRS-2 with the Janus family kinases in Cos cells and analyzed the IRS-2 immunoprecipitates. IRS-2 did not exhibit basal tyrosine phosphorylation when expressed alone or with the IL-2 receptor chain γ c (Fig. 2C, lanes 1 and 2, respectively). In contrast, IRS-2 tyrosine phosphorylation was readily detected in cells expressing IRS-2 in combination with JAK1 or JAK3 (Fig. 2C, lanes 3 and 4, respectively). Tyrosine-phosphorylated JAK1 and JAK3 were detected in the IRS-2 immunoprecipitates from these cells indicating again that these kinases associate with IRS-2 (Fig. 2C). Interestingly, in cells expressing JAK1, higher levels of IRS-2 tyrosine phosphorylation were observed than in cells expressing JAK3, even though JAK1 and JAK3 were expressed at comparable levels (data not shown). Likewise, higher levels of JAK1 protein were observed in the IRS-2 immunoprecipitates. Taken together, these data indicate that IRS-1 and IRS-2 can interact directly with the JAKs and may be substrates for these kinases, suggesting that JAK/IRS association may be important for IRS-1 and IRS-2 tyrosine phosphorylation in response to IL-2 and IL-4.

IL-2-induced Recruitment of PI 3-Kinase by IRS-1—The 85-kDa subunit of PI 3-kinase binds to IRS-1 following insulin, IL-4, and growth hormone stimulation (16, 23, 27). While it has been demonstrated that PI 3-kinase is activated in response to IL-2 (13), the mechanism by which PI 3-kinase is recruited is unclear. To determine whether IL-2 and IL-4 induce association of the 85-kDa subunit of PI 3-kinase with IRS-1, lysates from IL-2- and IL-4-stimulated human peripheral blood T cell blasts were immunoprecipitated with anti-IRS-1 and immunoblotted with anti-p85. Low basal levels of p85-associated IRS-1 were detected (Fig. 3, lane 1). However, an IL-2- and IL-4-dependent co-precipitation of the 85-kDa subunit of PI 3-kinase with IRS-1 was detected by immunoblotting (Fig. 3, lanes 2–5). These data suggest that the tyrosine phosphorylation of IRS-1 in response to IL-2 in T cells leads to the rapid recruitment of the 85-kDa subunit of PI 3-kinase and suggests a possible mechanism for IL-2-induced activation of PI 3-kinase.

DISCUSSION

The recent identification and cloning of the IRS-1-like molecule, IRS-2, has confirmed that both proteins exhibit substantial structural homology and indicates that they may have similar functional roles (17). Although, mice lacking IRS-1 have a reduced ability to uptake glucose and are diminished in

size, they are otherwise normal (22, 37), suggesting that IRS-2 may compensate for the absence of IRS-1 in many signaling pathways. Also, data reported from 32D cells showing that IRS-1 and IRS-2 can be used interchangeably by IL-4 and insulin indicate that they have similar roles in mediating a mitogenic response (35). Our data establish that IRS-1 and IRS-2 are both tyrosine-phosphorylated by IL-2, IL-4, and IL-15 in primary human lymphocytes. This suggests that IRS-1 and IRS-2 may have similar functions; that they both are utilized for cytokine signaling and that they may have important roles in the IL-2- and IL-4-induced response.

The functional importance of IL-2-induced IRS-1 and IRS-2 phosphorylation has yet to be defined. However, IRS-1 has been shown to be important for mediating insulin and IL-4-induced proliferative effects (23). IRS-1 phosphorylation has been shown to enhance insulin-stimulated MAP kinase activation. It has also been suggested that the binding of IRS-1 to Grb-2 may enhance insulin-stimulated mitogenesis via the activation of the Ras/Raf pathway (16). However, as the adaptor molecule Shc is recruited by IL-2 in T cells (8), and as Shc-Grb-2 complexes are known to form (16), it is possible that Shc and IRS-1 have synergistic roles in the IL-2 response. Therefore, IRS-1 phosphorylation is likely to represent an alternative pathway for IL-2-induced proliferation in T cells. In addition, as the tyrosine phosphorylation of IRS-1 by cytokines such as growth hormone (GH), IL-9, IFN α , insulin, and now IL-2 induce association of IRS-1 with PI 3-kinase, this may be a commonly used mechanism for PI 3-kinase activation. In summary, IRS-1 probably acts conjointly with other pathways of IL-2-mediated signaling, thereby amplifying the IL-2 proliferative response.

The mechanism by which cytokines that bind hematopoietin family receptors (*i.e.* IL-2 and IL-4) induce tyrosine phosphorylation of IRS-1 has been unclear (34). Our findings suggest that JAK1 and JAK3 can bind to and phosphorylate IRS-1. In addition, it is clear that growth hormone, which activates JAK2 (36), also phosphorylates IRS-1 (27), and that a number of cytokines, such as IFN α , IFN γ , and IL-13 phosphorylate IRS-1 but do not activate JAK3 (26, 27). Therefore, while the tyrosine phosphorylation of IRS-1 may result from Janus kinase activity, it appears to be independent of the particular JAK activated. However, the finding that the JAKs can directly phosphorylate and are physically associated with the IRS-1 and IRS-2 proteins suggests that the activation of JAK kinases may be a common mechanism by which many growth factors phosphorylate IRS-1 and IRS-2.

Although IL-4-induced IRS-1 phosphorylation has been reported previously (23), IL-2-induced IRS-1 phosphorylation has not. This is surprising since IL-2 signal transduction is an intensively investigated area. A possible explanation for this is that IRS-1 and IRS-2 are not expressed in certain T cell lines commonly used to examine IL-2 signaling.² However, it should be re-emphasized that the present study employed normal peripheral blood lymphocytes, thus reinforcing the physiological relevance of the observations.

The findings described in this paper strongly suggest that IRS-1 and IRS-2 may be important docking molecules recruited in response to IL-2, IL-4, and IL-15 in human T cells. The results further indicate that the JAKs associate with and phosphorylate IRS-1 and IRS-2, and that IL-2 and IL-4 induce association of PI 3-kinase with these docking molecules and thus provide a means of amplifying the cytokine-dependent signal in a manner that may not rely on recruitment of the signaling molecules to the phosphorylated receptor. Therefore, IL-2-dependent IRS-1 and IRS-2 phosphorylation is likely to have an important role in T lymphocyte activation.

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² J. A. Johnston, unpublished observation.