Identification and functional characterization of a second chain of the interleukin-10 receptor complex

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Interleukin-10 (IL-10) is a pleiotropic cytokine which signals through a specific cell surface receptor complex. Only one chain, that for ligand binding (IL-10R α or IL-10R1), was identified previously. We report here that, although human IL-10 binds to the human IL-10R1 chain expressed in hamster cells, it does not induce signal transduction. However, the co-expression of CRFB4, a transmembrane protein of previously unknown function belonging to the class II cytokine receptor family, together with the IL-10R1 chain renders hamster cells sensitive to IL-10. The IL-10:CRFB4 complex was detected by cross-linking to labeled IL-10. In addition, the IL-10R1 chain was able to be co-immunoprecipitated with anti-CRF antibody when peripheral blood mononuclear cells were treated with IL-10. These results demonstrate that the CRFB4 chain is part of the IL-10 receptor signaling complex. Thus, the CRFB4 chain, which we designate as the IL-10R2 or IL-10R β chain, serves as an accessory chain essential for the active IL-10 receptor complex and to initiate IL-10-induced signal transduction events.

Keywords: CRFB4/interferon- γ /interleukin-10/receptor/signal transduction

Introduction

Interleukin-10 (IL-10) is a pleiotropic immunomodulatory cytokine controlling the functions of myeloid and lymphoid cells (for reviews, see Moore *et al.*, 1993; Ho and Moore, 1994; O'Garra and Murphy, 1994). IL-10 plays an important role in the establishment and maintenance of a class of immune responses by suppressing T_H1-dependent cell-mediated immune responses, and supporting T_H2-dependent antibody-mediated immune responses. IL-10 exerts its anti-inflammatory action by blocking macrophage activation through suppression of proinflammatory cytokine synthesis [IL-1, IL-6, tumor necrosis factor (TNF)- α and IL-12] and suppression of the ability of lymphocytes to serve as antigen-presenting or co-stimulatory cells (Bogdan *et al.*, 1991; de Waal Malefyt *et al.*, 1991; D'Andrea *et al.*, 1993). Through prevention of macrophage activation and also directly, IL-10 inhibits proliferation and stimulation of T and natural killer (NK)

cells, blocking their ability to secrete cytokines such as interferon- γ (IFN- γ) and IL-2 (Fiorentino *et al.*, 1991; Moore *et al.*, 1993; Taga *et al.*, 1993), which mediate cellular immunity. On the other hand, IL-10 is a co-stimulator of proliferation and differentiation of B cells, mast cells and thymocytes (MacNeil *et al.*, 1990; Suda *et al.*, 1990; Chen and Zlotnik, 1991; Thompson-Snipes *et al.*, 1991; Rousset *et al.*, 1992).

The Hu-IL-10 receptor-binding chain, designated Hu-IL-10R or Hu-IL-10R1 in this report, was cloned and characterized (Liu *et al.*, 1994). Hu-IL-10R1 is a cell surface receptor with a single transmembrane domain and is a member of the class II cytokine receptor family (Bazan, 1990a,b; Thoreau *et al.*, 1990). The IL-10R1 chain can be considered comparable with other cytokine chains designated alpha subunits (IL-10R α).

Although the expression of Hu-IL-10R1 alone in heterologous cells allows Hu-IL-10 to exert some biological activities, other observations such as lower sensitivity to Hu-IL-10 of mouse Ba/F3 cells expressing the recombinant Hu-IL-10R1 than of Ba/F3 cells expressing the recombinant Mu-IL-10R1 or inability of the viral analog of IL-10 (vIL-10) to compete effectively for IL-10 binding to IL-10R1 (Ho and Moore, 1994; Liu *et al.*, 1994) suggested the possibility of involvement of another IL-10 receptor subunit in IL-10 signaling.

Human CRFB4 is an orphan receptor encoded on human chromosome 21 (Lutfalla *et al.*, 1993). Its cDNA was cloned by screening of a cDNA library from Daudi cells with a partial cDNA probe corresponding to the D21S58 locus (Neve *et al.*, 1986; Pearson *et al.*, 1987). The cloned cDNA encoded a member of the class II cytokine receptor family (Lutfalla *et al.*, 1993). The CRFB4 gene structure was characterized and the gene was linked to the IFN- α receptor region on human chromosome 21 (Lutfalla *et al.*, 1993, 1995a). Because of its proximity to the IFNAR1 locus, the involvement of CRFB4 in the IFN- α receptor complex was proposed (Lutfalla *et al.*, 1993). However, a function of CRFB4 in the Type I interferon receptor complex has not been demonstrated.

IL-10 exerts its biological functions through the activation of Jak1 and Tyk2, the members of the receptor-associated Janus tyrosine kinases (Jak) family (Ziemiecki *et al.*, 1994; Ihle *et al.*, 1995), and Stat1, Stat3 and, in certain cells, Stat5 (Finbloom and Winestock, 1995; Ho *et al.*, 1995; Weber-Nordt *et al.*, 1996), the members of signal transducers and activators of transcription (Stat) family (for reviews, see Darnell *et al.*, 1994; Fu, 1995; Ihle *et al.*, 1995; Ihle, 1996). Recently, we showed that the intracellular domain of CRFB4 associates with Tyk2 (Kotenko *et al.*, 1996). Since the activation of two different Jaks during signal transduction usually reflects the involvement of two different receptor chains associated with the two respective Jaks and since Tyk2 is one of the kinases

Fig. 1. Structure of chimeric receptors. Hu-IL-10R (IL-10R or IL-10R1) represents the intact ligand-binding chain of the IL-10 receptor complex. Hu-CRFB4 (CRF) is a receptor from the class II cytokine receptor family (Lutfalla *et al.*, 1993). The IL-10R1/γR1 and CRF/γR2 chimeric receptors have the extracellular domains of Hu-IL-10R1 and Hu-CRFB4 chains, respectively, and the transmembrane and intracellular domains of Hu-IFN-γR1 and Hu-IFN-γR2 chains, respectively. The CRF receptor and CRF/γR2 chimera were constructed with and without a FLAG epitope at the amino-terminus.

activated by IL-10, we evaluated the involvement of CRFB4 in the IL-10 receptor complex.

Results

Plasmid construction

The following receptors and receptor chimeras were used in this work. Hu-IL-10 receptor chain one (IL-10R1), the ligand-binding chain of IL-10 receptor complex (Liu *et al.*, 1994), Hu-IL-10R1/Hu-IFN-γR1 chimeric receptor (IL-10R1/γR1), where the transmembrane and intracellular domains of the Hu-IL-10R1 were substituted by the transmembrane and intracellular domains of the Hu-IFN-γ receptor chain one, Hu-IFN-γR1 (Aguet *et al.*, 1988); Hu-CRFB4 receptor (CRF) and FLAG epitope-tagged Hu-CRFB4 (FL-CRF), CRFB4/Hu-IFN-γR2 (CRF/γR2) and FLAG epitope-tagged Hu-CRFB4/Hu-IFN-γR2 (FL-CRF/γR2) chimeric receptors, where the extracellular domain of Hu-CRFB4 was fused to the transmembrane and intracellular domains of the second chain of the human IFN-γ receptor, designated AF-1 or Hu-IFN-γR2 (Soh *et al.*, 1994) (Figure 1). All receptor cDNAs were cloned into a mammalian expression vector pcDEF3 (Goldman *et al.*, 1996) carrying a *neo^R* marker where the transcription of the cDNAs are driven by the powerful promoter of human elongation factor 1α (eEF1α) (Uetsuki *et al.*, 1989; Mizushima and Nagata, 1990). Vectors containing the human EF1α promoter produced high levels of expression of the following receptors: Hu-IFN-αR2c (Domanski *et al.*, 1995; Lutfalla *et al.*, 1995b), Hu-IFN-αR1 (Uzé *et al.*, 1990), Hu-IFN-γR1 and Hu-IFN-γR2 (Goldman *et al.*, 1996; S.V.Kotenko, unpublished data).

Expression in COS cells and electrophoretic mobility shift assay

To express both IL-10R1 and CRFB4 receptor chains in a single transfected cell, a tandem vector expressing both chains was created. Expression of both cDNAs is controlled by separate promoters and separate polyadenylation signals on a single plasmid. The plasmids encoding either the CRFB4 chain or the IL-10R1 chain, or a tandem vector encoding both receptors, were transiently transfected into COS-1 cells. Three days after transfection,

Fig. 2. Electrophoretic mobility shift assay (EMSA) in monkey COS-1 and hamster 16-9 cells. EMSAs were performed as described (Kotenko *et al.*, 1995) with detergent-free total cellular lysates from COS-1 cells untreated or treated with Hu-IL-10 after transient transfection with the receptor expression plasmids indicated on the figure. Hamster cells were stably transfected with the receptor expression plasmids indicated on the figure, and EMSAs were performed as with COS-1 cell lysates with and without treatment with IL-10. Specific anti-Stat1α and anti-Stat3 antibodies were used for supershift assays. The positions of Stat DNA-binding complexes are indicated by arrows.

the COS-1 cells were treated with Hu-IL-10 (100 U/ml) or left untreated and the detergent-free total cellular lysates were prepared for electrophoretic mobility shift assays (EMSAs). It was shown previously that IL-10 activates Stat1α, Stat3 and, in certain cells, Stat5 during signal transduction and that these DNA-binding complexes can be detected with the IFN-γ activation sequence (GAS)-specific probe (Finbloom and Winestock, 1995; Weber-Nordt *et al.*, 1996). Thus, EMSAs were performed with a 22 bp sequence containing a Stat1α-binding site corresponding to the GAS element in the promoter region of the human IRF-1 gene. We detected the formation of Stat DNA-binding complexes in IL-10-treated COS-1 cells transiently transfected with the plasmid expressing both CRFB4 and IL-10R1 chains (Figure 2). With anti-Stat1α and anti-Stat3 antibodies, the major DNA-binding complexes (a, b and c Figure 2) were shown to consist of two Stats: Stat1α and Stat3. With anti-Stat1α antibody, complexes a and b were supershifted and with anti-Stat3 antibody complexes b and c were supershifted, suggesting that complexes a and c consist of Stat1α and Stat3 homodimers, respectively, and complex b consists of a heterodimer of Stat1α and Stat3. These results correlate with the pattern of Stat activation characteristic for IL-10 signaling (Finbloom and Winestock, 1995; Weber-Nordt *et al.*, 1996). We did not observe the activation of Stats in IL-10-treated COS-1 cells transfected with the plasmid expressing the CRFB4 chain only. However, after longer exposure (72 h instead of 36 h), Stat DNA-binding complexes can be observed in IL-10-treated COS-1 cells transiently transfected with the plasmid expressing only the IL-10R1 chain. This result and the results of others

Fig. 3. Expression of receptors on the cell surface and induction of HLA-B7 surface antigen in hamster cells by IL-10. The expression of IL-10R1/γR1 and/or FL-CRF or FL-CRF/γR2 (top panels) or induction of HLA-B7 antigen by IL-10 (bottom panels) were analyzed by flow cytometry. Top panels: cells were harvested and incubated with either anti-IL-10R1 (A, C and D, stippled areas) or anti-FLAG (B, C and D, thin lines) monoclonal antibodies or were incubated directly with secondary antibody only (A, B, C and D, thick lines). Bottom panels: cells were treated with 100 U/ml of IL-10 (stippled areas) or left untreated (open areas). HLA-B7 antigen was detected by treatment of cells with anti-HLA monoclonal antibody. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was used as the secondary antibody. Cells were the hamster cells stably transfected with: the IL-10R1/γR1 (A and E), the FL-CRF (B and F), both IL-10R1/γR1 and FL-CRF (C and G) and both IL-10R1/γR1 and FL-CRF/γR2 (D and H). The data represent results with clonal cell populations.

showing that the expression of Hu-IL-10R1 alone in mouse Ba/F3 or monkey COS-7 cells (Liu *et al.*, 1994) was sufficient to reconstitute some biological activities of IL-10 suggest that a second chain of the IL-10 receptor complex, but at low levels, is expressed in these cells and/or is not strongly species-specific between mouse, monkey and human cells.

Expression of IL-10 receptor chains and chimeric receptors in hamster cells

We then repeated a similar series of experiments in hamster cells. We did not observe the IL-10-induced activation of Stats in 16-9 hamster cells stably transfected with the plasmid expressing only the IL-10R1 chain, even after longer exposure of the EMSA (Figure 2). These cells were shown to express the IL-10R1 chain on the cell surface by flow cytometry with anti-IL-10R1 monoclonal antibodies (data not shown). No IL-10-induced Stat activation was observed in hamster cells stably transfected with the plasmid expressing only the CRFB4 chain. However, in IL-10-treated 16-9 cells transfected with the plasmid encoding both IL-10R1 and CRFB4 chains, activation of the Stats by IL-10 was observed (Figure 2). As with COS-1 cells, the data demonstrated that the major DNA-binding complexes activated in hamster cells by IL-10 are formed by combination of two Stats: Stat1α and Stat3 (Figure 2).

As IL-10 activities are restricted to certain cell types (Ho and Moore, 1994), we used chimeric receptors to detect IL-10-induced activities in hamster cells (Kotenko *et al.*, 1996; Muthukumaran *et al.*, 1996). The transmembrane and intracellular domains of the IFN-γR1 chain were substituted for the transmembrane and intracellular

domains of the IL-10R1 chain (Figure 1). Thus, we expected that IL-10 would induce an IFN-γ-like response in cells expressing CRFB4 and the IL-10R1/γR1 chimeric receptor. Since IFN-γ induces MHC class I antigen expression, we used this cell surface marker to determine if the IL-10R1/γR1 chain alone or together with the CRFB4 receptor chain would support MHC class I antigen expression in response to IL-10 (Figure 3). The EMSA was used to determine activation of Stats (Figure 4). To overcome the lack of antibodies against CRFB4 and enable detection of the CRFB4 chain on the cell surface, we introduced a FLAG epitope at the amino-terminal end of the CRFB4 extracellular domain (FL-CRF). The FLAG-tagged CRFB4 was detectable by anti-FLAG-specific monoclonal antibodies, as shown by flow cytometry (Figure 3B). Expression of the IL-10R1/γR1 chain was detected by anti-IL-10R1-specific monoclonal antibodies as shown by flow cytometry (Figure 3A). When the IL-10R1/γR1 chimera was expressed alone, it was not able to support signaling upon IL-10 treatment as measured by MHC class I antigen induction (Figure 3E) and EMSA (Figure 4). The same negative results were observed in cells expressing the FL-CRF chain alone (Figures 3F and 4). However, hamster cells stably transfected with the plasmid expressing both IL-10R1/γR1 and FL-CRF or FL-CRF/γR2 chains (Figure 3C and D) demonstrated IL-10-dependent MHC class I antigen induction (Figure 3G and H) and Stat1α activation as measured by EMSA (Figure 4). With specific anti-Stat1α antibodies, the DNA-binding complexes were shown to consist of Stat1α homodimers characteristic for IFN-γ signaling (Shuai *et al.*, 1992; Kotenko *et al.*, 1996). To confirm that the activation of the Stat1α DNA-binding complexes observed in hamster

Fig. 4. Electrophoretic mobility shift assay (EMSA) in hamster 16-9 cells. Cellular lysates were prepared from untreated or IL-10-treated cells stably transfected with receptor expression plasmids indicated on the figure and defined in the legend to Figure 3. Specific anti-Stat1 α and anti-Stat3 antibodies were used for supershift assays as noted. Neutralizing anti-IL-10R1 or anti-IL-10 antibodies were used as described under Results. The positions of the Stat DNA-binding complexes are indicated by arrows. The portion of the gel with samples obtained from cells expressing IL-10R1 and FL-CRF/ γ R2 chains (right four lanes) was exposed 10 times longer than the portion of the gel with samples from cells expressing the IL-10R1/ γ R1 chain and either the FL-CRF chain or the FL-CRF/ γ R2 chain (left 12 lanes) in order to detect a comparable amount of Stat DNA-binding complexes in the EMSA.

cells expressing IL-10R1/ γ R1 and FL-CRF or FL-CRF/ γ R2 chains were specific for IL-10 treatment, we performed EMSAs with lysates of cells that were either pre-treated for 30 min with neutralizing anti-IL-10R1 antibodies before IL-10 treatment or treated with IL-10 pre-incubated for 30 min with neutralizing anti-IL-10 antibodies before addition to the cells. In both cases, the activation of Stat1 α DNA-binding complexes was suppressed. Thus, the CRFB4 transmembrane chain supports IL-10-induced signal transduction with the chimeric IL-10R1/ γ R1 chain, confirming our prediction that the CRFB4 chain is the second subunit of the IL-10 receptor complex (Kotenko *et al.*, 1996).

Cross-linking

Four hamster cell lines were used for cross-linking experiments: cells expressing only the FL-CRF chain; cells expressing only the IL-10R1/ γ R1 chain; cells expressing both IL-10R1/ γ R1 and FL-CRF chains; and cells expressing the IL-10R1/ γ R1 chain and a FL γ R2 chain, the FLAG epitope-tagged second subunit of the IFN- γ receptor complex, Hu-IFN- γ R2 chain. The IFN- γ R2 chain is unrelated to the IL-10 receptor complex and was tagged with the FLAG epitope to serve as a negative control to test whether expression of the FLAG epitope on the cell surface would influence IL-10 binding or cross-linking. The level of expression of IL-10R1/ γ R1 and FL γ R2 chains in these cells was evaluated by flow cytometry and was

comparable with the level of expression of IL-10R1/ γ R1 and FL-CRF chains in cells used for cross-linking (data not shown). After cross-linking of radiolabeled IL-10 to the cells, the cells were lysed and cellular lysates were left untreated or treated with anti-FLAG antibodies. The cross-linked complexes from untreated cellular lysates or precipitated with anti-FLAG antibodies were resolved on 5% SDS-PAGE (Figure 5). The appearance of a few labeled cross-linked complexes was observed and their specificity was shown by competing with an excess of unlabeled IL-10 (Figure 5). Extracted cross-linked complexes from cells expressing the IL-10R1/ γ R1 chimera alone or with either the FL-CRF chain or the FL γ R2 chain migrated similarly on SDS-PAGE, with two major cross-linked complexes migrating in the region of 130 and 150 kDa (Figure 5, left panel), which are likely to consist of the IL-10R1 chain cross-linked to one or two molecules of IL-10. We did not observe any cross-linked complexes in cells expressing the FL-CRF chain alone.

After immunoprecipitation with anti-FLAG antibodies, two complexes migrating in the region of 80 and 200 kDa were observed only in cells expressing both IL-10R1/ γ R1 and FL-CRF chains, but not in cells expressing the IL-10R1/ γ R1 chain alone or with the FL γ R2 chain, or the FL-CRF chain alone (Figure 5, right panel). Based on the sizes of immunoprecipitated complexes, we can propose that the 80 kDa complex is an IL-10:FL-CRF complex and the 200 kDa complex is probably formed by three proteins: IL-10, IL-10R1 and FL-CRF.

Interaction between the IL-10R1 chain and the CRFB4 chain in peripheral blood mononuclear cells (PBMCs)

PBMCs were treated with IL-10 or left untreated and then 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP), a water-soluble, membrane-impermeable, thiol-cleavable cross-linker was added, after which the cells were lysed. Anti-CRF antibodies were added to the cellular lysates and the resultant immunoprecipitated complexes were resolved by SDS-PAGE (Figure 6). The cross-linker was cleaved with β -mercaptoethanol in the loading buffer before electrophoresis. Resolved proteins were transferred to a PVDF membrane and probed with anti-IL-10R1 antibodies. The IL-10R1 chain was co-immunoprecipitated with anti-CRF antibodies only in IL-10-treated cells (Figure 6B). After partial transfer, the gel was stained and the 100 kDa protein band was observed only in cells treated with IL-10 (Figure 6A). Since the CRF protein migrates as a 60 kDa protein on SDS-PAGE and, thus, co-migrates with antibody heavy chains, the portions of the original lysates were used for Western blotting to demonstrate that equal quantities of proteins were used for both IL-10 plus and minus lanes (Figure 6C). In parallel, EMSA was performed with PBMCs to demonstrate their responsiveness to IL-10 (Figure 6D). Specific Stat DNA-binding complexes were induced in cells treated with IL-10 (Figure 6A-D), as reported by Finbloom and Winestock (1995). Experiments with anti-Stat1-, anti-Stat3- and anti-Stat5-specific antibodies demonstrated that Stat DNA-binding complexes were supershifted with anti-Stat1 and anti-Stat3 antibodies, but were not shifted with anti-Stat5 antibody (Figure 6D). Thus, isolated PBMCs responded to IL-10 treatment by activation of Stat1 α and

Fig. 5. Cross-linking. Cells were incubated with ^{125}I -labeled Hu-IL-10 with or without addition of a 100-fold excess of unlabeled IL-10, washed, harvested and cross-linked. The extracted cross-linked complexes were analyzed on 5% SDS-PAGE directly (left panel) or after immunoprecipitation with anti-FLAG antibodies (right panel). The indicated cell lines are hamster cells expressing: FL-CRF or IL-10R1/ γ R1 alone; both IL-10R1/ γ R1 and FL- γ R2; and both IL-10R1/ γ R1 and FL-CRF. The arrows designate the cross-linked complexes of IL-10 with the indicated receptors.

Stat3 DNA-binding activities, which is characteristic for IL-10 signal transduction (Finbloom and Winestock, 1995; Weber-Nordt *et al.*, 1996). The experiments with primary cells demonstrated that both the IL-10R1 and the CRFB4 proteins can be co-immunoprecipitated only in the presence of IL-10.

The role of CRFB4 in IL-10 signaling

To characterize further the role of the CRFB4 chain in the IL-10 receptor complex, we created chimeric receptor FL-CRF/ γ R2, where the transmembrane and intracellular domains of CRFB4 were substituted by the transmembrane and intracellular domains of IFN- γ R2 (Figure 1). Expression of this receptor together with the IL-10R1/ γ R1 chimera (Figure 3D) renders hamster cells responsive to IL-10, as measured by MHC class I antigen induction and EMSA (Figures 3 and 4). We further showed that in cells expressing the FL-CRF/ γ R2 chimera along with IL-10R1/ γ R1, the activation of Jak2 occurs upon IL-10 treatment instead of activation of Tyk2, which was observed in cells expressing IL-10R1/ γ R1 and FL-CRF chains (Figure 7). The activation of Tyk2 occurs normally during IL-10 signal transduction (Finbloom and Winestock, 1995; Ho *et al.*, 1995). To determine if the ability of IL-10 to use the CRFB4/ γ R2 chimeric receptor for signal transduction is not an effect of the presence of the IFN- γ R1 intracellular domain in the IL-10R1/ γ R1 chimeric receptor, we repeated this experiment with the intact IL-10R1 chain. IL-10 activated the same Stats in cells expressing FL-CRF/ γ R2 and IL-10R1 chains as it did in cells expressing CRFB4 and IL-10R1 chains, as shown by EMSA (Figures 3 and 4).

Discussion

IL-10 is an immunomodulatory cytokine, which activates the Jak-Stat signal transduction pathway and exerts its pleiotropic activities through binding to the IL-10 receptor chain (IL-10R1) (Liu *et al.*, 1994). Although the expression of the Hu-IL-10R1 chain alone in mouse Ba/F3 or monkey COS-7 cells renders these cells responsive to Hu-IL-10, Ba/F3 cells expressing the recombinant Hu-IL-10R1 chain were ~10 times less sensitive to Hu-IL-10 than Ba/F3 cells expressing the recombinant Mu-IL-10R1 chain, despite comparable levels of receptor expression (Ho and Moore, 1994; Liu *et al.*, 1994). One possible explanation

Fig. 6. Immunoprecipitation and EMSA experiments with peripheral blood mononuclear cells (PBMCs). PBMCs were left untreated or treated with IL-10 and were either used for co-immunoprecipitation experiments (A, B and C) or for EMSA (D) as described in Materials and methods. Cell lysates were either untreated or immunoprecipitated with anti-CRF antibodies. The lysates or immunoprecipitates were resolved on SDS-PAGE, then transferred to PVDF membranes. The membrane with the lysates was probed with anti-CRF antibodies to demonstrate equal loadings in the lanes (C); the membrane with the immunoprecipitates was probed with anti-IL-10R1 antibodies (B). The protein gel was also stained with Coomassie brilliant blue (A).

for these results could be a multicomponent structure of the IL-10 receptor complex, as mouse Ba/F3 cells may express a second subunit of the IL-10 receptor complex which interacts more efficiently with the Mu-IL-10R1 chain than with the Hu-IL-10R1 chain (Liu *et al.*, 1994). Other observations also suggest a multicomponent structure of the IL-10 receptor complex. The viral analog of IL-10 (vIL-10), which shares some biological activities with cellular IL-10, did not compete effectively for Hu-IL-10 binding to the Hu-IL-10R1 chain expressed in mouse Ba/F3 cells (Liu *et al.*, 1994). In addition, expression of the Mu-IL-10R1 chain (Ho *et al.*, 1993) in murine L929 fibroblasts rendered these cells able to bind to Mu-IL-10;

Fig. 7. Phosphorylation of Jaks. Untreated or IL-10-treated cells were harvested, lysed and immunoprecipitated with anti-Tyk2 (A) and anti-Jak2 (B) antibodies. The cell lines are hamster cells expressing both IL-10R1/γR1 and either FL-CRF or FL-CRF/γR2 as indicated on the figure. Immunoprecipitates were resolved on SDS-PAGE, transferred to PVDF membranes, probed with anti-phosphotyrosine antibodies and then re-probed with either anti-Tyk2 or anti-Jak2 antibodies as described (Kotenko *et al.*, 1995, 1996).

however, Mu-IL-10 did not induce Stat activation in these cells (Weber-Nordt *et al.*, 1994).

We recently proposed (Kotenko *et al.*, 1996) that one possible candidate for the second subunit of the IL-10 receptor complex is the CRFB4 chain, the orphan receptor from the class II cytokine receptor family (Lutfalla *et al.*, 1993). With the use of chimeric receptors, we showed that the intracellular domain of the CRFB4 chain associates with Tyk2 (Kotenko *et al.*, 1996) and, thus, may be a component of a ligand-receptor system which activates Tyk2 during signal transduction. Tyk2 was shown to be activated by IFN-α and ciliary neurotrophic factor-related cytokines, IL-10 and IL-12 (Velazquez *et al.*, 1992; Bacon *et al.*, 1995; Barbieri *et al.*, 1994; Lütticken *et al.*, 1994; Stahl *et al.*, 1994; Finbloom and Winestock, 1995; Ho *et al.*, 1995). Since the subunits for a given cytokine-receptor complex belong to the same class of the cytokine receptor family, we proposed that the CRFB4 chain could be involved in IFN-α or IL-10 receptor complexes, as all cloned subunits of these receptors are members of the same class as the CRFB4 chain (Uzé *et al.*, 1990; Lutfalla *et al.*, 1993, 1995b; Liu *et al.*, 1994; Novick *et al.*, 1994; Domanski *et al.*, 1995). We were not able to reveal any involvement of the CRFB4 chain in the IFN-α ligand-receptor complex. In the case of IL-10, only one chain (IL-10R1), the ligand-binding chain, had been identified (Liu *et al.*, 1994). The activation of two different Jaks during signal transduction usually requires the involvement of two different receptor components associated with two distinct Jaks. Since IL-10 activates Jak1 and Tyk2, we tested the idea whether the CRFB4 chain is involved in the IL-10 receptor complex.

In monkey-derived COS-1 cells transiently transfected with the plasmid expressing both IL-10R1 and CRFB4 chains, the pattern of Stat activation characteristic of IL-10 signaling was observed (Figure 2). The low level of Stat activation detected in cells transfected with only the plasmid expressing the IL-10R1 chain may be explained by a low level of expression of the monkey IL-10 receptor second chain in COS-1 cells and/or that this second chain

is not species specific for Hu-IL-10. This assumption is in agreement with previous observations that the Hu-IL-10R1 chain expressed in COS-7 cells or mouse Ba/F3 cells renders them responsive to Hu-IL-10 (Liu *et al.*, 1994). However, the expression of the IL-10R1 chain in hamster cells failed to enable IL-10 to induce Stat DNA-binding complexes (Figure 2), suggesting that the hamster IL-10 receptor second chain either is not expressed in these cells or has a species-specific barrier for Hu-IL-10 binding and/or signaling. However, the expression of both human receptor chains, IL-10R1 and CRFB4, in hamster cells allowed Hu-IL-10 to induce activation of Stat1 and Stat3 (Figure 2) with the pattern of Stat DNA-binding complexes characteristic of IL-10 signal transduction (Finbloom and Winestock, 1995; Weber-Nordt *et al.*, 1996).

Since IL-10 activities are restricted to certain cell types (Ho and Moore, 1994), we could not determine the biological activities of IL-10 in the 16-9 hamster cell transfectants. Thus, we used chimeric receptors to provide an assay to detect IL-10-induced activities in hamster cells as we previously used chimeric receptors to demonstrate that CRFB4 associates with Tyk2 (Kotenko *et al.*, 1996). The chimeric IL-10R1/γR1 chain was used (Figure 1). The IFN-γR1 chain is a signal-transducing chain of the IFN-γ receptor complex, whose intracellular domain recruits Stat1α to the complex (Greenlund *et al.*, 1994). It was shown that to enable the IFN-γR1 chain to initiate signaling, the IFN-γR1 chain requires an additional tyrosine kinase activity which is brought into the normal IFN-γ receptor complex by the intracellular domain of the IFN-γR2 chain (Soh *et al.*, 1994; Bach *et al.*, 1996; Kotenko *et al.*, 1996). In normal cells, this function is performed by Jak2 associated with the IFN-γR2 intracellular domain (Kotenko *et al.*, 1995; Sakatsume *et al.*, 1995; Bach *et al.*, 1996). We showed that, if the IFN-γR2 intracellular domain is replaced with the intracellular domains of other receptors, Hu-IFN-γR1, Hu-IFN-αR2c, Hu-IFN-αR1, Hu-CRFB4 or Hu-IL-2R γ_c chains (Takeshita *et al.*, 1992), which are associated with other Jaks, thus bringing other Jaks into the IFN-γ receptor complex upon IFN-γ treatment, other tyrosine kinases of the Jak family, including Tyk2, can substitute for Jak2 in IFN-γ signal transduction (Kotenko *et al.*, 1996). Since Tyk2 is associated with the CRFB4 intracellular domain (Kotenko *et al.*, 1996), we postulated that the chimeric IL-10R1/γR1 chain and the CRFB4 chain would interact upon IL-10 binding so that the intracellular domains of these two chains would mimic the interaction which occurs between the intracellular domains of the IFN-γR1 and IFN-γR2 chains in the normal IFN-γ receptor complex and would initiate signal transduction characteristic of the IFN-γ receptor complex. Thus, IL-10 was expected to produce an IFN-γ-like response in cells expressing CRFB4 and IL-10R1/γR1 chains. Indeed, in these cells we detected an IL-10-dependent IFN-γ-like response as measured by MHC class I antigen induction and Stat1α activation (Figures 3 and 4). Thus, the evidence is compelling that the CRFB4 chain is the second chain of the IL-10 receptor complex.

To confirm that the CRFB4 chain is a component of the IL-10 receptor complex and in apposition to the IL-10R1 chain, we performed cross-linking experiments.

Cross-linking of labeled Hu-IL-10 to hamster cells expressing the Hu-IL-10R1/ γ R1 and FL-CRF chains resulted in formation of a few cross-linked complexes with two major complexes migrating in the region of 130 and 150 kDa (Figure 5). In cells expressing only the FL-CRF chain, no cross-linked complexes were observed. The distribution of radioactivity between 130 and 150 kDa complexes shifted toward the 150 kDa complex with an increase in concentration of cross-linker (data not shown). This and results of others indicating that IL-10 forms a non-covalently linked dimer under native conditions and binds to its receptor as a homodimer (Tan *et al.*, 1993) suggest that these two major radiolabeled complexes can be attributed to the IL-10R1/ γ R1 chain cross-linked to one or two molecules of IL-10 (20 kDa). The pattern of the cross-linked complexes in our experiments correlates well with patterns obtained earlier, where cross-linking of labeled Hu-IL-10 to Burkitt lymphoma cell line BJAB, or monkey COS-7 cells expressing the recombinant Hu-IL-10R1 chain resulted in formation of major complexes of 120–140 kDa (Liu *et al.*, 1994); or 100 kDa for the human B-lymphoma cell line JY (Tan *et al.*, 1993).

The pattern of cross-linking with cells expressing IL-10R1/ γ R1 and FL-CRF chains was similar to the pattern obtained with cells expressing the Hu-IL-10R1/ γ R1 chain alone or Hu-IL-10R1/ γ R1 and FL γ R2 chains. However, when extracted complexes were immunoprecipitated with anti-FLAG antibodies, two complexes with M_s of 80 and 200 kDa were observed only in cells expressing the IL-10R1/ γ R1 chain together with the FL-CRF chain but not in cells expressing the IL-10R1/ γ R1 chain alone or together with the FL γ R2 chain. The molecular mass of the FL-CRF chain, determined by Western blotting with lysates of hamster cells expressing this protein, was ~60 kDa. The size of IL-10 is ~20 kDa. Thus, the 80 kDa complex is the IL-10:FL-CRF complex, and the higher molecular weight complex can be attributed to a multimeric complex of IL-10, IL-10R1/ γ R1 and FL-CRF chains. It is interesting to note that cross-linking of labeled human IL-10 to mouse mast cell line MC/9 or human B-lymphoma cell line JY revealed the formation of high molecular weight complexes (190–210 kDa) only in human cells (Tan *et al.*, 1993), allowing the speculation that a species-specific component of the human IL-10 receptor complex, probably IL-10R2 (CRFB4), is necessary for oligomerization of human IL-10 receptor subunits and is absent in mouse cells. It correlates with our finding that the 200 kDa complex was observed only in hamster cells expressing both subunits of the human IL-10 receptor complex. The appearance of radiolabeled complexes immunoprecipitated with anti-FLAG antibodies indicates that the CRFB4 chain is in close proximity to IL-10 to enable cross-linking in cells expressing both receptors.

The experiments demonstrated that only in the presence of IL-10 can the IL-10R1 chain be co-immunoprecipitated with anti-CRF antibodies from lysates of PBMCs (Figure 6). This indicates that the CRFB4 chain, the IL-10R1 chain and IL-10 form a complex, providing additional evidence that the CRFB4 chain is a second subunit of the IL-10 receptor complex. We propose that the CRFB4 chain be designated IL-10R2, as it is the second chain of the IL-10 receptor complex. Preliminary results obtained

Fig. 8. Model of the IL-10 receptor complex and signal transduction. The IL-10 homodimer binds to the IL-10R1 chain expressed alone (A) or together with the IL-10R2 (CRFB4) chain (B). Only when both IL-10 receptor chains are present in the complex (B) can IL-10 initiate signal transduction events in the Jak–Stat pathway.

from experiments with CRFB4-deficient mice are consistent with our data (M. Aguet, personal communication).

To investigate further the role of the CRFB4 chain in IL-10 signaling, we created the chimeric receptor FL-CRF/ γ R2 (Figure 1). We proposed earlier that the CRFB4 chain can be designated as a helper receptor (Kotenko *et al.*, 1996) the only function of which is to bring an additional tyrosine kinase activity to the IL-10 receptor complex upon ligand binding, but does not determine the specificity of signal transduction in the Jak–Stat pathway. Ordinarily this function is performed by Tyk2 associated with the CRFB4 intracellular domain. However, we showed with the use of the FL-CRF/ γ R2 chimeric chain that Jak2 can functionally substitute for Tyk2 in IL-10 signaling (Figures 3, 4 and 6). Thus, the primary function of the CRFB4 chain is to bring an additional tyrosine kinase activity to the IL-10 receptor complex upon ligand binding and thus initiate signal transduction as we have shown for the IFN- γ R2 chain in the IFN- γ receptor complex (Kotenko *et al.*, 1995, 1996).

The results of this study and the common architecture of the IL-10 and IFN- γ receptor complexes allow us to propose that IL-10 signaling involves the same basic events as IFN- γ signaling (Figure 8). Both ligands are homodimers which bind to two molecules of their own ligand-binding receptor subunits (the R1 chains) which also serve as the signal-transducing (Stat-recruiting) receptor chains. However, these events alone are not sufficient for signaling. We recently showed that the configuration of the extracellular domain of the cytokine receptors controls the architecture of the intracellular domains and defines the necessity for recruitment of additional chains to the receptor complex (Muthukumaran *et al.*, 1996). The model (Figure 8A) illustrates that the binding of the ligand homodimers to the R1 chains results in formation of a non-functional intracellular receptor complex. The second helper chains (the R2 chains) are required to assemble an active intracellular receptor complex and thus to initiate the signal transduction events (Figure 8B). It is interesting

to note that the formation of complexes of two IL-10 dimers and four soluble IL-10R1 chains was shown (Tan *et al.*, 1995). Although the IFN- γ receptor complex was proposed to consist of one IFN- γ homodimer, two IFN- γ R1 chains and two IFN- γ R2 chains (Fountoulakis *et al.*, 1992; Kottenko *et al.*, 1995; Marsters *et al.*, 1995; Walter *et al.*, 1995), radiation inactivation studies of IFN- γ suggest that a functional tetramer of IFN- γ is required for activity (Pestka *et al.*, 1983; Langer *et al.*, 1994). Despite these common features in the architecture of receptor-ligand binding complexes, these two cytokines act antagonistically, particularly in the regulation of T_H1- and T_H2-dependent immune responses. It is possible that both cytokines use a similar approach to control T cells: the differential expression of the IL-10 or IFN- γ receptor second chains in T cell subsets, as already shown in the case of the IFN- γ R2 chain (Bach *et al.*, 1995; Pernis *et al.*, 1995; Novelli *et al.*, 1996; Sakatsume and Finbloom, 1996; Skrenta *et al.*, 1996). In addition, macrophage activation by IFN- γ is inhibited by IL-10 (Moore *et al.*, 1993). This led Ho *et al.* (1993) to propose that their respective receptor components or downstream signal transduction elements may be shared. The identification of the second subunit of the IL-10 receptor complex as the CRFB4 chain and initial characterization of its function in the IL-10 receptor complex and in signal transduction will facilitate further investigation of IL-10 signaling as well as cross-talk between IL-10, IFN- γ and other molecules.

Materials and methods

Reagents, restriction endonucleases and other enzymes

Taq polymerase, T4 DNA ligase and all restriction endonucleases were from Boehringer Mannheim Biochemicals or New England Biolabs. All radioisotopes were from New England Nuclear. The cross-linker bis(sulfosuccinimidyl)suberate (BS³) was from Pierce Chemical Co. All other chemical reagents were analytical grade and purchased from United States Biochemical Corp.

Plasmid construction

The Hu-IL-10R1 cDNA (Liu *et al.*, 1994) was digested with BssHII, incubated with the large fragment of DNA polymerase I and dNTPs and then digested with AvrII. The AvrII and blunt-ended BssHII fragment of the Hu-IL-10R1 cDNA was ligated into the EcoRV and XbaI sites of the pcDEF3 vector (Goldman *et al.*, 1996). The expression vector was designated pEF3-IL10R1. The Hu-CRFB4 cDNA was recloned from the pCRF vector (Kottenko *et al.*, 1996) into the pcDEF3 vector with KpnI and XbaI. The expression vector was designated pEF3-CRFB4.

To construct chimera IL-10R1/ γ R1, the PCR reaction was performed with 5'-CGGGGTACCCAGGATGCTGCCGTGCC-3' and 5'-ATCGCTAGCCAGTTGGTACGGTGAAATAC-3' primers and the pEF3-IL10R1 as a template. The PCR product was digested with NheI and KpnI and ligated into the NheI and KpnI sites of the plasmid pEF3- γ R2/ γ R1, which was created earlier by recloning the γ R2/ γ R1 fragment from the p γ R2/ γ R1 vector (Kottenko *et al.*, 1996) into the pcDEF3 vector with KpnI and XbaI. The plasmid was designated pEF3-IL10R1/ γ R1.

To introduce the FLAG epitope after the signal peptide of Hu-IFN- γ R2, two primers 5'-CGACTACAAGGACGACGATGACAAGGC-3' and 5'-CTTGTCATCGTCGTCCTGTGTAGTCGGC-3' were annealed and cloned into the SacII site of the p γ R2 vector (Kottenko *et al.*, 1995). The resultant plasmid was designated pFL γ R2.

To create the FLAG-tagged CRFB4 expression vector, two primers 5'-CCCAAGCTTGGTATCGAGCTCGGATCCGGGCC-3' and 5'-GTCTGGCGGTACCGCCTTGTC-3' and the pFL γ R2 plasmid as a template were used for PCR to amplify the signal peptide-FLAG fragment. The PCR product was digested with HindIII and KpnI and ligated into the HindIII and KpnI sites of the plasmid pCRF vector (Kottenko *et al.*, 1996). The plasmid was designated pFL-CRF. The pEF3-FL-CRF

plasmid was constructed by recloning of the FL-CRF fragment from the pFL-CRF vector into the pcDEF3 vector with BamHI and XbaI. To construct chimera FL-CRF/ γ R2, the PCR reaction was performed with 5'-CCCAAGCTTGGTATCGAGCTCGGATCCGGGCC-3' and 5'-ATCGCTAGCCAGGAGGGGACCGTTTCG-3' primers and the pFL-CRF as a template. The PCR product was digested with NheI and BamHI and ligated into the NheI and BamHI sites of the plasmid pEPOR/ γ R2 (Muthukumaran *et al.*, 1996). The plasmid was designated pFL-CRF/ γ R2. The FL-CRF/ γ R2 fragment was then recloned from the pFL-CRF/ γ R2 vector into the pcDEF3 vector with BamHI and XbaI. The plasmid was designated pEF3-FL-CRF/ γ R2.

Tandem vectors containing two receptor chains were created as follows. The fragment containing the EF-1 α promoter, IL-10R1 or IL-10R1/ γ R1 coding sequence and bovine growth hormone (BGH) polyadenylation signal was released from the pEF3-IL10R1 or pEF3-IL10R1/ γ R1 vector by digestion with AatII and SmaI. A partial digestion of the pEF3-IL10R1/ γ R1 vector with SmaI was performed. The fragments were ligated into the AatII and NruI sites of the pEF3-CRF, pEF3-FL-CRF and pEF3-FL-CRF/ γ R2 plasmids. The resulting plasmids were designated pEF3-IL10R1+CRF, pEF3-IL10R1/ γ R1+FL-CRF, pEF3-IL10R1/ γ R1+FL-CRF/ γ R2 and pEF3-IL10R1+FL-CRF/ γ R2.

To create an expression vector for the GST-CRFB4 extracellular domain fusion protein (GST-CRFB_{EC}) two primers 5'-CGGGATCCCACCTCCCGAAAATGTC-3' and 5'-GGGCTCGAGTCAGGAGGGGACCGTTTCG-3' and the pCRF plasmid as a template were used for PCR to amplify the extracellular domain of the CRFB4 chain. The PCR product was digested with BamHI and ligated into the BamHI and SmaI sites of the pGEX-3X vector (Pharmacia).

The nucleotide sequences of the modified regions of all the constructs were verified in their entirety by DNA sequencing.

Cells, media, transfection and cytofluorographic analysis

The 16-9 hamster $\times$ human somatic cell hybrid line is the Chinese hamster ovary cell (CHO-K1) hybrid containing a translocation of the long arm of human chromosome 6 encoding the human IFNGR1 (Hu-IFN- γ R1) gene and a transfected human HLA-B7 gene (Soh *et al.*, 1993). The 16-9 cells were maintained in F12 (Ham) medium (Sigma) containing 5% heat-inactivated fetal bovine serum (FBS; Sigma) (complete F12 medium). COS-1 cells, an SV40-transformed fibroblast-like simian cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% heat-inactivated FBS.

Leukocytes were obtained from a normal donor by leukapheresis. PBMCs were then isolated by density centrifugation with polysucrose and sodium diatrizoate, according to the manufacturer's suggested protocol (Sigma, HISTOPAQUE-1077).

The 16-9 cells were stably transfected with the expression vectors (1–3 μ g of supercoiled plasmid DNA per 10^5 – 10^6 cells) with the liposomal method as described (Campbell, 1995). For co-transfection, we used 1–3 μ g of plasmid DNA with the *neo*^R gene and a 10-fold excess of plasmid DNA without the *neo*^R gene per 10^5 – 10^6 cells. All cell lines transfected with plasmids carrying the *neo*^R gene were selected and maintained in complete F12 medium containing 450 μ g/ml of antibiotic G418. COS-1 cells were transiently transfected with the expression vectors by the DEAE-dextran procedure with a dimethyl-sulfoxide (DMSO) shock step (Sussman and Milman, 1984; Seed and Aruffo, 1987).

Cell surface expression of IL-10R1 and chimeras, FL-CRF and chimeras, or the HLA-B7 antigen was detected by treatment of cells with mouse anti-IL-10R1, anti-FLAG (see below, Cytokines and antibodies) or anti-HLA (W6/32) (Barnstable *et al.*, 1978) monoclonal antibodies, respectively, followed by treatment with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc., Cat# SC-2010). The cells then were analyzed by cytofluorography as described (Cook *et al.*, 1992; Hibino *et al.*, 1992; Soh *et al.*, 1993, 1994; Kottenko *et al.*, 1995, 1996). To detect IL-10-induced MHC class I antigen (HLA-B7) expression, cells were treated with Hu-IL-10 (100 U/ml) for 72 h and analyzed by flow cytometry as described above.

Cytokines and antibodies

Recombinant Hu-IL-10 with a specific activity of 1.4×10^7 U/mg and neutralizing anti-Hu-IL-10 antibody were provided by Schering-Plough Corp. Rabbit anti-Jak2, anti-Tyk2 and anti-IL-10R1 antibodies were from Santa Cruz Biotechnology, Inc. (Cat# SC-294, SC-169 and SC-986, respectively). Rabbit anti-Stat1 α antibody, raised against the C-terminus of Stat1 α , was a gift from James Darnell. Rabbit anti-Stat3 and anti-Stat5 antibodies were a gift from James Ihle. Rabbit anti-CRF antibody was raised against the GST-CRFB_{EC} fusion protein. Monoclonal anti-

phosphotyrosine antibody was from Sigma (Cat# P3300). Anti-Hu-IL-10R1 monoclonal and polyclonal antibodies were from R&D Systems, Inc. (Cat# MAB274 and AF-274-NA, respectively). Anti-FLAG epitope-specific M2 monoclonal antibody was from Eastman Kodak Company (Cat# IB13010).

Iodination and cross-linking of IL-10

Recombinant Hu-IL-10 was iodinated as reported (Cruz *et al.*, 1987). The [¹²⁵I]Hu-IL-10 was bound to cultured cells and then cross-linked to cells as described previously for [³²P]Hu-IFN- γ (Kotenko *et al.*, 1995).

For cross-linking of unlabeled IL-10 to the receptor components of PBMCs, 10⁷ cells were resuspended in 0.2 ml of phosphate-buffered saline (PBS), 0.1% bovine serum albumin and then treated with IL-10 (500 U/ml) or left untreated. After 60 min incubation at 22°C, the DTSSP cross-linker was added to a final concentration of 2 mM and incubation continued for 20 min at 22°C. After the reaction was terminated by addition of glycine to a final concentration of 0.1 M, the cells were washed twice with ice-cold PBS and used for immunoprecipitation experiments.

Immunoprecipitations, blottings and electrophoretic mobility shift assays (EMSAs)

Cultured cells were starved overnight in serum-free media, subsequently stimulated with Hu-IL-10 (100 U/ml) for 15 min at 37°C and used for immunoprecipitation or EMSA experiments. Isolated PBMCs were treated directly with Hu-IL-10 (100 U/ml) for 15 min at 37°C and used for EMSA.

Preparations of cell lysates, immunoprecipitation and blotting were performed as described (Kotenko *et al.*, 1995).

EMSAs were performed with a 22 bp sequence containing a Stat1 α -binding site corresponding to the GAS element in the promoter region of the human IRF-1 gene (5'-GATCGATTTCCTCCGAAATCATG-3') as described (Kotenko *et al.*, 1995).

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