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Induction of interleukin-2 receptor alpha (IL-2Ralpha) expression by interleukin-2: important role of the interleukin-2 receptor beta chain region between the two Stat5 docking sites



Abstract

The interleukin-2 receptor alpha (IL-2Ralpha) forms, together with IL-2Rbeta and gamma chains, a high affinity IL-2 receptor that is important for IL-2

responsiveness and normal T cell function. Expression of the IL-2Ralpha gene by T cells is regulated mainly at the transcription level which is transiently activated by antigen and upregulated and then prolonged by stimulation with IL-2. The effect of IL-2 on the IL-2Ralpha gene depends on the activation of the transcription factor Stat5, which acts on an IL-2- responsive enhancer that consists of two Stat5 and an Elf1 binding site. To identify the functional domains of the IL-2 receptor required for the stimulation of IL-2Ralpha gene expression, we introduced, into the CTL44 T cell line, receptor chimeras between the extracellular domain of the IL-9 receptor and the cytoplasmic region of IL-2Rbeta. Analyzing the effect of mutations in the intracellular IL-2Rbeta segment, we found that a minimal receptor containing the Jak boxes and one intact Stat5 docking site (i.e. tyrosine 392 or 510) can, as expected, mediate Stat5 activation, but is unable to stimulate IL-2Ralpha expression. However, when this minimal receptor includes the region between the two tyrosines, its capacity to mediate IL-2Ralpha cell surface expression is restored. These data suggest that the segment between the two Stat5 docking sites of the IL-2Rbeta chain mediates signaling events that, together with Stat5 activation, are essential for the stimulation of IL-2Ralpha gene transcription.

Full text

INTRODUCTION

Interleukin-2 (IL-2) influences several components of the immune system, including T and B cells, natural killer cells and monocytes. Three classes of IL-2 receptors are known: low affinity receptors containing only the alpha chain, intermediate affinity receptors consisting of beta and gamma chains, and high affinity receptors that contain all three chains (for review [1]).

In T lymphocytes, IL-2Rbeta and gammac are constitutively present at the cell surface [2, 3] while IL-2Ralpha only appears upon activation by antigen. Maximal and prolonged IL-2Ralpha expression by T cells depends on stimulation by IL-2 itself [4]. IL-2 activates the transcription factor Stat5 and regulates IL-2Ralpha transcription through an IL-2- responsive enhancer that contains two Stat5 binding sites and an Elf-1 binding site [5-8]. Mice lacking either one or both of the Stat5 isoforms, Stat5A or Stat5B, exhibit defective IL-2- induced expression of IL-2Ralpha [9, 10]. This indicates that either isoform can upregulate IL-2Ralpha. Signal transduction by the IL-2 receptor depends on the IL-2Rbeta and

gamma ϵ cytoplasmic domains. IL-2 activates three major pathways : the MAPK cascade [11], the Jak-Stat pathway [12] and PI3K [13]. The features of the intracytoplasmic tails of IL-2Rbeta and gamma ϵ chains required for triggering these pathways have been identified. The membrane proximal regions of IL-2Rbeta and gamma ϵ contain domains called Box 1, Box 2 and variable box (vBox) common to several type I cytokine receptors and involved in the recruitment and activation of Jak1 and Jak 3, associated respectively with IL-2Rbeta and gamma ϵ [14]. The other functional regions that have been delineated in the cytoplasmic portion of IL-2Rbeta include 1) the serine rich domain (S domain) between amino acids 267 and 322, which is required for the proliferative response to IL-2 [15], and 2) the acidic region (A domain) that includes the first four tyrosine residues (Y338-355-358-361). Its deletion does not prevent IL-2- driven proliferation or Stat5 activation but eliminates physical association of p56lck [16], induction of c-fos transcription (2), shc binding [17] and ras activation [18]. Finally, the C terminal tail contains two key tyrosine residues, Y392 and Y510, that are docking sites for Stat5 [19].

The cytoplasmic portion of the gamma ϵ chain contains conserved Boxes 1 and 2 and the variable vBox, and is required for IL-2 induced growth [20]. No other functional domains have been identified in this chain. Thus, the main gamma ϵ function appears to be to bring Jak3 into the signaling complex.

The aim of the present work was to test whether signals other than those required for Stat5 activation are involved in the stimulation of IL-2Ralpha expression. To this end, we wanted to map the domains of the IL-2Rbeta chain necessary for the enhancement of IL-2Ralpha expression. For this purpose we used chimeric receptors in which the intracellular part and the transmembrane domain of the human IL-2Rbeta chain were fused to an extracellular ligand binding domain not present in the host cell line (Figure 1A). For the latter we chose the IL-9R since, like IL-2Rbeta, this receptor combines with the gamma ϵ chain to transduce signals [21]. The chimeras were expressed in the CTL44 cell line which can be grown in IL-2 or in mIL-4. IL-2Ralpha expression declines in cells maintained in mIL-4, and it can be stimulated by IL-9 in cells expressing an IL-9R/IL-2Rbeta chimera. Using this system, we identified a region of 100 amino acids between the two Stat5 docking sites, Y392 and Y510, required for full induction of IL-2Ralpha cell surface expression by IL-2.

METHODS

Plasmid constructions and mutants

To construct the wild type hIL-9R/hIL-2Rbeta chimera (chim9-2), a full length hIL-2Rbeta cDNA fragment was excised from pHIL-2RbetaCDM8 [22] with HindIII and NotI, filled in with T4 DNA polymerase and inserted into EcoRV cleaved pBluescript SK +/- (Stratagene GmbH, Zürich, Switzerland). cDNA coding for hIL-9R in pEFBos was obtained from JC Renauld [23]. An exact fusion between the extracellular part of hIL-9R and the transmembrane region of IL-2Rbeta was made by overlap extension PCR [24]. Two specific primers p9Eco 5'-CAGAATTCAGGTTGGGGATGCCTCAG-3' and pE9-2 5'-GCCGAGCCACGGAATTGGCCACCCCCAGGGTGGG-3' (underlined bases are complementary to the IL-9R) were used to amplify the extracellular part of the IL-9R, whereas pT2-9 5'-CCCTGGGGGTGGCCAATTCCGTGGC-TCGGCCACC-3' (underlined bases are complementary to the IL-2Rbeta) and T7 primers were used to amplify transmembrane and intracellular regions of IL-2Rbeta.

An EcoRI-Sall fragment of the fusion receptor was inserted into pGem3ZF (Promega Corporation, Madison, WI, USA) and the sequence was checked by DNA sequencing. The fragment was then subcloned into the MSCVneo retroviral vector kindly provided by Dr. R. G. Hawley (Toronto, Canada) [25]. All deletion and substitution mutations in the IL-2Rbeta cytoplasmic domain were prepared using oligonucleotide primers and the polymerase chain reaction. Each mutant was confirmed by DNA sequencing.

Cell culture

CTL44 is a mouse cytotoxic T cell line growing in IL-2 or in mIL-4. Cells were maintained in Dulbecco's modified Eagle medium (DMEM, Life Technologies Basel, Switzerland) supplemented with 5% bovine calf serum, 2 mM glutamine, 50 µM beta-mercaptoethanol, and 100 U/ml of either IL-2 or mIL-4.

The amphotropic packaging cell line Phoenix (kindly provided by G. Nolan, Stanford University, CA, USA) was cultured in Dulbecco's modified Eagle medium containing 10% bovine calf serum and 2 mM glutamine.

Reagents and antibodies

Polybrene and chloroquine were from Sigma (St Louis, USA), neomycin from Life Technologies, Protein A Sepharose 4fast flow from Pharmacia (Dübendorf, Switzerland).

Recombinant human IL-2 and IL-9 were kindly provided by Glaxo IMB SA (Geneva, Switzerland) and by Dr J C Renauld (LICR, Brussels, Belgium), respectively.

Murine IL-4 was produced as culture supernatant from X63 transfected cells [26]. Antibodies against Stat5A and B isoforms were purchased respectively from Upstate Biotechnology (Lake Placid, NY, USA) and R&D systems (Abington, UK), whereas anti-phosphotyrosine antibodies (PY20 clone) were from Transduction Laboratories (Lexington, Kentucky, USA). Antiserum against the phosphorylated form of Stat5 was from Cell Signaling (Beverly, MA, USA).

Retroviral-mediated gene transfer

Retroviral supernatant was produced by transfecting Phoenix cells according to the protocols on the web site of the Nolan lab (<http://www.stanford.edu/group/nolan/index.html>).

2×10^6 CTL44 cells were infected, in the presence of polybrene (8 $\mu\text{g/ml}$), with 1 ml of filtered virus- containing supernatant in a final volume of 4 ml culture medium supplemented by 100 U/ml IL-2. After 16 hours, the culture fluid was replaced by fresh medium. Forty eight hours post-infection, neomycin was added to a final concentration of 2 mg/ml.

Seven days later resistant cells were tested for chimera expression by FACS analysis. The efficiency of transduction was near by 20%.

Immunoprecipitation and Western blotting

Cells were cultured for one week in DMEM supplemented with mIL-4. After extensive washing in culture medium, 10^7 cells were incubated for 18 hours with either IL-2 or IL-9. Whole cell lysates were prepared as previously described [27]. For immunoprecipitation, lysates were incubated with 4 μg anti-Stat5A or B purified antiserum for 3 hours at 4°C, followed by an one-hour incubation with Protein A Sepharose beads. After washes in lysis buffer, immunoprecipitates were dissolved in Laemmli buffer and separated on a 7.5% SDS-PAGE gel.

Proteins were then transferred to nitrocellulose membranes (Hybond ECL), which were incubated with anti-phosphotyrosine mAb or anti-Stat5 antiserum. Immunoreactive bands were visualised with the chemiluminescence western blotting system (Amersham).

EMSA (Electrophoretic mobility shift assay)

Nuclear extracts were prepared as described by Schreiber *et al.* [28]. Briefly, 10^7 cells were lysed in 200 μ l of buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, phosphatases and proteases inhibitors), in the presence of 0.6% NP40. The lysates were centrifuged, and nuclear proteins extracted from pellets in buffer B (20 mM Hepes pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT).

Stat DNA binding activity was assessed by EMSA using a probe derived from the GAS site (GRR) of the FcgammaR gene promoter (5'-GTATTTCCCAGAAAAGGAAC-3') as previously described [29].

FACS analysis

For immunofluorescence analysis of IL-2Ralpha cell surface expression, the anti-mouse IL-2Ralpha mAb, 5A2, was used as culture supernatant [30]. FITC-conjugated goat anti-mouse immunoglobulin was used as second step reagent (Tago, Burlingame, CA, USA). Chimera expression were detected with biotinylated anti-hIL-9R antibody AH9R1 [23] followed by phycoerythrin-conjugated streptavidin (Tago, Burlingame, CA, USA). Samples were analysed with a FACScan flow cytometer (Becton Dickinson, Basel, Switzerland) using the Lysis II program.

RESULTS AND DISCUSSION

Stimulation of IL-2Ralpha expression through a IL-9R/IL-2Rbeta chimeric receptor

CTL44 cells were infected with an ecotropic retrovirus coding for an IL-9R/IL-2Rbeta chimeric receptor and the bacterial neomycin resistance gene. During selection for neomycin resistance, cells were maintained in mouse IL-4. Seven days after infection we monitored expression of the chimeric receptor on the

survivors by staining cells with an antibody specific for the human IL-9R. As shown in Figure 1B, FACS profiles showed a unimodal population of positive cells, cells transduced with different receptor constructs expressing similar amounts of the chimera. The parental CTL44 cells grow in IL-2 or mIL-4 but not at all in IL-9, whereas this cytokine efficiently stimulated proliferation of the cells expressing the chimeric receptor (data not shown). Figure 1C shows that when cells were cultured in mIL-4, no IL-2Ralpha chain could be detected at the cell surface. Addition of IL-9 to parental cells cultured in mIL-4 had no effect on IL-2Ralpha expression. By contrast, IL-9 induced the same level of IL-2Ralpha expression on cells carrying the IL-9R/IL-2Rbeta chimera as did IL-2. In the present study, cells were maintained in medium supplemented with mIL-4 before stimulation with IL-2 or IL-9. mIL-4 culture conditions thus correspond to basal levels of IL-2Ralpha expression.

Stat5 activation is not sufficient for the stimulation of IL-2Ralpha expression

Previous works from this and other laboratories have shown that Stat5 activation is required for the stimulation of IL-2Ralpha gene transcription *via* the IL-2 responsive enhancer (IL-2rE) [5-8]. To explore whether Stat5 activation is the only IL-2-triggered signal required for the stimulation of IL-2Ralpha expression, we constructed two chimeras (Chim9-2 M2 and M3) which contained either one of the two tyrosine-containing peptides that can function as Stat5 docking sites [19], as well as the segment between amino acid 251 and amino acid 306, previously shown to be required for the activation of Jak kinases [31], but which lacked the rest of the IL-2Rbeta cytoplasmic domain (Figure 1A). Chim9-2 M2 and M3 contain the Stat5 docking sites around tyrosine 510 and tyrosine 392 [32], respectively. These mutants were expressed, as well as the wild type construct at the surface of CTL44 cells (Figure 1B), and both of them could support IL-9 - stimulated proliferation as efficiently as the wild type IL-9R/IL-2Rbeta receptor (data not shown). These observations are in agreement with previous results from other groups [20, 33, 15]. When cell populations expressing Chim9-2 M2 or M3 were cultured in mIL-4, they ceased to express IL-2Ralpha, like the parental cells (Figure 2A, upper histograms). Exposure to IL-2 reinduced similar levels of IL-2Ralpha expression in all populations (middle histograms). Triggering of wt chimera receptor by IL-9 induced IL-2Ralpha expression to a level comparable to that obtained with IL-2. In marked contrast, IL-9 - mediated IL-2Ralpha induction in cells carrying M2 and M3 chimeras was barely detectable (lower histograms). In the same series of experiments, we

tested a mutant, M1, that contained both Stat5 docking sites, as well as the segment between them. As expected, cells expressing this chimera proliferated in response to IL-9 as well as the other cell populations carrying chimeric receptors. In addition, IL-9 stimulated IL-2Ralpha expression by cells carrying the M1 mutant to similar levels as those found in cells expressing the wild type chimera (Figure 2A, lower histograms).

One simple explanation for the inability of the M2 and M3 chimeric receptors to stimulate IL-2Ralpha expression could be that they activate lower levels of Stat5 because they contain only a single Stat5 docking site. To explore this possibility, we compared Stat5 activation in the cells expressing the different chimeras. Stat5 activation by IL-2 depends on the phosphorylation of a conserved tyrosine residue in the C terminal part of the protein. The phosphorylation is mediated by activated Jaks, allowing dimerisation of STAT proteins *via* an SH2 domain interaction. Activated Stat5 dimers translocate into the nucleus and display specific DNA binding activity. This sequence of events usually occurs within several hours of cell stimulation. In the context of IL-2Ralpha gene regulation, the onset of Stat5 activation is as rapid as expected for Stat factors but its DNA binding activity continues to increase for 48 hours in parallel with increasing IL-2Ralpha expression [7]. We thus analyzed Stat5 DNA binding activity following 18 hours of cell stimulation time corresponding to detection of IL-2Ralpha-cell surface expression. For that we performed band shift assays with a probe that contains a high affinity binding site for both Stat5 and Stat6 (Figure 2B). Control experiments confirmed that the complex formed by nuclear extracts from CTL44 cells cultured in IL-2 was supershifted by antibodies to Stat5 (left panel, compare lanes 1 to 3), whereas the complex formed by the same cells cultured in mIL-4, presumably due to activated Stat6, migrated more slowly and was not affected by the anti-Stat5 antibodies (compare lanes 2 to 4). Considering this point, in the following bandshift experiments, cells were maintained in mIL-4 before stimulation with IL-2 or IL-9 to turn off Stat5 activation, and the absence of Stat5 DNA binding activity was systematically tested. Comparing nuclear extracts of CTL44 cells expressing different forms of IL-9R/IL-2Rbeta receptor chimeras and cultured in IL-2 or in IL-9, we found that IL-9 induced, in all populations, at least as much Stat5 binding activity as did IL-2 in control CTL44 cells (right panel, compare lanes 6-9 to lane 1). Clearly, expression of the receptor chimeras did not affect Stat5 DNA binding activity induced upon triggering of endogenous IL-2R (lanes 2 to 5). These results indicate that

deletions in the cytoplasmic part of the IL-2Rbeta chain do not affect Stat5 DNA binding activity as long as Stat5 docking sites and Jak boxes are preserved.

As demonstrated by knock-out experiments [9, 10], both Stat5A and Stat5B isoforms contribute to the IL-2-dependent activation of IL-2Ralpha expression. Since the binding specificity of the two isoforms cannot be distinguished by band shift assays [34], we could not discriminate between the activities of these two proteins by this assay.

To distinguish Stat5A from Stat5B activation, we used antibodies specific for one or the other isoform to precipitate the Stat5 molecules (Figure 2C). Their phosphorylation statuses were tested by incubating western blots of the immunoprecipitates with an anti-phosphotyrosine antibody. As expected, treatment of parental cells with IL-2 induced tyrosine phosphorylation of both Stat5A and B isoforms, whereas mIL-4 had no effect (compare lanes 2 and 9 to 7 and 14). Comparison of the signals observed in immunoprecipitates from cells expressing different receptor chimeras (lanes 3 to 6 and 10 to 13) indicates that IL-9 triggers similar levels of Stat5A and Stat5B phosphorylation in all of these populations, and that these levels are not lower than the ones induced by IL-2. Stat5B phosphorylation could also be assessed through mobility shift of the protein in SDS-PAGE after activation as previously observed in [35]. We thus examined the pattern of migration of Stat5B by western blot (Figure 3D). When CTL44 cells were cultured in mIL-4, Stat5B migrates with an apparent molecular weight of 92 kDa (lane 13). By contrast in the presence of IL-2, Stat5B undergoes a pronounced mobility shift (lanes 1 to 6). This mobility shift is also induced upon triggering of chim9-2 wt chimera by IL-9 (lane 7). Most importantly, the M1, M2 and M3 mutations do not affect this IL-9-induced shift of Stat5B (lanes 8 to 10).

These data indicate that the inability of M2 and M3 mutants to drive IL-2Ralpha expression is not due to a defect in Stat5 activation, as measured by tyrosine phosphorylation or DNA binding activity.

The IL-2Rbeta receptor segment between residues 392 and 510 is required for the stimulation of IL-2Ralpha expression

While previous studies have shown that Stat5 activation is required for the stimulation of IL-2Ralpha expression by IL-2 [10], the results described here suggest that activation of Stat5 DNA binding activity is not sufficient. It appears

that, in addition to Stat5 docking sites, the IL-2Rbeta segment between residues 392 and 510 is necessary for this response. To test whether this segment, combined with a single Stat5 docking site, was sufficient to drive IL-2Ralpha expression, we derived mutants from the M1 receptor in which either tyrosine 392 (M1F392) or tyrosine 510 (M1F510) or both (M1FF) were changed into phenylalanine (Figure 3A). Testing the activity of these receptors in CTL44 cells we found, not surprisingly, that the M1FF chimera was unable to stimulate proliferation, while cells expressing M1F510 and M1F392 receptors grew equally well in medium containing IL-9, IL-2 or mIL-4 (data not shown).

Analyzing IL-9-stimulated IL-2Ralpha expression of cells expressing the M1 receptor and the two derivatives, M1F392 and M1F510 with a single Stat5 docking site, we found that all three receptors gave comparable responses, similar in level to that induced by IL-2 (Figure 3B, compare middle to upper histograms). On the other hand, IL-9 was unable to induce IL-2Ralpha expression in M1FF receptor-expressing cells maintained in mIL-4 (Figure 3B, right part).

As expected, triggering of the two M1-derived receptors induced Stat5 DNA binding as did triggering of the M1 receptor (Figure 3C, compare lanes 5 and 6 to 4) and to levels comparable to those induced by IL-2 (Figure 3C lanes 1, 2, 3). In the same way, stimulation of M1F510 and M1F392 receptors by IL-9 induced Stat5B phosphorylation as assessed by the mobility shift in SDS-PAGE (Figure 3D, lanes 11 and 12).

The finding that either of the two Stat5 docking sites can induce IL-2Ralpha expression when combined with the 392/510 segment suggests that the only role of these sites in this response is Stat5 activation. Thus, we do not favor the hypothesis put forward by Ascherman *et al.* that IL-2Ralpha induction depends on additional pathways triggered by one or the other of these sites [36]. Indeed, they observed that simultaneous mutations of tyrosine 392 and tyrosine 510 completely blocked the induction of CD25 mRNA and that in the context of full length IL-2Rbeta, only one of the tyrosines is sufficient for this process. They thus concluded that the two tyrosines direct both Stat5 activation and an additional signaling pathway leading to induction of the IL-2Ralpha gene. We show here that stimulation of chim9-2M2 and M3 minimal receptors induces Stat5 activation but not IL-2Ralpha expression. The cytoplasmic part of these receptors consists of the tyrosine consensus motif thought to activate Stat5 [32], flanked by 10 more amino acids. This sequence surrounding tyrosines 392

and 510 should be enough to couple additional pathways and therefore to induce IL-2Ralpha expression according to Ascherman's model. Our data rather indicate that, besides a Stat5 docking site, the IL-2Rbeta segment between residues 392 and 510 is required for the enhancement of IL-2Ralpha expression by IL-2. It is important to note that this region is always present in the different mutants analyzed by Ascherman et al. This could explain why their model differs from ours.

Our results also raise the question of the requirement of the 392-510 IL-2Rbeta segment for the regulation of IL-2/Stat5 target genes other than IL-2Ralpha. The recruitment of unique sets of Stat to each cytokine receptor family, as well as the selective binding of Stat to specific DNA sequences, constitute critical steps in defining the specificity of the biological responses to cytokines. However, additional pathways leading to modifications of Stat such as phosphorylation or recruitment of coactivators, may contribute to this specificity. Indeed, the 392-510 IL-2Rbeta segment may transduce a signaling event which cooperates with Stat5 activation to drive IL-2Ralpha gene expression but not other Stat5 target genes. Recent data on the role of Stat5 and Sp1 in the regulation of cyclin D2 gene transcription in response to IL-2 argue in this way [37]. They showed that a minimum region of IL-2Rbeta, up to residue 325 and including only 13 residues surrounding tyrosine 510, is sufficient to drive cyclin D2 expression.

The function(s) of this 392-510 segment is (are) not known. One possibility is that it affects the *in vivo* activity of Stat5, e.g. by mediating Stat5 serine phosphorylation, which in the human Kit225 cell line is necessary for full Stat5 activation [38]. In COS cells, serine phosphorylation of Stat5A prolongs the duration of Stat5A activation through the prolactin receptor [39]. In CTLL44 cells expressing chim9-2 M1, M2 or M1F392 receptors, we observed no significant differences in the time course of Stat5 DNA binding activity induced by IL-9 (Figure 4A, lanes 1-3, 5-7, 9-11). Similar kinetics are obtained with the stimulation of parental cells by IL-2 (lanes 4, 8, 12). In parallel, we compared the content of phosphorylated Stat5 in the nucleus of the three mutants during the same period of time. For that we used antibodies specific for the tyrosine phosphorylated form of Stat5. As expected, in the presence of mIL-4, no phospho-Stat5 is detected (Figure 4B, lanes 1, 5, 12, 19). As a positive control, IL-2 induces nuclear accumulation of the phosphorylated form of STAT5 in parental and mutant CTL44 cells as soon as 1 hour of treatment (lanes 2-4, 6-8, 13-15, 20-21). Most importantly, triggering of the three mutants receptors leads to the

same time course of Stat5 tyrosine phosphorylation (lanes 9-11, 16-18, 23-25). Thus, the 392/510 segment of the IL-2Rbeta chain may not control the duration of Stat5 activation. Alternatively, this segment could trigger an independent signal transduction pathway that synergizes with activated Stat5 in the regulation of IL-2Ralpha expression. One good candidate is the MAPK pathway known to be involved in IL-2 signaling [40]. However we found no differences in the activation of the three MAPK family members (ERK1-2, JNK, p38) upon triggering of the chimeric receptors (not shown). To finally understand the function of the 392-510 segment, we are currently attempting to identify proteins that interact with this segment using a yeast two-hybrid screen.

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Figures

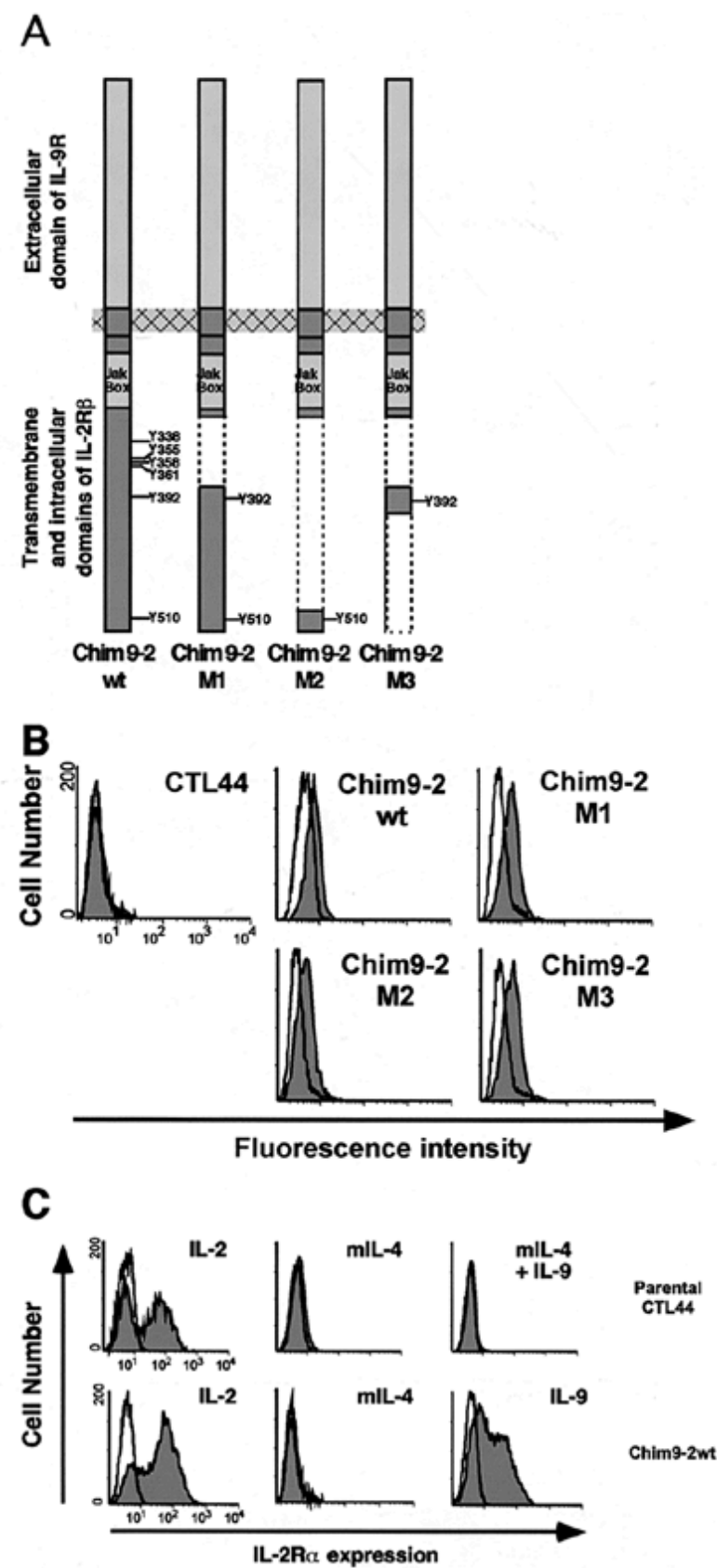


Figure 1

A) Schematic view of the IL-9R/IL-2Rb wild type (Chim9-2 wt) chimera and its mutants. M1 and M2 constructs lack the region from aa 314 to 380 or from aa 314 to 501, respectively. In M3, the two regions between aa 314 and 380 and between 407 and the C-terminal are deleted. **B)** Cell surface expression of chimeric receptors. CTL44 cells resistant to neomycin were subjected to FACS analysis to monitor IL-9R cell surface expression (filled histogram: anti-IL9R antibody; open histograms: irrelevant antibody). Parental CTL44 were used as negative control. **C)** Triggering of wild type chimeric receptor induces IL-2Ralpha expression. The T cell line, CTL44, which grows in IL-2 or mIL-4, was cultured in murine IL-4. After extensive washes, cells were cultured for 3 days with IL-2 (100 U/ml), mIL-4 (100 U/ml) , mIL-4 + IL-9 (100 U/ml) or IL-9 and IL-2Ralpha expression was measured by FACS analysis. Upper histograms show parental CTL44 cells, lower histograms cells expressing the wild type chimeric receptor, chim9-2wt.

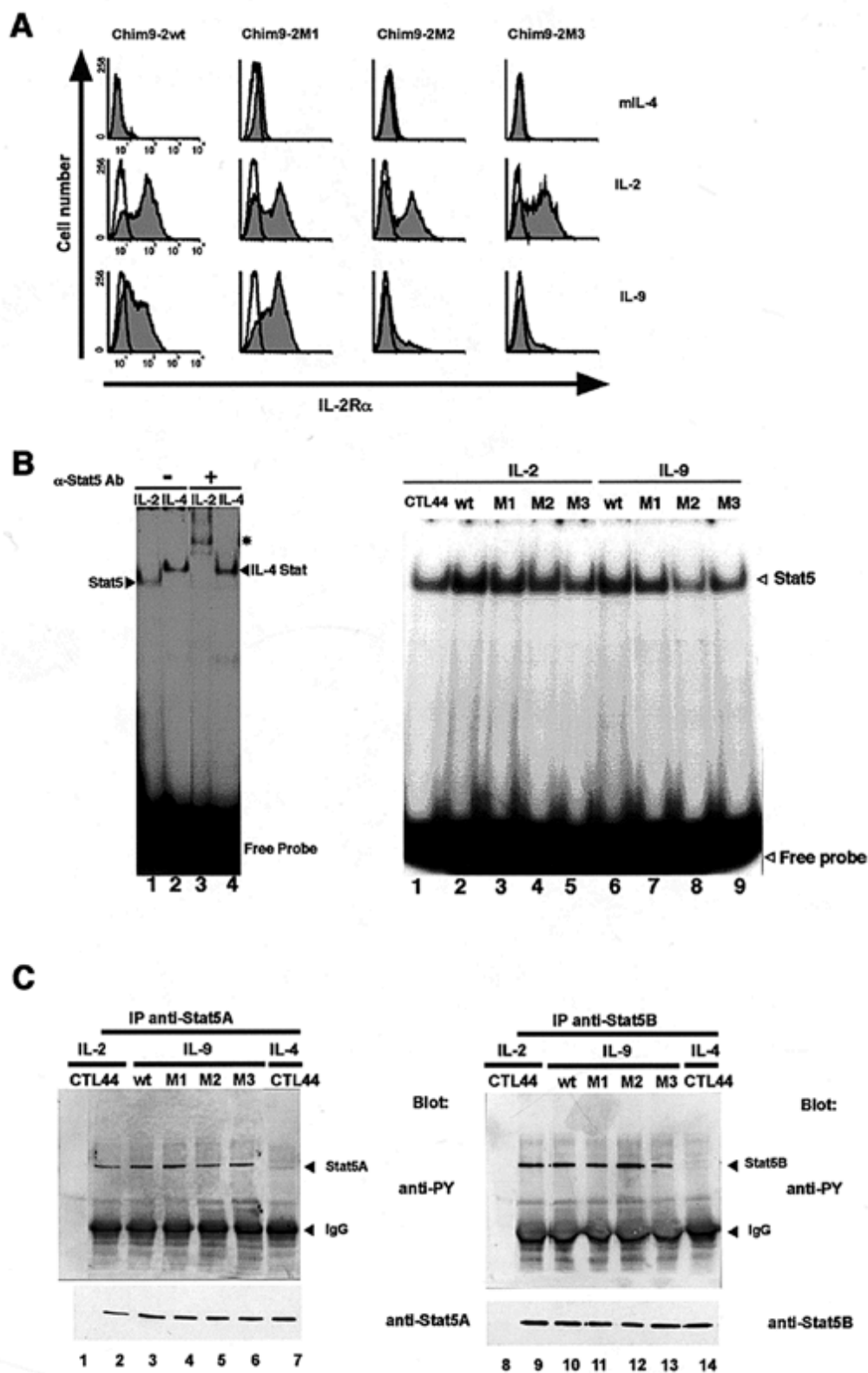


Figure 2

Biochemical events mediated by M1, M2 and M3 chimeric mutants.

Cells were maintained in mIL-4 to downregulate IL-2Ralpha cell surface expression, and then cultured in IL-2 or IL-9.

A) IL-2Ralpha expression was measured by FACS analysis after 48 h of culture in IL-2, IL-9 or mIL-4.

B) Stat5 DNA binding activity. Nuclear extracts were prepared and Stat5 DNA binding activity was assessed by bandshift using an FcgammaR probe.

Left panel: CTL44 parental cells were cultured for 3 days in the presence of IL-2 (lanes 1-3) or mIL-4 (lanes 2-4). Nuclear extracts were subjected to supershifting with antibodies directed against Stat5 (lanes 3-4). The asterisk shows the anti-Stat5 supershifted complex. Right panel: Parental CTL44 (lane 1) and chimeric receptors expressing cells were cultured for 48 h in IL-2 (lanes 1 to 5) or IL-9 (lanes 6 to 9).

C) Stat5A and B tyrosine phosphorylation.

Cells were cultured for 18 hours in IL-2 (lanes 1, 2, 8 and 9) or IL-9 (lanes 3-6 and 10-13). Stat5A (lanes 2 to 7) or anti-Stat5B (lanes 9 to 14) was immunoprecipitated from whole cell lysates. Lanes 1 and 8 are controls without anti-STAT antibodies. After washing, immune complexes were separated on a 7.5% acrylamide gel under reducing conditions and transferred to nitrocellulose membranes. Tyrosine phosphorylated proteins were revealed with anti-phosphotyrosine antibodies (anti-PY). Levels of immunoprecipitated Stat5 proteins were checked by stripping and reprobing the membranes with anti-Stat5A or Stat5B specific antibodies. IgG means heavy chains of IgG.

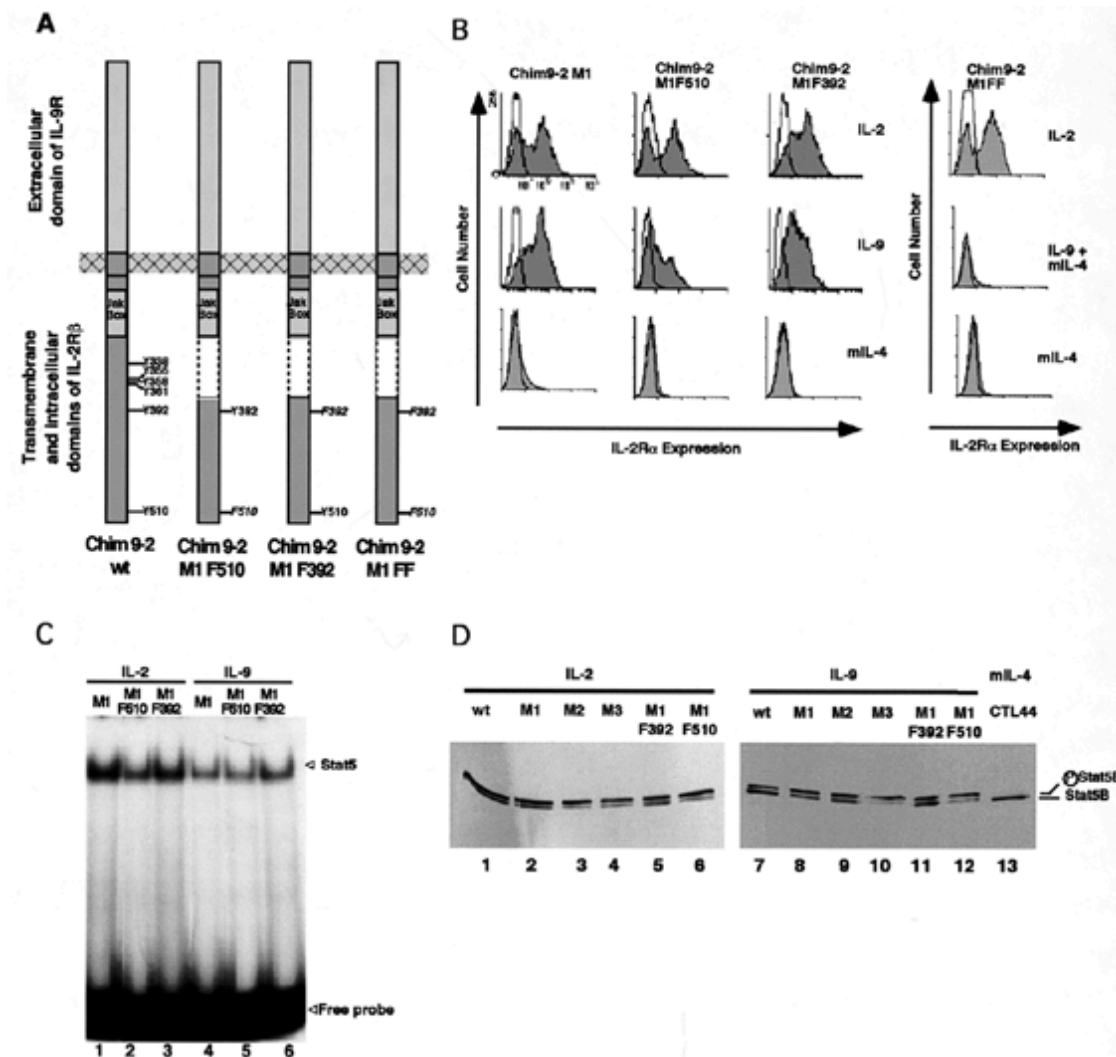


Figure 3

A) Schematic view of M1 derived chimera mutants.

M1F510, M1F392 and M1FF were obtained from M1 mutants. They contain point mutations in the Stat5 docking sites, which transform tyrosine 392 and/or tyrosine 510 into phenylalanine.

B), C) and D) IL-2Rα expression and Stat5 activation.

(B), Cells were stimulated for 48 hours in IL-2 or IL-9. IL-2Rα expression was measured by FACS analysis. (C), In parallel, nuclear extracts were prepared and Stat5 DNA binding activity was assessed by bandshift experiments. (D), Cells incubated for 24 h with IL-2 (lanes 1 to 6) or IL-9

(lanes 7 to 12) or kept in mIL-4 (lane 13). Whole cell lysates were prepared and analysed by western blot with anti-Stat5B antibodies. The change in the electrophoretic migration of Stat5B is related to its phosphorylation.

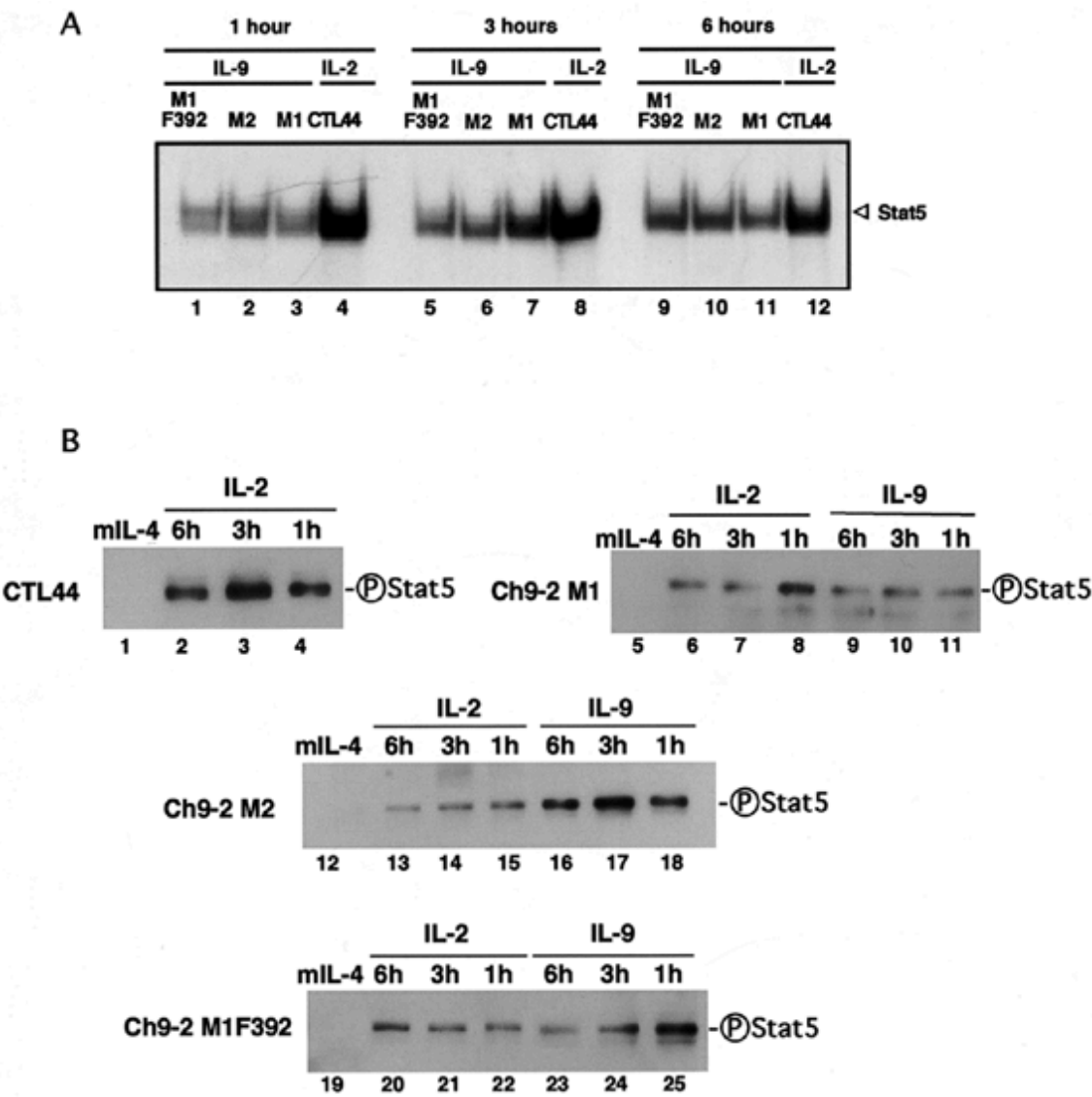


Figure 4

Kinetics of induction of Stat5 activation.

Cells expressing M1, M2 and M1F392 mutant receptors were stimulated for 1, 3 or 6 hours with IL-9. Time "zero" corresponds to cells cultured in mIL-4 i.e. without Stat5 activation. As control, parental CTL44 cells were

	incubated for the same period of time with IL-2. Nuclear extracts were prepared and assessed for A) Stat5 DNA binding activity by bandshift experiments or B) for Stat5 tyrosine phosphorylation by western blotting with phospho-specific antibodies.

Authors

Véronique Imbert, Roger Rezzonico, Patrick Reichenbach, Markus Nabholz

U526 INSERM, Faculté de Médecine-Pasteur, 06107 Nice Cedex 02.

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