Jak1 Expression Is Required for Mediating Interleukin-4-induced Tyrosine Phosphorylation of Insulin Receptor Substrate and Stat6 Signaling Molecules*

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The Jak1, Jak2, Jak3, and Fes tyrosine kinases have been demonstrated to undergo tyrosine phosphorylation in response to interleukin (IL)-4 stimulation in different cell systems. However, it is not clear which, if any, of these kinases are responsible for initiating IL-4-induced tyrosine phosphorylation of intracellular substrates in vivo. In the present study, we have utilized a mutant Jak1-deficient HeLa cell line, E1C3, and its parental Jak1-expressing counterpart, 1D4, to analyze the role of Jak1 in mediating IL-4-induced tyrosine phosphorylation events. IL-4 treatment rapidly induced tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2 in 1D4 but not in E1C3 cells. IL-4-mediated tyrosine phosphorylation of Stat6 was pronounced in 1D4 cells, while no IL-4-induced Stat6 phosphorylation was detected in E1C3 cells. IL-4 also induced Stat6 DNA binding activity from lysates of 1D4 but not E1C3 cells utilizing a radiolabeled immunoglobulin heavy chain germline promoter sequence (Ie) in an electrophoretic mobility shift assay. Reconstitution of Jak1 expression in E1C3 cells restored the ability of IL-4 to induce IRS and Stat6 tyrosine phosphorylation. These results provide evidence that Jak1 expression is required for mediating tyrosine phosphorylation and activation of crucial molecules involved in IL-4 signal transduction.

Interleukin (IL)-4 is known to play a critical role in determining the nature of an immune response to a given pathogen. In addition, IL-4 has been demonstrated to mediate a diverse array of proliferative and functional effects in cells of hematopoietic origin (1). The multifunctional role of IL-4 is reflected by the ubiquitous expression of IL-4 receptors (IL-4Rs) in both hematopoietic and nonhematopoietic cell types (2–4). The cDNA encoding a high affinity subunit for the human IL-4Rα sequence revealed that it is a member of the hematopoietin receptor superfamily. While IL-4Rα possesses a large cytoplasmic domain of approximately 500 amino acids, no consensus sequences for tyrosine or serine/threonine kinases have been identified. It has been demonstrated that IL-4 can also utilize the γ chain of the IL-2 receptor (IL-2Rγ) in a complex with IL-4Rα to enhance ligand binding and signal transduction (7, 8).

Despite the lack of a tyrosine kinase domain in the cytoplasmic region of either IL-4Rα or IL-2Rγ, IL-4 stimulation leads to tyrosine phosphorylation of IL-4Rα and certain intracellular signaling molecules. IL-4 treatment has been demonstrated to result in tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), IRS-2 (formerly termed 4PS), Stat6, and other undefined molecules (9–16). We have previously established that expression and tyrosine phosphorylation of IRS-1 or IRS-2 are required for efficient IL-4-mediated mitogenesis in 32D myeloid cells (17, 18). Recent studies characterizing the phenotype of Stat6−/− mice have provided evidence that Stat6 expression is required for multiple functional responses induced by IL-4 and strongly implicated Stat6 involvement in IL-4-induced proliferative effects (19–21). It is well established that tyrosine phosphorylation of Stat proteins is essential for their dimerization and translocation to the nucleus where they activate transcription (22, 23). These studies substantiate that tyrosine kinase-mediated phosphorylation events play an integral role in implementing IL-4 signal transduction.

Three members of the Janus kinase family, Jak1, Jak2, and Jak3, and the Fes proto- oncogene product are the only tyrosine kinases that have been reported to become tyrosine phosphorylated and/or activated in response to IL-4 stimulation in different cell systems (24–28). While IL-4-mediated tyrosine phosphorylation and activation of Jak1, Jak3, and Fes has been demonstrated in many cell systems (24–27), phosphorylation of Jak2 has only been reported to occur in human colon carcinoma cells (28). It has been demonstrated that IL-4 stimulation induces Jak1 and Fes association with IL-4Rα, while Jak3 has been shown to interact with IL-2Rγ (24–27). Cotransfection studies in COS cells and in vitro experiments have indicated that both Jak1 and Jak3 are able to associate with and mediate tyrosine phosphorylation of IRS-1 and IRS-2 (29, 30). However, it has not been determined which, if any, of these kinases are involved in mediating IL-4-induced phosphorylation of downstream signaling substrates under physiological conditions. In the present study, we have utilized a mutant HeLa cell line, originally isolated based on its lack of interferon responsiveness, that is deficient in Jak1 expression and its parental counterpart that does express Jak1 (31, 32) to determine if this...
tyrosine kinase is responsible for mediating IL-4-induced tyrosine phosphorylation events in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—The 1D4 and E1C3 HeLa cell lines have been previously described (31, 32). The human T lymphoid Jurkat cell line was a kind gift from Dr. L. Samelson, and the human monocytic U937 and B lymphoid Ramos cell lines were obtained from the ATCC. Human recombinant IL-4 was obtained from Peprotech and insulin was from Upstate Biotechnology, Inc. Murine anti-Jak1 monoclonal antibody (mAb) was obtained from Signal Transduction Laboratories, anti-phosphotyrosine mAb, anti-Jak2, and anti-Jak3 sera were from Upstate Biotechnology, Inc., and anti-Fes mAb was from Oncogene Sciences. The phycocyanin (PE)-conjugated IL-4 fluorochrome receptor reagent was obtained from R & D Systems. Rabbit anti-Jak-1 and anti-JAK-2 sera were generated by immunization of rabbits with baculovirus-expressed rat IRS-1 protein or an IRS-2-specific peptide comprised of amino acid residues 1310 to 1322 (LSSHKLKEAVVKE), respectively. The anti-JAK-1 serum very weakly recognizes IRS-2, while anti-JAK-2 serum does not recognize IRS-1. The anti-Stat6 peptide serum used for immunoprecipitation and immunoblot analysis was raised against amino acid residues 787–804 (GEDIFPPLLPPTEQDLTK) of human Stat6.

**Flow Cytometry**—The adherent 1D4 and E1C3 HeLa cell lines were treated with 0.5 mM EDTA to facilitate their removal from the substrate. HeLa and Ramos cells were washed in phosphate-buffered saline (PBS) and resuspended at 5 × 10^6 cells/ml. PE-conjugated IL-4 (10 μg) was added to 25 μl of washed cells. As a negative control, 20 μl of anti-human IL-4 neutralizing antibody was mixed with 10 μg of PE-conjugated IL-4 and incubated for 15 min at room temperature before addition to the cell suspensions. Samples were incubated for 2 h at 16°C and washed twice with PBS containing 0.1% NaNO₂. Flow cytometry was performed on a FACScan (Becton-Dickenson).

**Immunoprecipitation and Immunoblot Analysis**—HeLa cells were washed twice and starved for 4 h in Dulbecco’s modified Eagle’s medium containing 5 μg/ml transferrin and 10 nm selenium. After stimulation with IL-4 (100 ng/ml) for 10 min at 37°C, cells were diluted in PBS containing 100 μM NaVO₄. The cells were then lysed in buffer containing 50 mM Tris-Cl (pH 7.5), 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM NaFIP, 5 mM EDTA, 1 mM NaVO₄, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. The total protein content of the lysates was determined by the Bio-Rad protein assay. Equal amounts of clarified cell lysates (0.5–1 mg/ml) were directly resolved by SDS-PAGE without prior serum starvation. Immunoblot analysis was performed by transferring separated proteins onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore) in Tris-glycine buffer containing 20% methanol. The membrane was then treated for 1–2 h with 3% non-fat dry milk in TTBS (20 mM Tris-Cl (pH 7.5), 154 mM NaCl, 0.05% Tween, 0.05% NaNO₂, incubated with antibodies in TTBS containing 0.5% BSA (TTBS-BSA) for 1 to 2 h (concentration of antibodies: 2 μg/ml for anti-Tyr(P) and 1-500 for the remaining antisera) and reacted with 125I-labeled protein A (3 × 10^9 cpm/ml) in TTBS-BSA for 1 h. Alternatively, bound antibody was detected by enhanced chemiluminescence according to the manufacturer’s protocol (Amershams Corp.). All procedures were done at room temperature, and membranes were washed extensively with TTBS after each treatment. After the final wash, the membrane was rinsed with distilled water, air dried and autoradiographed with an intensifying screen at ~70°C.

**Electrophoretic Mobility Shift Assay**—DNA oligonucleotide probes used in the electrophoretic mobility shift assay (EMSA) were annealed, labeled, purified, and whole cell extracts were prepared for EMSA essentially as described previously (14–16, 33). The sequence of one strand of the double-stranded probe used for EMSA was 5’-GATCTA-AC TTCCTCAAAGAAAGC-3’. Briefly, 1D4 and E1C3 cells were serum-starved overnight and treated for 10 min with IL-4 (100 ng/ml). Cells were washed once with PBS containing 100 μM NaVO₄ and solubilized in gel shift lysis buffer (50 mM Tris-Cl (pH 8.0), 0.5% Nonidet P-40, 10% glycerol, 100 μM EDTA, 50 mM NaF, 167 mM NaCl, 100 μM NaVO₄, 1 mM dithiothreitol, 400 μM dimethyl sulfoxide, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) by incubation on ice for 1 h and vortexing three to five times. Lysates were cleared by centrifugation at 14,000 rpm for 10 min, and protein concentrations were determined. For EMSA, 5 μg of whole cell lysate were incubated with the 32P-labeled 1e probe in 20 mM HEPES (pH 7.9), 40 mM KCl, 1 mM MgCl₂, 100 μM EDTA, 500 μM dithiothreitol, 6.0% glycerol, 1 mg/ml BSA, and 100 μg/ml poly(dI-dC) for 15 min. Complexes were subsequently fractionated on 0.22 × TBE (100 mM Tris borate (pH 8.0), 2 mM EDTA) 4.5% acrylamide gels.

**Transient Transfection Assay**—E1C3 cells (4 × 10⁶) were plated in a 10-mm tissue culture dish 24 h before transfection in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were transfected with 10 μg of a retroviral expression vector containing murine Jak1 cDNA (a kind gift from Dr. Warren Leonard) and 40 μg of carrier DNA using the calcium phosphate method. Eighteen hours after transfection, cells were washed and incubated in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum for 7–12 h. Cells were then serum-starved as described previously and used for subsequent experiments.

**RESULTS AND DISCUSSION**

To confirm that parental 1D4 HeLa cells expressed Jak1 and that mutant E1C3 HeLa cells were Jak1-deficient, whole cell lysates were prepared and subjected to immunoblot analysis utilizing an anti-Jak1 mAb. A 130-kDa protein was readily expressed in 1D4 cells, while no protein in this size range was observed in E1C3 cells (Fig. 1A). The 130-kDa protein was also detected in Jurkat cells that are known to express Jak1 (Fig. 1A). The status of Jak2, Jak3, and Fes expression in the two HeLa cell lines was also examined by immunoblot analysis, since these tyrosine kinases have also been demonstrated to become tyrosine phosphorylated in response to IL-4 treatment (24, 25, 27, 28). Anti-Jak2 serum recognized a 130-kDa protein in Jurkat, 1D4, and E1C3 cells (Fig. 1A). A Jak2 degradation product of 97 kDa was also observed in all three lines. Anti-Jak3 serum specifically detected the expression of a 120-kDa protein in Jurkat cells, while no protein in this size range was observed in either 1D4 or E1C3 cells (Fig. 1A). A 92-kDa protein was detected in human U937 myeloid cells that are known to express Fes but not in 1D4 or E1C3 cells utilizing an anti-Fes mAb (Fig. 1A). Thus, mutant E1C3 cells do not detectably express Jak1, Jak3, or Fes but do express Jak2.
IL-4Rs have been detected on a wide variety of hematopoietic and nonhematopoietic cell types at levels ranging from 100 to 5000 sites per cell (2–4). In order to compare IL-4-induced signal transduction events in the 1D4 and E1C3 cell lines, it was necessary to determine whether these lines expressed receptors capable of binding IL-4. As shown in Fig. 1B, 1D4 and E1C3 cells specifically bound PE-conjugated human IL-4. The human B Ramos cell line, which is known to express high levels of IL-4Rs (2), was also found to specifically bind PE-conjugated human IL-4. These results demonstrate that mutant E1C3 cells and their 1D4 parental counterpart express detectable levels of cell surface IL-4Rs.

The ability of IL-4 to induce tyrosine phosphorylation of IRS-1 and IRS-2 in 1D4 and E1C3 was next examined. Cells were serum starved and either untreated or stimulated with IL-4 or insulin. Insulin treatment was performed because it is known to mediate potent tyrosine phosphorylation of IRS molecules, and evidence suggests that activated insulin receptors directly associate with and phosphorylate these substrates (34–36). Cell lysates were immunoprecipitated with antiserum specific for either IRS-1 or IRS-2 and subsequently subjected to immunoblot analysis utilizing anti-phosphotyrosine. IL-4 treatment of 1D4 cells resulted in pronounced tyrosine phosphorylation of both IRS-1 and IRS-2, while no detectable phosphorylation of either substrate was observed in E1C3 cells in response to IL-4 stimulation (Fig. 2A and B). However, insulin treatment led to equivalent increases in the phosphotyrosine content of IRS-1 and IRS-2 molecules in both cell lines. Immunoblot analysis of the same samples with either anti-IRS-1 or anti-IRS-2 serum revealed that equivalent amounts of IRS proteins were present in each immunoprecipitate and that IRS expression was similar in both cell lines.2 These results strongly suggest that IL-4-induced IRS-1 and IRS-2 phosphorylation is mediated through activation of Jak1, while insulin-induced IRS phosphorylation does not require Jak1 expression.

It has been established that IL-4 induces tyrosine phosphorylation of Stat6 and binding of Stat6 to a specific DNA consensus sequence, TTTG(A > T,N)GGAA (13–16). To determine if Jak1 expression is required for mediating these events, we first analyzed whether IL-4 could induce tyrosine phosphorylation of Stat6 in the two HeLa cell lines. Lysates prepared from untreated or IL-4-stimulated cells were immunoprecipitated with antisera specific for human Stat6, proteins were separated by SDS-PAGE, and transferred proteins were immunoblotted (Blot) with anti-phosphotyrosine (anti-PY), as designated. Molecular mass markers are shown in kilodaltons. The positions of IRS-1 and IRS-2 are indicated by arrowheads.

IL-4 stimulation of 1D4 cells resulted in evident tyrosine phosphorylation of a 100-kDa protein specifically recognized by anti-Stat6 serum, no tyrosine-phosphorylated protein in this size range was detected in response to IL-4 treatment of E1C3 cells (Fig. 3A). Immunoblot analysis of the same immunoprecipitated lysates with anti-Stat6 serum demonstrated that Stat6 expression levels were comparable in the 1D4 and E1C3 cell lines (Fig. 3B).

Stat6 can be distinguished from other Stat proteins by its ability to bind to a specific consensus sequence found in the immunoglobulin heavy chain germ line promotor of the IL-4-responsive human Ce gene, designated Ie. To determine whether IL-4 could induce Stat6 binding activity in 1D4 and E1C3 cells, whole cell extracts from untreated or IL-4-treated cells were prepared and assayed for the induction of 32P-labeled Ie DNA binding activity by EMSA. IL-4 stimulation of 1D4 cells led to rapid induction of Ie binding, while no detectable binding activity could be observed after IL-4 treatment of E1C3 cells (Fig. 3C). Thus, Jak1 expression appears to be required for mediating IL-4-induced Stat6 activation.

Since the E1C3 cell line was derived by mutagenesis with ethylmethane-sulfonate (31), it remained possible that other functional defects could account for the inability of IL-4 to mediate tyrosine phosphorylation events in these cells. Therefore, a mammalian expression vector containing murine Jak1 cDNA was transiently transfected into E1C3 cells (E1C3/Jak1). Immunoblot analysis revealed that Jak1 protein levels attained in E1C3/Jak1 cells were equivalent to those in 1D4 cells (Fig. 4A). The E1C3/Jak1 transfected cells were untreated or

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2 X. H. Chen and L.-M. Wang, unpublished data.

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Fig. 2. IL-4 mediates tyrosine phosphorylation of IRS-1 and IRS-2 in 1D4 but not in E1C3 cells. 1D4 or E1C3 cells were untreated (O) or stimulated with IL-4 or insulin (INS). Lysates were immunoprecipitated (IP) with A, anti-IRS-1 or B, anti-IRS-2 serum, proteins were separated by SDS-PAGE, and transferred proteins were immunoblotted (Blot) with anti-phosphotyrosine (anti-PY), as designated. Molecular mass markers are shown in kilodaltons. The positions of IRS-1 and IRS-2 are indicated by arrowheads.

Fig. 3. IL-4 induces Stat6 tyrosine phosphorylation and activation in 1D4 but not in E1C3 cells. 1D4 or E1C3 cells were untreated (O) or treated with IL-4. Lysates were immunoprecipitated (IP) with anti-Stat6 serum and subsequently immunoblotted (Blot) with A, anti-phosphotyrosine (anti-PY) or B, anti-Stat6 serum. Molecular mass markers are shown in kilodaltons, and the positions of Stat6 are indicated by arrowheads. C, whole cell lysates from untreated (O) or IL-4-stimulated 1D4 or E1C3 cells were incubated with 32P-labeled Ie for 10 min and assayed by EMSA as indicated under “Experimental Procedures.” The position of the IL-4-induced Ie mobility shift is indicated by an arrowhead.
Jak1 Requirement for IL-4 Signal Transduction

4 The phosphorylation of IRS molecules requires Jak1 expression. It will be necessary to determine if these kinases can mediate IL-4-directed tyrosine phosphorylation of IRS molecules or other substrates under physiological conditions. If so, this would suggest that Jak1 plays a central role in mediating signal transduction through multiple non-tyrosine kinase-containing receptors.

4 It will be of interest to determine whether other cytokines require Jak1 to direct tyrosine phosphorylation of IRS molecules or other substrates under physiological conditions. If so, this would suggest that Jak1 plays a central role in mediating signal transduction through multiple non-tyrosine kinase-containing receptors.

Fig. 4. Transient expression of Jak1 in E1C3 cells restores the ability of IL-4 to mediate IRS-1, IRS-2, and Stat6 tyrosine phosphorylation. E1C3 cells were transfected with a retroviral expression vector containing Jak1 cDNA as described under "Experimental Procedures." A, equivalent amounts of proteins from lysates of 1D4, E1C3, Jurkat, and E1C3/Jak1 cells were immunoblotted (Blot) with anti-Jak1 serum, as designated. The Jak1 protein is indicated by an arrowhead and the molecular mass marker is in kilodaltons. B, E1C3/Jak1 cells were unstimulated (0) or treated with IL-4. Lysates were immunoprecipitated (IP) with anti-phosphotyrosine (anti-PY) and immunoblotted (Blot) with anti-IRS-1 or anti-IRS-2 serum or C, immunoprecipitated (IP) with anti-Stat6 and immunoblotted (Blot) with anti-phosphotyrosine (anti-PY).

stimulated with IL-4 and IRS, and Stat6 phosphorylation was examined. As shown in Fig. 4, B and C, the ability of IL-4 to induce tyrosine phosphorylation of IRS-1, IRS-2, and Stat6 was restored in the E1C3/Jak1 transfected cells. The ability of IL-4 to induce binding of Stat6 to IκB has been demonstrated by EMSA and also reconstituted in the E1C3/Jak1 cells. These data provide conclusive evidence that Jak1 expression is required for mediating IL-4-induced tyrosine phosphorylation of both IRS and Stat6 molecules in the HeLa cell system.

A recent study demonstrated that Jak1 but not Jak2 or Tyk2 expression is crucial for IL-4-induced tyrosine phosphorylation of IRS molecules in a series of mutant derivatives of a human fibroblast cell line, HT 1080 (37). E1C3 cells express Jak2 (see Fig. 1A) and Tyk2 (32), further excluding the possibility that these Janus kinases are involved in IL-4-induced tyrosine phosphorylation of cellular substrates. However, our results do not rule out the possibility that Fes or Jak3 may be capable of evoking IL-4-induced tyrosine phosphorylation events in other cell types, since these kinases do not appear to be expressed in 1D4 or E1C3 lines. It has been reported that Fes expression appears to be limited to cells of the myeloid lineage (38). Since it is well established that IL-4 affects numerous cell types other than those of myeloid origin, the role of Fes in mediating IL-4-induced tyrosine phosphorylation of intracellular substrates would be severely restricted. Jak3 is also preferentially expressed in hematopoietic cells (39). Moreover, several recent studies have indicated that IL-2R expression is not necessarily required for IL-4 function (40-43). Since Jak3 has been demonstrated to associate with IL-2R (24, 27), this further suggests that it is unlikely that Jak3 is the primary kinase utilized by IL-4 for eliciting its biological functions. We are presently attempting to express Jak3 and Fes in the E1C9 line to determine if these kinases can mediate IL-4-directed tyrosine phosphorylation.

It has been demonstrated that a variety of cytokines and growth factors activate Jak1 (23). We have obtained preliminary evidence that interferon-α- and -γ-induced tyrosine phosphorylation of IRS molecules requires Jak1 expression. It will be of interest to determine whether other cytokines require Jak1 to direct tyrosine phosphorylation of IRS molecules or other substrates under physiological conditions. If so, this would suggest that Jak1 plays a central role in mediating signal transduction through multiple non-tyrosine kinase-containing receptors. In summary, our study strongly suggests that Jak1 is the primary kinase that couples with the IL-4R to initiate tyrosine phosphorylation of critical effector molecules involved in mediating IL-4 elicited signal transduction.

3 B. K. R. Patel, unpublished data.

4 X. H. Chen, B. K. R. Patel, and L.-M. Wang, unpublished data.
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