Regulation of Jak Kinases by Intracellular Leptin Receptor Sequences*

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Leptin signals the status of body energy stores via the leptin receptor (LR), a member of the Type I cytokine receptor family. Type I cytokine receptors mediate intracellular signaling via the activation of the associated Jak family tyrosine kinases. Although their COOH-terminal sequences vary, alternatively spliced LR isoforms (LRa–LRd) share common NH2-terminal sequences, including the first 29 intracellular amino acids. The so-called long form LR (LRb) activates Jak-dependent signaling and is required for the physiologic actions of leptin. In this study, we have analyzed Jak activation by intracellular LR sequences under the control of the extracellular erythropoietin (Epo) (Epo receptor/LRb chimeras). We show that Jak2 is the requisite Jak kinase for signaling by the LRb intracellular domain and confirm the requirement for the Box 1 motif for Jak2 activation. A minimal LRb intracellular domain for Jak2 activation includes intracellular amino acids 31–48. Although the sequence requirements for intracellular amino acids 37–48 are flexible, intracellular amino acids 31–36 of LRb play a critical role in Jak2 activation and contain a loose homology motif found in other Jak2-activating cytokine receptors. The failure of short form sequences to function in Jak2 activation reflects the absence of this motif.

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Leptin is a 16-kDa adipocyte-derived hormone that communicates the status of body energy stores to the central nervous system, regulating appetite, metabolic rate, and neuroendocrine function (1, 2). Leptin mediates these effects by binding to the leptin receptor (LR) (3); the structure of leptin is homologous to that of the IL-6 family of cytokines, and the LR is a member of the IL-6 receptor family of class I cytokine receptors (3). Alternative splicing of RNA from a single LR gene produces multiple LR isoforms that share a common ligand-binding extracellular domain (4, 5). LR lacks a transmembrane domain and is secreted. LRa–d each contain the same transmembrane domain and 29 membrane-proximal amino acids including the highly conserved, proline-rich Box 1 sequence that is required for Jak kinase activation by cytokine receptors. The number and identity of the subsequent amino acids varies among murine LRa–d, as well as the three human LR isoforms. LRb, which is highly conserved across species, contains a 282-amino acid extension (total 301-amino acid intracellular tail), robustly activates intracellular signaling, and is required to mediate the physiologic actions of leptin. Murine LRa, LRc, and LRd are the “short forms” of the leptin receptor with unclear physiological roles; these receptors contain 5, 3, and 11 amino acid extensions for 34–32, and 40-amino acid intracellular tails, respectively.

LRb, like other cytokine receptors, signals via associated Jak family tyrosine kinases, which autophosphorylate and become activated upon ligand binding and subsequently mediate phosphorylation of Tyr985 and Tyr1138 on LRb (6–11). Phosphorylated Tyr1138 recruits the transcription factor STAT3, whereas phosphorylated Tyr985 recruits the SH2 (Src homology 2) domain-containing protein-tyrosine phosphatase, SHP-2, as well as recruiting SOCS-3 to mediate feedback inhibition of LRb signaling (9–12). LRb-associated tyrosine-phosphorylated Jak2 mediates additional phosphotyrosine-dependent signals (11).

A number of questions surround the issue of LR-Jak kinase interaction, including the specificity of Jak kinase utilization by the LR, the role of other cytokine receptor homology motifs in Jak activation, and the ability of short forms to mediate signaling. Of the three ubiquitously expressed Jak kinases (Jak1, Jak2, and Tyk2) (13), Tyk2 has not been examined, and overexpression studies have suggested that LRb may signal via both Jak1 and Jak2 (14). Furthermore, under similar conditions, LRa may mediate some Jak2 activation and signaling (14), whereas LRc and LRd have not been studied. Indeed, Box 1 (the proline-rich membrane-proximal motif that is conserved among cytokine receptors) is required for the recruitment of all Jak kinase isoforms and is present at intracellular amino acids 6–17 in all transmembrane forms of the LR (4, 13, 15). The striking phenotype of db/db mice, in which LRa replaces LRb, is indistinguishable from that of entirely leptin-deficient ob/ob mice, however (16), suggesting that the short LR forms fail to mediate Jak activation and signaling. Indeed, the Box 2 motif, COOH-terminal to Box 1, may be important for the recruitment of Jak kinases by cytokine receptors (15). Two Box 2 homologous regions of LRb (both absent in other LR forms) have been proposed (7, 15).

Here we show that Jak2 is the only Jak kinase activated during and required for LRb signaling and that LRb sequences within and immediately surrounding Box 1 are required for the activation of Jak2. Although neither potential Box 2 motif is required, amino acids 31–36 of LRb are essential for Jak2 tyrosine phosphorylation. The sequences of the LR isoforms
diverge in this region, and none of the short forms mediate activation of Jak2 at physiologic levels of Jak2.

**MATERIALS AND METHODS**

*Antibodies, Growth Factors, and Reagents—*Rabbit αLRb has been described previously (11); rabbit αJak2 was raised against a synthetic peptide corresponding to amino acids 758–770 of murine Jak2. Polyclonal αSTAT3 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant murine IL-3, αTyk2, and αJak1, and monoclonal αphosphotyrosine (αPY) (4G10) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies directed against the phosphorylated (activated) form of extracellular signal-regulated kinase (ERK) and STAT1 were purchased from New England Biolabs (Beverly, MA) and BD Biosciences, respectively. Recombinant mouse erythropoietin (Epo) was purchased from BD Biosciences. Bovine serum albumin fraction V was purchased from Calbiochem. Protein A-Sepharose 6MB, 125I-Epo, and 125I-protein A were from Amersham Biosciences.

*Generation of Epo Receptor/LRb (ELR) Mutants—*The ELR chimera in pcDNA3 has been described previously (11). The ELR chimera consists of the extracellular domain of the Epo receptor joined to the intracellular domain of the long form of the leptin receptor, LRb; the junction occurs at a silent A/III site at the 3’-end of the transmembrane domain-encoding sequence. We generated four sets of ELR deletion mutants. The first set of ELR deletion mutants contained large COOH-terminal deletions: Δ219c, Δ35c, Δ37c, Δ43c, Δ49c, Δ51c, and Δ65c (deletions beginning at the indicated intracellular amino acid), and VNN (in which the conserved PNP motif in Box 1 was mutated to VNN). These mutations were made by PCR mutagenesis of the intracellular domain and subcloning into pcDNA3ELR using A/III and NotI as described previously (11). The second series of ELR constructs also contained large COOH-terminal deletions and included the following mutants: Δ29c, Δ33c, Δ35c, Δ43c, Δ49c, Δ51c, and Δ65c (deletions beginning at the indicated intracellular amino acid). The mutations were generated by PCR using a 5’-oligonucleotide containing an A/III site at the 5’-end and the sequence for the intracellular 28 amino acids of LRb. The 3’-oligonucleotide used in this reaction overlapped with the 5’-oligonucleotide by 21 base pairs and contained a sequence complementary to the desired 3’-terminus of that mutation including a stop codon and a NotI site. The PCR product was subcloned into pcDNA3ELR using A/III and NotI. The third panel of ELR constructs contained six amino acid internal deletions within the mutant Δ65c described previously and included the following mutants: Δ1–6, Δ7–12, Δ13–18, Δ19–24, Δ25–30, Δ31–36, Δ37–42, Δ43–48, Δ49–54, Δ55–60, and Δ61–64. These mutations were generated by PCR using pcDNA3Δ65c as a template and 5’-oligonucleotides containing an A/III site with sequences encoding the first 24 intracellular amino acids of LRb (containing the appropriate deletions). The 3’-oligonucleotides for these reactions contained sequences encoding the next 40 amino acids of LRb (including the appropriate deletions) followed by a NotI site. For each mutant either the 5’- or 3’-oligonucleotide contained an internal deletion of six amino acids. The PCR product was subcloned into pcDNA3ELR using A/III and NotI. ELR mutants corresponding to the additional isoforms of the murine LR (LRa, LRb, and LRd) were generated by PCR using the same 5’-oligo as for the COOH-terminal deletions and specific 3’-oligos containing a NotI site and sequences specific for each particular isoform. Subcloning was again by A/III and NotI. The identity of each ELR variant was confirmed by DNA sequence analysis.

*ELR Adenovirus—*Recombinant adenoviruses were generated using the AdEasy system (17). The ELR cDNA was excised from pcDNA3 and subcloned into pAdTrack-CMV using HindIII and XhoI. The resulting pAdTrack-CMV ELR was linearized and cotransfected with pAdEasy into BJ5183 bacteria by electroporation, and kanamycin-resistant colonies were analyzed for correct recombination. Correctly recombined pAdEasyTrack-CMV ELR was linearized and transfected into 293 cells (as below) for the generation of virus. The resulting adenovirus (Ad-ELR) was propagated in 293 cells, which were harvested 5 days post-infection by scraping and two cycles of freeze/thaw. Clarified supernatants were used to infect cells.

*Cell Lines—*All cells were transfected and maintained in a humidified atmosphere containing 5% CO2 and 95% air at 37°C. 32D cells were grown in 15% FBS and 15% horse serum. 1640 medium supplemented with 10% FBS and 5% WEHI-3-conditioned medium (a source of IL-3). 32D cells stably expressing ELR have been described (11). Jak kinase-deficient fibrosarcoma cell lines (kindly provided by Dr. George Stark, Cleveland Clinic, Cleveland, OH) were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated FBS (18).

293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. ELR constructs in pcDNA3 were transiently cotransfected with pcDNA3Jak2 into 293 cells using LipofectAMINE (Invitrogen); for the generation of stably expressing clones ELR constructs were transfected alone and split the following day for selection in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and 750 μg/ml G418. 293 clones expressing Epo on the cell surface were identified for further experiments by 125I-Epo binding (11).

Cell surface 125I-Epo binding was also determined in conjunction with each experiment performed to ensure the similar expression cell surface expression of each receptor isoform studied. Briefly, cells were plated to 10-cm dishes in parallel with experimental plates and were treated identically to experimental plates until the time of stimulation, at which time they were incubated for 30 min with ~25000 counts per minute/dish of 125I-Epo for 30 min at room temperature before washing three times with phosphate-buffered saline. Washed cells were lysed with 1% SDS, and bound radioactivity was quantified in a γ-counter. In each experiment, between 10 and 30% of total counts were bound by experimental lines, with the exception of untransfected control cells, which bound less than 1% of total counts.

*Preparation of Cell Lysates for Immunoprecipitation—*Prior to each experiment, subconfluent cells were made quiescent by incubation in Dulbecco’s modified Eagle’s medium containing 0.5% bovine serum albumin (32D cells, 4 h; 293 and fibrosarcoma cells, overnight) before stimulation with 50 ng/ml Epo at 37°C for 5 min. Cells were lysed in 20 mM Tris pH 7.4, containing 157 mM NaCl, 5 mM EDTA, 10% glycerol, 50 mM β-glycerophosphate, 50 mM Na3PO4, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, and 2 mM sodium orthovanadate (lysate buffer). Insoluble material was removed by centrifugation at 16,000 × g at 4°C for 5 min. Protein concentrations of the resulting lysates were determined, and equivalent amounts of protein were added to the appropriate antibodies for immunoprecipitation or denatured in 2× Laemmli buffer for direct resolution by SDS-PAGE. For immunoprecipitations, lysates were incubated with antisera at 4°C overnight followed by incubation with protein A-Sepharose for 30 min. Immunocomplexes were collected by centrifugation and washed three times in lysate buffer before denaturation in Laemmli buffer and separation by SDS-PAGE.

*Immunoblotting—*SDS–polyacrylamide gels were transferred to nitrocellulose membranes (Schleicher and Schuell) in Towbin buffer containing 0.2% SDS and 20% methanol. Membranes were blocked for 1 h at room temperature or overnight at 4°C in buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 0.01% Tween 20 (wash buffer) supplemented with 3% bovine serum albumin (block buffer). Membranes were incubated in primary antibody in block buffer for 1 h, rinsed three times with wash buffer, and incubated for 30 min in block buffer. Detection was by incubation for 1 h with 125I-protein A in block buffer (preceded by a 1-h incubation with rabbit anti-mouse antisera followed by washing in the case of 4G10 immunoblotting). Blots were rinsed four times in wash buffer before overnight exposure on Kodak X-Omat AR film or a PhosphorImager.

**RESULTS**

*Jak Kinase Activation by the Intracellular Tail of LRb—*To assess the Jak kinase selectivity of the intracellular domain of LRb, we examined the ability of a chimeric protein described previously consisting of the extracellular domain of the Epo receptor and the intracellular domain of LRb (ELR) (11) to stimulate tyrosine phosphorylation of the common Jak kinases (Jak1, Jak2, and Tyk2). We performed these assays in 32D myeloid progenitor cells stably expressing ELR (32D/ELR cells), because 32D cells abundantly express Jak1, Jak2, and Tyk2 in addition to functional IL-3 receptors (Fig. 1). The IL-3 receptor mediates tyrosine phosphorylation of Jak1, Jak2, and (weakly) Tyk2 and thus serves as a positive control. Because activation of Jak kinases results in their tyrosyl autophosphorylation, we assayed their stimulation by αPY immunoblotting of αJak1, αJak2, or αTyk2 immunoprecipitates prepared from 32D/ELR cells that had been incubated in the absence or presence of erythropoietin or IL-3 (Fig. 1, A–C). The activation of STAT3 was also determined by immunoblotting lysates with anti-activated specific for the tyrosyl-phosphorylated (activated) form of STAT3 (Fig. 1D). Although Epo and IL-3 both stimulated tyrosine phosphorylation of STAT3 and Jak2, only IL-3 mediated tyrosine phosphorylation of Jak1 (and weakly) Tyk2.
Thus, of the three common Jak kinases, ELR mediates activation of Jak2 but not Jak1 or Tyk2.

To confirm the specificity of and requirement for Jak2 in ELR signaling, we employed mutant human fibrosarcoma cell lines lacking various Jak kinase isoforms (18). U1A (lacking Jak1), U4A (lacking Tyk2), and 2A (lacking Jak2) cells were infected with an adenovirus that co-expresses ELR and green fluorescent protein. Green fluorescent protein fluorescence and 125I-Epo binding confirmed similar infection and cell surface expression in each cell line (data not shown). The various ELR-expressing cells were incubated in the absence or presence of Epo for 5 min, and ELR signaling was assessed by aPY immunoblotting of aELR immunoprecipitates and aSTAT3(PY) immunoblotting of cell lysates (Fig. 2, A and B). This analysis demonstrated tyrosine phosphorylation of STAT3 and ELR in Jak1-deficient U1A and Tyk2-deficient U4A cells but not in Jak2-deficient 2A cells. No signal was detected in uninfected cells (data not shown). A tyrine-phosphorylated protein species that migrated at the approximate molecular weight of Jak proteins was also detected during Epo stimulation in aELR immunoprecipitates from all but 2A cells (Fig. 2A). Thus, the tyrosine phosphorylation of ELR and STAT3 correlates with the ability to mediate tyrosine phosphorylation of associated Jak proteins, presumably Jak2. These data suggest that Jak2 is the unique Jak kinase activated by and required for signaling by the intracellular tail of LRb, and the remainder of our analysis thus focuses on Jak2.

**Box 1 and Membrane-proximal Sequences of LRb Mediate Jak2 Tyrosine Phosphorylation**—To define the approximate region of the intracellular LRb required for Jak2 interaction, we generated a set of mutant LRb constructs containing large COOH-terminal deletions (Fig. 3). Mutants include Δ219c, Δ65c, and Δ1c, which contain 218, 64, and 0 amino acids from the 301-amino acid LRb intracellular domain, respectively. Also included is VNV (which substitutes the conserved proline residues in Box 1 with valine residues). 293 cells were co-transfected with Jak2 and these LRb mutants. Cell surface 125I-Epo binding assay demonstrated similar cell surface expression of the LRb isoforms (data not shown). We assessed the ability of each of these receptor forms to mediate tyrosine phosphorylation of Jak2 during ligand stimulation by immunoprecipitating Jak2 from cells incubated in the presence or absence of Epo and analyzing the precipitated protein by aPY immunoblotting (Fig. 3). As expected, tyrosine phosphorylation of Jak2 and the associated ELR were detectable during stimulation of ELR-expressing cells. Additionally, tyrosine phosphorylation of Jak2 and a truncated ELR (retaining Tyr985) were detected during stimulation of Δ219c. Deletion of the entire LRb intracellular domain (Δ1c) or mutation of the conserved proline residues in Box 1 (VNV) abrogated Epo-stimulated Jak2 tyrosine phosphorylation of Jak2, whereas Δ65c mediated the tyrosine phosphorylation of Jak2. This analysis demonstrated that although intracellular sequences of LRb (and specifically the conserved PNP motif within Box 1) were required for Jak2 activation, the cellular sequences of LRb (and specifically the conserved PNP motif within Box 1) were required for Jak2 activation, the membrane-proximal potential sequences common to all LR isoforms are shown in black, and LRb-specific sequences are shown in gray. The locations at which various deletion mutations truncate are indicated (arrows), along with the PNP → VNV mutation in Box 1. Bottom Panel, the ELR constructs diagrammed in the top panel were expressed in 293 cells by cotransfection with Jak2. Transfected cells were incubated in the absence or presence (+) of 50 ng/ml Epo for 5 min before being lysed. Lysates were clarified, normalized for protein content, and immunoprecipitated (IP) with aLRb. Precipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting (IB) with aPY. Migration of phosphorylated Jak2 proteins, ELR, and STAT3 is indicated to the right of the autoradiograms.

**Minimal LRb Region for Jak2 Tyrosine Phosphorylation**—To assess the requirement for the membrane-proximal potential Box 2 motif (intracellular amino acids 49–60) in the phosphorylation of Jak2 and to more specifically define the sequences of LRb that are required for Jak2 activation, we generated a second panel of ELR mutants containing COOH-terminal deletion mutations (Fig. 4, top panel). In this series of mutants, we serially truncated a small number of amino acids at a time from the COOH terminus beginning with intracellular amino
LRb Sequences Specify Jak2 Activation

![Diagram](image)

Fig. 4. Minimal LRb region for Jak2 tyrosine phosphorylation. Top panel, the amino acid sequence of the first 64 intracellular amino acids of LRb is shown. The end of the transmembrane domain (TM), Box 1, and the membrane-proximal potential Box 2 motif are indicated. Sequences common to all LR isoforms are shown in gray, and LRb-specific sequences are shown in black. The locations at which various deletion mutations truncate is indicated (arrows). Lower panels, the indicated ELR constructs (diagrammed in the upper panel) were expressed in 293 cells either by (A and C) transient co-transfection with Jak2 or (B and D) selection of stably expressing clones. A and B, quiescent cells were incubated in the absence (–) or presence (+) of 50 ng/ml Epo for 5 min and lysed. Clarified lysates were normalized for protein content and immunoprecipitated with αJak2. Precipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting (IB) with αPY. Migration of tyrosine-phosphorylated Jak2 is indicated to the right of the autoradiograms. The data shown are representative of at least three independent experiments; in the case of stable cell lines, at least two independent clones were analyzed for each receptor mutant. C and D, cell-surface receptor expression of experimental cells was assayed by 125I-Epo binding. Data are representative of at least three independent determinations and are expressed as % of the ELR control ± S.E.

Acid 65 (Δ65c, above) (e.g., Δ61c, Δ49c, Δ43c, Δ37c, etc.). To assess the ability of these mutants to activate Jak2, we transiently co-transfected 293 cells with Jak2 and the various ELR mutants and assessed the ability of the various mutants to mediate ligand-stimulated tyrosyl phosphorylation of Jak2 (Fig. 4A). 125I-Epo binding demonstrated similar cell-surface expression of the ELR isoforms (Fig. 4C). We assessed the ability of each of these receptor forms to mediate tyrosine phosphorylation of Jak2 during ligand stimulation by immunoprecipitating Jak2 from cells incubated in the presence or absence of Epo and analyzing the precipitated protein by αPY immunoblotting (Fig. 4A). Truncation of LRb sequences COOH-terminal to intracellular amino acid 36 in ELR, Δ37c, Δ43c, Δ49c, Δ61c, and Δ65c, did not prevent ligand-stimulated tyrosine phosphorylation of Jak2. In contrast, deletion of sequences NH2-terminal to amino acid 37 in Δ29c and Δ31c abrogated the tyrosine phosphorylation of Jak2, suggesting an important role for LRb sequences between intracellular amino acids 31–36 in Jak2 activation. Moreover, these data suggest that the LRb sequences COOH-terminal to intracellular amino acid 37, including the membrane-proximal potential Box 2 motif, are not essential for tyrosine phosphorylation of Jak2.

It is possible that in the preceding assay the artificially high intracellular Jak2 levels achieved by transient co-transfection of Jak2 and the various ELR mutants masked a requirement for Box 2 or other sequences COOH-terminal to intracellular amino acid 36. Because transient transfection of ELR alone does not result in ELR expression in a high enough fraction of cells to analyze signaling effectively in the absence of co-expressed Jak2, we generated stably expressing 293 cell lines in which the ELR mutants were expressed in 100% of the cell population to facilitate the analysis of signaling. We confirmed similar cell-surface ligand binding of each stably expressing cell line (Fig. 4D) and repeated the analysis of the COOH-terminal truncations at endogenous levels of Jak2 in these 293 clones (Fig. 4B). The results of this analysis differed importantly from those in 293 cells co-transfected with Jak2 and the various ELR constructs in Fig. 4A, in that the activation of Jak2 by ELR isoforms Δ37c and Δ43c were greatly reduced; activation by Δ49c remained normal. Similar results were obtained in 32D cell lines expressing the various COOH-terminal truncations (data not shown). These results confirm that the region of LRb between amino acids 31–36 contains a domain that is critical for Jak2 activation. Furthermore, the residues immediately COOH-terminal to amino acids 31–36 (i.e. between 37 and 48) function in Jak2 activation at physiologic levels of Jak2, whereas the region COOH-terminal to intracellular amino acid 48 is not necessary for Jak2 activation even at endogenous levels of Jak2 expression.

Internal Deletions Pinpoint LRb Sequences Required for Jak2 Activation—To identify other potentially important LRb sequences NH2-terminal to intracellular amino acid 37 of LRb, we generated a panel of mutant ELR constructs containing six amino acid internal deletions within the mutant Δ65c (Fig. 5,
LRb Sequences Specify Jak2 Activation

To resolve this issue, we repeated the analysis of the internal deletion mutants in 293 cells stably expressing several ELR internal deletion mutants (Fig. 5, B and D). The results of this analysis were for the most part similar to those observed in the 293 cells transiently co-transfected with Jak2; Δ25–30, Δ37–42, Δ43–48, and Δ49–55 mediated Jak2 tyrosine phosphorylation. Similar results were obtained in stably expressing 32D cells (data not shown). In contrast to the results in transiently transfected cells, however, Δ31–6 failed to mediate the tyrosine phosphorylation of Jak2 in stably transfected cells. Thus, the region of LRb between intracellular amino acids 13–24 contains sequences essential for the ligand-stimulated tyrosine phosphorylation of Jak2 even at overexpressed levels of Jak2. Intracellular amino acids 31–36 of LRb also mediate Jak2 phosphorylation, but sequences COOH-terminal to intracellular amino acid 36 can substitute for this region at higher Jak2 levels. In the absence of COOH-terminal sequences, as in Δ31c, intracellular residues 31–36 are absolutely required. In contrast, although the presence of residues 43–48 amino acids inside the membrane seems to be important at physiologic levels of Jak2, the exact sequence requirements are loose.

Activation of Jak2 by Short LR Isoforms—The preceding analysis suggests that amino acids 31–36 of LRb are critical for the LRb-mediated tyrosine phosphorylation of Jak2 and that amino acids 37–48 are required in the absence of downstream sequence elements that may substitute for them. Interestingly, the various LR isoforms diverge in this region. The various LR isoforms have the same first 29 amino acids as LRb but have varying additional sequences of five (LRα), three (LRc), and 11 (LRd) amino acids (Fig. 6A). Hence, the short murine LR isoforms may differ in the ability to mediate Jak2 tyrosine phosphorylation and downstream signaling. To determine the ability of these other LR isoforms to activate Jak2, we generated a
set of ELR constructs that contained the internal sequence of each murine LR isoform (Fig. 6). As above, we initially analyzed these constructs in 293 cells by co-transfection of Jak2 and the various ELR forms with intracellular tails corresponding to LRA, B, C, and D (Fig. 6, A and C). 125I-Epo binding demonstrated similar cell surface expression of the ELR isoforms (Fig. 6C). We assessed the ability of each of these receptors to mediate tyrosine phosphorylation of Jak2 during ligand stimulation by immunoprecipitating Jak2 from cells incubated in the presence or absence of Epo and analyzing the precipitated protein by αPY immunoblotting. As before, ELR Δ29C, which contains only 28 intracellular amino acids, did not activate Jak2, whereas ELR robustly activated Jak2. ELRA, ELRC, and ELRD each mediated reduced tyrosine phosphorylation of Jak2 at ~10–30% of the level mediated by ELR. Hence, at high Jak2 levels, the short forms of LRA, C, and D are able to mediate some phosphorylation of Jak2, although to a lesser degree than ELR.

Because LRb amino acids 31–36 were not required for Jak2 activation when assayed by cotransfection with Jak2 (Fig. 5A, Δ31–36), we examined the possibility that the Jak2 tyrosine phosphorylation that was mediated by the short LR isoforms was secondary to artificially high Jak2 levels. We thus assessed the ability of the ELR short forms to mediate Jak2 phosphorylation at the physiological Jak2 levels in 293 cells stably expressing the various ELR isoforms (Fig. 6, B and D). Indeed, although ELR robustly activated Jak2 in these systems, ELR Δ29C, ELRA, ELRC, and ELRD failed to mediate the tyrosine phosphorylation of Jak2.

We confirmed that co-expression of Jak2 increased levels of
Jak2 and signal intensity in these cells by directly comparing in the same experiment Jak2 levels and activation among 293 cells stably expressing ELR or ELRa and cells cotransfected with Jak2 and ELR or ELRa (Fig. 6E). This analysis demonstrated a severalfold overexpression of Jak2 with transient transfection in addition to more robust Jak2 tyrosine phosphorylation in Jak2-transfected cells. Thus, our analysis suggests that the short forms of the leptin receptor do not mediate downstream signaling at physiologic Jak2 levels.

DISCUSSION

Cytokine receptors contain no intrinsic enzymatic activity but transmit signals via non-covalently associated Jak family tyrosine kinases (6, 8). Ligand binding to the leptin receptor mediates tyrosyl phosphorylation of the constitutively LRb-associated intracellular Jak kinase molecules (7, 20, 21). Phosphorylation of paired tyrosine residues within the Jak tyrosine kinase domain function to alter the conformation of the molecule, activating the tyrosine kinase (13). Hence, tyrosine phosphorylation of Jak kinases reflect tyrosine kinase activation. In addition to mediating tyrosine phosphorylation of residues on the cytokine receptor, cytokine receptor-associated Jak kinases mediate downstream signals independent of receptor tyrosine phosphorylation (13, 18). In the case of LRb, it is presumably the activated Jak kinase that mediates residual downstream signaling (e.g. to extracellular signal-regulated kinase activation) by a mutant LRb devoid of intracellular tyrosine residues (11). A number of data indirectly suggest that LRb-activated Jak2 may mediate important leptin-regulated neural signals (11). A number of data indirectly suggest that LRb-activated Jak2 may mediate downstream signals independent of receptor tyrosine phosphorylation in Jak2-transfected cells. Thus, our analysis suggests that the short forms of the leptin receptor do not mediate downstream signaling at physiologic Jak2 levels.

Fig. 7. Sequence homology among cytokine receptors. Shown are the membrane-proximal amino acid sequences of eight murine cytokine receptors, including LRb, with known Jak kinase preference (indicated on the right). Also shown are consensus homology sequences (CONS.) and the sequences of alternate LR isoforms. Conserved residues are underlined in all receptors. The residues known to be important for Jak2 interaction with EpoR (27) and LRb (this analysis) are indicated in gray boxes, and the putative residues in the region homologous to intracellular amino acids 31–36 of LRb are italicized in Jak2-associating receptors and in the consensus motif.

Jak2 kinases, the intracellular domain of LRb fails to mediate tyrosine phosphorylation/activation of Jak1 or Tyk2, although it robustly mediates the tyrosyl phosphorylation of Jak2. Our analysis using a panel of fibrosarcoma cell lines devoid of various Jak kinase isoforms demonstrates that the intracellular domain of LRb is tyrosine-phosphorylated and mediates STAT3 activation during ligand stimulation in the absence of Jak1 or Tyk2 but not in the absence of Jak2. Hence, Jak2 is the unique Jak kinase isoform activated by and required for signaling by the intracellular tail of LRb. The LRb-mediated activation of other Jak kinases observed by others likely represents an artifact of Jak kinase overexpression (14); indeed, this is consistent with our observation that the requirement for sequences outside of Box 1 can be overcome by high level Jak kinase overexpression. Because the Box 1 motif is common to all cytokine receptors regardless of Jak kinase choice, receptor sequences COOH-terminal to Box 1 determines Jak kinase specificity, and we show that the requirement for specific COOH-terminal sequences can be overcome by overexpression of Jak2.

Our present data confirm that Box 1 and the immediately surrounding residues are essential for mediating the tyrosine phosphorylation of Jak2. Although the first 12 intracellular amino acids of LRb (including the first half of the Box 1 motif) are not required for Jak2 activation, the poorly conserved intracellular amino acids 19–24 are clearly important for Jak2 activation. The normal function of Box 2 and subsequent residues is consistent with our observation that the requirement for Box 2 is not present in Box 1. Jak2 is the unique Jak kinase isoform activated by and required for signaling by the intracellular tail of LRb.

The so-called Box 2 motif also plays a critical role in Jak kinase recruitment by numerous cytokine receptors (including gp130) (28); two potential Box 2 homology motifs on LRb have been identified (intracellular amino acids 49–60 and 202–213) (7, 15). In contrast, the majority of the COOH-terminal tail of LRb, including both putative Box 2 motifs, is not required for the activation of Jak2; only the first 37 intracellular amino acids of LRb are required under conditions where Jak2 is overexpressed. These results are consistent with the results of Bahrenberg et al. (29), who also showed that Box 2 is not required for signaling by LRb. Indeed, data from other cytokine...
receptors suggest that Box 2 is required for the binding and activation of Jak1 and Tyk2 but not Jak2 (28, 30). We identify the region of LRb between intracellular amino acids 31–36 as critical for mediating the tyrosine phosphorylation of Jak2 at physiologic levels of intracellular Jak2. Indeed, others have suggested a role for the first 15 LRb-specific amino acids (to intracellular residue 44) in the activation of Jak2 (29). Interestingly, COOH-terminal truncations that delete intracellular residues 31–36 abrogate the ability of the receptor to mediate Jak2 tyrosine phosphorylation even in the context of Jak2 overexpression, whereas internal deletions that remove these residues demonstrate a phenotype only at lower, more physiologic levels of Jak2. Similarly, intracellular amino acids 37–48 also contribute to Jak2 activation as assessed by COOH-terminal truncation but are dispensable when deleted internally. One reasonable interpretation of these results is that at supraphysiologic levels of Jak2, sequences COOH-terminal to intracellular amino acids 31–36 can weakly substitute for the important motif within amino acids 31–36. Even at physiologic levels of Jak2, the function of residues 37–48 can be replaced by other sequences. Similarly, analysis of internal deletions within the EpoR suggest that the region homologous to LRb residues 31–36 are important for Jak2 binding but that COOH-terminal sequences are not required for Jak2 signaling (27). The flexible sequence requirements for amino acids 37–48 suggest that the peptide backbone in the region between amino acids 43–48 may be involved in interaction with Jak2.

This analysis suggests the presence of three elements of LRb sequence that are required for Jak2 activation: 1) The Box 1 PXPF motif that is common to all cytokine receptor/Jak kinase interactions and indispensable for these interactions. 2) Amino acids 31–36, containing a secondary element that mediates the activation of Jak2 by LRb and that cannot be substituted at physiologic levels of Jak2 expression. 3) Amino acids 37–48, which appear to increase the strength of the Jak2 signal but that can be substituted by other LRb sequences.

Consistent with the requirement for intracellular residues 31–36 of LRb and sequences beyond 43 intracellular amino acids for Jak2 activation by LRb, we show that the alternate (short) isoforms of the murine LR with different primary sequences in this region fail to mediate the phosphorylation of Jak2 at physiologic intracellular Jak2 levels. At high intracellular Jak2 levels the short forms of the LR were able to weakly activate Jak2; such phosphorylation likely reflects weak substitution of the short form sequences for LRb intracellular amino acids 31–36 as observed for COOH-terminal LRb sequences in Δ31–6 perhaps because of the lack of COOH-terminal sequences to and beyond 43 amino acids. Interestingly, when co-expressed with Jak2, these short LR forms perform more poorly than Δ31–36. These data are consistent with a model of short LR function that is independent of Jak2 phosphotyrosine-dependent signaling for all of the murine short LR forms. Although our present analysis was conducted with chimeric receptors, the results of others (28–33) that have used chimeric receptors suggest that extracellular sequences do not alter Jak kinase interactions. Furthermore, although we have studied transfected cells, we have endeavored to analyze the mutant receptors in as physiologic a context as possible by conducting much of the analysis at endogenous Jak kinase levels.

Although it is clear from our present analysis that intracellular amino acids 31–36 of LRb are critical for the efficient tyrosine phosphorylation of Jak2, the critical element(s) missing from COOH-terminal LRb sequences and alternate LR sequences remain somewhat unclear. Alignment of the juxtamembrane sequences of numerous cytokine receptors suggested the presence of a conserved Pro/Gly residue (LRb intracellular amino acid 30) (Fig. 7), but our present analysis suggests that the region containing intracellular amino acids 25–30 are not required, and our mutational analysis of this residue of LRb confirmed that this residue is not required for Jak2 tyrosine phosphorylation by δ57C (data not shown). A more limited analysis of cytokine receptors specific for Jak2 suggests the presence of a loosely conserved motif in this region that contains multiple di/Pro/Gln residues preceding a terminal Leu/Ile residue, (ENX)X₁₋₂(ENQ)X₋₄(L/D) (where X is any amino acid). Indeed, receptors that predominantly signal via Jak1 and/or Tyk2 (such as gp130, IFNRe, and IL2Rβ) do not contain this motif and generally require sequences COOH-terminal to this region (e.g. Box 2) for association with signaling by these Jak kinase isoforms (31, 33). In contrast, as for LRb, the residues that make up this (ENQ/R)–rich motif in the Epo receptor are required for Jak2 association, whereas residues immediately COOH-terminal to this motif are not required (27). This motif, even by its loosest definition, is absent in all LR isoforms other than LRb. It is reasonable to hypothesize that this (ENQ/R)–rich motif specifies the binding and activation of Jak2 instead of other Jak kinases.

Our present analysis demonstrates that Jak2 is the primary Jak kinase involved in signaling by the intracellular domain of LRb. This finding is important to our understanding of LR signaling, because tyrosine phosphorylation sites on Jak kinases engage downstream signals that may vary among Jak family members. LRb contains a conserved motif within intracellular amino acids 31–36, as well as sequences extending the tail to and beyond 43–48 intracellular residues. Both of these properties are critical for Jak2 binding and are absent from murine short LR isoforms, explaining the inability of these forms to mediate intracellular or physiologic signaling.

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