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Homodimerization of IL-2 receptor β chain is necessary and sufficient to activate Jak2 and dowstream signaling pathways

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Abstract Cytokine receptor signaling involves the Jak/Stat pathways. Heterotrimeric IL-2R (α, β, γ_c chains) activates Jak1 and Jak3, whereas homodimeric PRLR activates Jak2. The requirements directing such specificity of Jak activation are unknown. We show that chimeric receptors containing the intracellular domain of IL-2Rβ chain fused to the extracellular domain of either EPOR or Kit, a non-cytokine receptor, activate Jak2. This observation provides evidence that IL-2Rβ intrinsically possesses the ability to activate Jak2, but that this property is only displayed in homodimerized complexes. Our data suggest a role for the stoichiometry of cytokine receptors in selective activation of Janus kinases.

Key words: Cytokine receptor; Janus kinase; Specificity; Dimerization; Signal transduction

1. Introduction

The stoichiometry of activated cytokine receptor complexes is highly variable. For instance, PRLR and EPOR are homodimers, IL-2R is a heterotrimeric complex involving α, β and γ_c chains, whereas signaling IL-6R is a hexameric complex consisting of two molecules each of IL-6, IL-6R (α chain) and gp130 [1]. Cytokine receptors are intrinsically devoid of any enzymatic activity and signal through the Jak/Stat cascade [2]. To date, four Jak kinases (Jak1, Jak2, Jak3, Tyk2) and seven Stats (Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, Stat6) have been identified. Although it is widely assumed that the specificity of cytokine receptor signaling is partly directed by the subset of Jaks and Stats that are activated by each receptor, the molecular basis of such specificity is not yet completely understood. Based on mutational studies of tyrosines within several cytokine receptors, it has been suggested that first the receptor-Stat interaction involves phosphotyrosines of the former and the SH2 domain of the latter, and second the panel of Stats that are specifically recruited by receptors is directed by consensus sequences in the near environment of these phosphotyrosines [3–6]. Although it is clear that the associated Jaks interact with the membrane-proximal part of the receptor cytoplasmic domains, which involves the proline-rich box 1 [2,7], the molecular features directing the specific recruitment of a given Janus kinase by a given receptor are still unknown. Homodimeric receptors for PRL, EPO or GH recruit Jak2 [8–10], whereas heterotrimeric IL-2R selectively recruits Jak1 and Jak3 through its β and γ_c subunits, respectively [11,12]. In a recent study [13], however, we have shown that a chimeric receptor composed of the extracellular domain of the PRLR fused to the cytoplasmic domain of the IL-2Rβ chain (so-called PRLR/2β) is able to activate both Jak1 and Jak2, despite the fact that the kinase associated with the IL-2Rβ chain within the wild type IL-2R is Jak1. This observation provided evidence that the type of Jak naturally activated by IL-2Rβ does not exclusively depend, if at all, on intrinsic sequences within its cytoplasmic domain. In view of this unexpected observation, we addressed the question whether the extracellular domain of PRLR per se or, alternatively, the homodimeric stoichiometry of the activated PRLR/2β chimeric receptor might participate in directing activation of Jak2. To test these hypotheses, we have analyzed the signaling properties of two other chimeric receptors, both containing the cytoplasmic domain of the IL-2Rβ chain linked to the extracellular domain either of the EPOR (so-called EPOR/2β chimera) or of the receptor tyrosine kinase Kit (so-called Kit/2β chimera; Fig. 1). Our results show that homodimerization of IL-2Rβ is necessary and sufficient to activate Jak2 and downstream signaling pathways.

2. Materials and methods

2.1. Materials

Ovine PRL (NIAMDD) was obtained from the National Hormone and Pituitary Program/NIDDK (Baltimore, MD, USA). SCF was a generous gift from Genzyme (Cambridge, MA, USA) and recombinant human EPO from Dr. P. Mayeux (Paris, France). The following antibodies were obtained from commercial sources: anti-phosphotyrosine (4G10) and anti-Jak2 (Upstate Biotechnology), rabbit polyclonal anti-Stat5a/Stat5b (Santa Cruz Biotechnology), rat monoclonal anti-Kit extracellular domain (ACK-2) and fluorescein-conjugated anti-rat IgG antibody (Gibco-BRL). Other reagents were obtained from commercial sources as previously described [13].

2.2. Expression vectors

An *Eco*RI/*Eco*RV cDNA fragment encoding the murine EPOR extracellular domain [14] was obtained by PCR and substituted for that encoding the PRLR extracellular domain in the pECE-PRLR/2β expression vector [13]. Kit/2β, cloned into the eukaryotic expression vector pHβAPr-1-neo [15], has been previously described [16].

2.3. Cell culture and stable transfection

CHO K1 cells were routinely cultured as described [13]. CHO cells stably expressing EPOR/2β or Kit/2β chimeras were selected by neomycin resistance after transfection with 8 μg of DNA using the calcium-phosphate precipitation procedure. The CHO stable clone expressing the PRLR/2β chimera was previously described [13].

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Abbreviations: R, receptor; PRL, prolactin; EPO, erythropoietin; SCF, stem cell factor; IL, interleukin; Jak, Janus kinase; Stat, signal transducer and activator of transcription; RTK, receptor tyrosine kinase; LIF, leukemia inhibitory factor; OSM, oncostatin M; CNTF, ciliary neurotrophic factor

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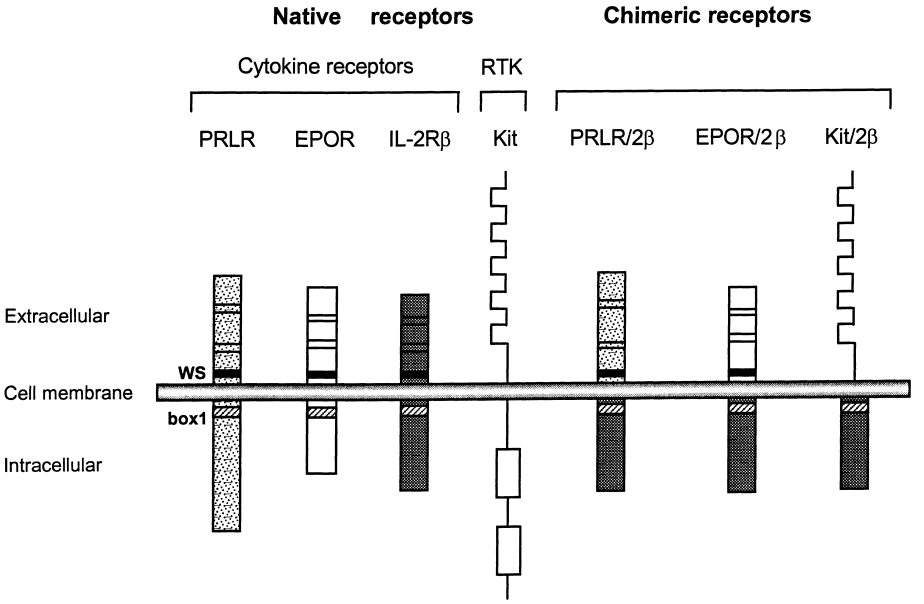

Fig. 1. Schematic representation of native and chimeric receptors. Thin lines in the extracellular domain of PRLR, EPOR and IL-2Rβ indicate cysteine residues and the black boxes the WSXWS motif; both motifs are conserved structural features among cytokine receptors [41]. Semi-squares of the extracellular domain of Kit represent immunoglobulin-like structures. The hatched rectangle in the cytoplasmic domain symbolizes box 1, a proline-rich region highly conserved within the cytokine receptor superfamily. Kit contains a separated cytoplasmic tyrosine kinase domain symbolized by two white rectangles.

2.4. Cross-linking

Confluent monolayer CHO cells (2×10^6) expressing the EPOR/2β chimeric receptor were incubated with 1 nM ^{125}I-labeled EPO with or without 100-fold molar excess of unlabeled EPO in 1 ml of ligand binding buffer (Ham's F-12, 0.5% BSA, 0.1% azide) for 1 h at room temperature. Cells were washed four times with PBS followed by incubation with 0.5 mM disuccinimidyl suberate (DSS) for 15 min at 20°C. The cross-linking reaction was quenched by the addition of 50 mM Tris-HCl, pH 7.4. Cells were boiled in 50 μl SDS sample buffer and subjected to 7.5% SDS-PAGE prior autoradiography of dried gel.

2.5. Cell labeling

CHO cells transfected with Kit/2β (10^6 cells) were immunostained with ACK-2 antibody (10 μg/ml) or with staining medium alone (PBS, 5% FCS, 0.2% azide) for 30 min at 4°C. After washing, cells were incubated with fluorescein-conjugated anti-rat IgG monoclonal antibody (1/10 dilution) for 30 min at 4°C, washed and resuspended. Fluorescence analysis was performed using a FACScan analyzer (Becton Dickinson, Grenoble, France).

2.6. Immunoprecipitation and Western blots

Cell lysates were immunoprecipitated with appropriate antibodies (see Section 2.1); experimental procedures for immunoprecipitation, electrophoresis and immunoblots were as described [13].

2.7. Transient transfection and luciferase assay

Transient transfection of stable clones with β-casein promoter-luciferase reporter plasmid and β-galactosidase plasmid was performed as described [13]. Transfected cells were incubated in serum-free medium containing 250 nM dexamethasone and various concentration of PRL, EPO or SCF for 48 h at 37°C. Luciferase activity was measured as previously reported [13] and values were normalized to the β-galactosidase activity. Results are means ± S.E.M. from three independent experiments.

3. Results

3.1. Selection of stable clones

Although some data relating to the PRLR/2β chimeric receptor have been previously published [13], all the experiments performed in the present study included the PRLR/2β clone for direct comparison of the three chimeras within the same experiments. Electrophoretic mobility of the ^{125}I-EPO-EPOR/2β cross-linked complex corresponded to a relative molecular mass of ~104 kDa, in good agreement with the predicted size of the chimeric receptor (68 kDa, including ~10 kDa glycosylation [17]) and the 35 kDa contribution of monomeric EPO

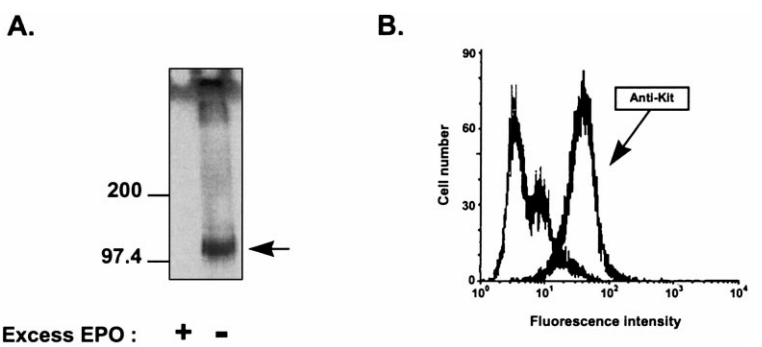

Fig. 2. Identification of EPOR/2β and Kit/2β stable CHO clones. A: Autoradiography of EPOR/2β chimera cross-linked to ^{125}I-labeled EPO in stably transfected CHO cells. Binding was performed with (+) or without (−) excess unlabeled EPO. Numbers on the left indicate molecular masses (kDa) of protein markers. The arrow on the right indicates the position of the EPOR/2β chimera-EPO complex. B: Flow cytometric analysis of Kit/2β expression on CHO cells. CHO cells transfected with Kit/2β were immunostained with or without the rat antibody ACK-2, specific to the extracellular region of the murine Kit, followed by a secondary staining with fluorescein-conjugated anti-rat IgG antibody.

(Fig. 2A). Band specificity was assessed by displacement of the labeled complex with 100-fold molar excess of unlabeled EPO. CHO clones expressing Kit/2β were selected by flow cytometry and the integrity of the Kit/2β transfectant was confirmed by a positive shift in fluorescence intensity (Fig. 2B).

3.2. Tyrosine phosphorylation of Jak2 and Stat5

Kit/2β and EPOR/2β chimeras were first tested for their ability to induce Jak2 tyrosine phosphorylation. In the presence of the appropriate ligand (EPO, SCF or PRL, 10 min stimulation), similar strong ligand-induced phosphorylation of immunoprecipitated Jak2 was observed for the three clones (Fig. 3A). Control immunoblot analysis revealed a similar quantity of immunoprecipitated Jak2 for all conditions (Fig. 3B). We next investigated the ability of the chimeras to activate one of the known targets of Jak2, namely Stat5 tyrosine phosphorylation [18]. Anti-phosphotyrosine immunoblot of immunoprecipitated Stat5 showed a strong and similar ligand-dependent phosphotyrosine signal of Stat5 for the three stable clones (Fig. 4A). Control anti-Stat5 immunoblot analysis revealed a comparable quantity of immunoprecipitated Stat5 (Fig. 4B).

3.3. Transcriptional activation of β-casein reporter gene

Activation of the β-casein gene promoter is a known target of the Jak2/Stat5 cascade [18]. For all chimeras, dose-dependent activation of this construct was observed (Fig. 5). In agreement with proliferation studies performed on lymphoid cells transfected with Kit/2β or EPOR/2β, a maximal effect was achieved at concentrations of 200 ng SCF/ml [16] and 10 U EPO/ml [19], respectively. Higher EPO concentrations did not result in any increase of the response (not shown). Due to a limited supply, we could not assay higher doses of SCF; however, the same induction observed at 100 ng/ml and 200 ng/ml (Fig. 5) indicates that these concentrations correspond to the plateau of the response. Maximal activation of PRLR/2β was achieved at PRL concentrations around 400 ng/ml [13]. Transcriptional activation of the reporter gene was similar for both PRLR/2β and EPOR/2β, with a maximal induction around 8–10-fold. Kit/2β hybrid receptor was approximately two-fold less active, with a maximal luciferase induction of 4–5-fold.

4. Discussion

Our previous observation that the PRLR/2β chimera is able to mediate tyrosine phosphorylation of Jak2 was unexpected [13], since the IL-2Rβ chain in the context of wild type IL-2R recruits Jak1, and not Jak2 [12]. The aim of the present work was thus to investigate whether this property might be due to some specific features intrinsically contained in the PRLR extracellular domain, as previously suggested for the extracellular domain of other receptors [20]. As shown, substitution of the EPOR extracellular domain for that of PRLR did not alter the signaling properties of the IL-2Rβ chimeric receptor, suggesting that parameters other than the intrinsic composition of the extracellular domain are involved in directing Jak2 activation by the IL-2Rβ cytoplasmic domain.

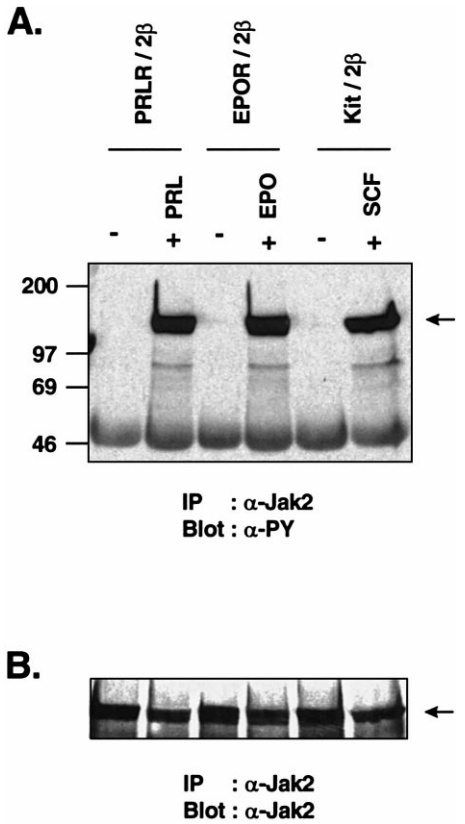

Fig. 3. Tyrosine phosphorylation of Jak2. Serum-starved stable CHO clones were stimulated (+) or not (−) with 400 ng PRL/ml, 10 U EPO/ml or 200 ng SCF/ml for 10 min. Cell extracts were immunoprecipitated with Jak2 antiserum and analyzed by anti-phosphotyrosine (A) and anti-Jak2 (B) immunoblot. Numbers on the left indicate molecular masses (kDa) of protein markers. The arrow on the right indicates the position of Jak2.

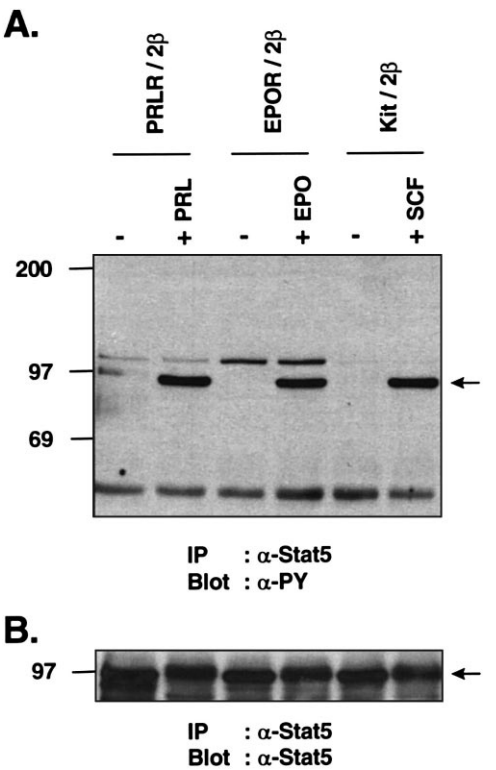

Fig. 4. Tyrosine phosphorylation of Stat5. Serum-starved stable CHO clones were stimulated (+) or not (−) with 400 ng PRL/ml, 10 U EPO/ml or 200 ng SCF/ml for 10 min. Cell extracts were immunoprecipitated with Stat5 antiserum and analyzed by anti-phosphotyrosine (A) and anti-Stat5 (B) immunoblot. Numbers on the left indicate molecular masses (kDa) of protein markers. The arrow on the right indicates the position of Stat5.

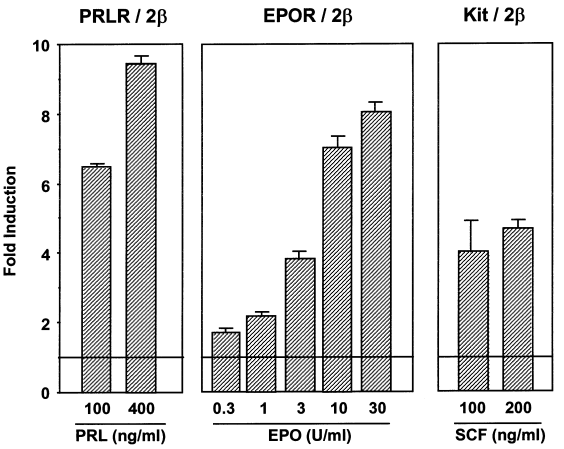

Fig. 5. Induction of the β-casein gene promoter. Stable CHO clones expressing chimeric receptors were transiently transfected with β-casein-luciferase plasmid (see Section 2). Cells were stimulated with various concentrations of PRL, EPO or SCF in the presence of 250 nM dexamethasone. The fold induction is calculated as described in Section 2. Results represent the mean ± S.E.M. of three independent experiments.

One major difference between the wild type IL-2R and PRL and EPO receptors is the stoichiometry of the activated complexes. IL-2R is a heterotrimer, whereas PRL and EPO receptors are homodimerized upon ligand binding [1]. Hence, PRLR/2β and EPOR/2β are also assumed to homodimerize after ligand binding to the extracellular region. In order to test whether the receptor stoichiometry per se, in the present case a homodimer, is sufficient to give IL-2Rβ the ability to activate Jak2, we analyzed the signaling properties of Kit/2β. Kit, a member of the RTK family [21], does not signal through the Jak/Stat pathways and like all RTKs is activated by ligand-induced homodimerization [22]. Kit/2β exhibits a qualitatively identical ability to stimulate Jak2 and Stat5 tyrosine phosphorylation compared to both other chimeras, strengthening the observation that these properties are independent of the intrinsic composition of the extracellular domain. Rather, this indicates that the IL-2Rβ cytoplasmic domain intrinsically possesses the ability to activate Jak2, but that this property can only be displayed in a homodimerized complex.

Although the reduced transcriptional activity of Kit/2β compared to the other chimeras was not investigated in detail, one can postulate it is related to the overall structure of the complexes. Indeed, crystallographic structures of genetically engineered PRLR [23] and EPOR [24] extracellular domains have shown that they are almost superimposable, suggesting that homodimerized PRLR/2β and EPOR/2β complexes adopt overall structures that are very similar to each other as well as to wild type PRLR. The overall conformation of Kit/2β, although still unknown, presumably markedly differs from that of cytokine receptors, since the Kit extracellular domain is composed of five immunoglobulin-like domains [21,22]. The conformation of homodimerized Kit/2β might not allow optimal activation of the Jak/Stat signaling cascade leading to β-casein transactivation. For instance, induction of molecular event(s) other than tyrosine phosphorylation of Jak2 and Stat5, such as serine phosphorylation of Stat5 [25–27], might not be fully achieved by Kit/2β hybrid receptor. Interestingly, SCF binding to Kit/2β expressed in D10 lymphoid cells did not induce detectable tyrosine phosphorylation of cellular proteins, despite the presence of Jak kinases in these cells [16]. This observation leads to the hypothesis that the homodimerization step, the assumed prerequisite for signal transduction by this chimera, may not be sufficient to activate phosphorylation pathways in some cell types, possibly because other membrane components/features are also required. This underlines the importance of the cell type in which experiments are carried out.

By merging receptor domains from two wild type receptors that are both unable to activate Jak2 (Kit and IL-2Rβ), we have generated an artificial PRL-like receptor able to stimulate the Jak2/Stat5/β-casein cascade. In a general fashion, our data suggest that the stoichiometry of a cytokine receptor complex, and not only their intrinsic composition (i.e. the type of subunits), can affect their signaling properties and, more particularly, that the homodimerization process of cytokine receptor subunits plays a major role in providing the ability to activate Jak2 (Table 1). Interestingly, all cytokine receptors activated by homodimerization (GHR, PRLR, EPOR, G-CSFR, Mpl) activate Jak2 [1,2,28]. Cytokine receptors of the IL-3/IL-5/GM-CSF subfamily are also able to activate Jak2, despite the fact that ligand-bound complexes are formed by heterodimerization of the common βc and a ligand-specific α chain. However, the α chains of these receptors contain a box 1-like motif [29] whose deletion appears detrimental to signaling [30–33], including Jak2 tyrosine phosphorylation [34]. Although the α chain per se is not able to associate with Jak2, βc does [35], thus it is possible that dimerization of the box 1(-like) motifs following ligand-induced clustering of α and βc subunits allows recruitment of

Table 1
Summary of some functional characteristics of activated native and chimeric receptors

Functional characteristic		Wild type receptors				Chimeric receptors		
		Cytokine receptors			RTK			
		PRLR	EPOR	IL-2R	Kit	PRLR/2β	EPOR/2β	Kit/2β
Stoichiometry		homodimer	homodimer	heterotrimer	homodimer	homodimer	homodimer	homodimer
Jak2 tyrosine phosphorylation		++	++	−	−	++	++	++
Stat5 tyrosine phosphorylation		++	++	++	−	++	++	++
β-Casein gene:	DNA binding	++	++	++	ND	ND	ND	ND
	transactivation	++	−	ND	ND	++	++	+
Reference		[13]	[42,43]	[42]	ND	this study		

++: strong activation; +: moderate activation; −: no activation; ND: not determined.
The table indicates the following parameters: stoichoimetry of ligand-bound receptors, tyrosine phosphorylation of Jak2, tyrosine phosphorylation of Stat5 and activation of the β-casein gene promoter. For the latter, DNA binding in gel shift assay experiments and transactivation of a β-casein-reporter construct are distinguished. The predicted stoichiometry of chimeric receptors is based on that of native receptors from which the extracellular domain originates. Receptor homodimerization in the context of both wild type and hybrid receptors parallels the ability to activate Jak2; Kit/2β displays reduced ability to induce β-casein gene transcription.

a Jak kinase (Jak1 and Jak2) by each subunit, and further activation by transphosphorylation. The gp130 subfamily (LIF, OSM, CNTF and IL-6 receptors) also activates Jak2. The three former complexes contain a β chain (gp130) and a γ chain that both contain a box 1 motif able to interact with a Jak protein. The IL-6R does not contain a γ chain, but activation of a signaling complex requires homodimerization of the gp130 transducer, leading to clustering of two box 1 motifs [36,37]. Finally, IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15 are the only class 1 cytokine receptors that do not activate Jak2. Remarkably, although the β chains of these receptors contain a box 1 motif, which associates with Jak1, their γ chain is devoid of box 1 motif and associates with Jak3 through the C-terminus of the cytoplasmic domain [12].

In conclusion, we postulate that the activation of Jak2 requires the presence of two copies of box 1(-like) motifs, which can be achieved by homodimerized receptors (EPOR, PRLR, etc.) or by hetero-oligomerized receptors (IL-6R or IL-3R subfamilies) involving subunits having box 1 motifs. The presence of two box 1 is not always sufficient for activating Jak2, however, since heterodimerization of the short and intermediate PRLR isoforms, despite the fact that both contain a box 1 and activate Jak2 in homodimerized PRLR [38], leads to inactive complexes [39,40]. This suggests that in some [39,40], but not all [7] cases, the involvement of additional motifs downstream of box 1 might also be involved or, alternatively, that some degree of structural compatibility between clustered subunits is required. Further studies on Jak-receptor interaction will be required to better understand the molecular requirements of these associations.

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