

Interleukin-6-induced serine phosphorylation of transcription factor APRF: evidence for a role in interleukin-6 target gene induction

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Abstract The cytokine interleukin-6 (IL-6) rapidly activates a latent cytoplasmic transcription factor, acute-phase response factor (APRF), by tyrosine phosphorylation. Activation and DNA binding of APRF are inhibited by inhibitors of protein tyrosine kinases but not serine/threonine kinases. However, immediate-early gene induction by IL-6 and, as we show here, stimulation of the promoters of the genes for α_2 -macroglobulin, Jun-B, and intercellular adhesion molecule-1 (ICAM-1) are blocked by the serine/threonine kinase inhibitor H7. We now show that IL-6 triggers a delayed phosphorylation of APRF at serine residues which can be reversed in vitro by protein phosphatase 2A and is also inhibited by H7. Therefore, APRF serine phosphorylation is likely to represent a crucial event in IL-6 signal transduction leading to target gene induction.

Key words: Interleukin-6; Signal transduction; Transcription factor; Acute-phase response factor; Stat; Phosphorylation

1. Introduction

IL-6 is a pleiotropic cytokine controlling growth, differentiation, and other cellular functions of a variety of cell types [1]. As a major mediator of the acute-phase response in the liver, IL-6 induces the expression of many acute-phase plasma protein genes in hepatocytes [2,3]. Furthermore, the actions of IL-6 include the rapid induction of several immediate-early response genes, e.g. *jun-B*, interferon regulatory factor 1 (IRF-1), intercellular adhesion molecule 1 (ICAM-1), and TIS11, in target cells [4–6].

Major components of the IL-6 signal transduction pathway to the nucleus have recently been identified. IL-6 binds to a plasma membrane receptor composed of a ligand-binding subunit, gp80, and a signal transducer, gp130 [7,8]. The latter component is also part of the receptors for the cytokines interleukin-11 (IL-11), leukemia inhibitory factor (LIF), oncostatin M, and ciliary neurotrophic factor (CNTF) [9–11]. Neither

gp80 nor gp130 contain cytoplasmic protein tyrosine kinase domains, but recent data from our laboratory as well as from others demonstrate that gp130 is constitutively associated with members of the Jak family of protein tyrosine kinases [12,13]. After binding of IL-6 to gp80, three members of this family, i.e. Jak 1, Jak 2, and Tyk 2, are rapidly tyrosine phosphorylated and activated [12,13].

We have previously identified a ubiquitous transcription factor, APRF, which, in response to IL-6, transiently associates with gp130 and then is tyrosine phosphorylated and translocated to the nucleus [12,14]. On the basis of immunological cross-reactivity and DNA-binding specificities we proposed a relationship of APRF to the interferon- γ -activated transcription factor Stat 1 [15]. In fact, recent cloning of murine and human APRF cDNA clones revealed an about 50% overall homology of APRF with Stat 1 [16,17]. Thus, APRF, now also called Stat 3, belongs to a growing family (the Stat family) of signal transducing and transactivating proteins which all are recruited by tyrosine phosphorylation from latent cytoplasmic forms in response to cytokines, hormones, and growth factors [16,18–21]. In the nucleus, APRF binds to IL-6 response elements (IL-6REs) in the flanking regions of many acute-phase protein genes [14]. Furthermore, we could demonstrate its binding to IL-6REs in the promoters of the immediate-early genes IRF-1, ICAM-1, and *junB* [22,23]. Therefore, APRF is likely to represent an important regulator of IL-6 target gene expression.

The induction of the *Jun-B* and IRF-1 immediate-early genes by IL-6 was shown to be blocked by H7, and inhibitor of various protein serine/threonine kinases [5,6]. APRF tyrosine phosphorylation, nuclear translocation, and DNA binding, however, are not sensitive to H7 [15]. Therefore, an as yet unknown H7-sensitive event must be involved in the induction of at least some IL-6 target genes.

We now demonstrate that, subsequent to the rapid phosphorylation of APRF at tyrosine, IL-6 also induces the serine phosphorylation of the factor, albeit with a delayed time-course. This latter modification is inhibited by H7 and is reversed upon incubation with protein phosphatase 2A. These data provide evidence that APRF activity is regulated at two levels: tyrosine phosphorylation triggers the translocation of APRF to the nucleus and its binding to DNA while serine phosphorylation may be involved in the regulation of other functions of the factor, e.g. its transactivating potential, and therefore represent an important step in IL-6 signal transduction to the nucleus.

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Abbreviations: APRF, acute-phase response factor; CAT, chloramphenicol acetyltransferase; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; ICAM-1, intercellular adhesion molecule 1; IL-6, interleukin-6; IL-6RE, IL-6 response element.

2. Materials and methods

2.1. Materials

Recombinant human IL-6 was a generous gift from Drs. T. Kishimoto and T. Taga, Osaka, Japan. Amounts of IL-6 used to stimulate cells are given in B-cell stimulatory factor 2 units. The protein kinase inhibitors H7 and genistein were purchased from Sigma and Gibco-BRL, respectively. Purified protein phosphatases 1 and 2A were generously provided by Dr. Goris, Leuven; and expression vectors for the dominant negative Ras-mutant ASN-17 and the dominant-negative mutant of Raf kinase, N4Raf, by Dr. J. Bos, Utrecht. A vector containing 1014 bp of the 5' flanking region of the human ICAM-1 gene fused to the luciferase gene was a gift from Dr. Johnson, Munich, Germany. Construction of the vectors used for transient transfection has been reported previously. The following plasmids were used: p α_2 M-215CAT contains 215 bp of the rat α_2 -macroglobulin promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) gene [22]; pAPREtkCAT contains the IL-6RE (APRE) of the rat α_2 -macroglobulin promoter fused to the Herpes virus thymidine kinase (tk) promoter and the CAT gene [22]; pIC-1014 consists of 1014 bp of the human ICAM-1 promoter fused to the fire-fly luciferase gene [24]; 2 $\times$ pIREtkluc contains two copies of the IL-6RE (pIRE) of the ICAM-1 promoter in front of the tk promoter and the luciferase gene [24]; pJB1 contains 848 bp of the murine Jun-B promoter fused to CAT [23]; and pJI-tkCAT the IL-6RE of the Jun-B promoter fused to the tk promoter and the CAT gene [23]. The polyclonal antiserum raised against the NH₂-terminal 60 amino acids of Stat 1 has been described [25].

2.2. Transient transfections

Cultivation and transient transfection of human hepatoma HepG2 cells were as previously described [22]. Briefly, HepG2 cells were transfected using calcium phosphate precipitates [26] containing 6 μ g of reporter plasmids and 4 μ g of the expression vector for β -galactosidase pSV-LacZ [23] as an internal control for transfection efficiency, were stimulated without or with IL-6 one day after transfection for an additional 4 h, and harvested thereafter. Protein extracts prepared by freeze-thawing [27] were assayed for β -galactosidase [28], CAT [29], and luciferase activities [30] as described. Units of CAT or luciferase activities were normalized to β -galactosidase activity. All transfections were carried out three times in independent experiments.

2.3. Gel retardation analysis

Nuclear proteins of HepG2 cells were extracted as previously described [14]. A double-stranded synthetic oligonucleotide comprising the IL-6RE of the ICAM-1 promoter (upper strand: 5'-AGCTTAG-GTTTCGGGAAAGCAC-3') was ³²P-labelled by filling in the 5'-protruding ends with Klenow enzyme. Gel retardation analysis using a native 4% polyacrylamide gel was performed as previously described [14].

2.4. Immunoprecipitations and immunoblot analysis

HepG2 cells (10⁷) were rinsed with cold phosphate-buffered saline (PBS) and lysed in 0.5 ml lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM sodium orthovanadate, 1 mM NaF, 0.75 mM phenylmethylsulfonyl fluoride, 15% glycerol, and 10 μ g/ml each of aprotinin, pepstatin, and leupeptin) for 20 min at 0°C. Proteins in the lysates were immunoprecipitated with anti-Stat 1-NH₂ (4 μ l). Immune complexes were separated by SDS-PAGE on a 7% gel, transferred to polyvinylidene difluoride membrane (Qiabran; Diagen), and probed with monoclonal antibodies to phosphotyrosine (PY20; ICN). The bands were visualized by enhanced chemiluminescence (ECL; Amersham).

3. Results

3.1. IL-6-stimulation of the α_2 -macroglobulin, Jun-B, and ICAM-1 promoters is inhibited by H7

The induction of the immediate-early response genes *jun-B*, *IRF-1*, and *TIS11* in B-cell hybridoma and myeloid leukemia cells is blocked by both protein tyrosine kinase inhibitors and the serine/threonine kinase inhibitor H7 [5,6]. To determine

whether this observation reflects a general feature of IL-6 signalling we analyzed the effects of protein kinase inhibitors on the stimulation of IL-6-responsive promoters in the human hepatoma cell line, HepG2. We used the promoters of three IL-6 target genes, the α_2 -macroglobulin gene, a major acute-phase protein gene in the rat, and the murine Jun-B and human ICAM-1 genes. HepG2 cells were transiently transfected with constructs containing 215 bp of the α_2 -macroglobulin promoter or 846 bp of the Jun-B promoter fused to the bacterial CAT reporter gene, or 1000 bp of the human ICAM-1 promoter fused to the fire-fly luciferase gene. Transfected cells preincubated either without or with H7 were then stimulated with IL-6 for 4 h. Measurement in cell homogenates of CAT and luciferase enzyme activities showed strong inhibitory effects of H7 on the IL-6 stimulation of all three promoters (Fig. 1A).

3.2. H7 inhibits the transactivation from IL-6REs

The IL-6REs of the α_2 -macroglobulin, Jun-B, and ICAM-1 promoters have all been shown to contain APRF binding sites and to confer IL-6 responsiveness to heterologous promoters [22,23]. In order to analyze whether the H7 sensitivity of target gene induction reflects the action of IL-6 onto these IL-6REs or involves other *cis*-acting elements present in the promoters we next transfected constructs containing either one or two copies of the three IL-6REs fused to the Herpes virus thymidine kinase (tk) promoter and the luciferase gene. Again, the stimulatory effect of IL-6 was found to be inhibited by H7 (Fig. 1B) while the activity of the tk promoter alone was not affected by the inhibitors (data not shown). We conclude that the action of an H7-sensitive protein kinase is required for the induction of acute-phase protein and immediate-early genes in hepatoma cells and that this effect is mediated through the IL-6REs present in their promoters.

3.3. α_2 -Macroglobulin and ICAM-1 promoter stimulation by IL-6 does not require activation of the Ras pathway

In several cell lines, an activation of p21^{ras} and the MAP kinase pathway by IL-6 has been observed [31,32]. NF-IL6, a member of the C/EBP family of transcription factors, is likely to be phosphorylated by MAP kinase in response to IL-6 [33]. Therefore, an H7-sensitive protein kinase involved in IL-6 target gene induction may be activated via this pathway. In fact, we observed a significant activation of p21^{ras} by IL-6 in HepG2 cells as well [23]. To study whether the Ras pathway is involved in the early induction of IL-6 target genes we analyzed the effect of dominant negative mutants of c-Ha-Ras (ASN-17) and the Raf-1 kinase (N4Raf) on the stimulation of the ICAM-1, Jun-B, and α_2 -macroglobulin promoters by IL-6. Expression vectors coding for ASN-17 or N4Raf were co-transfected with ICAM-1, Jun-B, and α_2 -macroglobulin promoter constructs into HepG2 cells. As shown in Fig. 1C, expression of the mutant proteins did not block the IL-6 effect on these promoters while under the same conditions both mutants inhibited serum induction of the serum response element (SRE)-containing CAT-reporter construct [23]. Similarly, expression of ASN-17 showed no effect on the IL-6 stimulation of a construct containing two copies of the ICAM-1 IL-6RE fused to the tk promoter (Fig. 1C). We conclude that the activation of p21^{ras} and Raf-1 kinase is not required for the induction of the ICAM-1 and α_2 -macroglobulin genes by IL-6.

3.4. IL-6-induced APRF binding to the ICAM-1 IL-6RE is not inhibited by H7

In contrast to gene induction, the IL-6-induced tyrosine phosphorylation and nuclear translocation of APRF are not

inhibited by H7 [15]. Furthermore, we have previously demonstrated that H7 pretreatment does not prevent APRF from binding to the α_2 -macroglobulin and Jun-B IL-6REs [15,23]. As shown in Fig. 2, the same holds true for binding of APRF to

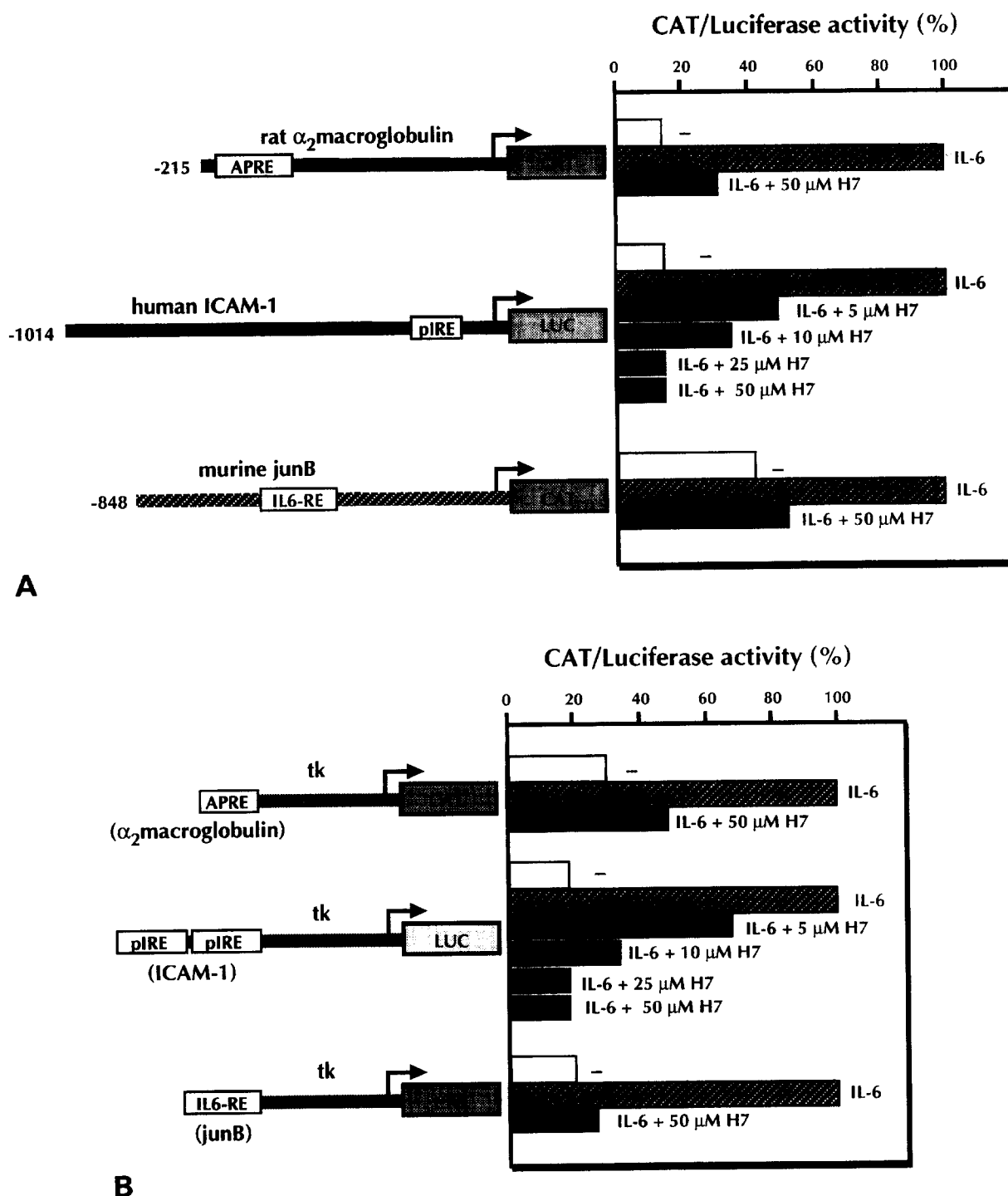


Fig. 1. Activation of IL-6 target gene promoters is inhibited by H7. HepG2 cells were transfected by the calcium-phosphate co-precipitation method with 6 μ g of the indicated constructs and 4 μ g of the β -galactosidase expression vector pSV-LacZ (A, B), or with 6 μ g of the receptor constructs, 2 μ g pSV-LacZ, and 4 μ g of either N-Raf, Ras(ASN-17), or pUC18 (C). One day after transfection, the cells were stimulated with human recombinant IL-6 (100 units/ml) with or without preincubation (20 min) with the indicated amounts of H7. After 4 h, the cells were harvested and CAT, luciferase, and β -galactosidase activities were measured in the homogenates. CAT and luciferase activities were normalized to β -galactosidase activities as an internal standard for transfection efficiency. Values are given in percent of the relative activities measured in IL-6-treated cells in the absence of the inhibitor H7. All transfections were carried out in three independent experiments, standard errors never exceeded 10% of the given values. For details about the transfected constructs refer to section 2. APRE, acute-phase response element; pIRE, palindromic IL-6 and IFN- γ response element; LUC, fire-fly luciferase gene.

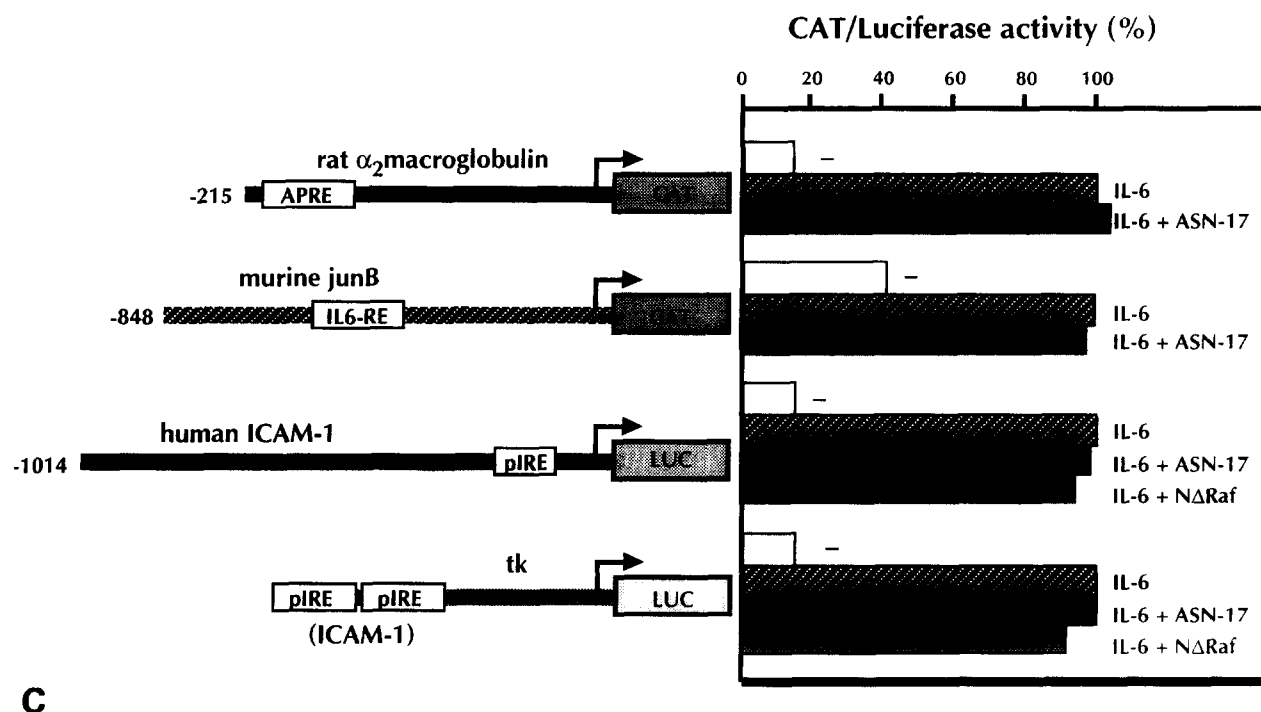


Fig. 1 (continued).

the IL-6RE of the ICAM-1 promoter. In gel retardation assays using a radiolabelled probe containing this element, the formation of APRF–DNA complexes with nuclear extracts from IL-6-treated HepG2 cells preincubated with H7 was not reduced when compared to extracts from cells treated with IL-6 alone. Therefore, although IL-6-induced transactivation from IL-6REs is strongly inhibited by H7, binding of APRF to these elements is not prevented by the inhibitor.

3.5. IL-6-induced serine phosphorylation of APRF

As shown previously, activated APRF is phosphorylated at both tyrosine and serine residues [12]. APRF tyrosine phosphorylation was shown to be induced by IL-6 but it was still unknown whether the serine phosphorylation represents a constitutive modification of APRF or is induced by IL-6 as well. We next considered the possibility that APRF itself may be substrate of an IL-6-activated and H7-sensitive protein serine/threonine kinase. Recently, we had demonstrated the existence of two APRF forms which slightly differ in mobility upon SDS-PAGE [15]. When APRF was analyzed by immunoprecipitation from HepG2 cell lysates and anti-phosphotyrosine immunoblotting it became apparent that the two tyrosine phosphorylated forms of APRF appear with different kinetics (Fig. 3). Whereas the 87-kDa form was found to be tyrosine phosphorylated as early as 2 min after IL-6 stimulation, the 89-kDa form appeared only after 10 min. Since phosphorylation of many proteins results in a reduced electrophoretic mobility upon SDS-PAGE, we examined whether an additional phosphorylation step converts the 87-kDa into the 89-kDa APRF form. In fact, when APRF immunoprecipitated from HepG2 cells treated with IL-6 for 20 min was incubated with protein phosphatase 2A the predominant 89-kDa form of APRF in this sample was converted back to the 87-kDa form

(Fig. 4A). This effect was specific for phosphatase 2A as protein phosphatase 1 was unable to dephosphorylate the 89-kDa APRF. Treatment with alkaline phosphatase eliminated both bands due to the removal of phosphotyrosine. We conclude that between 5 and 15 min after addition of IL-6 the tyrosine phosphorylated APRF undergoes a second phosphorylation, now at serine residues, causing its reduced electrophoretic mobility. A phosphorylation at threonine is not likely to occur since phosphoamino acid analysis of activated APRF did not yield any detectable phosphothreonine [12].

Interestingly, the IL-6-activated Stat 1 α protein does not undergo a comparable delayed mobility shift nor is its mobility affected by the treatment with the phosphatases 1 or 2A. Therefore, Stat 1 α is either not the target of such an IL-6-induced serine phosphorylation or such a phosphorylation if it occurs does not change the mobility of that protein. It is worth noting in this context that Stat 1 α undergoes a mobility shift upon tyrosine phosphorylation [19] whereas APRF does not (unpublished observation).

3.6. APRF serine phosphorylation is H7 sensitive

To investigate whether the IL-6-induced APRF serine phosphorylation could correlate to the H7-sensitive step required for IL-6 target gene induction we analyzed whether the addition of H7 is able to prevent it. We preincubated HepG2 cells with H7, stimulated the cells with IL-6, and then immunoprecipitated APRF and Stat 1 α from the cell lysates. As shown in Fig. 4B, H7 prevented the appearance of the 89-kDa form of APRF, indicating that the serine phosphorylation of APRF is inhibited by H7. This finding strongly suggests that the APRF serine phosphorylation represents the missing link between APRF activation by tyrosine phosphorylation at the plasma membrane and the induction of IL-6 target genes in the nucleus.

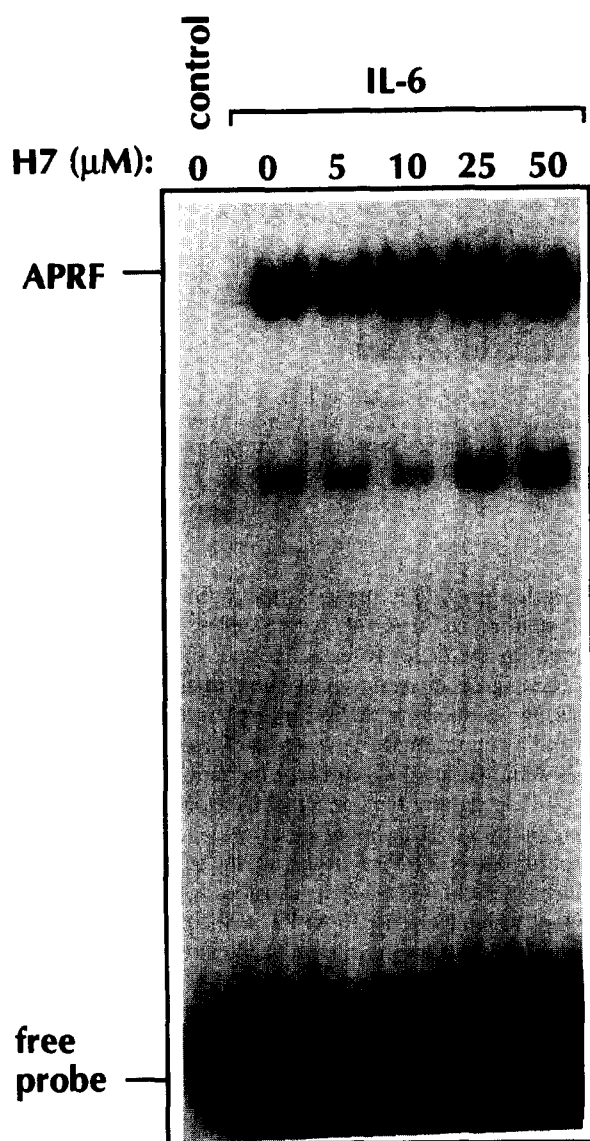


Fig. 2. Binding of APRF to the IL-6RE of the ICAM-1 promoter is unaffected by H7 pretreatment. HepG2 cells were pretreated for 1 h with the indicated amounts of H7 and then incubated for further 15 min without or with IL-6 (100 units/ml). The cells were harvested, and nuclear extracts (5 μ g protein) analyzed in a gel-retardation assay using 32 P-labelled oligonucleotide comprising the palindromic IL-6/IFN- γ response element of the ICAM-1 promoter. After incubating nuclear extract with the probe, free DNA and DNA-protein complexes were separated by electrophoresis through a native polyacrylamide (4%) gel. The gel was fixed, dried, and autoradiographed.

4. Discussion

Our recent work has demonstrated the existence of a remarkably direct signalling pathway from the IL-6 plasma membrane receptor to the nucleus [12,14,15,22]. The transcription factor APRF rapidly associates with the IL-6 signal transducer gp130 upon binding of IL-6 to the receptor, and is then tyrosine phosphorylated and translocated to the nucleus [12]. These data suggested that APRF tyrosine phosphorylation is sufficient for the induction of IL-6 target genes. Accordingly, binding of APRF to the IL-6 response element (APRE) of the rat α_2 -

macroglobulin gene was inhibited by the protein tyrosine kinase inhibitors genistein and tyrphostin but not by the serine/threonine kinase inhibitors sphingosine, H7, or W7 [15]. Furthermore, APRF DNA-binding activity could be demonstrated in cytosolic fractions of IL-6-treated cells indicating that the early events in IL-6 signal transduction yield an active APRF protein [14]. In fact, APRF activation could even be achieved by incubation of cell homogenates with IL-6 in the presence of ATP [15].

On the other hand, several groups have reported that the induction of immediate-early genes by IL-6, i.e. the genes for Jun-B, IRF-1, and TIS11, in hybridoma and myeloma cells can be blocked by both tyrosine kinase inhibitors and H7 [5,6]. Therefore, the action of an H7-sensitive serine/threonine kinase is likely to be involved in the induction of these genes by IL-6. We had shown earlier that APRF is likely to play an important role in the induction of the Jun-B, IRF-1, and ICAM-1 immediate-early genes by IL-6 [22,23]. The promoters of all three promoters contain IL-6 response elements containing APRF binding sites which are able to confer IL-6 inducibility to control promoters [22–24].

Therefore, despite the direct IL-6 signalling pathway which leads to a rapid accumulation of DNA-binding APRF in the nucleus, the induction of genes regulated by this transcription factor requires an additional, H7-sensitive step. Two alternative explanations seem possible: either that an additional transcription factor, which is regulated by serine/threonine phosphorylation, is required, or that the APRF activation pathway itself harbours an as yet unrecognized step. The results presented here strongly favour the latter explanation.

By transient transfection of reporter gene constructs into hepatoma cells we could demonstrate that the activation of three immediate-early gene promoters by IL-6 is also sensitive to H7. Even more intriguing, when the IL-6 response elements of these promoters were fused to the thymidine kinase promoter the induction of these constructs was completely blocked by H7 as well. Since all three IL-6REs were shown by us to represent APRF binding sites this observation indicates the unknown H7-sensitive step to involve either APRF itself or a protein closely cooperating with APRF in the transactivation of target promoters.

Our earlier and present results clearly indicate, however, that APRF tyrosine phosphorylation is sufficient to induce the DNA-binding capacity of the factor and that H7 does not interfere with this process, neither when binding to the α_2 -macroglobulin APRE [15], the Jun-B IL-6RE [23], nor the ICAM-1 pIRE (this paper) is concerned.

In this context, the observation was of interest that after IL-6-stimulation of HepG2 cells two tyrosine phosphorylated APRF bands of apparent molecular sizes of 87 and 89 kDa were detected by immunoprecipitation and immunoblot analyses [15]. These two bands are observed with antibodies directed against the amino-terminal portion of Stat 1 which cross-react with APRF (as used here) as well as with specific antibodies to APRF (unpublished data). Furthermore, this effect is not restricted to hepatoma cells but also observed in all other cell types studied so far, i.e. in COS7 cells and in fibrosarcoma, hybridoma, and preadipocyte cell lines (unpublished data). Since in contrast to the situation with Stat 1 [19] APRF tyrosine phosphorylation does not account for this mobility shift upon SDS gel-electrophoresis we considered the possibility that the

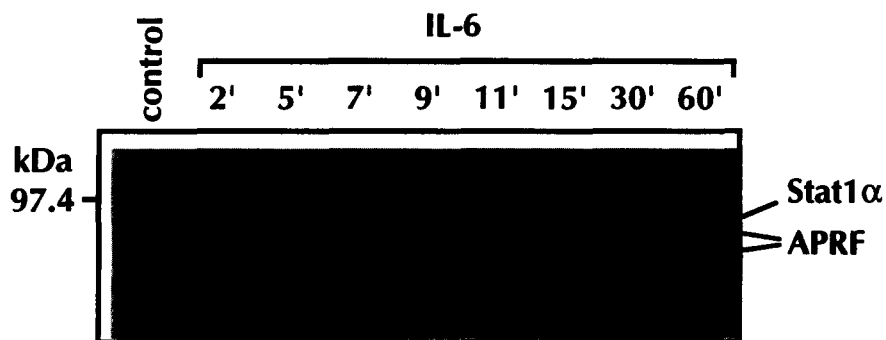


Fig. 3. Time-course of APRF tyrosine phosphorylation. HepG2 cells were treated for the times indicated with IL-6 (100 units/ml) and lysed. The lysates were immunoprecipitated with an antiserum raised against the amino-terminal portion of Stat 1 [25] which cross-reacts with APRF [15]. The immunoprecipitates were separated by SDS-PAGE (7% gel) and electroblotted to polyvinylidene difluoride membrane. Tyrosine-phosphorylated proteins were detected on the blot using monoclonal antibodies to phosphotyrosine and visualized by enhanced chemiluminescence. The band corresponding to Stat 1 α and the doublet of bands representing APRF are indicated.

phenomenon reflects an additional post-translational modification of APRF.

The time-course of APRF tyrosine phosphorylation (Fig. 3) demonstrates that the shift in APRF mobility occurs only about 10 min after the tyrosine phosphorylation had started. Therefore, the two phenomena can be discriminated between. Earlier results had shown that APRF from IL-6-treated HepG2 cells contains both phosphotyrosine and phosphoserine while phosphothreonine could not be detected [12]. Since phosphorylation of many proteins causes considerable mobility shift in SDS gel-electrophoresis we considered the possibility that the APRF mobility shift may be due to an additional phosphorylation. In fact, our results show that the shift can be reversed by treatment with protein phosphatase 2A and is prevented by pretreatment of the cells with H7. Hence the data clearly indicate that APRF undergoes an additional IL-6-induced phosphorylation which, for the lack of the occurrence of phosphothreonine in the protein, presumably occurs at serine residues, and which is catalyzed by an H7-sensitive protein kinase.

The coincidence of an H7-sensitive step required for the induction of IL-6 target genes and an H7-sensitive serine phos-

phorylation of APRF suggests that both represent the same phenomenon, i.e. that the APRF serine phosphorylation is required for the factor to be active as a transactivating factor. It will be of great interest to prove this conclusion by identifying the serine residue(s) of APRF phosphorylated in response to IL-6 and by mutational analysis of these residues. The data also clearly demonstrate that both gene induction and APRF activation by IL-6 require the convergent action of at least two signalling pathways. The Jak/Stat pathway accounts for tyrosine phosphorylation of APRF triggering the activation of its DNA-binding potential and nuclear translocation whereas the other pathway, which gives rise to APRF serine phosphorylation and presumably is important in target gene induction, still needs to be identified. IL-6 activates the Ras/MAP kinase pathway in various target cells [31,32]. Furthermore, the p90^{rk} serine/threonine kinase activated via this pathway is known to be H7 sensitive [31]. However, our data using dominant-negative Ras and Raf-1 kinase mutants do not support the involvement of this pathway in IL-6 target gene induction. Therefore, the protein kinase responsible for APRF serine phosphorylation as well as the exact function of this modification will be important issues for future research.

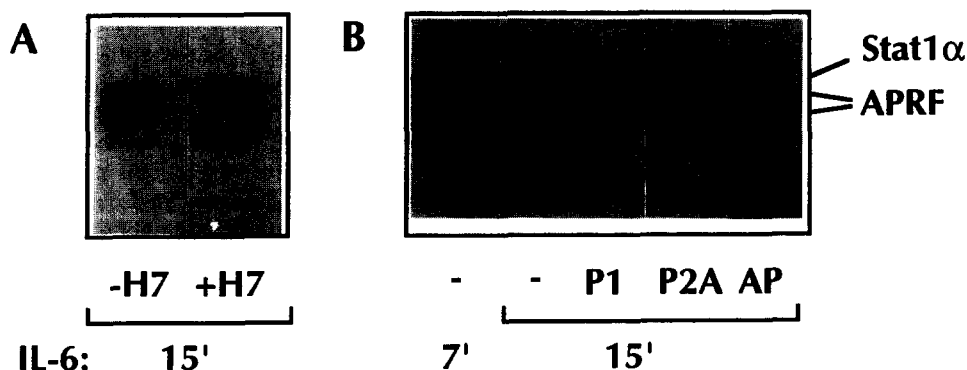


Fig. 4. Effects of phosphatases and H7 on the IL-6-induced APRF mobility shift. (A) HepG2 cells were stimulated with IL-6 (100 units/ml) without or with preincubation with H7 (50 μ M, 1 h) as indicated. The cells were lysed, immunoprecipitated, and immunoblotted with anti-phosphotyrosine as described in the legend to Fig. 3 and in section 2. (B) HepG2 cells were stimulated as indicated with IL-6 (100 units/ml), lysed, and immunoprecipitated as described above. The protein A-Sepharose-coupled immunoprecipitates were incubated without or with purified protein phosphatase 1 (P1), the catalytic subunit of protein phosphatase 2A (P2A), or intestine alkaline phosphatase (AP) for 30 min at 30°C. The immunoprecipitates were then separated by SDS-PAGE, blotted to polyvinylidene difluoride membrane, and detected by anti-phosphotyrosine as described for Fig. 3.

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