The role of the cytoplasmic regions of interleukin-12 receptors (IL-12R) β1 and β2 in stimulating proliferation was examined. The transmembrane and cytoplasmic regions of IL-12Rβ1 or IL-12Rβ2 were fused to the extracellular domain of the epidermal growth factor (EGF) receptor, yielding chimeric receptors E12R1 and E12R2, respectively. These chimeras were stably transfected into BaF3 cells, a factor-dependent murine pro-B cell line. Only E12R2 or E12R1+E12R2 transfectants were capable of EGF-dependent proliferation. EGF-dependent phosphorylation of E12R2, JAK2, Tyk2, and STAT3 was observed. JAK2 was phosphorylated in E12R1-, E12R2-, and E12R1+E12R2-expressing cells. However, direct associations were detectable only between E12R2 and JAK2. Tyk2 phosphorylation was observed only in cells expressing E12R1 or E12R1+E12R2. In parallel with this activation pattern, direct interactions only between Tyk2 and E12R1 were demonstrable. Phosphorylation of STAT3 was observed in cells expressing E12R1, E12R2, and E12R1+E12R2. The expression levels of STAT4 protein in BaF3 cells are undetectable by the methods employed here; therefore, STAT4 phosphorylation was not observed. Taken together, the data indicate that differential interactions take place between the cytoplasmic regions of the two IL-12R subunits and JAK2/Tyk2 and that the cytoplasmic region of IL-12Rβ2 alone is capable of delivering a proliferative signal.

Interleukin-12 (IL-12) is a 75-kDa heterodimeric cytokine composed of two disulfide-bonded subunits, p35 and p40 (1, 2). IL-12 is primarily produced by macrophages and dendritic cells. It has pleiotropic effects on T and natural killer cells, including the induction of interferon-γ secretion, the stimulation of cell proliferation, and the promotion of a Th1-type response (3).

IL-12 manifests its biological functions through interaction with cell-surface IL-12 receptors (IL-12R). Three classes of IL-12-binding sites were identified on the surface of PHA-activated T lymphoblasts and the human T cell line Kit225/K6 (4, 5): high affinity (Kd = 5–20 pM), intermediate affinity (Kd = 50–200 pM), and low affinity (Kd = 2–6 nM). The cloning of a cDNA encoding a human IL-12R subunit that belongs to the gp130 subgroup of the cytokine receptor superfamily (IL-12Rβ1; see below) has been reported (6). However, when expressed in COS cells, IL-12Rβ1 binds IL-12 with only low affinity (Kd = 2–5 nM). We have recently cloned the cDNA for a second IL-12R subunit (7). This newly identified IL-12R subunit is also strongly related to gp130. These IL-12R subunits have therefore been classified as IL-12Rβ1 and IL-12Rβ2 (7). Similar to IL-12Rβ1, IL-12Rβ2 binds IL-12 with only low affinity when transfected into COS cells. However, coexpression of β1 and β2 subunits in COS cells gives rise to both high and low affinity IL-12-binding sites and a receptor complex capable of signaling (7).

The signal transduction pathways utilized by IL-12 have not been fully characterized. Previous studies indicate that IL-12R can signal through the JAK/STAT signaling pathways. IL-12 induces tyrosine phosphorylation of JAK2 and Tyk2 in PHA-activated T lymphocytes (8). It has been reported that IL-12 induces tyrosine phosphorylation of JAK2 and Tyk2 in PHA-activated T lymphocytes (8). Previous reports suggest that IL-12 signaling may involve more than one pathway since IL-12 was shown to induce tyrosine phosphorylation of Lck in natural killer cells (11) and of the 44-kDa mitogen-activated protein kinase in T cells (12). Using EGF/IL-12 chimeras, we now report an initial analysis of the interactions between the cytoplasmic regions of the two known IL-12R subunits and molecules belonging to the JAK/STAT signaling pathways.

Materials and Methods

Reagents—Recombinant human EGF; polyclonal rabbit antisera against JAK1, JAK2, and JAK3; and mouse monoclonal antibodies against phosphorytrosine (clone 4G10) and against human EGF (clone LA22) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal rabbit IgG preparations directed against human and mouse Tyk2, STAT1, STAT2, STAT3, and STAT4 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse mAb 13A9 specific for human EGF (13) and the EGF cDNA were the generous gifts of Brian Fendly (Genentech, South San Francisco, CA). Recombinant IL-12 was supplied by Dr. A. Stern (Hoffmann-La Roche).

Cell Lines—The BaF3 cell line, an IL-3-dependent murine pro-B cell line, has been described (14). Transfection of BaF3 cells was performed by electroporation (15) with linearized plasmids (800 μg of the plasmid encoding the EGF/IL-12 chimera and 4 μg of the plasmid encoding a puromycin resistance marker (16). Transfected cells were selected by culture in medium containing either 3 μg/ml puromycin or 10 ng/ml EGF. Transfections of the COS-7 cell line were performed as described previously (17).

Construction of Plasmids Encoding EGF/IL-12 Chimeras—A polymerase chain reaction-based overlap extension technique (18) and Pfu polymerase (Stratagene) were used for this approach. A cDNA encoding the 645-amino acid-long extracellular domain of EGF was fused in frame to cDNA fragments encoding the transmembrane and cytoplasmic domains of either human IL-12Rβ1 (122 amino acids; see Ref. 6) or human IL-12Rβ2 (216 amino acids; see Ref. 7) to yield the chimeric receptors E12R1 (767 amino acids) and E12R2 (861 amino acids), respectively. The chimeric DNAs were subcloned into the expression vector pCDNA3 (Invitrogen).
vector pEF-BOS (19), and the sequences were confirmed by DNA sequencing.

**Proliferation Assays**—A conventional [3H]thymidine incorporation assay was performed as described (20). Cells were incubated for 48 h with various concentrations of EGF before an 8-h thymidine pulse was performed. All samples were assayed in triplicate.

**Detection of EGFR/IL-12R Chimeras Expressed on BaF3 Cells by Flow Cytometry**—A modification of a previously described method (4) was employed. Briefly, 5 × 10^5 cells were washed twice with fluorescence-activated cell sorting buffer (phosphate-buffered saline, 3% fetal calf serum, and 0.01% NaN₃) and incubated on ice for 1 h with 5 μg/ml mouse mAb 13A9. After a wash step, the cells were incubated with a 200 μg/ml concentration of an R-phychorytin-conjugated goat anti-mouse IgG (Cappel, Durham, NC) for 30 min on ice before the flow cytometric analysis was carried out using a FACSort instrument (Becton Dickinson Advanced Cellular Biology, San Jose, CA).

**Immunoprecipitation and Immunoblotting**—Cells (10⁶ BaF3 transfected or PHA-activated lymphoblasts/100 ml) were washed in acidified RPMI 1640 medium (pH 6.4) and incubated overnight in RPMI 1640 medium containing 1% fetal calf serum as described (8). The cells were then resuspended in 10 ml of RPM 1640 medium and 1% fetal calf serum and stimulated with 100 ng/ml EGF or 10⁵ units/ml IL-12 for 10 min at 37 °C. Subsequently, the BaF3 transfected cells were washed with cold phosphate-buffered saline containing 2 mM NaVO₄ and then lysed in 1 ml of lysis buffer (1% Triton X-100 (or 1% Brij 96 for the analysis of phosphorylation between JAK kinases and IL-12R), 50 mM KCl, 50 mM Tris (pH 8), 4 mM EDTA, 10 mM Na₃PO₄, 2 mM Na₄P₂O₇, 100 mM NaF, 0.02% NaN₃, 0.1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin A). For experiments shown in Fig. 5, an additional chemical cross-linking step was introduced. Cells were resuspended at 5 × 10⁶ cells/ml in cold phosphate-buffered saline (pH 8.3) and 1 mM MgCl₂, 10 mM EDTA, 100 mM NaCl, 50 mM Tris (pH 7.5) and 5 mM EDTA. The cells were then lysed as described above. Clarified lysates were incubated with the specific antibodies and protein G-coupled Sepharose beads (Pharmacia Biotech Inc.). The immunoprecipitates were fractionated on 6 or 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes.

Probing of membranes with anti-phosphotyrosine mAb 4G10 was performed using a modification of a previously described procedure (21), enhanced chemiluminescence (ECL, Amersham Corp.) was used as the detection system for all the immunoblots.

**RESULTS AND DISCUSSION**

**Construction and Expression of EGFR/IL-12R Chimeras**—To study the functional role of the cytoplasmic domains of IL-12R subunits β1 and β2, we constructed two chimeric receptors in which the extracellular domain of EGFR was fused to the transmembrane and cytoplasmic domains of either IL-12R1 or IL-12R2. EGF is expected to dimerize the chimeric receptors, thereby triggering an intracellular IL-12-specific signal. The chimeric receptors were termed E12R1 and E12R2 and can be detected using anti-EGFR antibodies. E12R1 is composed of 767 amino acids (predicted molecular mass of ~84 kDa), and E12R2 is composed of 861 amino acids (predicted molecular mass of ~95 kDa). As shown in Fig. 1, the receptors, when expressed in COS cells, had apparent molecular masses of ~140 kDa (E12R1) and ~140 kDa (E12R2). The differences between predicted and observed molecular masses are most likely due to glycosylation. In COS cells, the expression efficiency tended to be higher for E12R1 than for E12R2 (data not shown). Therefore, to equalize expression levels, only one-tenth the amount of E12R1 plasmid was used in the experiment shown in Fig. 1. Fig. 1 also shows that COS cells express an endogenous EGFR with a molecular mass of ~175 kDa (22). The EGFR/IL-12R chimeras were subsequently transfected alone or in combination into the IL-3-dependent pro-B cell line, BaF3. Stable transfecteds were selected either in EGF-supplemented medium or in medium containing puromycin and IL-3. Flow cytometric analysis demonstrated that the chimeric receptors could be detected on the surface of the BaF3 transfecteds, again with different expression efficiencies as previously observed in COS cells (Fig. 2). In cells cotransfected with E12R1+E12R2, the anti-EGFR monoclonal antibody cannot distinguish between the two different chimeric receptors. Flow cytometric analysis did not detect any EGFR expression on the parental BaF3 cells.

**E12R2 Alone Is Sufficient to Transmit a Proliferative Signal in Transfected BaF3 Cells**—Only transfecteds expressing either E12R1+E12R2 or E12R2 were able to maintain long-term growth in medium containing EGF alone, while BaF3 transfected expressing E12R1 by itself were not capable of long-term proliferation under these conditions. After the initial cell selection period, more quantitative cell proliferation assays were performed. These results are shown in Fig. 3. Again, cells expressing either E12R1+E12R2 or E12R2 alone undergo a dose-dependent proliferation in response to EGF, with a very similar EC₅₀. In contrast, transfecteds expressing E12R1 alone respond only very weakly and transiently to EGF, while parental BaF3 cells do not respond to EGF at all. In control experiments, all transfecteds proliferated equally well in the presence of WEHI-3-derived IL-3 (data not shown). The inability of E12R1 to signal a proliferative response in EGF-containing medium was not due to a lack of expression of the chimeric receptors at the cell surface (Fig. 2). Overall, these findings support and extend our earlier results that showed that the wild-type IL-12Rβ2 subunit can indeed signal and sustain long-term IL-12-induced proliferation in the absence of IL-12Rβ1 (7).

**Tyrosine Phosphorylation of Tyk2 Occurs Only in Cells Transfected with E12R1**—It was previously reported that IL-12 induces tyrosine phosphorylation of Jak2 and Tyk2 (8). Thus, the phosphorylation patterns of Jak2 and Tyk2 were examined in the chimera-expressing BaF3 cells. As shown in Fig. 4A, EGFR induced tyrosine phosphorylation of Jak2 in all the transfecteds (E12R1, E12R2, and E12R1+E12R2). In contrast, Tyk2 was phosphorylated only in cells expressing E12R1 or E12R1+E12R2 and not in cells expressing E12R2 alone (Fig. 4C). The amounts of Jak2 or Tyk2 were roughly equivalent in the transfecteds analyzed. These findings therefore suggest that Tyk2 interacts directly with E12R1 and not with E12R2. It is tempting to speculate that because E12R1 cells do not proliferate, Tyk2 activation might not be necessary for a proliferative signal. However, additional data will be needed to confirm this hypothesis.

In control experiments, tyrosine phosphorylation of both Tyk2 and Jak2 were induced by IL-12 in human PHA-activated lymphoblasts as expected (Fig. 4, B and D). In agreement with
a previous report (8), tyrosine phosphorylation of JAK1 or JAK3 was not induced either in IL-12-treated PHA-activated lymphoblasts or in EGF-treated E12R1 or E12R2 transfectants (data not shown).

Specific Interactions between E12R1 and Tyk2 and between E12R2 and JAK2 Are Detectable—We next set out to determine if the observed phosphorylation patterns for JAK2 and Tyk2 were the results of direct receptor subunit/kinase interactions, using coimmunoprecipitation techniques and lysates from the BaF3 cells that had been transfected with either E12R1 or E12R2 alone. To increase the likelihood of detecting receptor-kinase complexes, cell lysates were cross-linked with dithio-bis(succinimidyl propionate) prior to the immunoprecipitations. We first examined the proteins associated with E12R1. E12R1 protein was immunoprecipitated with anti-EGFR mAb and subjected to immunoblotting. Blots were probed with antibodies against EGFR, phosphotyrosine, Tyk2, and JAK2. As expected, tyrosine phosphorylation of E12R1 protein (~125 kDa) was present in BaF3 transfectants whether or not the cells had been stimulated with EGF. However, a protein corresponding in size to Tyk2 was strongly tyrosine-phosphorylated when E12R1 transfectants were stimulated with EGF (fourth lane). Bands of equal intensity in samples prepared with or without EGF stimulation were detected using anti-Tyk2 antibodies (fifth and sixth lanes). JAK2 protein was not detected (seventh and eighth lanes), even though EGF-dependent phosphorylation of JAK2 had been observed previously (Fig. 4). Overall, the results suggest that Tyk2 was associated with E12R1 prior to EGF stimulation and became tyrosine-phosphorylated upon stimulation with EGF. The failure to detect any E12R1/JAK2 associations could be because (i) the amounts of JAK2 are too small to detect or (ii) the interactions between E12R1 and JAK2 are too weak or too short-lived to allow cross-linking and coimmunoprecipitation. It is also conceivable that a third, as yet unidentified signaling component acts as an intermediate between JAK2 and E12R1.

We next examined the association between JAK2/Tyk2 and E12R2 in transfectants expressing E12R2 (Fig. 5B). Consistent with the flow cytometric results (Fig. 2), expression of E12R2 was barely detectable when probing blots with the anti-EGFR antibody (Fig. 5B, first and second lanes). However, the phosphotyrosine blot showed ligand-dependent phosphorylation of E12R2 (third and fourth lanes). We can rule out that the slowest migrating band in the fourth lane is phosphorylated Tyk2 because Tyk2 is not activated in E12R2 cells (Fig. 4C, third and fourth lanes). A protein corresponding in size to JAK2 was tyrosine-phosphorylated when cells were stimulated with EGF (Fig. 5B, fourth lane). The amounts of JAK2 associated with E12R2 appear to be equivalent in lysates from cells with or without EGF stimulation (fifth and sixth lanes). Reprobing
The failure to detect Tyk2 is in agreement with the observed absence of phosphorylation of Tyk2 in cells expressing E12R2 and becomes phosphorylated upon ligand stimulation. The failure to detect Tyk2 is in agreement with the expected absence of phosphorylation of Tyk2 in cells expressing E12R2 (Fig. 4).

The results suggest that JAK2 preassociates with E12R2 and becomes phosphorylated upon ligand stimulation. The failure to detect Tyk2 is in agreement with the observed absence of phosphorylation of Tyk2 in cells expressing E12R2 (Fig. 4C).

To compare these findings with the IL-12R complex naturally expressed on PHA-activated lymphoblasts, we used the same techniques and the 2B10 antibody that reacts with IL-12Rb1, when fused to the extracellular domain of EGFR, can signal sustained proliferation. The situation is identical to that observed for the EGFR/IL-12R complex.

The use of this receptor subunit-specific antibody allows us to conclude that both JAK2 and Tyk2 are preassociated with the IL-12R complex. However, since the IL-12R complex is composed of both IL-12Rb1 and IL-12Rb2 and was cross-linked prior to the immunoprecipitations, this experiment does not distinguish among the various subunit/kinase interactions.

Only Phosphorylation of STAT3 Is Detectable in the BaF3 Transfectants—Both STAT3 and STAT4 have been shown to be part of the IL-12 signaling pathway. Interestingly, IL-12 is so far the only cytokine that has been reported to induce tyrosine phosphorylation of STAT4 (10, 24), suggesting a specific role of STAT4 in IL-12 signaling (9, 10). We therefore examined tyrosine phosphorylation of STAT1–STAT4 in the BaF3 transfectants. Cell lysates were immunoprecipitated with antibodies against STAT1–STAT4, and the resulting blots were probed with anti-phosphotyrosine antibodies. The phosphorylation patterns detected for STAT3 are shown in Fig. 6. STAT3 phosphorylation is strictly dependent on ligand stimulation in BaF3 transfectants expressing E12R1, E12R2, or E12R1+E12R2. Tyrosine phosphorylation of STAT1, STAT2, and STAT4 was not detected (data not shown). Thus, the activation patterns observed for JAK2 and STAT3 appear indistinguishable for E12R1-, E12R2-, or E12R1+E12R2-expressing cells, despite the fact that only cells containing E12R2 are capable of long-term proliferation in response to EGF. The crucial importance of STAT4 in IL-12-induced signaling of proliferation was recently highlighted (25, 26). Expression of STAT4 protein and mRNA was thus analyzed in more detail. Levels of expressed STAT4 protein appear to be very low in BaF3 cells, as they were undetectable by our standard immunoblotting procedures. In control experiments, the same antibody easily detected levels of STAT4 protein in lysates from PHA-activated lymphoblasts. In contrast to the Western blotting results, however, reverse transcription-polymerase chain reaction analysis did detect STAT4 mRNA in the parental BaF3 cell line (data not shown). Therefore, the inability to detect an interaction between phosphorylated STAT4 and the receptor subunits is likely due to these very low levels of STAT4 protein present in BaF3 cells. The interactions of these “catalytic” residues of STAT4 with the E12R2 chimera must still be sufficient to transmit a proliferative signal. It should also be pointed out that the participation of non-JAK/STAT components in the transmission of this signal cannot be excluded at this point.

It is interesting to note that in E12R1-expressing cells, STAT3 is phosphorylated upon EGF stimulation, even though the cytoplasmic region of E12R1 does not contain any tyrosine residues. Similarly, an IL-4R mutant devoid of cytoplasmic tyrosines has been described that is able to activate receptor-associated JAK kinases and still transmit a proliferative signal (27). Therefore, the current thinking that such tyrosine residues serve as docking sites for STAT proteins that are in turn activated by receptor-associated kinases may have to be reconsidered.

In conclusion, we have shown the following. (i) The cytoplasmic region of only IL-12Rb2, but not IL-12Rb1, when fused to the extracellular domain of EGFR, can signal sustained proliferation. This situation is identical to that observed for the wild-type IL-12Rb1 and IL-12Rb2 subunits. (ii) Only the cytoplasmic region of IL-12Rb2 is phosphorylated upon ligand binding. (iii) Ligand stimulation gives rise to phosphorylation of STAT3 and analyzed by immunoblotting. Blots were probed with an antibody specific for phosphotyrosine (upper panel). Afterwards, the blots were stripped and reprobed with an antibody specific for STAT3 (lower panel).
of Tyk2 in the presence of the IL-12Rβ1 cytoplasmic region only. In contrast, JAK2 is phosphorylated in the presence of either IL-12Rβ1- or IL-12Rβ2-derived cytoplasmic tails. (iv) Consistent with these phosphorylation patterns, we can detect specific and differential interactions between E12R1 and Tyk2 and between E12R2 and JAK2. Direct interactions between E12R1 and JAK2, however, could not be detected. These results should lead the way to further clarification of what components of the IL-12 receptor complex are absolutely required for IL-12-mediated signaling and what other signaling pathways may be involved.

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