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Modulation of growth factor receptor function by isoform heterodimerization

(prolactin receptor/granulocyte-macrophage colony-stimulating factor receptor/chimeric receptor/cell proliferation/Sos)

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ABSTRACT **Activation of prolactin (PRL)-dependent signaling occurs as the result of ligand-induced dimerization of receptor (PRLr). Although three PRLr isoforms (short, intermediate, and long) have been characterized and are variably coexpressed in PRL-responsive tissues, the functional effects of ligand-induced PRLr isoform heterodimerization have not been examined. To determine whether heterodimeric PRLr complexes were capable of ligand-induced signaling and cellular proliferation, chimeras consisting of the extracellular domain of either the α or β subunit of human granulocyte-macrophage colony-stimulating factor receptor (GM-CSFr) and the intracellular domain of the rat intermediate or short PRLr isoforms (PRLr-I or PRLr-S) were synthesized. Because high affinity binding of GM-CSF is mediated by the extracellular domain of one α and β GM-CSFr pair, use of GM-CSFr/PRLr chimera specifically directed the dimerization of the PRLr intracellular domains within ligand-receptor complexes. Stable transfection of these constructs into the Ba/F3 line was demonstrated by Northern blot and immunoprecipitation analyses. Flow cytometry revealed specific binding of a phycoerythrin-conjugated human GM-CSF to the transfectants, confirming cell surface expression of the chimeric receptors. When tested for their ability to proliferate in response to GM-CSF, only chimeric transfectants expressing GM-CSFr/PRLr-I homodimers demonstrated significant [^{3}H]thymidine incorporation. GM-CSF stimulation of transfectants expressing either GM-CSFr/PRLr-S homodimers or GM-CSFr/PRLr-S+I heterodimers failed to induce proliferation. Consistent with these data, the GM-CSF-induced activation of two phosphotyrosine kinases, Jak2 and Fyn, was observed only in homodimeric GM-CSFr/PRLr-I transfectants. These results show that the PRLr-S functions as a dominant negative isoform, down-regulating both signaling and proliferation mediated by the receptor complex. Thus, structural motifs necessary for Jak2 and Fyn activation within the carboxy terminus of the PRLr-I, absent in the PRLr-S, are required in each member of the dimeric PRLr complex.**

The neuroendocrine hormone prolactin (PRL) is an important growth factor in the development of mammary tissues and immune cells (1–4). The immunoregulatory effects of PRL are believed, in part, to be mediated by its cell surface receptor (PRLr) (5, 6). Three PRLr isoforms have been cloned: the short (PRLr-S), the long (PRLr-L), and the intermediate mutant (PRLr-I) in the rat (7–9). The three isoforms are identical in their ligand-binding extracellular domain and differ in the length of their intracellular domains, with 57, 160, or 358 amino acids (aa) within the PRLr-S, PRLr-I, or PRLr-L, respectively. The PRLr-S and PRLr-L isoforms are widely coexpressed at various levels in different PRL-responsive tissues (10). Synthesis of PRLr-S mRNA occurs through alternative splicing, creating a protein that is truncated at its carboxy-terminus (8). The PRLr-I isoform lacks 198 aa (i.e., amino acids 323–520) from the central portion of the intracellular domain found within the PRLr-L (9). Like other members of the growth factor receptor family (11), PRLr lacks an intrinsic tyrosine kinase catalytic domain. Analogous to the growth hormone receptor (12), signaling by the PRLr complex is thought to occur as the result of ligand-induced dimerization and activation of associated signaling factors such as Jak2 (13–15), Fyn (16), Grb2/Sos (17), Raf (18), and Vav (19). Initial data have indicated that three structural motifs within the intracytoplasmic domain of the PRLr, namely the box 1, variable box, and box 2, may contribute to the interactions with these transduction factors. The box 1 motif consists of a hydrophobic proline-rich region (20) and presents some similarity with the Src homology 3 binding sites (21–23). Although it may serve as a binding site for signaling factors, the fact that half of the proline residues in box 1 must be mutated before receptor function is impaired, suggests a structural role for this region (21, 24). The box 2 motif, present in PRLr-I and PRLr-L but absent in PRLr-S, is rich in hydrophobic and acidic amino acid residues. The intervening region between box 1 and box 2 is the variable box; only a partial sequence of this motif is found within the PRLr-S, secondary to this isoform's alternative splice site. Structure/function studies with other related receptors (i.e., growth hormone and erythropoietin) have revealed that conserved residues within each of these domains are required for receptor function (25–27). Whether these amino acids directly interact with PRLr-associated signaling factors, or contribute to critical receptor structure, however, remains uncertain.

Transfected reporter genes have been used to examine the function of the structural motifs within the PRLr isoforms (28, 29). These studies have found that only PRLr-L and PRLr-I, but not PRLr-S, can induce transcription from a β-lactoglobulin gene promoter in Chinese hamster ovary cotransfectants when stimulated with PRL. When transfected into the cytokine responsive line Ba/F3, the PRLr-L and PRLr-I isoforms were comparable at stimulating PRL-driven cell proliferation and gene expression (30), while the PRLr-S isoform lacked such activity. These functional differences in the cotransfectants were attributed to the differential splicing of amino acid sequence from the PRLr-S isoform, that includes all of the box 2 and a portion of the variable box. Similar studies, using mutant PRLr isoforms, have demonstrated that PRLr-driven

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Abbreviations: PRL, prolactin; PRLr, prolactin receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GM-CSFr, GM-CSF receptor; hGM-CSFr, human GM-CSFr; PRLr-L, PRLr long isoform; PRLr-I, PRLr intermediate isoform; PRLr-S, PRLr short isoform; α, the extracellular domain of GM-CSFr α subunit; β, the extracellular domain of GM-CSFr β subunit; I, the transmembrane and intracellular domains of PRLr-I; S, the transmembrane and intracellular domains of PRLr-S; IL-3, interleukin 3; PE, phycoerythrin.

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proliferation and activation of Jak2 requires the presence of the box 1 motif and a tyrosine residue within the carboxyl terminus of the PRLr (14, 31). In contrast, a single recent report has indicated that homodimers of the PRLr-S isoform were capable of stimulating the proliferation of NIH 3T3 cells (32), suggesting that the PRLr isoforms exert effects that are tissue- and/or cell-specific. Unlike these *in vitro* transfectants, however, no PRL-responsive tissue has been shown to express only a single PRLr isoform (10). Thus, given that the ligand-binding and transmembrane domains of the PRLr isoforms are identical, heterodimeric PRLr complexes *in vivo* may be more of a rule than an exception. Unfortunately, the functional significance of heterodimerization between such isoforms has not been examined. This has been due largely to technical considerations, since *in vitro* cotransfection with two PRLr isoforms should produce mixtures of homo- and hetero-dimers in response to ligand stimulation, precluding definitive functional analysis.

In this study, we addressed whether PRLr isoform heterodimerization modulated ligand-driven signal transduction and cellular proliferation through the use of chimeric receptors. To this end, chimeras of the extracellular domain of human granulocyte-macrophage colony-stimulating factor receptor (hGM-CSFr) and the transmembrane and intracellular domains of rat PRLr isoforms were synthesized. GM-CSFr and PRLr belong to the growth factor receptor superfamily (33), which shares homologous extracellular features, including conservation of four periodically spaced cysteine residues and a Trp-Ser-Xaa-Trp-Ser (WSXWS) motif. There are two subunits of GM-CSFr—i.e., α subunit and β subunit: the α subunit binds GM-CSF with low affinity, whereas the β subunit fails to bind ligand by itself but converts the low affinity binding sites to high affinity receptors that are biologically active (34). When these chimeras are coexpressed, ligand-induced dimerization of the extracellular domains of GM-CSFr α and β subunits of the expressed chimeras should induce a specific, one-to-one pairing of their associated PRLr intracellular domains. By using such constructs, we now report the modulation of proliferation and signal transduction by heterodimerized receptor complexes in the cotransfected, cytokine responsive, Ba/F3 cell line.

MATERIALS AND METHODS

Chimeric Constructs. Cassettes of the extracellular domains of the hGM-CSFr α or β subunits, termed α or β, and the transmembrane and intracellular domains of the rat PRLr-I or PRLr-S isoforms, termed I or S, were generated by PCR from a full-length cDNA. A *Kpn*I site was engineered into the 3′ end of the α and β subunits and the 5′ end of the I and S subunits to permit ligation of the extra- and intracellular cassettes. One internal *Kpn*I site in α was conservatively altered by PCR-mediated site-directed mutagenesis with a synthetic oligonucleotide primer changing the *Kpn*I site to GGTACA. Ligation of α or β at the *Kpn*I site to S or I, yielded four chimeric constructs: αS, αI, βS, and βI (see Fig. 1). These chimeric constructs were subcloned into the pREP4 expression vector containing a hygromycin resistance gene or the pREP9 containing a neomycin (G418) resistance gene (InVitrogen). Sequences of the chimeric constructs were confirmed by the dideoxynucleotide chain-terminating method.

Cell Culture, Transfection, and Proliferation Assays. A mouse interleukin 3 (IL-3)-dependent pro-B cell line, Ba/F3 (35) was maintained in RPMI 1640 medium (GIBCO/BRL) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in the presence of 1 ng of IL-3 per ml (PeproTech, Rocky Hill, NJ). A hGM-CSF/IL-3-dependent cell line, TF-1 (36), was maintained in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1 ng of hGM-CSF per ml (PeproTech). Ba/F3 cells (1×10^7) were transfected with 50 μg of *Xmn*I-linearized chimeric construct cDNA by exposure to a single voltage pulse (0.6 kV, 25 μF for 0.1 sec) in a Gene Pulser electroporater (Bio-Rad). Selection utilized either 750 μg of G418 per ml (GIBCO/BRL) and/or 375 μg of hygromycin per ml (Boehringer Mannheim) and clones were obtained by limiting dilution. To assess ligand-induced cellular proliferation, 1×10^6 washed transfectants were aliquoted in medium consisting of RPMI 1640 medium supplemented with sodium selenide, linoleic acid, insulin, and transferrin (ITS+; Calbiochem) in the presence or absence hGM-CSF. After overnight culture, cells were pulsed with 0.5 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine at 37°C for 8 h. Incorporation of radiolabel was determined by scintillography.

Northern Blot Analysis. Total RNA from cells was isolated by extraction with guanidinium isothiocyanate (37), as described. Ten micrograms of total RNA was denatured and subjected to electrophoresis on 1% agarose formaldehyde gels and transferred onto a nylon membrane. Probe inserts were labeled with [α-^{32}P]dCTP by the random primer method. Membranes were hybridized under stringent conditions as described (38) with 2.5×10^7 cpm random labeled α or β cDNA probes.

Cell Metabolic Labeling and Immunoprecipitation. [^{35}S]Methionine incorporation and immunoprecipitation were performed and analyzed as described (39). Cells (1×10^7) were placed in methionine-free RPMI 1640 medium with 5% fetal bovine serum at 37°C for 1 h before the addition of 0.1 mCi of L-[^{35}S]methionine and cysteine (Dupont) per ml for 5 h. Cell lysates were prepared in immunoprecipitation buffer (10 mM Tris, pH 7.6/50 mM NaCl/30 mM sodium deoxycholate/50 mM NaF/1% Nonidet P-40/1 mM Na_3VO_4/1 mM phenylmethylsulfonyl flouride/10 μg of leupeptin per ml/2.5 mM EDTA/1 μM pepstatin/0.23 unit of aprotonin per ml) at 4°C for 10 min. Cleared lysates were immunoprecipitated with anti-α (Genzyme) or anti-β (Santa Cruz Biotechnology) GM-CSFr, followed by incubation with protein G-agarose beads. After washing, immunoprecipitates were analyzed by SDS/PAGE before autoradiography.

Flow Cytometry. Ligand binding by transfected cells was assessed by Fluorokine kit (R & D Systems). Washed cells (1×10^5) were incubated with 110 ng of phycoerythrin (PE)-conjugated recombinant hGM-CSF or 110 ng of PE-conjugated streptavidin per ml at 4°C for 1 h. After washing, 1×10^4 cells were analyzed at 488 nm by a FACSTAR flow cytometer (Becton Dickinson).

***In Vitro* Kinase Assay.** Autokinase activities were measured using a modification of a previously described method (40). Washed lysates, obtained as described above, were immunoprecipitated with anti-mouse Jak2 (5 μl; Upstate Biotechnology, Lake Placid, NY) or anti-mouse Fyn (10 μl; Santa Cruz Biotechnology). Washed immunoprecipitates were resuspended in kinase buffer (50 mM Hepes, pH 7.1/0.1 mM EDTA/0.1 mg of bovine serum albumin per ml/0.1% 2-mercaptoethanol/0.15 M NaCl/0.15 mM ATP/20 mM $MgCl_2$) containing 10 μCi [γ-^{32}P]ATP for 20 min. Beads were boiled in 2× SDS sample buffer for 5 min, resolved by SDS/10% PAGE, and visualized by autoradiography.

RESULTS

Expression of GM-CSFr/PRLr Chimeric Receptors in Ba/F3 Transfectants Induces GM-CSF Binding. To test whether the PRLr isoform heterodimerization modulated receptor signaling, four chimeric receptors were coexpressed in transfected cytokine-responsive Ba/F3 cells. These chimeric receptors contained the extracellular domains of the hGM-CSFr α or β subunit (i.e., α or β) and the transmembrane and intracellular domains of the rat PRLr intermediate or short isoform (i.e., I or S). PRLr-L was not examined in these initial studies, as significant functional differences between the

PRLr-I and -L isoforms *vis-a-vis* signaling and proliferation have not been observed (28, 30). Thus, the chimeric receptors used for these studies consist of αS, αI, βS, and βI (Fig. 1). Because high-affinity GM-CSFr binding requires the dimerization of one α and one β subunit, it was anticipated that GM-CSFr/PRLr chimeras would induce the specific juxtaposition of desired I and/or S domains of the PRLr in response to GM-CSF. Ba/F3 cells transfected with these chimeras revealed expression of chimeric transcripts of the expected size (Fig. 2*A*). To confirm synthesis at the protein level, the lysates from metabolically labeled transfectants were immunoprecipitated with the anti-α or anti-β chain of the hGM-CSFr antibody. Analysis of the immunoprecipitates demonstrated that chimeric receptors of the expected size were expressed at approximately equivalent levels in both single and double transfectants (Fig. 2 *B* and *C*). Thus, the chimeric constructs were expressed at both the RNA and protein levels in the transfected Ba/F3 cells.

The ability of the expressed chimeras to bind the ligand was examined by a flow cytometric assay that utilizes a PE-conjugated GM-CSF. As demonstrated in Fig. 3, all α/β cotransfectants demonstrated ligand binding, whereas the Ba/F3 parent line lacked the ability to bind PE-conjugated GM-CSF. Specific binding of ligand was observed in 70–90% of the cells within each clone when quantitated by flow cytometry. Since the GM-CSFr β subunit does not directly bind ligand, but confers the high affinity state, it was anticipated that βS or βI single transfectants would not be capable of binding GM-CSF. Indeed, no specific binding of ligand by the β single transfectants was observed; however, under the saturating conditions used for these experiments, GM-CSF-PE binding was noted in the single transfectants expressing the low affinity αS or αI chimera (data not shown).

Heterodimerization of the PRLr Intracellular Domains Down-Modulates Ligand-Stimulated Signaling and Proliferation. Single and double chimeric receptor transfectants were tested for their ability to proliferate in response to exogenous hGM-CSF (Fig. 4). Only those cotransfectants that received both the αI and βI chimeric receptors showed a significant induction of cellular proliferation observed in response to 100 ng hGM-CSF per ml. The induction of αI/βI cotransfectant proliferation was dose-dependent and comparable to that observed with IL-3 stimulation (data not shown). Ligand stimulation of the single transfectants (αS, αI, βS, or βI) or other double transfectants (i.e., αS/βS homodimers, or αS/βI or αI/βS heterodimers) failed to induce their proliferation. The ability of the dimerized intracellular PRLr domains to initiate signal transduction was also evaluated using *in vitro* kinase assay. These data demonstrated that Jak2 autokinase activity was significantly increased only in αI/βI double transfectants after ligand stimulation (Fig. 5*A*). Other chimeric receptor combinations (i.e., αS/βS, αS/βI, αI/βS) did not activate Jak2 following ligand stimulation. Similar to the data obtained for Jak2, the induction of significant Fyn autokinase activity by ligand was observed in only the αI/βI double transfectants (Fig. 5*B*). An unidentified protein (≈170 kDa) was coprecipitated and radiolabeled with Fyn in αI/βI double

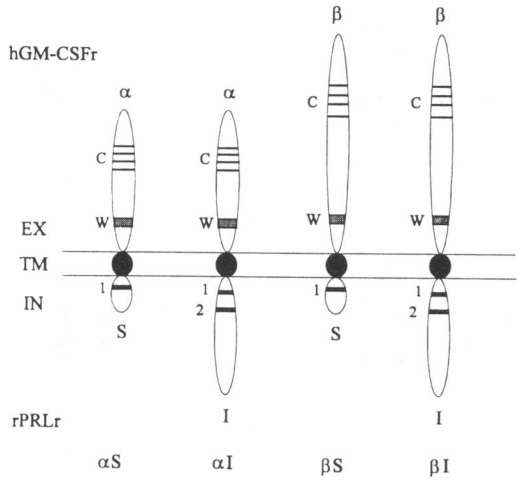

FIG. 1. Schematic representation of chimeric receptors. The extracellular, transmembrane, or intracellular domains are abbreviated to EX, TM, or IN, respectively. Chimeric receptors consist of the extracellular domains of the hGM-CSFr α or β subunit (i.e., α or β) and the transmembrane and intracellular domains of the rat PRLr-I or PRLr-S isoform (i.e., I or S). The α or β are 321 or 408 aa, respectively. There are conserved regions, such as four cysteine residues (as C) and a WSXWS motif (as W), in the extracellular domain among members of the growth factor receptor superfamily including GM-CSFr and PRLr. The I or S is 57 or 159 aa, respectively. Two thick lines labeled 1 and 2 indicate conserved regions called box 1 and box 2 motifs. The chimeric receptors are termed αS, αI, βS, and βI.

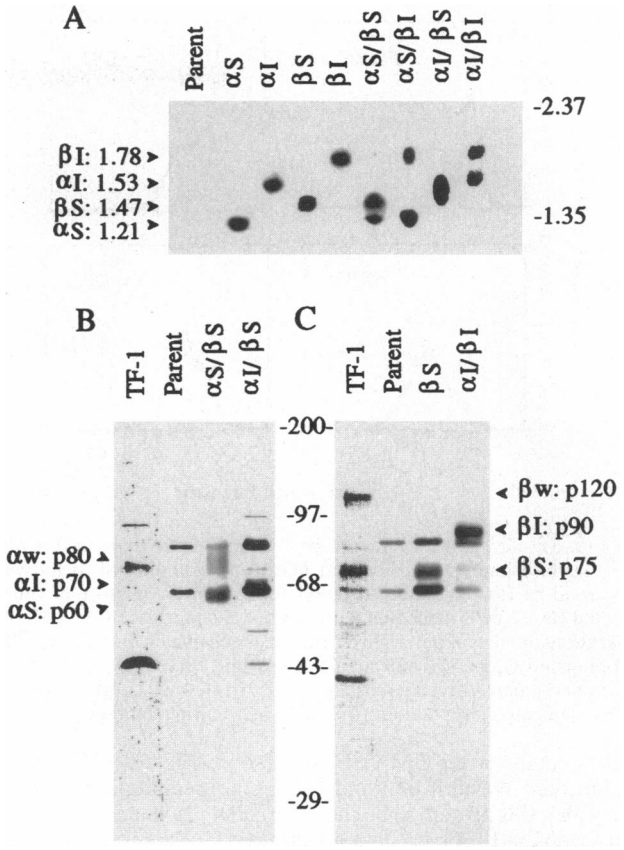

FIG. 2. Expression of chimeric receptors in Ba/F3 transfectants at the RNA and protein level. As shown in *A*, Northern blot analysis of total cellular RNA from Ba/F3 cells transfected with GM-CSFr/PRLr chimeras. Ten micrograms per lane of total RNA was hybridized with the extracellular domains of α or β subunit of hGM-CSFr as probes. Transfectants are indicated on top of each lane. Lanes: 1, parental Ba/F3 line; 2–5, single transfectants; 6–9, double transfectants. The sizes of different chimeric transcripts in kb are indicated on the left; the molecular markers in kb are expressed on the right. As shown in *B* and *C*, metabolically labeled cell lysates were immunoprecipitated with an anti-hGM-CSFr α subunit antibody (*B*) or an anti-hGM-CSFr β subunit antibody (*C*), and immunoprecipitates were separated on SDS/10% PAGE. Lanes: 1, TF-1 line as a positive control; 2, parental Ba/F3 line; 3, αS/βS cotransfectant or βS transfectant; 4, αI/βS or αI/βI cotransfectant. The bands present in lysates from the parental Ba/F3 line were nonspecific, as they were present in parallel blots probed with both the anti-α and anti-β GM-CSFr. The sizes of the wild-type receptors (αw and βw) and chimeric receptors in kDa are indicated at the edges; the molecular markers in kDa are expressed within the middle lane.

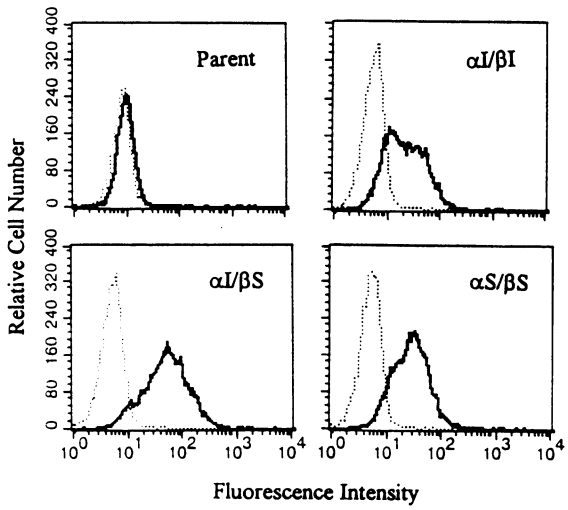

FIG. 3. GM-CSFr/PRLr receptor chimeras are expressed at the cell surface and specifically bind hGM-CSF. Binding of ligand was assessed by flow cytometric analysis of chimeric receptors of transfected Ba/F3 cells incubated with excess PE-conjugated GM-CSF. As indicated in the upper right corner of each panel, binding of PE-conjugated GM-CSF (indicated as thick line curves) by the cotransfectants greatly exceeded that of the irrelevant control protein, PE-conjugated streptavidin (indicated as thin line curves).

transfectants after GM-CSF stimulation (Fig. 5*B*). Data from additional Western blot and coimmunoprecipitation analyses revealed this protein to be the GTP/GDP exchange factor, son of sevenless 1 (Sos 1) (data not shown). These results revealed that the activation Jak2 and Fyn during PRLr signaling required the presence of two copies of the carboxy terminal motifs present in the PRLr-I. Since the PRLr-S isoform contains only an intact box 1, these data indicate that two copies of the box 1 motif alone are insufficient for the mediation of PRLr-induced signaling and mitogenesis.

DISCUSSION

PRLr, a receptor that utilizes homodimeric ligand binding domains, belongs to the growth factor receptor superfamily. All superfamily members have limited similarity in the membrane-proximal region, referred to as box 1, box 2, and variable box motifs (14, 41, 42). Each receptor also associates with one or more members of the Jak family of tyrosine kinases (43). The Jak family consists of Jak1, Jak2, Jak3, and Tyk2 (44, 45); ligand stimulation of a growth factor receptor initiates Jak autophosphorylation and activation, thereby coupling receptor dimerization with tyrosine phosphorylation of downstream signaling factors. As a sequela of Jak activation, the STAT family of transcriptional factors is phosphorylated, dimerized, and translocated into the nucleus, where they serve to induce receptor-associated gene expression (46, 47). Several laboratories have demonstrated that the protein tyrosine kinase Jak2, and to a lesser degree Jak1, are activated upon ligand stimulation of the PRLr (13–15, 48). Other signaling factors, such as the Src family phosphotyrosine kinase $p59^{Fyn}$, the serine/threonine kinase $p72–74^{Raf-1}$, and the protooncogene $p95^{Vav}$, also inducibly associate and/or are activated in response to PRL (16, 18, 19). Two salient questions, however, remained regarding the structure/function relationships within the PRLr complex: (*i*) what were the functional effects of PRLr isoform heterodimerization and (*ii*) were one or two copies of the variable box and box 2 motifs necessary for signaling via the PRLr complex?

Chimeric receptors have been successfully employed by several laboratories to study various aspects of growth factor receptor signaling. Transfection of growth factor receptor chimeras into the cytokine-responsive lines Ba/F3 or FDC-P1 have demonstrated that the functions of the extracellular (i.e., ligand binding/dimerization) and intracellular (i.e., signaling) domains were specific and interchangeable. Thus, when stimulated with the corresponding ligand, the extracellular domain of one growth factor receptor was capable of dimerization and activation of signaling specific to the intracellular domains. Examples of such chimera have included those between the extra- and intracellular domains of the following receptors: growth hormone and granulocyte colony-stimulating factor (49), erythropoietin and colony stimulating factor 1 (50), erythropoietin and the IL-3 receptor β subunit (51), GM-CSFr α/β subunits, and the IL-2 receptor β/γ subunits (52). In the study presented here, the function of heterodimeric PRLr complexes was examined through the use of the extracellular

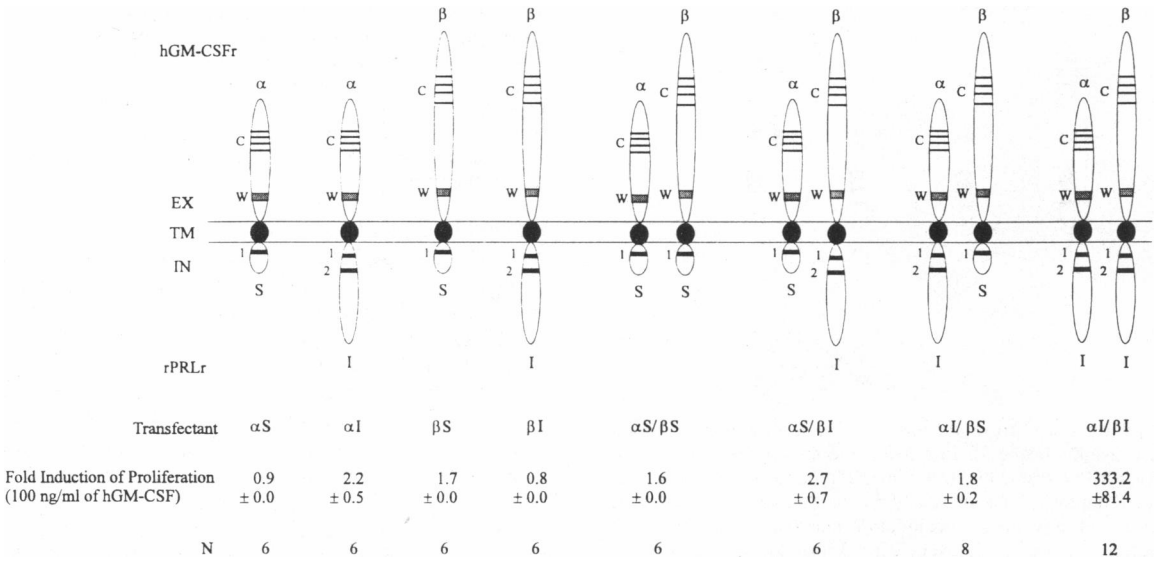

FIG. 4. Ligand-induced proliferation is observed only in $\alpha I/\beta I$ cotransfectants. The ratio of GM-CSF (100 ng/ml) stimulated/unstimulated [^{3}H]thymidine incorporation (cpm) was used to calculate the fold induction of proliferation by ligand. The mean incorporation of [^{3}H]thymidine by the parental Ba/F3 line in the presence or absence of hGM-CSF was 685 ± 45 or 737 ± 147 cpm per 1×10^6 cells, respectively. Each value represents the mean ± SEM of 6 to 12 separate experiments (shown as the experimental number, N). Statistical analysis of these results using the Student's *t*-test demonstrated a significant difference between the $\alpha I/\beta I$ cotransfectants versus the other transfectants ($P \leq 0.01$).

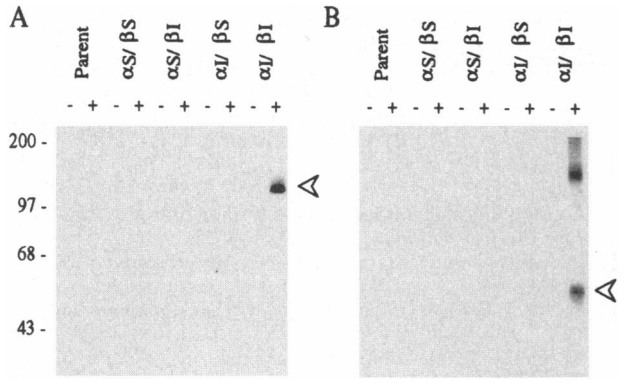

FIG. 5. Ligand-induced signal transduction is observed only in αI/βI cotransfectants. Recombinant hGM-CSF induced phosphorylation of Jak2 and Fyn through chimeric receptors in Ba/F3 transfectants was assessed by *in vitro* autokinase assay. Resting transfectants (2×10^6) (the name is indicated on top of each lane pair) were stimulated with 0 or 100 ng/ml of hGM-CSF (indicated as − or +, respectively). Cell lysates were immunoprecipitated with 5 μl rabbit polyclonal antibody against murine Jak2 (*A*) or 10 μl rabbit polyclonal antibody against Fyn (*B*) and immobilized on protein G-agarose beads, followed by incubation *in vitro* with 10 μCi [γ-^{32}P]ATP in the presence of 20 mM Mg^{2+} for 20 min. Proteins were resolved on SDS/10% PAGE followed by autoradiography. The relative positions of molecular standards are indicated in kDa. Arrows indicate $p130^{Jak2}$ and $p59^{Fyn}$ in *A* and *B*, respectively.

domains of the α and β subunits of the GM-CSFr. Coupling of these extracellular domains to the intracellular PRLr isoforms permitted one-to-one pairing of the PRLr signaling domains. As anticipated, transfectants expressing any single chimeric construct were incapable of initiating cellular proliferation in response to ligand. Similarly, transfectants expressing GM-CSFr/PRLr-S homodimers did not proliferate or demonstrate Jak2 or Fyn activation when stimulated with GM-CSF. In contrast, as a positive control, GM-CSFr/PRLr-I homodimers were fully capable of GM-CSF-induced proliferation and activation of Jak2 and Fyn in the Ba/F3 transfectants. Thus, the functional activities of the homochimeric GM-CSFr/PRLr-I complexes observed here are consistent with those previously observed with homodimeric complexes of wild-type PRLr isoforms transfected into cytokine-responsive hematopoietic cell lines (48).

The novel data obtained from these studies indicate that heterodimerization of the PRLr-S with the PRLr-I isoforms inhibits both ligand-driven proliferation and receptor-associated signaling. Like PRLr-I, PRLr-S contains a box 1 motif and a partial variable box, but lacks a box 2 motif. Thus, these data demonstrate that two copies of the carboxyl-terminal domain of PRL-I, absent in PRLr-S, are necessary for PRLr-associated signaling and proliferation. Carboxyl-terminal deletions of other growth factor receptors have demonstrated that the contribution of the box 2 motif to signaling and proliferation within this superfamily is variable. Prior study of PRLr has revealed that both box 1 and 2 were necessary for proliferation mediated by homodimerized receptor complexes (14). Although the box 1 and 2 motifs were necessary for erythropoietin receptor-associated Jak2 activation, an additional 60 aa ("extended box 2") carboxyl to the box 2 were also required for erythropoietin-driven proliferation (53). gp130-associated proliferation also required the box 2 sequence but did not require an extended box 2 motif (54). Removal of box 2 from the growth hormone receptor markedly reduced growth hormone-induced proliferation (55). In marked contrast to the erythropoietin receptor mutants, however, growth hormone receptor mutants lacking the box 2 motif demonstrated constitutive Jak2 activation and STAT signaling (42). Thus, from a structure/function perspective, PRLr most closely resembles gp130, in that a loss of a single box 2 motif precludes ligand-induced proliferation and does not lead to a constitutive activation of receptor associated signaling factors.

Other motifs within the PRLr intracellular domain, however, may contribute to PRL-driven proliferation and signaling. Unlike the PRLr-I/-S heterodimers, it is possible that heterodimeric complexes of PRLr-L/-S isoforms may be functional. Although we believe that this possibility is unlikely given the similar functionalities of the PRLr-I and PRLr-L homodimeric complexes (30), an examination of the function of PRLr-L/-S heterodimers is currently underway in our laboratory. One recent report has implicated a tyrosine residue within the carboxyl terminus of the PRLr-I (Y^{382}), absent in the PRLr-S, as necessary for Jak2 activation and PRL-stimulated gene expression in a human kidney cell line (31). The necessity of this tyrosine for proliferation and signaling, however, was not observed when PRLr-L mutants with truncations of the carboxy terminal region were transfected into a the IL-3-dependent, murine 32D line (14). Indeed, this study implicated the box 2 motif, a subdomain also absent from PRLr-S, as a critical structure in PRLr-associated transduction. Thus, while the data presented here do not distinguish between the relative importance of the box 2 and Y^{382}, our findings demonstrate that two copies of this region (i.e. the variable box/box 2/carboxy terminus present in PRLr-I, but lacking in PRlr-S) are required for the activation of Jak2 and Fyn by the dimeric PRLr complex. Whether other signaling factors associated with PRLr signaling, such as protein kinase C, Sos, and Src, are activated by the GM-CSFr/PRLr heterochimers is under active examination in our laboratory.

Previous data from our laboratory has demonstrated that a Src-family tyrosine kinase, Fyn, was complexed with the PRLr in resting Nb2 cells (16). This Src homology 2/Src homology 3 domain containing tyrosine kinase was activated by PRL stimulation; however, the direct substrates for the activated Fyn kinase within Nb2 cells had not yet been identified. The results of *in vitro* autokinase assays presented here indicate that a protein of 170 kDa is phosphorylated during these assays, using lysates from GM-CSF stimulated transfectants (Fig. 5*B*). Data from Western blot and bidirectional coimmunoprecipitation analyses have revealed this protein to be the guanine nucleotide exchange factor, Sos 1. Sos associates with the PRLr through the ubiquitous adapter protein Grb-2 (17). Our data indicate that Sos may serve as a substrate for Fyn, which could in turn activate the GDP-GTP exchange activity of this factor, required for Ras activation. Recent studies of T-cell receptor signaling have confirmed an association between Fyn and Sos and indicate that Fyn may directly contribute to the activation of this exchange factor (56). Whether the association of the Fyn with Sos occurs directly or is mediated by PRLr and/or other adapter/docking proteins awaits further structure/function studies. Thus, Src family kinases may regulate signaling through the Ras-Raf pathway, mediated by their phosphorylation of receptor/exchange factor complexes.

The data presented here demonstrate that PRLr-isoform heterodimerization modulates signal transduction from this receptor complex. This may be a generalized phenomenon for other growth factor receptors that engage ligand through homodimeric extracellular domains. Erythropoietin (57) and growth hormone (58, 59) receptor isoforms with truncated intracellular domains have been observed within human tissues. Like the PRLr-S, neither of these truncated isoforms was capable of initiating signaling when expressed as a homodimer. Whether these receptor isoforms are capable of down-regulating heterodimeric receptor complex signaling will require studies similar to those presented here. Thus, the observations presented here provide another mechanism through which members of the growth factor receptor family may modulate signaling function. Further studies using similar

chimeric methodology should determine the stoichiometric requirement for associated signaling factors and specific amino acid residues within the PRLr complex.

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