Granulocyte-macrophage colony-stimulating factor receptor signals by a complex which includes the ligand and two different receptor subunits: a low affinity α receptor binding chain (granulocyte-macrophage colony-stimulating factor receptor α subunit (GM-Rα)) and a signal-transducing β chain (GM-Rβ). To investigate two unresolved issues in the initiation of signaling, the role of receptor extracellular domains and receptor stoichiometry, we replaced the mouse GM-Rα and GM-Rβ extracellular domains with the leucine zipper domain of either the Fos or Jun molecule. We co-transfected combinations of chimeric receptors into Ba/F3 cells and found that both simple heterodimers of the GM-Rα and GM-Rβ intracellular domains and homodimers of the GM-Rβ intracellular domain signaled for proliferation. Surprisingly, homodimers of the GM-Rα intracellular domain also signaled for prevention of apoptosis in transfected cells. We similarly engineered dimers of the intracellular domain of the human interferon γ receptor β subunit and found that homodimers of the intracellular domain signaled for proliferation. When Fos peptide was added to Ba/F3 cells expressing the human interferon γ receptor β subunit construct, thereby preventing homodimer formation, the cells no longer proliferated in the absence of mouse interleukin 3.

The cytokine receptor superfamily is a large group of structurally related proteins that together with their ligands, the hematopoietic cytokines, regulate important cellular processes such as differentiation and proliferation. Characteristic three-dimensional folds have been identified for both receptor and ligand families. The ligands have been characterized as four antiparallel α helix bundle proteins (for review, see Ref. 1). For the receptors a common two domain immunoglobulin-like fold was suggested, based on the low but significant homology seen in the extracellular ligand binding domain of these proteins (2). Recent structures of the growth hormone receptor and the IFNγ1 receptor have confirmed these predictions (3, 4).

It is generally accepted that common structural principles underlie hematopoietic cytokine receptor recognition and signaling. As shown for growth hormone, binding and signal transduction involve formation of a receptor subunit dimer that is dependent on binding of the ligand first to the primary receptor subunit, followed by binding of this complex to the second receptor subunit (5, 6). Juxtaposition of the two cytoplasmic receptor domains is thought to initiate the signaling cascade and typically involves tyrosine phosphorylation of a number of proteins, including the receptor subunits themselves, by receptor-associated tyrosine kinases (7, 8).

Signaling induced by GM-CSF follows this paradigm and is mediated by a complex comprising the ligand and two receptor subunits, GM-Rα and GM-Rβ, which are members of the cytokine receptor superfamily (9–12). The primary subunit GM-Rα binds GM-CSF with low affinity, whereas GM-Rβ on its own has no detectable binding affinity for GM-CSF. In combination with GM-Rα, GM-Rβ confers high affinity binding and forms a heterodimeric receptor complex, a critical step in signal transduction induced by GM-CSF. However, several aspects remain unresolved, including the precise stoichiometry of the signaling complex and the contributions of each subunit to signaling. Several groups have suggested that the native GM-CSF receptor signaling complex may be comprised of oligomers of αβ dimers or β subunit multimers and may involve other as yet unidentified molecules (13–15).

Similar to the signaling complex for GM-CSF, the class II cytokine receptor IFNγ receptor complex has two subunits: a ligand binding α chain, which alone cannot signal, and a β chain, which is required for signaling. In vivo, the hIFNγ receptor-signaling complex consists of one IFNγ dimer, two α receptor subunits, and one or two β receptor subunits (16, 17). The α subunit has an extended cytoplasmic domain of 222 amino acid residues (18) and associates with the tyrosine kinase JAK1 (17, 19). The β subunit has a 64-amino acid cytoplasmic domain (20) that associates with JAK2 (17, 21). IFNγ stimulation of cells expressing both receptor subunits activates both JAK1 and JAK2 (19).

To investigate the precise role of the extracellular domains of the GM-CSF receptor in the activation process, we have used a novel means of inducing receptor dimerization. We replaced the extracellular domains of the GM-CSF receptor α and β subunits with a coiled coil motif or "leucine zipper" and examined these constructs for their capacity to confer factor-independent growth when transfected into the pro-B cell line Ba/F3. To form a coiled coil, we used the leucine zipper regions of the transcription factors Jun and Fos. When both leucine zipper domains are present, heterodimers of the two leucine zippers predominate, since this is the most energetically favorable combination (22). However, the Jun leucine zipper domain can form homodimers, although they are less favorable than heterodimers, and Fos homodimers are the least likely to occur (22). In previous studies, the Fos-Jun leucine zipper has been used to mediate the formation of bispecific antibodies (23), as well as to initiate aspartate receptor-mediated chemotaxis in Escherichia coli (24).

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1 The abbreviations used are: IFNγ, interferon γ; GM-CSF, granulocyte-macrophage colony-stimulating factor; GM-Rα, granulocyte-macrophage colony-stimulating factor receptor common β subunit; hIFNγ, human interferon γ; hIFNγRβ, human interferon γ receptor β subunit; mIL-3, mouse interleukin-3; JAK, Janus kinase; PCR, polymerase chain reaction; FACS, fluorescence-activated cell sorting.
In this report, we describe the use of the Fos-Jun leucine zipper to analyze the mechanism of signal transduction through the GM-CSF receptor and to test the potential of the IFNγRβ chain to signal as a homodimer. We find that the leucine zipper can functionally substitute for the combination of ligand and GM-Rα and GM-Rβ extracellular domains. Our findings bear on the question of GM-CSF signaling complex stoichiometry, since we find that simple heterodimers of GM-Rα and GM-Rβ are able to signal. We also observe signaling by homodimers of the GM-Rα cytoktoplasmic domain. In addition, we have created homodimers of the β subunit of the IFNγ receptor, a class II cytokine receptor subunit with a short cytoktoplasmic domain, via a Jun extracellular zipper domain to explore the potential of the β subunit to signal. We demonstrate that a hIFNγRβ homodimer can transduce a proliferative response in Ba/F3 cells.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Plasmids**—From the mouse GM-Rα and mouse GM-Rβ, the two membrane-proximal extracellular amino acids, transmembrane and intracellular domains (mouse GM-Rα amino acids 294–359 (25) and mouse GM-Rβ amino acids 439–896 (26)) were amplified by PCR from cDNA clones (mouse GM-Rα cDNA was a kind gift from Dr. Linda Park, Immunex). The 5′-PCR primers included an in-frame Bgl II site directly upstream of both receptor fragments, whereas the primers introduced immediately following the stop codon of each gene fragment. The PCR fragments were digested with Bgl II and Xba I and cloned in Bgl II- and Xba I-digested pME-18 carrying the CD8 secretion leader sequence followed by a FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Lys) and a unique in-frame Bgl II site. The 39-amino acid Jun and Fos leucine zipper domains were amplified by PCR from cDNAs (a kind gift from Dr. J. Yun Tso, Protein Design Labs (23) including two glycine residues either with or without a preceding cysteine residue directly upstream of the zipper domains. The various Fos and Jun zipper fragments all included 5′- and 3′-in-frame Bgl II sites. Bgl II-digested fragments were subcloned in the Bgl II-opened pME-18 vectors carrying the CD8 leader, FLAG epitope, and either mouse GM-Rα or mouse GM-Rβ fragments and screened for orientation. The Jun-hIFNγRβ construct was made by amplifying the hIFNγRβ transmembrane and intracellular domain (amino acids 274–337 (20)) by PCR from a cDNA clone (a kind gift from Dr. Michel Aguet, Genentech) and subcloning the product using existing restriction sites within the pME-18 cassette described. The final constructs carry the appropriate orientation. The Jun-hIFNγRβ construct was made by amplifying the hIFNγRβ transmembrane and intracellular domain (amino acids 274–337 (20)) by PCR from a cDNA clone (a kind gift from Dr. Michel Aguet, Genentech) and subcloning the product using existing restriction sites within the pME-18 cassette described. The final constructs carry the appropriate orientation.

**Cells and Transfected Lines**—The mouse pro-B line Ba/F3 (27) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10 ng/ml mIL-3. Ba/F3 cells were transfected with linearized DNA by electroporation essentially as described (11). The following modification was made in the protocol; cells were resuspended in potassium-phosphate-buffered saline (30.8 mM NaCl, 120 mM KCl, 8 mM Na2HPO4, and 1.5 mM KH2PO4) supplemented with 5 mM MgCl2 and electroporated at 200 V, 960 microfarads. Cells were allowed to recover for 2 days in growth medium and then transferred to selection medium containing 1:10,000 dilution. Both stains and washes were performed in the presence of 1 mg/ml CoCl2, which is required for anti-FLAG M1 antibody binding. Bound antibody was detected by an ECL chemiluminescence kit (Amersham) according to the manufacturer’s instructions.

**Expression of Leucine Zipper-Receptor Complexes**—We selected Ba/F3 cells for expression of our constructs because the line is dependent on the presence of mIL-3 for growth but is capable of transducing GM-CSF-induced proliferation signals when appropriate GM-CSF receptors are in place (11, 29). Stable clonal transfectants of the mouse pro-B-cell line Ba/F3 (27) were established co-expressing various leucine zipper-containing receptor constructs. From each transfection,
multiple stable transfectants were obtained and analyzed. We show results from one representative clonal line per transfection. The presence of the introduced receptor construct(s) in the genomic DNA of transfectant lines was confirmed by PCR (data not shown).

We determined cell surface expression levels of the hybrid receptors by flow cytometry (Fig. 2). Cells were stained with anti-FLAG M1-biotin as the primary antibody, followed by a secondary streptavidin-phycocerythrin detection step. From each transfectant at least five clonal lines were analyzed by flow cytometry, each displaying a slightly different staining intensity. For details of how clones were selected for further analysis, see “Experimental Procedures.” Although all lines expressed FLAG tag-containing hybrid receptors on their cell surface, the expression levels varied, for example, low for Fos-α (Fig. 2A) and high for Cys/Jun-α (Fig. 2B). Since both the Fos-α and Jun-β constructs included a FLAG tag, we could not determine from flow cytometry alone whether both constructs were simultaneously expressed in the double transfectant line (Fig. 2F).

We therefore performed Western blot analysis on transfectant line cell lysates to visualize dual expression Fos-α and Jun-β, as well as to demonstrate that the proteins detected by flow cytometry were of the anticipated molecular weights. FLAG fusion proteins were immunoprecipitated from cell extracts with M1 antibody-conjugated agarose beads followed by immunoblot analysis of the eluted proteins. All lines showed proteins of the expected sizes (Fig. 3A); lane 1 is the Ba/F3 control; lanes 2 and 3 show, respectively, the 14-kDa Fos-α and Cys/Jun-α protein products; lanes 4–6 show the 60-kDa proteins Cys/Fos-βα, Jun-β, and Cys/Jun-βα; and lane 7 shows the protein products of Ba/F3 cells expressing both Fos-α and Jun-βα. Two contaminating bands at ~25 and 110 kDa are most readily visible in Fig. 3A, lanes 5 and 6, because of differential loading of immunoprecipitates; the bands are nonspecific, as they are also observed in lysates from untransfected Ba/F3 cells when comparable amounts of protein were immunoprecipitated and immunoblotted (data not shown).

Several proteins include an extracellular cysteine residue preceding the Jun or Fos leucine zipper to allow for disulfide linkage of assembled coiled coil GM-CSF receptors. We visualized dimers by immunoblot analysis of lysates from the Cys'/Jun-α line (Fig. 3B). Although Cys/Jun-α was present only as a 14-kDa monomer in reducing conditions (lane 1), in nonreducing conditions the protein appeared both as a monomer and as a 28-kDa dimer (lane 2). Proteins lacking the additional extracellular cysteine residue did not form dimers under nonreducing conditions (data not shown).

Proliferative Response Induced by Leucine Zipper Receptors—Ba/F3 cells are normally mIL-3 dependent for their growth, and after 24 h in the absence of mIL-3 >95% of the cells are dead (data not shown). To determine whether the leucine-zippered GM-CSF receptors could signal for proliferation in the transfectant lines, we monitored the number of cells in culture over time in the absence of mIL-3. Proliferation in the presence of mIL-3 was also measured to ensure that the clonal lines selected were not impaired in their IL-3-dependent proliferative response. For comparison, we used the rate of cell number increase for untransfected Ba/F3 cells (Fig. 4A).

Ba/F3 cells expressing only Fos-α were not able to proliferate or survive in the absence of mIL-3 (Fig. 4B); by 49 h >90% of the cells were dead. Similarly, the expression of Jun-βα did not support long term survival or proliferation, but it did promote...
short term survival (Fig. 4C) for nearly 100 h in the absence of mIL-3. We attribute the increase in short term survival relative to untransfected cells to the formation of Jun homodimers. Long term proliferation does not occur, either because the number of Jun homodimers complexes is below a critical threshold or because the complexes are too short-lived. In contrast when Jun-βa was co-expressed with Fos-α, cells became factor-independent (Fig. 4D). Their long term growth behavior and proliferative capacity in the absence of mIL-3 was indistinguishable from that induced by mIL-3; we have maintained the line in the absence of mIL-3 for more than 18 months. We conclude that formation of the Jun-Fos leucine zipper functionally substitutes for the GM-CSF-induced dimerization of the two GM-CSF receptor extracellular domains.

Constructs bearing the additional extracellular cysteine residue displayed a phenotype quite different from that seen in transfectants lacking the cysteine. In the case of Jun-βa and Cys/Jun-βa, the cell surface expression levels were comparable (Fig. 2, D and E, respectively), but only the Cys/Jun-βa line proliferated in the absence of mIL-3 at a rate similar to that observed in the presence of mIL-3 (Fig. 4E). Cells expressing Cys/Fos-βa exhibited a similar phenotype, albeit with a slightly lower rate of proliferation (Fig. 4F). We attribute the differences between the Cys/Jun-βa and Cys/Fos-βa growth rates to the lower propensity of Fos to form homodimers compared with Jun (22). The phenotypes of the two cell lines demonstrate that stable dimerization of two intracellular β chain domains (via the extracellular disulfide bridge) triggers a proliferative response. Although the possibility of higher order aggregates cannot be excluded, the extracellular cysteine most likely induces formation of a covalent bond between domains that otherwise exist as transient Fos or Jun homodimers. The small but reproducible difference in proliferative signaling induced by Rαβ dimers (Fig. 4D) compared with that induced by Rββ dimers (Fig. 4E and F) suggests that the cytoplasmic domain of GM-Rα provides a unique contribution to signaling, since the role of the GM-Rα intracellular domain can only be partially replaced by the GM-Rβ intracellular domain.

The Cys/Jun-α transfectant line displayed an unexpected response to mIL-3 deprivation. Homodimers of Cys/Jun-α promoted the long term survival of the culture for the first 250 h in the absence of mIL-3 (Fig. 4G). With continued maintenance without mIL-3, the line exhibited a relatively constant total live cell count, whereas the viability of the culture gradually fell to 21% at 215 h from 94% at t = 0 (data not shown). From 215 h onward, the viability steadily increased, and the rate of proliferation increased starting at 400 h (Fig. 4H). By 600 h, the viability of the culture was 94%, the same as it had been initially, and the cells were proliferating at a rate similar to that observed for the Cys/Fos-β transfectant line. Several transfections of Ba/F3 cells with the Cys/Jun-α construct were performed. When clonal populations were deprived of mIL-3, each exhibited the growth characteristics described above, thus reducing the possibility that we had initially selected variants of Ba/F3 cells. We did not observe this pattern of growth (low proliferation rates for 14 days followed by a gradual increase in proliferative rates) in transfectant lines from any other constructs.

Disruption of Leucine Zipper Receptors with Fos or Jun Peptide—We have generated a similar leucine zipper-containing hIFN-γRβ (Jun-hIFN-γRβ; Fig. 1). Ba/F3 transfectants were assayed by FACSanalysis and immunoblot analysis to confirm expression (data not shown). Surprisingly, we found that the transfectant line proliferated in the absence of mIL-3 (Fig. 5A) at a rate equivalent to that in the presence of mIL-3. Clones derived from a separate transfection proliferated at a similar rate in the absence of mIL-3.

Proliferation of Jun-hIFN-γRβ transfectants suggested that in this case Jun homodimers were sufficient to drive signaling. This hypothesis was tested by attempting to disrupt the formation of Jun-receptor homodimers by the addition of either Fos or Jun peptides to the media in a proliferation assay. Each peptide was titrated at 0.75-fold dilutions, starting at a 240 μM final concentration. Proliferation of the Jun-hIFN-γRβ transfectant line decreased markedly in the presence of the Fos peptide but not the Jun peptide (Fig. 5B). We conclude that the Fos peptide can prevent the formation of Jun-receptor homodimers (by forming Fos-Jun heterodimers) and therefore can decrease the concomitant dimerization of the hIFN-γRβ cytoplasmic domains. The Jun peptide competed only slightly at a very high concentration. These observations are consistent with previous findings that Fos-Jun heterodimers are more energetically favorable than either homodimer. The finding that disruption of Jun-Jun homodimer formation reverses the factor-independent proliferation of the line provides direct evidence that the phenotypes observed in our transfectant lines result from Fos- and/or Jun-mediated associations.

DISCUSSION

We have investigated the requirement of GM-CSF receptor extracellular domains for one aspect of signaling, proliferation, by replacing the extracellular domain with the leucine zipper domain of either the Fos or Jun protein. Simultaneous expression of Fos-α and Jun-βa resulted in a proliferative response and conferred factor independence on Ba/F3 cells, suggesting that the extracellular domains contribute to signaling only by causing ligand-dependent association of the receptor intracellular domains. Since neither Fos nor Jun has structural similarities to receptor extracellular domains, yet they functionally substitute for the combination of ligand and GM-Rα and GM-Rβ extracellular domains, we conclude that specific extracellular domain structure is not crucial as long as specific dimer formation is ensured. Our observations suggest that specific ligand-induced allosteric changes in the receptor extracellular domains are unlikely to be required for initiation of signal transduction. Furthermore, the GM-CSF receptor extracellular domains do not depend on interactions with auxiliary proteins to initiate the signaling cascade that results in proliferation, since our simple Fos-α and Jun-βa heterodimers signal but exclude the possibility of interactions with additional extracellular domains.

We selected a leucine zipper to drive receptor dimerization based on the observation that in ligand-induced signaling by single transmembrane receptors, close parallel alignment of the α helical transmembrane segment of each of the two inter-
acting subunits appears to be crucial (30). Activating transmembrane domain mutations have been described for two single transmembrane receptors, the neu oncogene (31) and the common β subunit of the GM-CSF receptor (13). Both mutations act by inducing constitutive receptor dimerization (13, 32, 33). Structural evidence indicates that the basis for constitutive homodimerization in the mutant neu receptor is the formation of an energetically favorable coiled coil by two parallel transmembrane domains (34). Replacement of both GM-CSF receptor extracellular domains with a helices capable of forming a coiled coil heterodimer most likely allows for optimal positioning of the two parallel transmembrane and cytoplasmic domains to initiate the signal transduction process.

Our findings have implications for the question of signaling complex stoichiometry. The number of each receptor subunit present in the native GM-CSF receptor signaling complex remains unknown, although several groups have suggested that it may comprise oligomers of αβ dimers, which allow multimers of β subunits to come in contact with each other (14, 15). However, the leucine zippers Fos and Jun interact as a simple heterodimer in a 1:1 ratio. Therefore in the Fos-α/Jun-β transfectant line, the stoichiometry of intracellular domains in the signaling complex is one α and one β subunit. Although multiple receptor subunits may be present in the native complex, our results indicate that dimers consisting of one α and one β subunit are sufficient to initiate signaling for proliferation. We cannot rule out the possibility that αβ dimers may recruit additional β subunits or αβ dimers into the complex. If additional components do enter the complex, then in our reconstituted system (and possibly in the native complex) they must do so by interactions mediated by either the transmembrane or cytoplasmic domains, since our system excludes additional extracellular interactions.

Several studies with chimeric receptors have shown that dimerization of the intracellular portion of the GM-Rα subunit is sufficient to initiate proliferation (14, 15). Similarly, our transfectant lines expressing either Jun-β, or Cys/Jun-β, show a biological response. From our observed increase in the short-term survival of the Jun-Rβ transfectants over untransfected cells, we infer that the Jun-β construct promotes the dimerization of the β intracellular domain, albeit transiently and perhaps infrequently. Long term survival and proliferation probably require the formation of a greater number of intracellular dimers, longer lasting association, or both. These requirements are clearly met by the presence of a single additional extracellular cysteine residue. In Cys/Jun-β transfectants, the paired β intracellular domains are able to signal for long term proliferation, a response not observed with the equivalent construct lacking the additional cysteine.

More surprising are our findings regarding the biological effects of stable GM-Rα dimers formed by the Cys/Jun-α construct. The role of the intracellular domain of the GM-Rα subunit in proliferation is not well understood. Several studies point to its requirement for GM-CSF-induced initiation of proliferation (35, 36), but in at least one study, dimers of the α cytoplasmic domain (via hybrid GM-CSF receptors) did not result in a signal for proliferation (15). On detailed examination of our Cys/Jun-α transfectants, however, we found no net change in the live cell numbers during the first 14 days following mIL-3 deprivation. Since the line remains viable in the absence of growth factor, we propose that dimers of the GM-Rα cytoplasmic domain are signaling either to prevent apoptosis or to initiate cell proliferation at a rate equal to the rate of cell death. After approximately 2 weeks in the absence of mIL-3, the Cys/Jun-α cultures were able to proliferate at a rate that exceeded the rate of cell death, and these cultures were maintained for more than 6 months.

The growth characteristics of our Cys/Jun-α line closely resemble the phenotype observed with a mouse GM-Rβ variant that lacks an extracellular domain, except for a small novel sequence of 34 amino acids, which includes a single cysteine residue (37). When transfected into Ba/F3 cells this variant of the β chain does not immediately confer factor-independent proliferation but, rather, promotes the transformation of cells to factor independence after >14 days. A possible explanation for the delayed proliferative response is that during the 2-week period, cells with mutations that allow a more robust proliferative response have been selected. However, formation of either

**Fig. 4. Increase in cell numbers of Ba/F3 cell transfectants.** Cell lines maintained in mIL-3 plus selection (hygromycin or Geneticin) were washed in media lacking mIL-3 and then seeded at an initial density of 1 × 10⁴ cells/ml in 2 cm² wells in the presence (●) or absence (○) of mIL-3 as described under "Experimental Procedures." Live cells were counted by trypan blue exclusion every 20–30 h and split as necessary. Total live cell number accounts for splits. A, Ba/F3, untransfected; B, Fos-α; C, Jun-β; D, Fos-α and Jun-β; E, Cys/Jun-β; F, Cys/Fos-β; G, Cys/Jun-α; H, Cys/Jun-α, monitored over a 700-h time course. Each line was assayed at least two times; a single representative experiment is shown.
the presence of Fos peptide (representative of all clonal populations).

Blue exclusion every 20–30 h and split as necessary. Total live cell productivereceptor combinations in the absence of ligand. The dimer independence in Ba/F3 cells using a mutant to obtain the proliferative response. A similar attempt to in-

duce factor independence in Ba/F3 cells using a mutant domain and no intact domain isse to form dimers (as described here) or with mIL-3, the GM-R complex, but this complex only contains one cytoplasmic subunit did not give rise to factor independence, that forced dimerization of any two GM-CSF receptor subunits (αα, αβ, or ββ) can result in initiation of signaling, presumably by activation of cytoplasmic proteins associated with the cytoplasmic domains of the GM-CSF receptor subunits (for example GM-Rβ with JAK2). If these molecules come in close physical proximity for extended periods, as would be the case in our Cys/Jun-α transfectants, they can become activated and can initiate signaling.

We have demonstrated the general utility of using Fos and Jun domains to replace extracellular receptor domains to test productive receptor combinations in the absence of ligand. The FLAG tag allows easy detection, and the small extracellular domain is easy to manipulate as DNA and protein. Specifically we have shown that the extracellular domains of the mouse GM-CSF receptor subunits are functionally dispensable if dimers can be formed by other means, and that dimers consisting of only one α and one β receptor subunit can signal in a manner similar to the wild type receptor. Dimers of the α subunit also initiate signaling, either for prevention of apoptosis or for a low level of proliferation. Finally, we have demonstrated that the formation of hIFN-Rβ cytokilic domain dimers is sufficient to induce a proliferative signal. With our Jun-hIFN-Rβ line, we have defined a system in which one can use intact minimal cytokilic domains to further investigate the requirements for signaling that results in proliferation.

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