IL-10 induces DNA binding activity of three STAT proteins (Stat1, Stat3, and Stat5) and their distinct combinatorial assembly in the promoters of selected genes

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Abstract Interaction of IL-10 with its receptor leads to the activation of STAT transcription factors. Herein we report the IL-10 dependent simultaneous activation of three STAT transcription factors: Stat1, Stat3, and Stat5. Upon IL-10 treatment multiple Stat proteins become simultaneously activated, and bind to different promoters with equal kinetics but form distinct homo- and heterodimeric transcriptionally active complexes depending on the STAT-consensus elements of a selected gene promoter. Upon IL-10 treatment Stat1, 3, and 5 bind to the GRR of the FcγRI gene, activated Stat1 and 3 bind to the SIE sequence of the c-fos promoter and transcriptionally active Stat5 assembles at the PRL-STAT consensus sequence of the β-casein gene. Thus, functionally relevant STAT dimerization is influenced by the activated cytokine receptor as well as the specific STAT consensus sequence present in a specific gene promoter.

Key words: Interleukin-10; STAT protein; Signaling

1. Introduction

Recent studies demonstrated that multiple cytokines signal through activation of a novel family of transcription factors, termed STAT proteins [1,2]. To date, eight proteins have been identified belonging to the family of STAT transcription factors [3]. STAT activation is mediated by the interaction of these transcription factors at distinct sites of a cytokine receptor's intracellular domain and by the subsequent tyrosine and serine/threonine phosphorylation of the STAT proteins [4,5]. Phosphorylation of the STAT transcription factors is a function of the tyrosine kinase activity of a cytokine receptor itself and/or receptor associated Janus-kinases and serine/threonine phosphorylation has been shown to be mediated by members of the MAP-kinase family [6–9]. STAT proteins that are activated in the cytoplasm of a cytokine treated cell translocate to the nucleus and display DNA binding activity at the promoters of diverse genes forming homo- or heterodimeric complexes [7]. Taking into consideration that cytokines that serve opposite functions can activate one STAT protein, we sought to investigate further the role of STAT transcription factors in the signaling pathway that is initiated by a pleiotropic cytokine like IL-10 [10]. A recent report by Finbloom and Winestock [11] demonstrated the IL-10 dependent activation of Stat1 and Stat3 in human T-cells and monocytes. The results presented herein extend the initial studies of IL-10 dependent STAT activation and demonstrate that, in addition to Stat1 and Stat3, Stat5 becomes tyrosine phosphorylated by IL-10. IL-10 dependent signaling is shown to involve the tyrosine phosphorylation of multiple STAT proteins and IL-10 dependent transcriptional gene activation has been found to depend on specific STAT homo- and heterodimers that are recruited at the site of a specific promoter region.

2. Materials and methods

2.1. Reagents

IL-10 and IFNγ were purchased from Genzyme (Cambridge, MA), IL-3 was from Boehringer Mannheim, Germany. Streptavidin-Sepharose was obtained from Sigma (St. Louis, MO, USA). Horseradish-peroxidase-conjugated goat-anti-rabbit IgG was purchased from Amersham (Arlington Heights, IL, USA). Polyclonal antisera specific for Stat1–Stat4 were generously provided by Dr. J. Darnell, Rockefeller University, New York, NY, USA. Stat5 specific antisera were prepared as described [12]. Stat6 specific antisera was generously provided by Dr. S. McKnight, Tularic Inc., Palo Alto, CA.

2.2. Cells

IL-3 dependent murine preB-cells BaF3 lacking the mIL-10 receptor and BaMR cells (BaF3 stably transfected with the mIL-10 receptor) were cultured as described [13,14].

2.3. Oligonucleotides

An 18 base pair oligonucleotide probe GRR of the FcγRI gene promoter (sense strand, 5′-ATGTATTTCCCAGAAA; antisense strand, 5′-CTTTTCTGGGAAATA), the PRL STAT consensus element of the β-casein gene promoter (sense, 5′-AGATTTCTAGGAATTCAAATCCAC; antisense, 5′-GTGGATTTGAATTCCTAGAAATCT) [14], and the SIE of the c-fos promoter (sense, 5′-GTCGACAGTTCCCGTCAATC; antisense, 5′-GATTGACGGGAACTGTCGAC) were made by annealing the oligonucleotides as described [14,16]. Precipitation studies were performed using biotinylated GRR oligonucleotide that was immobilized on a streptavidin-agarose matrix (Sigma, St. Louis, USA).

2.4. Electromobility shift assay (EMSA)

Cells were harvested at distinct time points, nuclear extracts prepared and gel shift experiments performed as described [14].

2.5. STAT protein precipitation

1 ng of biotinylated GRR probe was coupled to 100 μl of streptavidin-Sepharose for 30 min at 4°C. Sepharose was washed in PBS and incubated with 1 ml of nuclear extracts derived from 7×10^7 BaMR cells treated for 10 min at 37°C with 100 ng/ml rmIL-10, 100 ng/ml rmIL-3 or 100 ng/ml rmIFNγ. After 2 h incubation the Sepharose was pelleted at $100\times g$ for 5 min and washed with 800 μl of washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM EDTA, 1 mM Na-orthovanadate, 10 mM NaF, 750 μM DTT, 1 mM PMSF) and 10 μg/ml leupeptin and aprotinin. The Sepharose was resuspended in 50 μl 2×Laemmli buffer and 180 mM 2-mercap-

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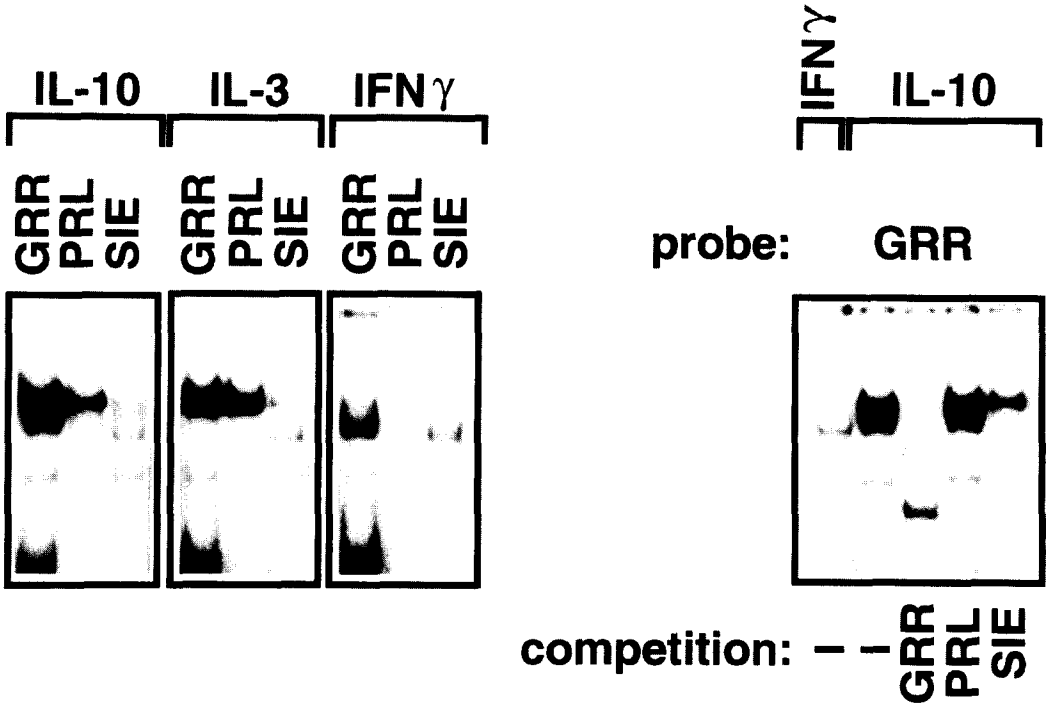

Fig. 1. IL-10 induces the DNA binding activity of distinct STAT proteins in the STAT consensus elements of the FcγRI, the β-casein, and the c-fos promoters. Nuclear extracts from IL-10, IL-3 or IFNγ treated BaMR cells were assayed by gel-shift analyses employing 25000 cpm (1 ng) of the GRR, PRL or SIE probes, respectively (left panels). Competition studies were performed by incubating 1 ng of the radiolabeled GRR probe with 3 μg of nuclear extracts derived from IL-10 treated cells in the presence of a 100-fold excess of the unlabeled GRR probe, the PRL or SIE probes (right panel). DNA binding activity was assayed by polyacrylamide electrophoresis and autoradiography.

toethanol (Sigma) and the suspension was heated to 70°C for 5 min. Samples were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes (BioRad Laboratories, Richmond, CA) and membranes were blocked for 18 h at 4°C with 50 mM Tris-buffered physiologic saline pH 8.0 (TBS) containing 2% BSA (Sigma) and 0.5% Tween 20 (TBST) (BioRad). Membranes were washed in TBST, incubated with STAT specific antisera at 1:3000 final dilution for 1 h, washed, incubated with HRPO-conjugated goat-anti-rabbit-IgG at 1:7500 for 20 min and developed by chemiluminescence using the ECL detection reagent (Amersham, Arlington Heights, IL).

2.6. Transient transfection assays

The pXM-PRL(−105) vector that contained 105 bp upstream the transcriptional start site of the β-casein promoter or a mutated version of that sequence were used as described [2]. 5×10^6 BaMR cells were transiently transfected by electroporation with 10 μg of the pXM-PRL(−105)/(mut) vectors, respectively, and 1 μg of a CMV-CAT-reporter construct [15,16]. 24 h later BaMR cells were either left untreated, or treated with 100 ng/ml IL-3 or IL-10 for 48 h. Cells were harvested and luciferase assays were performed as described [17]. CAT assays were performed using the Boehringer Mannheim ELISA system. Data are presented as the mean of triplicate determinations of relative light units (RLU) normalized for transfection efficiency by CMV-CAT reporter construct. Each experiment was repeated at least three times.

3. Results

3.1. IL-10 treatment of BaMR cells leads to the activation of multiple STAT proteins

To test for STAT activation gel-shift experiments were performed. IL-10 treatment of IL-10 receptor bearing BaMR cells led to the generation of three distinct DNA binding complexes in the GRR of the FcγRI promoter, to the generation of a single gel-shift complex with the PRL probe of the β-casein promoter and to the formation of three DNA binding complexes with the SIE probe of the c-fos promoter (Fig. 1). The latter complexes showed slightly higher mobilities and significantly reduced binding affinities compared to the IL-10 induced GRR binding gel shift complexes (Fig. 1). To check that the difference in the binding affinities was not caused by the amount of nuclear proteins or the radioactivity of the EMSA probes used, 3 μg of nuclear extracts were incubated with 25000 cpm (1 ng). Further control experiments using IL-3 or IFNγ treated BaMR cells demonstrated single IL-3 induced DNA complexes with comparable binding affinities in the GRR and the PRL probe. In addition, a Stat1-like gel shift complex was observed when the IL-3 treated BaMR extracts were probed with the SIE sequence. In nuclear extracts from IFNγ stimulated BaMR cells, one prominent gel shift band was obtained with the GRR probe, no DNA complex was formed with the PRL probe and a DNA complex with reduced binding intensity was obtained with the SIE probe (Fig. 1, left panels). In summary, IL-3 and IFNγ treatment of the cells leads to the formation of single DNA binding complexes in the GRR, PRL, SIE or the GRR and SIE consensus sequences, respectively. In contrast, IL-10 induced the combinatorial formation of multiple gel shift complexes depending on the STAT consensus probes used. To identify further the binding characteristics of the IL-10 induced complexes competition experiments were performed. Therefore, nuclear extracts from IL-10 treated cells were preincubated with a 100-fold excess of unlabeled GRR, PRL or SIE probe and EMSA complexes were detected thereafter by probing with 1 ng of the radiolabeled GRR probe (Fig. 1, right panel). Unlabeled GRR probe competed the binding of the IL-10

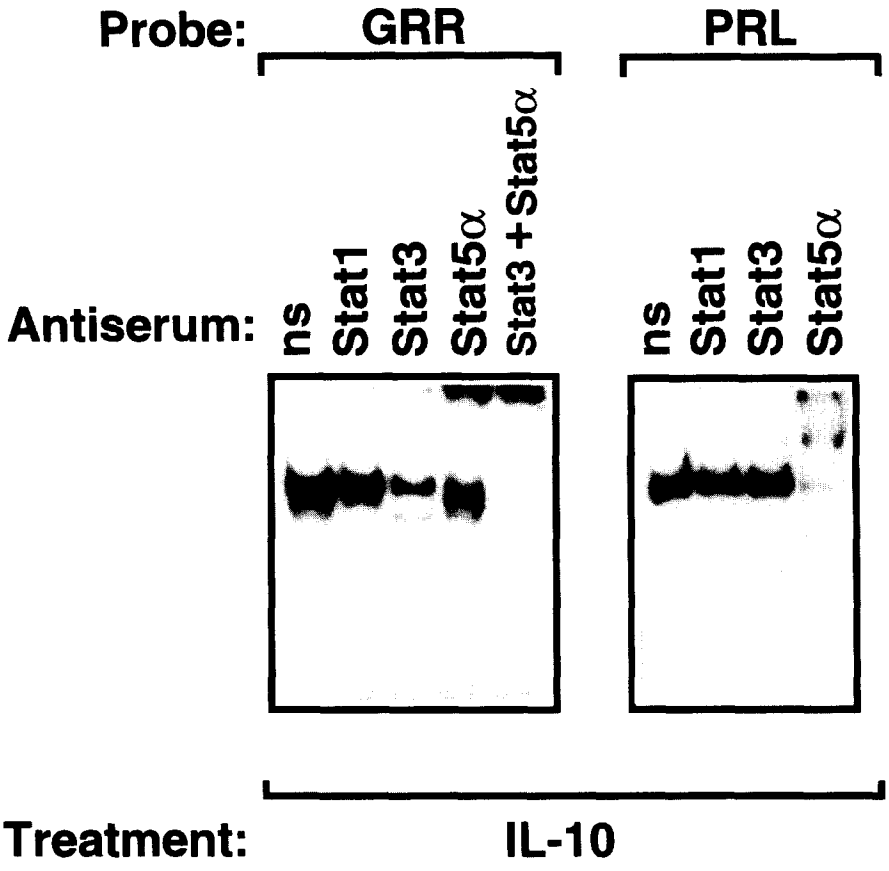

Fig. 2. IL-10 activates the DNA binding of multiple STAT proteins. Supershift experiments were performed employing 3 μg of nuclear extracts derived from IL-10 (100 ng/ml) treated cells in the presence of normal rabbit sera or STAT specific antisera using the GRR or PRL probes, respectively.

dependent DNA complexes indicating the specificity of the DNA-protein interaction (Fig. 1, right panel, lanes 2,3). An excess of the PRL probe only slightly reduced the intensity of the upper band A whereas preincubation of the nuclear extracts with a 100-fold excess of the SIE probe competed the binding of the lower band C, the middle band B and competition with unlabeled SIE probe reduced the intensity of the IL-10 induced GRR complex A (Fig. 1, right panel). These results indicated that multiple STAT proteins were activated in the nuclei of IL-10 treated cells that diversely bound to STAT consensus elements within the selected promoter sequences. To investigate the time characteristics of IL-10 induced gel shift complexes nuclear extracts were harvested at distinct time points and probed using the GRR or PRL probes, respectively. IL-10 induced gel shift complexes were first detectable after 1 min, were maximal after 7 to 30 min and disappeared after 120 min upon addition of IL-10. No differences in the IL-10 dependent kinetics were observed with regard to the binding characteristics to either one of the probes used and equal results were obtained with the SIE probe (data not shown). In contrast, IL-3 induced STAT activation was observed after 1 min and was maintained at maximal levels for more than 120 min up to a maximum of 4–5 h after addition of IL-3 (data not shown). Thus, IL-10 induced activation of STAT proteins in a very time-restricted manner whereas IL-3 dependent STAT activation was extended to as long as 4–5 h after cytokine treatment of the cells.

3.2. IL-10 activates the DNA binding of Stat1, Stat3, and Stat5

To identify the nature of the STAT proteins that were activated in response to IL-10, supershift experiments were performed that employed STAT specific antisera. Stat2, Stat4, and Stat6 specific antisera did not influence the formation of the IL-10 dependent EMSA complexes (data not shown) as did normal rabbit serum (Fig. 2). However, when the GRR sequence was used Stat1 specific antisera supershifted the lowest band C and reduced the intensity of the middle band B, Stat3 specific antisera supershifted the middle band B and reduced the intensity of the upper band A, and Stat5 specific antisera partially supershifted the upper band A of the EMSA complexes (Fig. 2, left panel). The activation of multiple STAT proteins by IL-10 was confirmed by the precipitation of phosphorylated forms of Stat1, Stat3, and Stat5 using Sepharose-coupled biotinylated double-stranded GRR probe (Fig. 3). Control experiments on the activation of Stat1 and Stat5 were performed using IFNγ or IL-3 treated nuclear extracts, respectively (Fig. 3). Despite the herein documented simultaneous activation of multiple STAT proteins upon IL-10 treatment of the cells, only Stat5 antisera supershifted the EMSA complex that was formed in the PRL sequence of the β-casein promoter (Fig. 2, right panel). Stat1 and Stat3 antisera supershifted the complexes obtained with the SIE sequence (data not shown). Thus, these data demonstrate the simultaneous activation of multiple STAT proteins by the

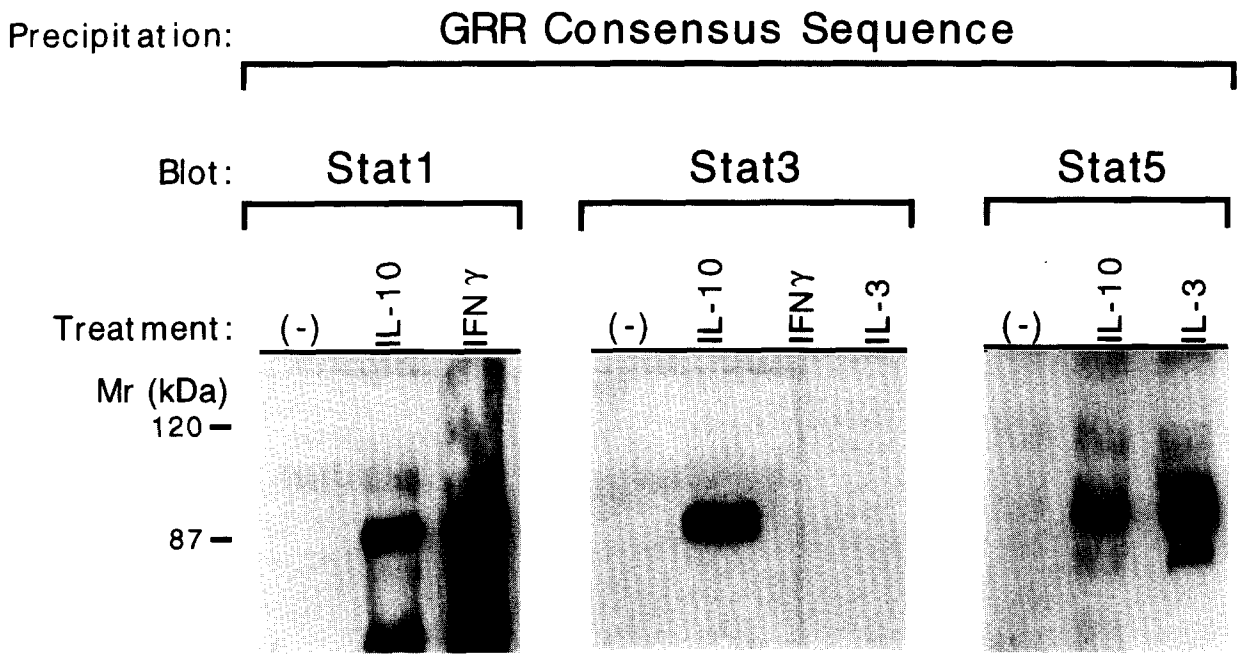

Fig. 3. IL-10 activates the nuclear translocation and DNA binding activity of Stat1, 3, and 5 to the GRR of the FcγRI gene. Nuclear extracts from IL-10, IL-3 or IFNγ treated BaMR cells were precipitated with Sepharose-coupled biotinylated GRR double stranded oligonucleotide. Proteins were separated by SDS-gel electrophoresis, Western-blotted and probed with Stat1, Stat3, and Stat5 specific antisera, respectively.

activated IL-10 receptor but further indicate the differential recruitment of these transcription factors at the sites of the specific promoters.

3.3. Stat1 and Stat5 do not form heterodimers

To further investigate the characteristics of the binding specificities and the nature of the heterodimer formation of STAT proteins, BaMR cells were treated with IL-3 and IFNγ individually or in combination or with IL-10. IL-10 activated the expected trimeric gel shift complex at the GRR site (Fig. 4, left, first lane). A single IL-10 induced gel shift band containing Stat5 was observed when nuclear extracts were probed with the PRL oligonucleotide (Fig. 4, right, first lane). Stat5 that became activated upon IL-3 treatment of the BaMR cells displayed binding affinity to the GRR as well as to the PRL oligonucleotide (Fig. 4, left and right, second lanes). Upon IFNγ treatment of the BaMR cells a gel shift complex containing Stat1 homodimers was induced as described that bound to GRR but not to the PRL sequence (Fig. 4, left and right, third lanes) [1]. To test further for heterodimer formation of Stat1 and 5, BaMR cells were simultaneously treated with IL-3 and IFNγ. When nuclear extracts of these BaMR cells were probed for DNA binding complexes by EMSA, two distinct gel shift bands to the GRR oligonucleotide were observed. In contrast, a single STAT complex was observed with the PRL sequence. Thus, the simultaneous treatment of BaMR cells with IL-3 and IFNγ led to the simultaneous activation of Stat1 and 5 that both bound in the promoter of the FcγRI gene. However, a third complex consisting of Stat1/Stat5 heterodimers was not formed independent of the ratio of activated Stat5 to Stat1 used in the assay (data not shown). Thus, these experiments indicated that simultaneous Stat1/Stat5 activation does not lead to the formation of Stat1/Stat5 heterocomplexes.

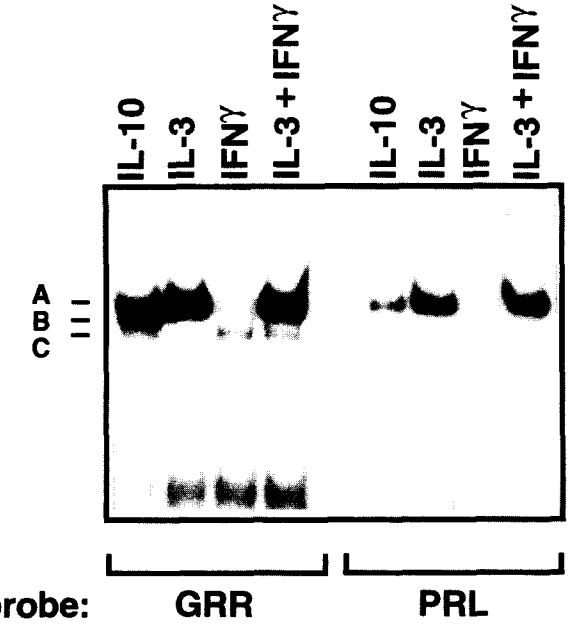

Fig. 4. Simultaneous activation of Stat1 and Stat5 does not lead to heterodimer formation. BaMR cells were treated with 100 ng/ml IL-10, 100 ng/ml IL-3, 10 ng/ml IFNγ or a combination of IL-3 and IFNγ. Nuclear extracts were probed for the presence of STAT DNA-binding activity by gel shift analyses using the GRR (left panel) or the PRL probe (right panel), respectively.

3.4. IL-10 dependent STAT protein activation leads to the recruitment of transcriptionally active Stat5 protein at the promoter of the β-casein gene

Our data have demonstrated that multiple STAT proteins (Stat1, 3, and 5) become activated by IL-10. However, of the IL-10 induced STAT proteins only Stat5 binds in the promoter of the β-casein gene. We next addressed the question of

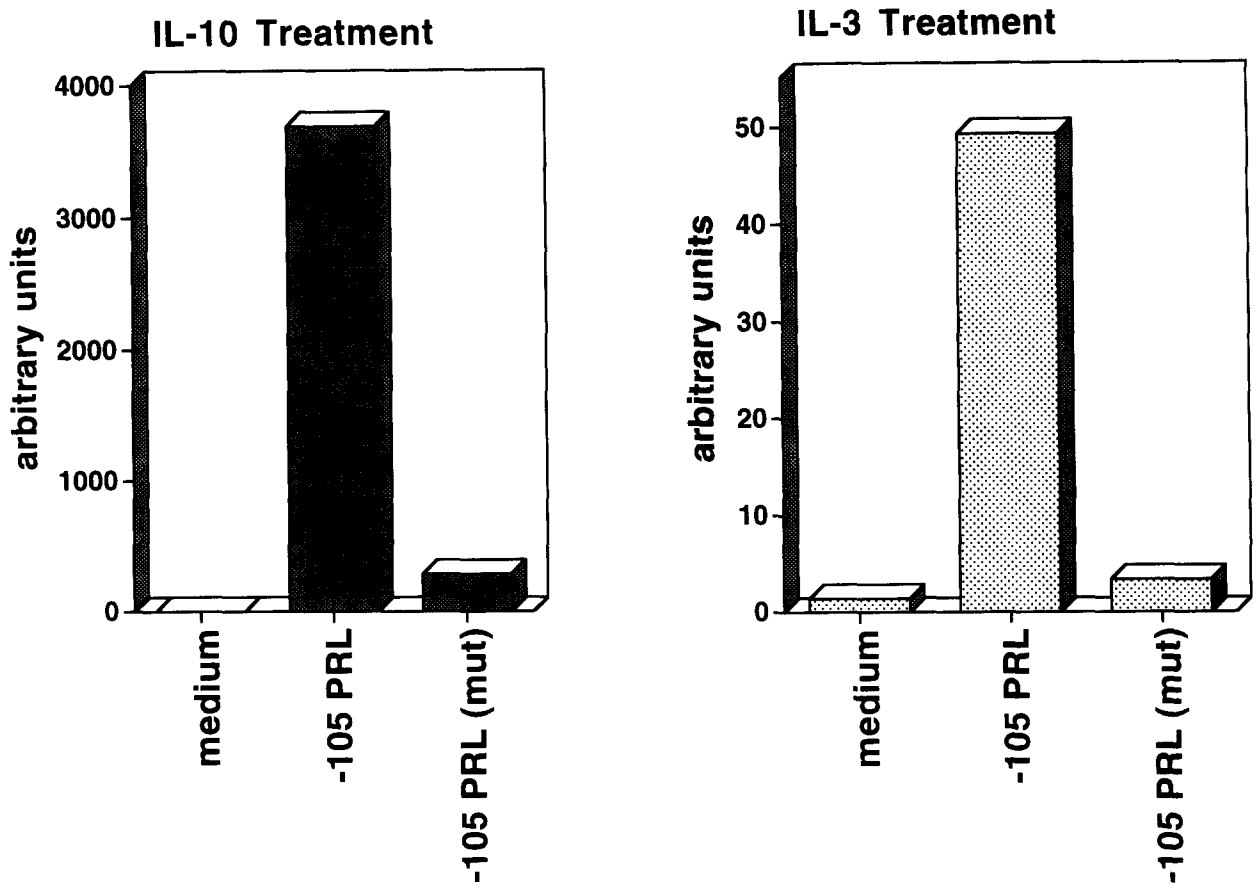

Fig. 5. IL-10 regulates the transcriptional activity of the β-casein promoter through the activation of Stat5. BaMR cells were transiently transfected with the pXM-PRL(−105) luciferase reporter construct, cells were treated with IL-10 (100 ng/ml) or IL-3 (100 ng/ml) and luciferase assays were performed after 48 h (middle bars). In control experiments transfected BaMR cells were left untreated (left bars) and basal activity of the construct was determined. To control for specificity, a reporter construct pXM-PRL(−105)mut mutated at the site of the PRL-STAT consensus element of the β-casein promoter was transiently transfected into BaMR cells and cells were treated as before (right bars). Arbitrary units are luciferase activity units normalized to CAT activity to account for differences in transfection efficiencies.

whether the IL-10 dependent recruitment of the Stat5 protein in the β-casein promoter was sufficient for transcriptional activation. Therefore, reporter constructs spanning the proximal part of the β-casein promoter 105 bp upstream of the transcriptional start site encompassing the Stat5 consensus sequence were transiently transfected into BaMR cells. Transfected cells were subsequently treated with IL-10 and IL-3, respectively, and luciferase activity was measured. These experiments revealed that the Stat5 consensus sequence in the proximal β-casein promoter was sufficient to transcriptionally activate the luciferase gene in response to IL-10 (Fig. 5). The transcriptional activation was specific for the Stat5 consensus sequence because a single base pair mutation within that sequence ablated not only IL-3 induced Stat5 activation but also the IL-10 induced transcriptional activation of the reporter gene (Fig. 5). Control experiments were performed in BaMR cells that were transfected with the luciferase construct but left untreated. In these cells no basal activity of the construct was obtained (Fig. 5). Luciferase activity was normalized to CAT activity to account for differences in transfection efficiencies of the cells. Thus, the binding of Stat5 in the promoter of the β-casein gene was sufficient to activate an IL-10 dependent transcriptional response. In summary, IL-10 activates multiple STAT proteins in IL-10 responsive cells. These activated STAT proteins bind in the promoters of distinct genes and become transcriptionally active in a combinatorial manner.

4. Discussion

4.1. Stat1, Stat3, and Stat5 activation by IL-10

While IL-10 is known to transduce its pleiotropic functions on cells of the immune system [10] by interacting with a specific IL-10 receptor that belongs to the interferon receptor family [13], little data exist that characterize IL-10's intracellular signalling on a molecular basis [18,19]. A recent report by Finbloom and Winestock [11] demonstrated the activation of Stat1 and 3 through IL-10. Here, we extend these data by showing that a third STAT protein Stat5 is activated by IL-10 in addition to Stat1 and 3. STAT proteins are known to bind in the promoters of genes by forming homo- or heterodimers [20,21]. One cytokine may thus activate one STAT protein like IFNγ, prolactin, erythropoietin (EPO), thrombopoietin (TPO), IL-4, IL-5 [19,22,23] or one cytokine may activate two STAT transcription factors like IL-3, IL-6, CNTF, OSM, LIF [24]. Here we first demonstrate that multiple STAT transcription factors become activated by a single cytokine, IL-10. Stat1, Stat3, and Stat5 simultaneously activated

by IL-10 subsequently form multiple STAT heterocomplexes (Stat1-Stat3 and Stat3-Stat5) that are detectable depending on the STAT consensus sequence used. However, the simultaneous activation of Stat1 and Stat5 upon treatment with IL-10 or upon combined treatment of BaMR cells with IFNγ and IL-3 did not lead to Stat1/Stat5 heterocomplex formation. Thus, the simultaneous tyrosine phosphorylation of different STAT proteins appears as a prerequisite but does not necessarily lead to the formation of STAT heterocomplexes. Our previous studies of the IL-10 receptor signalling pathway suggested a direct assembly of Stat3 at specific phosphotyrosine containing sequences YXXQ within the intracellular domain of the IL-10 receptor's α-chain [26]. Our current data indicate the IL-10 dependent activation of multiple STAT proteins that form hetero- or homodimers at the sites of different promoters in a combinatorial manner. Thus, two possible mechanisms of STAT dimerization might be discussed. First, upon STAT activation all possible STAT-dimer combinations could be randomly formed at the intracellular parts of the cytokine receptors, translocated to the nucleus and recruited at the different STAT consensus elements. To date, our data so far do not support this hypothesis. Second, STAT proteins could be activated at the receptor's intracellular domain and STAT dimerization could specifically be rearranged in the nucleus depending on the promoter sites. Heterodimer formation of STAT proteins might thus be influenced by the structure or the activation mode of the molecules [21]. Specific tyrosine residues of Stat1 (Y701) and Stat5 (Y694) have been identified that are necessary for transcriptional activity [2,21]. These tyrosine residues are known to be important for the formation of STAT homo- and heterodimers [1,21]. In addition, modulation of the DNA binding activity of the STAT proteins Stat1 and Stat3 is mediated through the serine/threonine phosphorylation within their carboxy-terminal part [8,9]. Thus, specificity of the STAT signaling events may subsequently be mediated through different mechanisms: (1) the potential of a cytokine's receptor intracellular domain to recruit distinct STAT proteins [20]; (2) the expression of specific STAT proteins in a cell [3]; (3) the activation of signaling cascades other than the JAK-pathway [23,25,26]; and (4) the STAT consensus sequence presented by a specific gene promoter [27]. Thus, specificity and selectivity in STAT dependent signal transduction appear to be achieved in a multifactorial and combinatorial manner.

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