FEBS Letters 345 (1994) 187–192

FEBS 14052

Human interleukin-13 activates the interleukin-4-dependent transcription factor NF-IL4 sharing a DNA binding motif with an interferon-γ-induced nuclear binding factor

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Received 4 March 1994; revised version received 19 April 1994

Abstract

The effects of interleukin-13 (IL-13) and interleukin-4 (IL-4) on cellular functions were shown to be quite similar. We provide evidence that in monocytes as well as in T lymphocytes both IL-4 and IL-13 activate the same recently identified transcription factor NF-IL4 which binds to the specific responsive element IL-4RE. In addition, we show that a nuclear factor activated by interferon-γ also interacts with the IL-4RE. It differs from NF-IL4 in the electrophoretic mobility of the complex with DNA, in its DNA-binding specificity and in the proteins interacting with the DNA sequence. Sensitivity against various enzyme inhibitors suggests that components of the signal transduction pathway are shared by all three cytokines.

Key words: Interleukin-4; Interleukin-13; Interferon-γ; Signal transduction; Nuclear binding factor; Responsive element

1. Introduction

Interleukin-4 (IL-4) is an important immunoregulatory cytokine which has pleiotropic effects on a variety of cell types [1]. It is predominantly secreted by activated Th2-'like' cells [2] and by mast cells [3] and is one of the major promoters of the 'Th2-type' immune response. IL-4 stimulates proliferation of B lymphocytes and promotes the production of certain immunoglobulin isotypes such as IgG4 and IgE in humans [1]. Another characteristic activity of IL-4 is the induction of the low affinity receptor for IgE (FcεRII/CD23) in various cell types such as B lymphocytes [4], monocytes [5] and T cells [6]. These effects are initiated by the binding of IL-4 to a specific surface receptor (IL-4R) consisting of a 140 kDa polypeptide chain [7] which can be associated with a common γ chain (γ_c) shared with other cytokine receptors such as IL-2 and IL-7 receptors [8–10]. The signalling pathway connecting the IL-4R with the activation of gene transcription has remained elusive so far. Recently, we identified an IL-4 responsive element in the promoter regions of the CD23b and Iε genes (IL-4RE) which is specifically bound by the novel IL-4-induced transcription factor NF-IL4 [11]. NF-IL4 is posttranslationally activated within a few minutes after IL-4 stimulation and its binding to IL-4RE represents the final step of the IL-4 signalling pathway. Lately, another cytokine, interleukin-13 (IL-13) has been described which has effects quite similar to IL-4 on the expression of various genes in a number of different cell types [12,13]. It has been shown to induce IgG4 and IgE synthesis by human B cells, to promote CD23 expression in B cells and monocytes and to inhibit IL-6 synthesis [12–16]. However, it differs from IL-4 by its inability to activate T lymphocytes [14,17]. Studies with a mutant form of the human IL-4 and with a transfected IL-4R ligand binding protein suggested that both the IL-4R and the IL-13R share a receptor subunit involved in signal transduction [17]. According to recent reports this additional receptor chain might be identical with the common cytokine receptor γ chain γ_c [8,9]. In this study we show that stimulation of monocytic cells and T cells with IL-13 activates a nuclear binding protein which binds to the same IL-4-responsive element IL-4RE as NF-IL4. We provide evidence, that both binding factors are identical. (1) The complexes formed by both factors have identical electrophoretic mobility; (2) complex formation by both factors is equally sensitive to competition by different binding motifs; (3) crosslinking of the IL-4RE with the binding proteins after complex formation revealed that the covalently bound proteins derived from both factors have the same relative molecular mass.

Whereas IL-4 is the principal promoter of the 'Th2-type' immune responses the 'Th1-type' response is guided by interferon-γ (IFN-γ) [2]. Depending on the cell type, the effects of IFN-γ on gene expression are either antagonistic or analogous to the activity of IL-4 [4,18,19]. Here we show that IFN-γ activates a nuclear factor which binds to the IL-4RE. However, the mobility of the resulting complex in EMSA is different from that of the NF-IL4/IL-4RE complex. In addition, we provide

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SSDI 0014-5793(94)00438-2

evidence for similarities between the signal transduction activated by IL-4 or IL-13 and the signalling pathway initiated by IFN-γ.

2. Materials and methods

2.1. Reagents

Human recombinant IL-4 (rIL-4) was prepared from the supernatant of CHO cells which had been stably transfected with the IL-4 gene in collaboration with Boehringer-Mannheim (Penzberg, Germany). Recombinant human IFN-γ (rIFN-γ) was obtained from Biogen (Cambridge, MA, USA). Recombinant human IL-13 (rIL-13) was prepared as described [12]. The following enzyme inhibitors were used: staurosporine as a broad spectrum kinase inhibitor, genistein as a specific inhibitor of tyrosine kinase, calyculin A as inhibitor of serine/threonine phosphatases, and H7 as inhibitor of protein kinase C. All inhibitors were purchased from Calbiochem (Bad Soden, Germany). The monoclonal anti-phosphotyrosine antibody IG2 was obtained from Amersham (Braunschweig, Germany).

2.2. Cells

Mononuclear blood cells (PBMC) were prepared by Ficoll/Hypaque density centrifugation of heparinized venous blood samples. T lymphocytes were isolated from PBMC at >99% purity by a direct monoclonal antibody rosetting technique using bovine erythrocytes coated with a monoclonal CD2 antibody essentially as described [20]. Monocytes were enriched by removal of T and B lymphocytes from PBMC using the same technique. The monocytic cell line U937 was obtained from the American Type Tissue Collection. Cells were cultured in RPMI 1640 medium containing 5% fetal calf serum, 1 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Electrophoretic mobility shift assay (EMSA)

For complex formation with nuclear binding factors the following IL-4RE containing DNA sequences were synthesized, the IL-4REs are underlined: (a) from the CD23b promoter [11,21] 5'-GGTGAATTTCTAAGAAAGG-3'; and (b) the interferon-γ responsive region (GRR) from the promoter of the Fcγ receptor I (FcγRI, CD64) [22] 5'-AGCATGTTTCAAGGATTTGAGATGTATTTCCCAGAAAA-G-3'. In addition, an oligonucleotide containing the IFN-γ activation site (GAS) as a binding motif for the IFN-γ-induced activation factor (GAF) was prepared for use in control experiments: 5'-ATTACTCTAAA-3' (sequence taken from [23]). The oligonucleotides were end-labeled using the Klenow polymerase (Boehringer Mannheim, Germany) together with [α-^{32}P]dATP (Amersham, 3000 Ci/mmol) and the three other dNTPs in unlabeled form. Nuclear extracts were prepared according to Dignam et al. [24]. The nuclear extracts (3–5 μg) were incubated on ice for 30 min with the ^{32}P-labeled oligonucleotide in 20 μl of binding buffer consisting of 2 mM HEPES (pH 7.9), 100 mM KCl, 10 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 1.5 mM EDTA, 15% glycerol, 1 mg/ml BSA, 15 μg/ml aprotinin, 0.5 mM PMSF and 2 μg of the carrier polymer poly(dI-dC) (Pharmacia, Freiburg, Germany). The DNA–protein complexes were separated in a 4% polyacrylamide gel in 0.5 × TBE which was then exposed to an X-ray film (Kodak XOMAT AR). Competition of complex formation was performed with the IL-4RE-containing sequences from the CD23b- and CD64-promoter (see above). In addition, the following two mutants M-1 and M-2 of the CD23b-IL-4RE were used, in which single base pair exchanges were introduced known to be essential for NF-IL4-binding (the mutated positions are underlined): M-1, 5'-GGTGAATTTCTAAAAAGG-3'; M-2, 5'-GGTGAATTTCTAGGAAAGG-3'. The nuclear extracts were incubated in a DNA binding mix containing a 20-fold molar excess of unlabeled double stranded competitor oligonucleotides for 15 min on ice. After addition of the ^{32}P-labeled IL-4RE-containing sequence of the CD64 promoter the incubation was continued for 30 min. The complexes were then separated as described above.

2.4. UV cross-linking

For cross-linking experiments the following double stranded oligonucleotide was generated by annealing two BrdU-containing (B = BrdU) oligonucleotides (Eurogentec, Seraing, Belgium) and labeling by incorporation of [α-^{32}P]dATP using Klenow polymerase (see section 2.3):

5'-A G C B B B C B B A G A A A T T C A A G C T-3'

3'-T C G A A A G A A B C B B B A A G B T C G A-5'.

Nuclear extracts (10 μg of protein) were incubated with 50 fmol of the BrdU-substituted oligonucleotide for 20 min at 20°C, then irradiated on ice with UV light (305 nm) at a distance of 5 cm for 20 min. The reaction mixture was separated on a 4% polyacrylamide gel. The gel was then irradiated by UV light (305 nm) at a distance of 5 cm for 20 min on ice before exposing to an X-ray film for 3 h. The detected radioactive bands were excised as indicated in Fig. 4, electroeluted and separated on a denaturing 10% SDS polyacrylamide gel which was then exposed to an X-ray film.

3. Results and discussion

3.1. Both IL-13 and IL-4 activate the transcription factor NF-IL4

IL-4 and IL-13 have similar biological effects on various cell types and use cellular receptors which supposedly share a receptor subunit. These findings strongly suggest that IL-4 and IL-13 initiate the same signalling cascade resulting in the activation of the IL-4-dependent transcription factor NF-IL4. In order to prove this hypothesis nuclear extracts were prepared from U937 cells stimulated either with 100 ng/ml of rIL-13 or 400 U/ml of rIL-4 for 15 min and then incubated with the radiolabeled IL-4RE from the CD23b promoter. Both types of nuclear extracts formed a complex with the same mobility in EMSA (Fig. 1a, lanes 2 and 3). Recent reports indicated that IL-4 and IL-13 differ in their effects on a

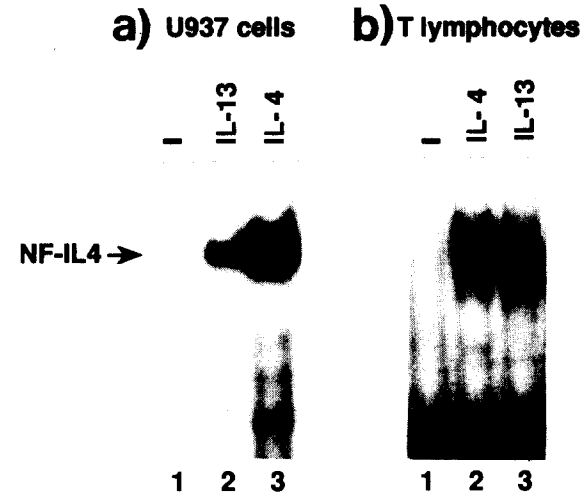

Fig. 1. Induction of a nuclear binding factor by IL-13 forming a complex with IL-4RE and displaying the same electrophoretic mobility as the NF-IL4/IL-4RE complex. (a) induction in U937 cells; (b) induction in purified blood T lymphocytes. 2 × 10^7 cells were incubated for 15 min without or with either 100 ng/ml rIL-13 or 400 U/ml rIL-4. Nuclear extracts were then incubated with the ^{32}P-labeled IL-4RE from the CD23b promoter and analysed in EMSA. The arrow indicates the band representing the NF-IL4 complex.

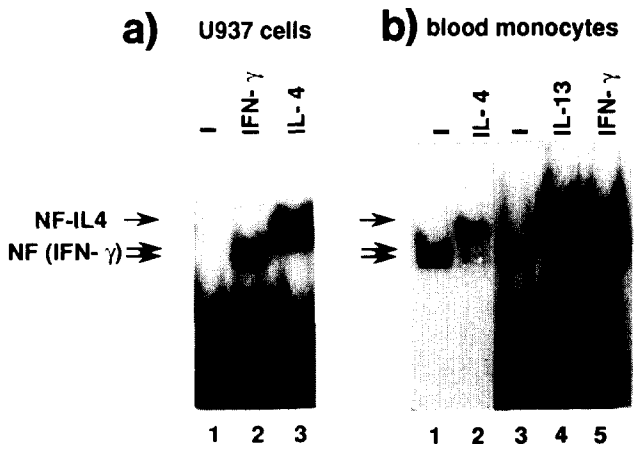

Fig. 2. (a) Induction of a nuclear binding factor by IFN-γ in U937 cells which forms a complex with IL-4RE. 2×10^7 U937 cells were incubated for 15 min without or with either 1000 U/ml rIFN-γ or 400 U/ml rIL-4. Nuclear extracts were incubated with the ^{32}P-labeled IL-4RE from the CD23b promoter and analysed in EMSA. (b) Presence of a nuclear factor binding to IL-4RE in freshly isolated blood monocytes. Nuclear extracts were prepared from enriched blood monocytes after incubation for 15 min with or without 400 U/ml rIL-4 or 100 ng/ml rIL-13 or 1000 U/ml rIFN-γ, incubated with the ^{32}P-labeled IL-4RE-containing sequence from the CD64 promoter and analysed in EMSA. The single arrow indicates the band representing the complex formed with NF-IL4, the double arrow shows the position of the IFN-γ-dependent complex.

number of T cell functions [14,17]. In contrast to IL-4, IL13 is not capable to promote growth of T cell clones or to induce expression of CD8α on CD4$^+$ T cell clones. These findings were taken as an indication for the lack of functional IL-13 receptors on T lymphocytes [17]. Hence, it was of particular interest to find out whether these cytokines differ also in their capability to activate NF-IL4 in T lymphocytes. To this end, highly purified blood T cells were treated with rIL-4 and rIL-13 for 15 min followed by analysis of the nuclear extracts in EMSA. As shown in Fig. 1b, lanes 2 and 3, both cytokines were able to activate NF-IL4 in T cells. Therefore, the diverse functional effects of IL-4 and IL-13 on T cells cannot be explained by a different capacity to induce the transcription factor NF-IL4 in T cells. Above all, our finding is at variance with the notion that T cells do not express functional IL-13 receptors. It is still possible that the induction of T cell functions by IL-4 require yet unknown cooperative factors which IL-13 is unable to activate in T cells.

3.2. *The IL-4- and IL-13-induced NF-IL4 shares a DNA binding motif with an IFN-γ activated nuclear binding factor*

IFN-γ has been shown to activate a transcription factor containing the p91 protein [25], which binds to the IFN-γ response region (GRR) in the promoter of the FcγRI (CD64) gene [26]. This region contains at the 3′ end a sequence which is homologous to the IL-4RE and which is recognized by NF-IL4 (unpublished observation). During our analysis of gene activation by Th1- and Th2-derived cytokines we realized that IFN-γ induces a nuclear binding factor in U937 cells which forms a complex with the IL-4RE derived from the CD23b promoter as well as with the IL-4RE from the CD64 promoter. In EMSA, this complex has a somewhat faster mobility when compared with the NF-IL4/IL-4RE complex (Fig. 2a, lanes 2 and 3). In the course of preparing this manuscript we became aware of a paper reporting similar results for THP-1 cells [27]. When we analysed freshly prepared blood monocytes which had not been intentionally treated with cytokines we frequently observed a nuclear factor forming a complex with the IL-4RE of the CD23b promoter. In EMSA this complex migrated with a mobility identical to that obtained for the complex after IFN-γ treatment (Fig. 2b, lanes 1, 3 and 5). When such cells were treated for 15 min with rIL-4 the lower band corresponding to the IFN-γ-induced binding factor disappeared in EMSA and predominantly NF-IL4 was extracted from the nuclei (Fig. 2b, lane 2). The same effect was seen when fresh monocytes were treated with rIL-13 (Fig. 2b, lane 4). Incubation of

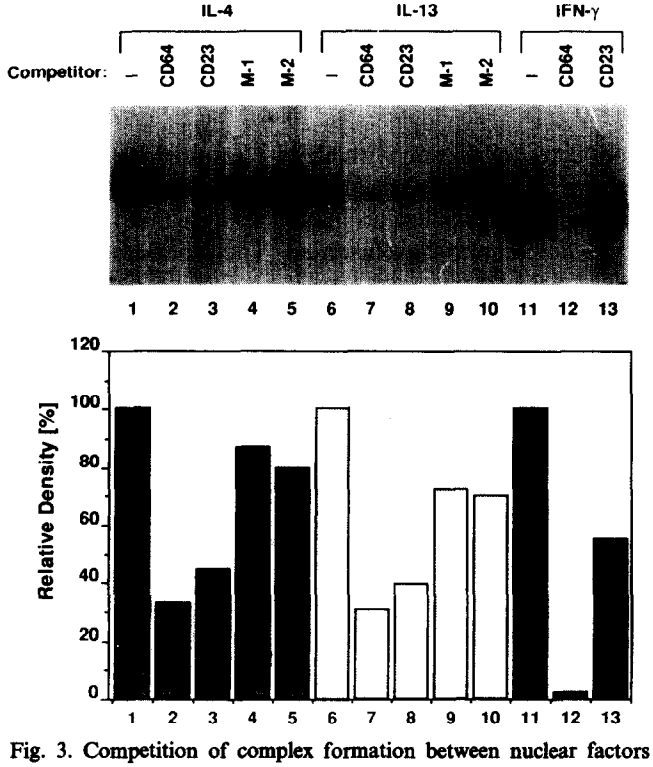

Fig. 3. Competition of complex formation between nuclear factors induced by IL-4, IL-13 or IFN-γ and IL-4RE analysed by EMSA. Nuclear extracts from U937 cells stimulated by the various cytokines were prepared as described in the legends to Figs. 1 and 2 and incubated with a 20-fold molar excess of different competitor DNAs as indicated on top of the gel before adding the ^{32}P-labeled IL-4RE of the CD64 promoter. Following EMSA the exposed X-ray film was scanned on a densitometer. The relative intensity of the bands corresponding to specific complexes is shown as a diagramm. For each stimulation (lanes 1, 6 and 11) the lanes without competitor were set as 100%.

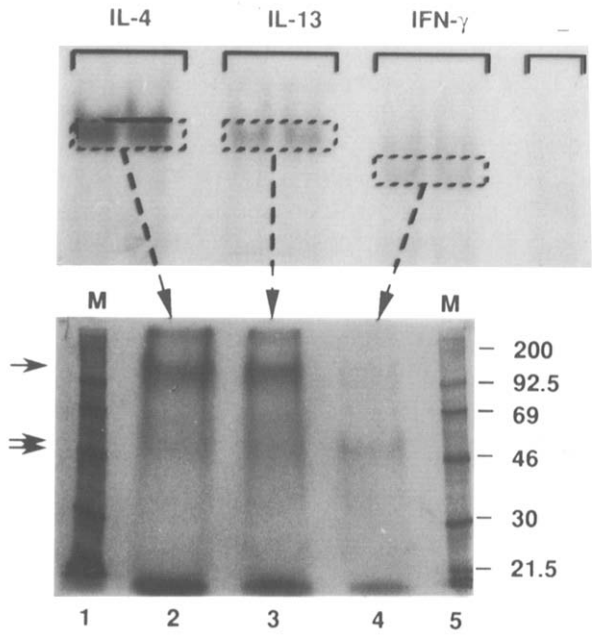

Fig. 4. UV-cross-linking of a BrdU-substituted oligonucleotide carrying the IL-4RE of the CD23b promoter with different nuclear extracts. The extracts of U937 cells stimulated without (−) or with IL-4, IL-13 or IFN-γ were crosslinked and separated on a 4% EMSA-γel (upper gel). The specific complexes were excized, eluted and separated on a 10% SDS-polyacrylamide gel (lanes 2–4), which was then exposed to an X-ray film (lower gel). M (lanes 1 and 5) indicates a ^{14}C-labeled molecular weight standard. The single and double arrows mark the separated proteins covalently bound to the labeled DNA.

monocytes for 15 min with rIFN-γ resulted in an amplification of the lower band. The reason for this constitutive factor expression in freshly isolated blood monocytes remains to be elucidated. These findings suggest that under certain experimental conditions, particularly in the absence of a continuous IFN-γ stimulus, the IFN-γ-induced binding factor can be displaced from its DNA motif by newly induced NF-IL4. Taken together, these data indicate that different nuclear binding factors activated by diverse cytokines can share a DNA binding motif and may thus compete with each other for DNA sequences or for DNA-binding protein components. This phenomenon may contribute to the antagonistic regulation of gene activation by certain cytokines. However, this hypothesis has to be proven by analyzing binding of purified nuclear factors.

3.3. *Comparative analysis of the nuclear binding factors*

The finding that IL-4 and IL-13 induce nuclear factors forming complexes with IL-4RE of corresponding electrophoretic mobility suggests that the factors are identical. To provide additional evidence for that a series of comparative analyses was performed. These experiments included a more detailed characterization of the IFN-γ-induced IL-4RE-binding nuclear factor. First, the binding specificity of the factors was tested by competing the complex formation with the radiolabeled IL-4RE from the CD64 promoter by various informative oligonucleotides. As shown in Fig. 3, the two CD23b and CD64 promoter motifs were similarly efficient in competing the binding of the radiolabeled CD64-IL-4RE to both the IL-4- and IL-13-induced factors. This indicates that the factors bind to the two motifs with comparable affinity. In contrast, the IFN-γ-induced factor appeared to have a much stronger affinity to the CD64-IL-4RE than to the CD23b-IL-4RE. Furthermore, complex formation of the IL-4- and IL-13-induced factors was equally resistant to competition by the two oligonucleotides M-1 and M-2 carrying point mutations within the IL-4RE. In a second set of experiments it was found that the DNA-binding proteins derived from both the IL-4- and IL-13-induced nuclear factors have the same size in a denaturing SDS gel after cross-linking to the IL-4RE (Fig. 4, lanes 2 and 3). One major band appeared between 110 and 130 kDa (single arrow in Fig. 4). An additional smaller band of about 50 kDa was detectable in both lanes (double arrow in Fig. 4). When the IFN-γ-induced nuclear factor was analysed by the same method only this band of about 50 kDa was found. IFN-α has been shown to induce a nuclear factor binding to the interferon stimulated response element (ISRE). This factor is composed of at least four proteins p113, p91, p84 and p48 with p48 as the DNA-binding component [28]. On the other hand, IFN-γ activates a nuclear factor binding to the γ-interferon activation site (GAS) where the p91 was shown to be the DNA-binding constituent. However, the IFN-γ-activated protein directly interacting with the IL-4RE has not been identified so far. Ac-

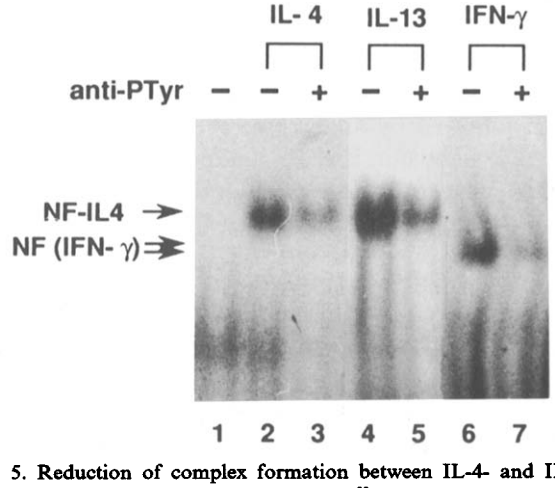

Fig. 5. Reduction of complex formation between IL-4- and IL-13-induced nuclear binding factors and the ^{32}P-labeled IL-4RE of the CD23b promoter by a monoclonal anti-phosphotyrosine antibody (anti-PTyr). As a control, reduction of complex formation between an IFN-γ-induced nuclear binding factor and the ^{32}P-labeled GAS sequence by the anti-phosphotyrosine antibody is shown. 1.8 μg of the anti-PTyr were added to 40 μl of nuclear extract solution in binding buffer for 60 min. Then the ^{32}P-labelled oligonucleotide was added for a further 30 min followed by EMSA. The single arrow indicates the band representing the complex formed with NF-IL4, the double arrow shows the position of the IFN-γ-dependent complex.

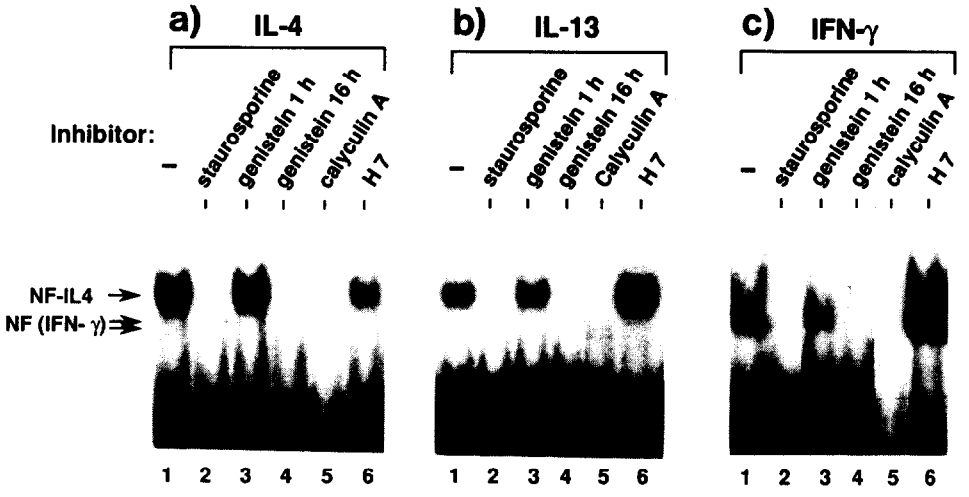

Fig. 6. Effect of various enzyme inhibitors on the activation of nuclear binding factors by (a) IL-4; (b) IL-13 and (c) IFN-γ. Cells were preincubated with the enzyme inhibitors as described in section 3 and then stimulated for 15 min with the cytokines. Nuclear extracts were incubated with the ^{32}P-labeled IL-4RE of the CD23b promoter (a) and (b) or with the radiolabeled GRR sequence (c) and then analysed in EMSA. The single arrow indicates the band representing the complex formed with NF-IL4, the double arrow shows the position of the IFN-γ-dependent complex.

cording to our data it could be the p48. This view is supported by the finding of a deficient IFN-γ-signalling in a cell line expressing a truncated p48 [29]. This p48 seems to be a constitutive cellular protein which is recruited to DNA-binding complexes after IFN stimulation. Based on our observation that the IL-4- and IL-13-activated nuclear factors contain a DNA-binding component of a corresponding size one might speculate that p48 is also involved in the binding of NF-IL4 to IL-4RE. The major DNA-binding element of NF-IL4, however, has a size between 110 and 130 kDa. It is not yet clear whether it corresponds to the p113, another component of the IFN-activated nuclear factor family. Since the IFN-γ-induced transcription factor is activated by a phosphorylation at Tyr^{701} of the p91 component [28] it was of interest to determine whether also the IL4- and IL-13- activated nuclear binding factors are phosphorylated on tyrosine. For this, in a third set of experiments the nuclear extracts obtained from IL-4- or IL-13-treated cells were incubated with a monoclonal anti-phosphotyrosine antibody before adding the ^{32}P-labelled IL-4RE motif for another 30 min. By this treatment, the complex formation by both factors was significantly reduced when compared with the untreated control (Fig. 5, lanes 2–5). The efficiency of the antibody was controlled by its effect on the complex formation between the GAS sequence and the IFN-γ-induced nuclear factor GAF which has been reported to be tyrosine phosphorylated [28]. The finding that the complex formation was inhibited by the anti-phosphotyrosine antibody and did not result in a supershift indicates that the phosphorylated tyrosine is related to the DNA binding site of NF-IL4. Taken together, these data provide strong evidence for the identity of the nuclear binding factors activated by IL-4 and IL-13.

3.4. *Similar enzymes are involved in signalling by IL-4, IL-13 and IFN-γ*

Little is known about the signal transduction pathway after binding of IL-4 or IL-13 to their membrane receptors. We had previously shown that classical second messengers such as inositol phospholipid metabolites, elevated cytoplasmic Ca^{2+}, protein kinase C or cAMP are not involved in the activation of the IL-4-dependent transcription factor NF-IL4 [11]. In order to test the contribution of kinases and phosphatases to the signalling cascade resulting in the activation of NF-IL4 U937 cells were treated with different enzyme inhibitors prior to stimulation with IL-4 or IL-13. Incubation of cells with the broadly reacting kinase inhibitor staurosporine at a concentration of 1 μM for 1 h blocked both the IL-4- and IL-13-dependent NF-IL4 activation (Fig. 6a, lane 2, and Fig. 6b, lane 2). Genistein, a specific inhibitor of tyrosine kinases, had little effect when applied at 100 μg/ml for 1 h, however, treatment of cells for 16 h with the same concentration of genistein resulted in complete inhibition of complex formation (lanes 3 and 4 in Fig. 6a and 6b). When calyculin A, a specific inhibitor of serine/threonine phosphatases, was applied to the cells for 1 h at a concentration of 10 nM, activation of NF-IL4 by the two cytokines was prevented (Fig. 6a, lane 5 and Fig. 6b, lane 5), whereas the inhibitor of protein kinase C, H7, at 3 μM for 1 h was unable to interfere with NF-IL4-induction (Fig. 6a, lane 6 and Fig. 6b, lane 6). The finding that activation of NF-IL4 is impeded by an inhibitor of tyrosine kinases is consistent with previous reports showing that a tyrosine kinase is involved in the IL-4 signal transduction pathway [27,30]. The involvement of serine/threonine phosphatases in the IL-4/IL-13 signalling is a new observation. The identity of inhibition patterns is in favour of a common pathway

for both IL-4- and IL-13-signalling. This view is supported by the notion that both the receptors for IL-4 and IL-13 share an affinity converting subunit that is critical for cellular signal transduction [17]. In a parallel set of experiments the IFN-γ signalling pathway which results in the activation of a nuclear factor binding to IL-4RE was analysed with regard to blockade by enzyme inhibitors. As shown in Fig. 6c, activation of this nuclear factor was sensitive to the same enzyme inhibitors as the induction of NF-IL4. This similarity of inhibition patterns suggests that the IL-4/IL-13-induced signalling belongs to a category of signal transduction pathways which has been described for the interferon-induced activation of transcription factors and for which the 'direct effector' model has been proposed [31].

Acknowledgements: We thank E. Kopp for skillful technical help. This work was supported by the Deutsche Forschungsgemeinschaft, SFB 217.

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