HEMATOPOIESIS

Heterodimerization of the α and β Chains of the Interleukin-3 (IL-3) Receptor Is Necessary and Sufficient for IL-3–Induced Mitogenesis

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The high-affinity receptor for interleukin-3 (IL-3) is a complex of the IL-3–binding subunit (αIL-3) and a larger β chain—βc, or, in the mouse, β1, or its close relative βIL-3. There is evidence that the critical event that initiates signaling is not the approximation of the cytoplasmic domains of αIL-3 and βIL-3, but rather, the formation of a β-β homodimer. Many of these studies involved the analyses of receptor chimeras where the cytoplasmic domains were derived from αIL-3, βc, or βIL-3, and the extracellular domains were derived from other cytokine receptors, such as the erythropoietin receptor (EpoR). However, evidence that the EpoR may also associate with other receptors clouds the interpretation of these experiments. Therefore, we reevaluated the structure of the functional IL-3R using chimeric receptors with extracellular domains derived not from members of the cytokine-receptor family, but from CD8 or CD16. We show, by expression of these chimeras in Ba/F3 or CTLL-2 cells, that mitogenic signals were only generated by heterodimerization of the cytoplasmic domains of αIL-3 and βIL-3. Homodimers of either αIL-3 or βIL-3 alone or in combination, were nonfunctional. Furthermore, the ability of heterodimers to stimulate mitogenesis correlated with their ability to induce tyrosine phosphorylation of JAK-2. These data suggest that the physiological activation of the IL-3R involves the generation of simple heterodimers of αIL-3 and βIL-3.

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THE CYTOKINE-RECEPTOR FAMILY can be divided into those which are homodimers of a single subunit (eg, receptors for growth hormone and erythropoietin [Epo]), and those which are heterodimers or higher-order oligomers made up of 2 or more different subunits (eg, interleukin-4 [IL-4] and IL-6). The receptors for IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) belong to the latter group. Each of these cytokines binds specifically with low or moderate affinity to a unique α chain termed, respectively, IL-3Rα, IL-5Rα, and GM-CSFRα. These complexes of the ligand and its respective α chain each bind with high affinity to a common beta chain (βc), which is required for activation of intracellular signals. In the mouse, while IL-3Rα, IL-5Rα, and GM-CSFRα and their ligands interact with the murine homolog of βc complexes of IL-3 and IL-3Rα can also form a functional IL-3 receptor by interacting with an IL-3–specific β chain (βIL-3) that is the product of a relatively recent duplication of βc.

Activation of JAKs follows rapidly after binding of cytokines to their receptors, and is essential for the initiation of intracellular signaling. Moreover, in some artificial systems in which the cytoplasmic domain of a transmembrane protein was replaced by JAK2, the induction of dimerization of the membrane-associated JAK was sufficient to deliver a proliferative signal. These observations suggest that ligand-induced dimerization or oligomerization of cytokine receptor subunits triggers signaling by bringing together JAK molecules associated with the receptor subunits, and allowing trans-phosphorylation and activation of JAKs.

Experiments on several of the multi-subunit cytokine receptors have suggested that dimerization of their respective long chains which, like βc, have multiple potential docking sites for signaling molecules, is sufficient for the initiation of a proliferative signal. Thus, it has been shown that homodimerization of gp130, the IL-2Rβ, the IL-4Rα, or the IL-4Rα are sufficient for the initiation of biochemical and biological events. These observations are consistent with the paradigm of the IL-6R where the function of the ligand-binding (α) chain is merely to induce dimerization of the β chain, which mediates all the intracellular signaling functions. However, because these observations have all involved overexpression of the receptor chains in cell lines, it is by no means clear that they accurately reflect physiological signaling processes. The situation in normal cells with smaller numbers of receptors and different levels of signal transduction molecules may be significantly different. In the case of IL-3 (or the closely related IL-5 or GM-CSF), it is unclear whether signaling is mediated by simple heterodimerization of the αIL-3 and βIL-3 chains, implying an active role for αIL-3, or alternatively, by analogy with the receptor for IL-6, is mediated by homodimerization of the β chain. Truncations of the relatively short cytoplasmic domains of the IL-3Rα, IL-5Rα, and GM-CSFRα do not affect ligand binding, but, in contrast with results with the IL-6Rα chain, abolish signaling and (and P.C. Orban, K.B. Leslie, unpublished data). Further, Stomski et al reported finding disulfide-linked heterodimers of human αIL-3 and βc after binding of IL-3. However, there is other evidence that βc, homodimers might be both necessary and sufficient for transduction of the mitogenic signal of IL-3. The βc exhibits extensive homology with the Epo receptor (EpoR), which forms a homodimer in the active state. Observations that a chimera composed of the extracellular and transmembrane portion of the EpoR and the cytoplasmic portion of the mouse βIL-3 chain (EpoR/βIL-3) conferred responsiveness to Epo in murine IL-3–dependent cell lines have been interpreted as showing that dimerization of the β chain was sufficient for signaling. Other evidence has come...
from the demonstration of the existence of “preformed” dimers of the βc in cell lines, as well as in primary leukemia cells.

However, a simple homodimer of the EpoR may not constitute a functional receptor, and may include additional subunits such as the steel ligand factor receptor (c-kit) and βc. Indeed, when cells are stimulated with Epo, βc undergoes tyrosine phosphorylation and is coimmunoprecipitated with the EpoR. Moreover, when expressed in the T-cell line CTLL-2, which does not express βc, neither Epo nor a chimeric receptor consisting of the extracellular and transmembrane portions of the EpoR together with the cytoplasmic portion of βc supported ligand-induced proliferation. In contrast, however, when the αIL-3 and βc were transfected into CTLL-2 cells, they were capable of transducing a ligand-induced mitogenic signal.

To avoid the confounding possibility that the extracellular component of a cytokine receptor chimera could qualitatively influence biochemical signaling, perhaps by recruiting additional receptor subunits to the receptor complex, we generated chimeras in which the cytoplasmic domains of mouse αIL-3 and βc were fused to extracellular domains derived from proteins that were unrelated to cytokine receptors.

MATERIALS AND METHODS

Plasmids. DNA manipulations were performed by standard methods. The plasmids pStu1 and pCD226 containing the mouse αIL-3 and mouse βc cDNAs, respectively, were the kind gift of Atsushi Miyajima (University of Tokyo, Tokyo, Japan). The plasmid pCD16tm7.syk was the kind gift of Brian Seed (Massachusetts General Hospital, Boston, MA), and the plasmid pMV7Fl.2, containing the pSut1 by removing the inserted cDNA. Polymerase-mediated amplification and introduce an EcoRI site to the 3' end of the EpoR. EpoR cytoplasmic domains.

Cell culture. Ba/F3 cells and transfected clones were maintained in RPMI 1640 medium (Stem Cell Technologies, Vancouver, BC, Canada), supplemented with 10% fetal calf serum (FCS) (Intergen, Purchase, NY), 50 µmol/L 2-mercaptoethanol, and 2% (vol/vol) 10X concentrated RPMI 1640 medium (Stem Cell Technologies, Vancouver, BC, Canada), supplemented with 10% FCS (Intergen, Purchase, NY), 50 µmol/L 2-mercaptoethanol, and 3% (vol/vol) IL-3-containing conditioned media derived from AgX063 cells transfected with the murine Eil-2 cDNA.

Transfections and screening of protein expression. Ba/F3 cells were transfected twice in phosphate-buffered saline (PBS) and 1 × 10⁶ cells were resuspended in 800 µL of transfection buffer (25 mMol/L HEPES, 0.75 mMol/L Na₂HPO₄, 140 mMol/L KCl, 5 mMol/L NaCl, 2 mMol/L MgCl₂, 0.5% Ficoll 400 [Pharmacia Biotech, Uppsala, Sweden]). For each transfection, cells were mixed with 1 µg of linearized pPGKNeo or pPGKPro with 15 µg of the linearized expression vector of interest, and subjected to electroporation using a Bio-Rad gene pulser (Bio-Rad, Mississauga, Ontario, Canada) at 960 µF and 270 V. After transfection, the cells were cultured in the appropriate media for 48 hours and then transferred to selection media in 96-well plates. Individual clones of neomycin- or puromycin-resistant clones were tested for expression of the appropriate receptor chain by flow cytometry. Either OKT8 (American Type Culture Collection [ATCC], Rockville, MD) or Leu2A (Becton Dickinson, Mississauga, Ontario, Canada) were used to detect cell-surface expression of human CD8α, 3G8 (Cedar Lane, Hornby, Ontario, Canada) was used to detect cell-surface expression of human CD16. 9D3 (Alice Mui, DNAX, Palo Alto, CA) was used to detect expression of βc. Rabbit polyclonal antiserum raised against the extracellular domain of IL-3Rα was used to detect expression of the IL-3Rα.

Cell proliferation assays. Proliferation of cells was assessed by either [3H]-thymidine incorporation into de novo synthesized DNA, or 3-(3-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) incorporation. Cells were washed 3 times with HEPES-buffered saline solution (HBSS) supplemented with 2% (vol/vol) FCS. Cells were plated at 1,000 cells/well in a Terasaki microtiter plate (Disposable Products, Adelaide, SA, Australia) for [3H]-thymidine incorporation or at 10,000 cells/well in a 96-well plate for MTT assays. Chemically

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synthesized IL-3 (Ian Clark-Lewis, The Biomedical Research Centre, Vancouver, BC, Canada) was used to assess maximal proliferation of Ba/F3 cells. Recombinant murine IL-2 (Genzyme, Cambridge, MA) was used to assess maximal proliferation of CTLL-2 cells. Recombinant human Epo was a kind gift from Dr Ross Hardison (Pennsylvania State University, State College, PA). For use in bioassays, the 3G8 (anti-CD16) antibodies were dialyzed against RPMI to remove azide and were added in serial dilutions to cells. Cells were incubated at 37°C for 40 hours, and then pulsed for a further 8 hours with either [3 H]-thymidine at a final concentration of 15 μCi/mL, or 0.75 μg/mL MTT (Sigma, Oakville, Ontario, Canada). In the [3 H]-thymidine uptake assays, cells were harvested onto filters and counted in a scintillation counter. In MTT assays, the reaction product was solubilized by addition of 100 μL of 10% sodium dodecyl sulfate (SDS), 50% dimethyl formamide (DMF), pH 4.5, and the plates were read at OD550 using a BioTek plate reader (BioTek, Winooski, VT).

**Cell-surface biotinylation.** Cells were washed twice in HBSS and resuspended at 2 × 10^7 cells/mL in HBSS supplemented with 0.8 mg/mL Sulfo-NHS-Biotin (Pierce, Rockford, IL). After 15 minutes on ice, the cells were washed 3 times with 50 mL of HBSS supplemented with 10% FCS to quench the biotinylation reaction, followed by 1 wash in HBSS. The cells were lysed in lysis buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, 0.7 μg/mL pepstatin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor). Before specific immunoprecipitation, lysates were “precleared” by absorption with protein A-Sepharose (Pharmacia, Uppsala, Sweden). Chimeric receptors with the extracellular domain of human CD8α were immunoprecipitated with OKT8, followed by adsorption onto protein A-Sepharose. Beads were washed 3 times with lysis buffer, boiled in SDS sample buffer, and the eluate was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The surface-biotinylated material precipitated with anti-CD8 was detected by immunoblotting with streptavidin-conjugated horseradish peroxidase (HRP; Calbiochem, La Jolla, CA).

**Cell stimulations.** To analyze the biochemical effects of using anti-CD16 antibodies to generate homodimers or heterodimers of the cytoplasmic domains of the IL-3Rα or β, the respective transfectants were placed in RPMI, 10% FCS, 0.2% WEHI-3B for 16 hours, washed 3 times with serum-free RPMI supplemented with 20 mmol/L HEPES (SFN), and incubated in SFM at 1 × 10^7 cells/mL at 37°C for 1 hour. The cells were then left untreated as a control or stimulated with synthetic IL-3 (10 μg/mL) for 10 minutes, or anti-CD16 as follows. The monoclonal anti-CD16 (3G8, 1 μg/mL) was allowed to bind to the cells on ice for 15 minutes, after which cells were washed once with SFM, and resuspended in 1 mL of SFM containing 1 μg/mL secondary goat α-mouse Ig (DAKO A/S, Glostrup, Denmark). The cells were then transferred to 37°C for 10 minutes and lysed in lysis buffer supplemented with phosphatase inhibitors (1 mmol/L sodium orthovanadate, 1 mmol/L sodium molybdate, 10 mmol/L sodium fluoride). Lysates were then incubated with antisera against JAK-2 (Upstate Biotechnology Inc, Lake Placid, NY), followed by adsorption onto protein A-Sepharose. Beads were washed and boiled as above, and the eluates were subjected to SDS-PAGE and immunoblotting with 4G10 (Upstate Biotechnology Inc) to detect tyrosine-phosphorylated JAK-2 and, after stripping, with antisera to JAK-2 to assess equivalency of loading.

**RESULTS**

**Epo-mediated homodimerization of βIL-3 leads to mitogenesis in Ba/F3 cells.** Given that the discrepancies in the literature about the function of homodimers of the cytoplasmic domain of βIL-3 may relate to differences between clones of cell lines, we first repeated the experiments of expressing full-length EpoR or the EpoR/βIL-3 chimera using our line of IL-3–dependent Ba/F3 cells and IL-2–dependent CTLL-2 cells (see Fig 1 for schematic diagrams of all chimeric receptors used in this work). Like others,38,39 we observed that the EpoR/βIL-3 chimera was capable of transducing a proliferative response to Epo in Ba/F3 cells (Fig 2). We noted, however, that untransfected Ba/F3 cells displayed some small but reproducible response to higher concentrations of Epo, consistent with the observations of Damen et al,42 and suggesting that Ba/F3 cells express some endogenous EpoR. Expression of the R129C mutant of the EpoR in Ba/F3 cells, which is expressed as a homodimer by virtue of a Cys-Cys bond in the extracellular region,26 also conferred factor independence to Ba/F3 cells (data not shown), as reported by others.16,26 In contrast, when these various constructs were expressed in IL-2–dependent CTLL-2 cells, which do not express any of the receptor chains known to be involved in Epo or IL-3 signaling, we failed to obtain clones that responded to Epo; nor, in the case of the R129C mutant, did we obtain factor-independent CTLL-2 transfectants (data not shown). These differences between Ba/F3 and CTLL-2 cells are consistent with previous observations.16,28,38

**Homodimers of βIL-3 fail to stimulate mitogenesis in CTLL-2 cells.** One approach to determining whether the generation of simple dimers of the βIL-3 is sufficient to generate signals is to investigate the activity of dimers of the cytoplasmic portion of βIL-3 formed when IL-3 brings together βIL-3 and a chimera of the extracellular portion of αIL-3 and the cytoplasmic domain of βIL-3. Clones of CTLL-2 cells were derived that expressed either full-length αIL-3 alone, full-length βIL-3 alone, both full-length chains together, or the full-length βIL-3 and the chimeric αIL-3/βIL-3 together. Expression of the exogenous receptor chains was assessed by flow cytometry (Fig 3). As shown in Table 1, CTLL-2 clones that expressed both full-length αIL-3 and βIL-3 proliferated in response to IL-3, whereas clones that...
THE IL-3 RECEPTOR IS A HETERODIMER

Fig 2. Ba/F3 cells transfected with the full-length EpoR or the EpoR/βb chimera proliferate in response to Epo. Cells were washed free of IL-3 and incubated with indicated concentrations of Epo. After 40 hours, the cells were pulsed with MTT for a further 4 hours, and MTT reduction was measured as optical density at 550 nmol/L (OD550) of solubilized cells. Data are plotted as the percentages of the maximal response of cells cultured in IL-3. Error bars represent SEM of triplicate samples. Similar results were obtained with several individual clones.

expressed either chain alone, the αIL-3/βIL-3 chimera alone, or the full-length βIL-3 together with the αIL-3/βIL-3 chimera, showed no growth in response to IL-3. These data suggested that simple dimers of the cytoplasmic domain of βIL-3 were not sufficient for the generation of mitogenic signals, at least in these cells.

Constitutive heterodimers of αIL-3 and βIL-3, but not homodimers of βIL-3, stimulate mitogenesis in Ba/F3 and CTLL-2 cells. Given that the use of extracellular domains of growth factor receptors in such chimeric proteins might be difficult to interpret because of the possibility of the recruitment of cytokine-receptor family subunits,4,14,16,37,39,41 we next generated chimeras in which the extracellular regions of an unrelated cell-surface molecule, human CD8α,42,43 were fused with the transmembrane and cytoplasmic portions of the respective IL-3 and Epo receptor chains (see Fig 1). Human CD8α has been shown to spontaneously form disulfide-linked dimers at the cell surface.45 Chimeric receptor chains—CD8/EpoR, CD8/αIL-3, and CD8/βIL-3—were each expressed in Ba/F3 cells, as confirmed by flow cytometric analysis (Fig 4A). In addition, we derived Ba/F3 clones that coexpressed both the CD8/αIL-3 and CD8/βIL-3 chimeras. To confirm expression of both of these chimeras in the same clone, we biotinylated the cell surface, lysed the cells, and immunoprecipitated the chimeric proteins with an antibody against CD8. The presence of the biotinylated chimeras in the anti-CD8 immunoprecipitates was assessed by SDS-PAGE and blotting with streptavidin-conjugated peroxidase. As shown in Fig 4B, biotinylated molecules corresponding to the predicted sizes of both chimeras were present.

Ba/F3 cells expressing the CD8/EpoR chimera grew independently of exogenous IL-3 or Epo (Fig 4C). This was despite the low levels of expression of the chimeric CD8/EpoR protein as judged by our inability to detect its expression by flow cytometry (Fig 4A). This observation suggests that the CD8 domains formed disulfide-linked dimers as predicted, and that the generation of simple dimers of the EpoR cytoplasmic domain in these cells was sufficient for their proliferation and survival. In contrast, neither the Ba/F3 cells expressing CD8/αIL-3, nor those expressing CD8/βIL-3 alone, were capable of factor-independent growth, though these clones continued to respond normally to IL-3. On the other hand, all clones that expressed both the CD8/αIL-3 and CD8/βIL-3 chimeras showed factor-independent growth. Clones expressing both chimeric receptors were obtained by transfecting a series of clones that expressed one chimeric chain, and were thus not factor-independent with the second CD8 chimera. Other clones were generated by cotransfection of parental cells with both chimeric chains simultaneously. All clones expressing one CD8 chimera could be rendered factor-independent by expression of the alternate CD8 chimera. This result also showed that, while the

Table 1. CTLL-2 Cells Expressing βIL-3 and the αIL-3/βIL-3 Chimera Fail to Survive in the Presence of IL-3

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Viability in IL-2</th>
<th>Viability in IL-3</th>
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<tbody>
<tr>
<td>CTLL-2</td>
<td>+</td>
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<tr>
<td>αIL-3</td>
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Cells were cultured in IL-2 or IL-3 in triplicate for 4 days and were scored for viability by light microscopy. + represents >99% viable cells and – represents the absence of any viable cells. In no cases were any intermediate responses observed.

Fig 3. Flow cytometric analyses of expression of chimeric receptors. CTLL-2 cells expressing both αIL-3 and βIL-3 (top), or αIL-3 and βIL-3 (bottom) were stained with antibodies against the extracellular domain of αIL-3 or βIL-3. Thin lines represent results with cells treated with fluorescein isothiocyanate (FITC)-coupled secondary antibody alone; thick lines represent results with cells incubated with anti-receptor antibodies and then FITC-coupled secondary antibody. CTLL-2 cells bearing αIL-3 alone or βIL-3 alone displayed levels of expression similar to those shown for CTLL αIL-3 + βIL-3.
clones expressing the individual CD8 chimeras remained factor-dependent, they were nonetheless fully capable of factor-independent growth when transfected with both chimeras.

These experiments with CD8/αIL-3 and CD8/βIL-3 chimeras were repeated in CTLL-2 cells with identical results (Fig 5A through C). These data clearly indicate that simple dimerization of βIL-3, at least when expressed at the levels we observed, is not sufficient to promote survival or mitogenesis in either cell line. In contrast, both cell types reproducibly exhibited factor-independent growth when the cytoplasmic domains of αIL-3 and βIL-3 were heterodimerized. These results suggest that heterodimers of αIL-3 and βIL-3 cytoplasmic domains are significantly more efficient in initiating signaling than dimers of βIL-3.

Inducible heterodimers of αIL-3 and βIL-3, but not homodimers of βIL-3, stimulate mitogenesis in Ba/F3 cells. These data showed a requirement for heterodimerization of the cytoplasmic
domains of αIL-3 and βIL-3 and contradicted a body of evidence suggesting that the dimerization of the cytoplasmic domain of βIL-3 is sufficient for mitogenic signaling. Therefore, we investigated this question using another system that involved the use of chimeras consisting of the extracellular portion of the human CD16 molecule, the transmembrane segment of human CD7, and the cytoplasmic portions of either the EpoR, αIL-3, or βIL-3. In this system, the chimeric proteins are expressed as monomers but dimerization can be induced using a monoclonal antibody (MoAb) (3G8) specific for the extracellular domain of CD16. These chimeras were expressed alone or together in Ba/F3 cells, and expression was confirmed by flow cytometry (Fig 6A). Proliferative responsiveness to the MoAb to hCD16 (3G8) was assessed by [3H]-thymidine incorporation (Fig 6C and D). Cells expressing only CD16/7/αIL-3 or only CD16/7/βIL-3 failed to respond to any concentration of the dimerizing antibody. However, in keeping with the results obtained with the CD8 chimeras, those cells that expressed both CD16/7/αIL-3 and CD16/7/βIL-3 exhibited proliferative responses to the dimerizing antibody.

Coincident formation of homodimers of cytoplasmic domains of αIL-3 and βIL-3 is not mitogenic. It was conceivable that the mitogenic responses to the anti-CD16 antibody, observed in cells expressing both CD8/αIL-3 and CD8/βIL-3, reflected a requirement for signals generated by the coincident formation of independent homodimers of αIL-3 or βIL-3 cytoplasmic domains. To investigate this possibility, we derived clones of Ba/F3 cells expressing both CD8/αIL-3 and CD16/7/βIL-3. Expression of both receptor chimeras was confirmed by flow cytometry (Fig 6B). In these cells, CD8/αIL-3 was constitutively dimerized and anti-CD16 antibodies were used to generate homodimers of the cytoplasmic domain of βIL-3. These clones were derived from the same CD8/αIL-3-expressing clones that generated factor-independent subclones when CD8/βIL-3 was also expressed (Fig 4C). However, addition of anti-CD16 did not induce proliferation at any dose tested (Fig 6D).

Mitogenic effects of heterodimers of the cytoplasmic domains of αIL-3 and βIL-3 correlate with tyrosine phosphorylation of JAK-2. Transmission of a proliferative signal through the IL-3 receptor is known to involve the tyrosine phosphorylation and activation of JAK-2. Therefore, we assessed phosphorylation of JAK-2 after antibody-induced dimerization of chimeric receptors in Ba/F3 cells expressing various combinations of CD16/7 chimeras. Clones expressing a single CD16/7 chimera (αIL-3 or βIL-3) were transfected with the alternate CD16/7 chimeras. Dimerization of CD16/7/αIL-3 alone or CD16/7/βIL-3
alone failed to stimulate phosphorylation of JAK-2 (Fig 7).
However, when dimerization of CD16 extracellular domains was
induced in cells expressing both CD16/7/αIL-3 and CD16/7/βIL-3,
phosphorylation of JAK-2 was clearly evident (Fig 7).
These data indicated that homodimerization of the cytoplasmic
domains of either αIL-3 or βIL-3 alone was ineffective in
activating JAK-2, and that only the generation of heterodimers of
the cytoplasmic domains of αIL-3 and βIL-3 resulted in tyrosine
phosphorylation of JAK-2. Furthermore, activation of JAK-2 in
cells expressing the various chimeras correlated with their
ability to proliferate.

DISCUSSION
We have shown that the transduction of mitogenic signals
from the murine IL-3 receptor requires the heterodimerization
of the cytoplasmic domains of the 2 receptor components αIL-3
and βIL-3. Our experiments were performed in murine cells well
characterized with respect to endogenous cytokine receptor
expression, and used murine cytokine receptor components to
minimize the likelihood of confounding molecular interactions.
We obtained qualitatively identical results using two different
means of generating dimers of the αIL-3 and βIL-3 cytoplasmic
domains (Figs 4 and 6). In the case of CD8, dimerization was
achieved via a single constitutive disulfide bond and, in the case
of CD16, through addition of a specific MoAb. The use in these
chimeras of the extracellular domains of CD8 or CD16, rather than
those of a cytokine receptor family member such as the
EpoR, obviated the possibility of recruitment of additional
cytokine receptor subunits, which clouded the interpretation of
previously reported experiments with chimeric receptors in
which the extracellular domains were derived from subunits of
cytokine receptors. Significantly, the same results were obtained
from experiments performed in IL-3–dependent Ba/F3 cells and
in IL-2–dependent CTLL-2 cells (Figs 4 and 5).
We could find no circumstance in which homodimerization of
either the cytoplasmic domains of αIL-3 or βIL-3 resulted in
growth. Thus, neither constitutive dimerization of the βIL-3
cytoplasmic region in a CD8/βIL-3 chimera nor the anti-CD16–
duced dimerization of CD16/7/βIL-3 supported proliferation of
Ba/F3 or CTLL-2 cells (Figs 4C, 5C, and 6D). All of these cells
expressed chimeras that were potentially functional, because
derivatives of these clones transfected with respective CD8/
αIL-3 or CD16/7/αIL-3 chimeras proliferated spontaneously or in
response to anti-CD16 antibody. Likewise, in CTLL-2 cells, the
generation of βIL-3 cytoplasmic domain homodimers by addi-
tion of IL-3 to cells expressing βIL-3 and an αIL-3/βIL-3 chimera
did not result in IL-3–dependent proliferation (Table 1), whereas
all clones we obtained that expressed both βIL-3 and full-length
αIL-3 proliferated in the presence of IL-3. In all cases, het-
erodimerization of the αIL-3 and βIL-3 cytoplasmic domains
resulted in proliferation (Figs 4C, 5C, and 6D). Moreover, the
simultaneous presence in the same cells of homodimers of the
αIL-3 and βIL-3 cytoplasmic domains was insufficient for mitogen-
esis (Fig 6D).
Our findings that coexpression of CD8/αIL-3 and CD8/βIL-3
results in mitogenesis suggest not only that dimerization of the
α and β cytoplasmic domains is required, but also that simple
heterodimers rather than higher-order oligomeric complexes
may be sufficient. Furthermore, dimerization of CD16 receptor
chimeras was induced by addition of an MoAb that can only
bind to 1 epitope on each CD16 monomer. Thus, each divalent
antibody can only link 2 CD16/cytoplasmic domain receptor
chimeras. Our data suggest that all that is required of the
extracellular domains for mitogenic signaling is that they
generate a simple heterodimer. However, we cannot exclude
the possibility that the cytoplasmic domains of α and β het-
erodimers drive the formation of higher-order multimers.
Importantly, we found that the ability of the various clones to
grow correlated with the tyrosine phosphorylation of JAK-2, the
key step in the biochemical events that lead from cytokine
receptor activation to proliferative responses.6,7,47 Thus, cell
lines expressing either 16/7/αIL-3 or 16/7/βIL-3 showed no
detectable JAK-2 phosphorylation when antibody-mediated
homodimerization was induced by anti-CD16. In contrast, when
the complementary CD16/7/α chimera was also expressed in
these lines, addition of anti-CD16 resulted in phosphorylation
of JAK-2 (Fig 7).
Our data on the failure of homodimerized βIL-3 to result in
growth (Table 1, Figs 4C, 5C, and 6D) or biochemical changes
associated with growth (Fig 7) contrast with a series of studies
reporting that the induction of homodimerization of the cytoplas-
mic domain of βIL-3 resulted in proliferation.15,16,28 However,
in all these cases the extracellular domains of the chimeric receptors were derived from cytokine receptors. Our
results and those of others35 show that the EpoR, or chimeras
that include the extracellular domain of EpoR, and the cytoplas-
mic domain of βIL-3 function in Ba/F3 cells (Fig 2) but not in
CTLL-2 cells (data not shown). Together, these data indicate
a requirement for an additional molecule, absent in CTLL-2
cells, for the function of the EpoR. Thus, in Ba/F3 cells, we
observed that the EpoR/βIL-3 chimera gave a mitogenic signal in
the presence of Epo, or when the chimera was constitutively
dimerized by virtue of an EpoR point mutation (Fig 2 and data
not shown).38,31 However, in CTLL-2 cells neither chimera was
functional (data not shown), suggesting that Ba/F3 cells express
an additional protein that was required for the function of
chimeras with EpoR extracellular domains and is not present in
CTLL-2 cells. Therefore, our results support the notion that

Fig 7. Antibody-induced heterodimerization of the cytoplasmic
domains of αIL-3 and βIL-3 induces tyrosine phosphorylation of JAK-2.
Factor- and serum-starved cells were incubated on ice with anti-CD16
antibodies for 10 minutes (α16). Cells were washed once with RPMI,
and after the addition of secondary goat–mouse, the cells were
stimulated at 37°C for 10 minutes. Alternatively, the cells were
stimulated with IL-3 for 10 minutes or left unstimulated as a control
(–). Cell lysates were subjected to immunoprecipitation (IP) with
antibodies against JAK-2, and the eluates were resolved by SDS-
PAGE. The membranes were immunoblotted (IB) with 4G10 (αPY) to
detect phosphorylation on tyrosine.
chimeras of the cytoplasmic domain of β<sub>IL-3</sub> and the extracellular portion of the EpoR are functional only if additional membrane-associated molecules are recruited. As discussed, there is evidence that the activated EpoR interacts functionally with other receptors not present in CTLL-2 cells, including c-kit and β<sub>3</sub>. 34-37

There is one report of homodimerization of β<sub>c</sub> leading to mitogenesis in the likely absence of the recruitment of other subunits of the cytokine receptor family. Patel et al 48 generated chimeras of the cytoplasmic domains of β<sub>c</sub> or GM-CSFβ<sub>c</sub> using as the extracellular domains, the leucine-zipper portions from c-fos and c-jun. In keeping with our observations, they showed that heterodimers of the GM-CSFβ<sub>c</sub> and β<sub>c</sub> cytoplasmic domains delivered the strongest mitogenic signal. However, in contrast to our results, they also observed a weak mitogenic signal from homodimers of the β<sub>c</sub> chain, and a still weaker, but detectable, signal from α<sub>c</sub> chain homodimers.

What, aside from recruitment of other cytokine receptor subunits, might explain the differences between our data, which imply that heterodimers of the cytoplasmic domains of α<sub>a</sub> and β<sub>IL-3</sub> are necessary for transduction of proliferative signals, and those that indicate that homodimers of β<sub>c</sub>, β<sub>IL-3</sub> 28-31 and even α<sub>a</sub> and β<sub>IL-3</sub> 48 can generate signals? The most parsimonious explanation would be that at sufficient levels of expression, in cells that also express at sufficient levels key intracellular signaling proteins, homodimers might generate mitogenic signals, but at lower levels of efficiency than αβ heterodimers. In the simplest model, JAK-2 is associated to the same degree with both α<sub>a</sub> and β<sub>IL-3</sub>, and homodimerization of either α<sub>a</sub> or β<sub>IL-3</sub> could then lead to apposition and trans-activation of JAK-2. The generation of mitogenic signals from the homodimers might depend on unphysiologically high levels of expression present in transfected cell lines, and at physiological levels of expression, only the generation of simple heterodimers of α<sub>a</sub> and β<sub>IL-3</sub> activate signal transduction. It could additionally be postulated that steric considerations, or differences in the affinity of JAK-2 for the different dimers could mean that the relative efficiency of JAK-2 activation decreases in the hierarchy of α<sub>a</sub>β<sub>IL-3</sub> > β<sub>a</sub>β<sub>IL-3</sub> > α<sub>α</sub>β<sub>IL-3</sub>.

Several lines of evidence support an active role for the cytoplasmic domain of α<sub>a</sub> in signal transduction. First, deletion or mutation of the short cytoplasmic tails of the IL-3R, IL-5R, and GM-CSFβ<sub>c</sub> chains abrogates signal transduction triggered by the receptor ligand 17,22-24,49 (and P.C. Orban, K.B. Leslie, unpublished data). In support of a direct role of these α<sub>a</sub> subunits in recruitment and activation of JAK-2, Ogata et al 40 have shown that JAK-2 can bind to IL-5Rα. Likewise, we have shown that the cytoplasmic domain of IL-5Rα binds directly to JAK-2 (P. Orchansky, J.W. Schrader, manuscript in preparation). Together with evidence that β<sub>c</sub> also binds JAK-2, 23 these data fit well with the model that both α<sub>a</sub> and β<sub>IL-3</sub> contribute to recruitment of 2 molecules of JAK-2 to a heterodimeric complex. The notion of a hierarchy of efficiency of activation of JAK-2 of α<sub>a</sub>β<sub>IL-3</sub> > β<sub>a</sub>β<sub>IL-3</sub> > α<sub>α</sub>β<sub>IL-3</sub> correlates well with the elegant experiments of Patel et al 48 with overexpressed proteins. We contend, however, that at the levels of receptor expression found in primary cells, typically of the order of a few hundred high-affinity receptors per cell, only α<sub>a</sub>β<sub>IL-3</sub> heterodimers generated by the presence of physiological ligand would deliver sufficient signal to trigger mitogenesis.

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REFERENCES

14. Jenkins BJ, D’Andrea R, Gonda TJ: Activating point mutations in the common beta subunit of the human GM-CSF, IL-3 and IL-5 receptors suggest the involvement of beta subunit dimerization and cell type-specific molecules in signalling. EMBO J 14:4276, 1995
15. Kuro T, Kikuchi Y, Kanazawa H, Hirokawa K, Harada N, Shiiba M, Wakao H, Takaki S, Takatsu K: Critical proline residues of the cytoplasmic domain of the IL-5 receptor alpha chain and its function...
in IL-5-mediated activation of JAK kinase and STAT5. Int Immunol 8:237, 1996


20. Lai SY, Molden J, Liu KD, Puck JM, White MD, Goldsmith MA: Interleukin-4-specific signal transduction events are driven by homotypic interactions of the interleukin-4 receptor alpha subunit. EMBO J 15:4506, 1996


27. Luo K, Lodish HF: Signaling by chimeric erythropoietin-TGF-beta receptors: Homodimerization of the cytoplasmic domain of the type I TGF-beta receptor and heterodimerization with the type II receptor are both required for intracellular signal transduction. EMBO J 15:4485, 1996


40. Karasuyama H, Melchers E: Formation of adhesion structures on mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. Eur J Immunol 18:97, 1988


Heterodimerization of the α and β Chains of the Interleukin-3 (IL-3) Receptor Is Necessary and Sufficient for IL-3–Induced Mitogenesis

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