The nuclear factor interleukin-6 (NF-IL6) and signal transducer and activator of transcription-3 (STAT-3) signalling pathways co-operate to mediate the activation of the hsp90β gene by interleukin-6 but have opposite effects on its inducibility by heat shock

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The levels of the 90 kDa heat-shock protein (hsp90) and the activity of the hsp90β gene promoter are increased in response to treatment by interleukin (IL)-6. The hsp90β gene promoter contains binding sites for the transcription factors nuclear factor IL-6 (NF-IL6) and signal transducer and activator of transcription 3 (STAT-3), which are activated respectively by the mitogen-activated-protein-kinase and Jak-kinase pathways following IL-6 treatment. Both these factors can activate the hsp90 promoter and have a strong synergistic effect on its activity, which appears to be critical for IL-6-mediated activation of the promoter. Interestingly, the two factors interact differently with the heat-shock factor (HSF) and a heat-shock stress. Thus STAT-3 reduces the stimulatory effect of heat shock whereas NF-IL6 enhances it. When applied together, heat shock and IL-6 produce only weak activation of the hsp90 promoter compared with either stimulus alone, indicating that the inhibitory effect of STAT-3 on HSF predominates under these conditions. In contrast, IL-1, which activates only the NF-IL6 pathway, synergizes with heat shock to produce strong activation of hsp90. These effects are discussed in terms of the multiple stimuli that may regulate the hsp90 promoter in unstressed cells and their interaction with its stress-mediated activation.

INTRODUCTION

Interleukin-6 (IL-6) is a multifunctional cytokine with pleiotropic activities that enable it to play a central role in host defence (for reviews see [1,2]). Thus for example IL-6 promotes the terminal differentiation and secretion of immunoglobulin by B lymphocytes, the differentiation and/or activation of T cells and macrophages, and the production of acute-phase proteins by the liver. The ability of IL-6 to induce these diverse phenomena is dependent upon its ability to stimulate two distinct signalling pathways, resulting in the activation of two different classes of cellular transcription factors (for reviews see [2,3]). Thus, initial studies showed that a variety of IL-6-inducible genes contained binding sites for a transcription factor named NF-IL6 (nuclear factor IL-6), which showed high homology with the rat-liver nuclear factor C/EBP (CCAAT-enhancer-binding protein), and is therefore also known as C/EBPβ [4]. Subsequently, a second member of the C/EBP family, known as NF-IL6β or C/EBPα, was identified and shown to form heterodimers with NF-IL6, resulting in a synergistic transcriptional effect [5]. After exposure of cells to IL-6, NF-IL6 is phosphorylated, resulting in its enhanced ability to stimulate transcription [6] whereas NF-IL6β is synthesized de novo [7].

As well as the activation of NF-IL6 and NF-IL6β by IL-6 treatment, subsequent studies showed that the DNA-binding ability of the STAT (signal transducer and activator of transcription)-3 transcription factor (also known as APRF) is rapidly activated by IL-6 at the post-translational level [8,9]. This factor is a member of the STAT family of DNA-binding proteins (for review see [10]). After exposure to IL-6, pre-existing inactive STAT-3 is activated to a form competent for DNA binding by tyrosine phosphorylation following association with tyrosine kinases of the Jak family, which are in turn associated with IL-6-receptor components (for review see [3]).

It is generally accepted that the NF-IL6/NF-IL6β and STAT-3-signalling pathways allow IL-6 to activate two distinct sets of genes, each of which is responsive to one of these pathways. Thus, class 1 acute-phase proteins (such as α2-acid glycoprotein, haptoglobin, C-reactive protein and serum amyloid) contain response elements for NF-IL6 and NF-IL6β and these factors have been shown to be involved in the activation of these genes following IL-6 treatment [4,6,11,12]. In agreement with this idea, these genes are stimulated by exposure of cells to IL-1 which also stimulates NF-IL6/NF-IL6β activity without affecting STAT-3 [4,11,13]. In contrast, type 2 acute-phase genes such as fibrinogen, thiostatin and α2-microglobulin are not inducible by IL-1 and lack binding sites for NF-IL6/NF-IL6β. Instead these genes contain binding sites allowing binding of STAT-3, which is responsible for activation of these genes in response to IL-6 [8,9].

We have recently reported [14] that IL-6 can induce increased expression of the 90 kDa heat-shock protein (hsp90) in a variety of different cell types. The hsp90β gene promoter was shown to be responsive to IL-6 and could also be activated by cotransfection with NF-IL6 or NF-IL6β expression vectors. Moreover, a short region of the promoter containing an NF-IL6-binding site was essential for activation of the promoter by both...
IL-6 and NF-IL6 and could confer responsiveness both to IL-6 and to overexpression of NF-IL6 on a heterologous promoter. These findings suggested that hsp90 was a member of the class of IL-6-responsive genes that were activated by NF-IL6/NF-IL6β. Here we report, however, that this short region of the promoter also contains binding sites for STAT-3 and that the hsp90 promoter can be activated also by overexpression of this factor. Interestingly, overexpression of NF-IL6 and STAT-3 has a synergistic effect on the hsp90 promoter and both these signalling pathways appear to be required for activation of the hsp90 promoter by IL-6. Despite their synergistic action in IL-6 signalling however, these two pathways have opposite effects on the heat-shock-mediated regulation of the hsp90 promoter.

MATERIALS AND METHODS

Plasmid constructs

The 5′ hsp90β gene-promoter constructs used in transfection experiments were kindly provided by Dr. N. Rebbe [15]. Construct A contains a fragment from –1044 to +36 bp relative to the transcriptional start site of the hsp90β gene coupled with the chloramphenicol acetyltransferase (CAT) gene, whereas construct C contains a fragment from –299 to +36 bp coupled with the CAT gene. The construct containing the region of the hsp90 gene promoter with STAT-3, NF-IL6- and HSF (heat-shock factor)-binding sites was prepared by synthesizing oligonucleotides, which when annealed would contain the region from –643 to –623 bp of the hsp90β promoter relative to the transcriptional start site. This double-stranded oligonucleotide was cloned upstream of the thymidine kinase promoter and the CAT reporter gene in the vector pBLCAT2 [16]. The wild-type NF-IL6 expression vector contains the full coding region for NF-IL6 cloned downstream of the strong cytomegalovirus immediate early promoter [4], while the dominant negative mutant ΔSp1 NF-IL6 lacks the region encoding amino acids 41–205 [17]. The STAT-3 mutant contains a phenylalanine residue at position 705 replacing the wild-type tyrosine residue [18]. The HSF expression vector contains the full-length human HSF1 cDNA inserted downstream of the cytomegalovirus immediate early promoter (a kind gift of Dr. J. Wisniewski and Professor C. Wu, Laboratory of Biochemistry, National Cancer Institute, National Institute of Health, Bethesda, MD 20892, U.S.A.).

DNA transfection and CAT assay

Transfection of DNA was carried out according to the calcium phosphate method of Gorman [19]. HuH7 hepatoma cells were plated at a density of 1 × 10⁶ cells/well in six-well plates. 10 µg of reporter plasmid and the indicated amounts of the expression vectors were transected for 4–6 h and the cells were then osmotically shocked with 15%, glycerol in PBS for 2 min and then washed in PBS. The cells were re-fed with complete medium and harvested 48 h later. The cells were then lysed by three cycles of repeated freeze-thawing. To normalize for transfection efficiency the β-galactosidase activity of a transfected control plasmid was determined and used to determine the amount of extract which was assayed for CAT activity. Assays of CAT activity were carried out according to the method of Gorman [19] using samples that had been equalized for protein content as determined by the method of Bradford [20].

DNA-mobility-shift assays

DNA-mobility-shift assays were carried out as previously described [21] using extracts from untreated or IL-6-treated HuH7 cells and a radiolabelled double-stranded oligonucleotide containing the sequence shown in Figure 1. The double-stranded oligonucleotide containing the STAT-3 consensus sequence used in the competition experiments had the sequence (top strand) 5′-TGATTAChGGAAATG-3′.

RESULTS

In our previous experiments [14] we showed that both IL-6 itself and overexpression of NF-IL6 or NF-IL6β could stimulate a reporter construct containing the hsp90β gene promoter from –1044 to +36 bp relative to the transcriptional start site coupled with the readily assayable CAT gene [15]. In contrast a similar construct containing only the region from –299 to +36 bp was not stimulated by either IL-6 or NF-IL6. The region from –1044 to –299 bp contains an element from –643 to –623 bp that contains a binding site for NF-IL6 (Figure 1; [15]). When linked to a heterologous promoter this short sequence could render the promoter inducible by IL-6 and by overexpression of NF-IL6 [14].

Further inspection of this sequence, however, revealed that it also contains two putative binding sites for STAT-3 (Figure 1). We therefore used this sequence in a DNA-mobility-shift assay with extracts from the HuH7 hepatoma cell line. In these experiments (Figure 2) the sequence from the hsp90 promoter bound a protein the DNA-binding activity of which was greatly increased in extracts from IL-6-treated cells compared with untreated cells, as expected for STAT-3 [8,9]. Moreover, this protein could be removed equally effectively by competition with excess unlabelled oligonucleotides containing either the identical hsp90 promoter sequence or the consensus DNA-binding sequence for STAT-3, but not with an unrelated oligonucleotide containing the Spl-binding site. Hence, this sequence element binds a protein whose DNA binding is IL-6-inducible and which binds to a STAT consensus sequence with high affinity, characteristics unique to STAT-3 [8,9].

We next tested the effect of co-transfecting HuH7 hepatoma cells with the various hsp90 reporter constructs and an expression...
vector directing enhanced expression of STAT-3. The construct containing hsp90 sequences from −1044 to +36 bp relative to the transcriptional start site was indeed activated by over-expression of STAT-3. Thus this construct containing the full promoter was activated approx. 8-fold by co-transfection with a STAT-3 expression vector, whereas the deleted construct with sequences from −299 to +36 was unaffected. Moreover, the oligonucleotide illustrated in Figure 1 was able to confer a response to STAT-3 when linked to the thymidine kinase promoter in the vector pBLCAT2 in which this promoter drives expression of the CATgene [16]. Thus this construct was activated 3-fold by co-transfection with the STAT-3 expression vector whereas the parental vector was unaffected. Hence the region of the hsp90 promoter from −643 to −623 can render a homologous promoter responsive to IL-6 and to over-expression of NF-IL6 and STAT-3.

The hsp90 promoter thus appears to represent a unique case in which the promoter is activated by both the IL-6-stimulated signalling pathways. To begin to investigate the significance of this effect, we co-transfected the hsp90 promoter with expression vectors encoding NF-IL6 or STAT-3, either alone or in combination [4,9]. In these experiments (Figure 3) overexpression of either NF-IL6 alone or STAT-3 activated the hsp90 promoter in accordance with our previous observations [14] as well as the data presented in the previous paragraph. However, in the presence of both factors a strong synergistic effect was observed, suggesting that the two factors co-operate together to maximally activate the hsp90 promoter at least in co-transfection assays.

To investigate whether this effect was also occurring during the stimulation of the hsp90 promoter by IL-6 itself, we made use of dominant negative mutants of NF-IL6 and STAT-3 that are able to inhibit the response to the functional factors. The NF-IL6 mutant ΔSpl NF-IL6 lacks amino acids 41–205 of NF-IL6, which contain the transcriptional activation domain. It is therefore capable of binding to DNA but cannot activate transcription [17]. Similarly, the STAT-3 mutant contains a phenylalanine residue in place of the tyrosine whose phosphorylation is essential for the activation of STAT-3 [18]. Cells were therefore transfected with different amounts of each of these dominant negative mutants and then treated with IL-6. As illustrated in Figure 4, IL-6 was, as expected, able to strongly stimulate the hsp90 promoter. Most interestingly however the dominant negative mutants of either NF-IL6 or STAT-3 were able to abolish this stimulation, resulting in high concentrations of either factor being able to totally abolish stimulation of the promoter by IL-6 in the absence of the other. Thus inhibition of either the NF-IL6 or STAT-3-mediated signalling pathway is able to totally abolish the response to IL-6 even though the other pathway is left intact. This indicates that the two pathways are likely to synergize strongly in IL-6-treated cells so that the observed response is virtually eliminated when either one factor or the other is eliminated.

We next investigated whether the overexpression of one or other of the IL-6-inducible factors could compensate for the inhibition of the other pathway. To do this, cells were transfected with the functional STAT-3 expression vector together with the expression vector encoding the dominant negative form of NF-IL6 and were subsequently treated with IL-6. Conversely, cells were also treated with IL-6 following transfection of expression vectors encoding functional NF-IL6 and the dominant negative mutant of STAT-3. In these experiments (Figure 5) the response to IL-6 was enhanced by overexpression of STAT-3 in the transfected cells. Nonetheless the NF-IL6 dominant negative
mutant was able to still virtually abolish the IL-6 response in a dose-dependent manner, although in this case a very small amount of residual stimulation remained in cells treated with the highest level of this mutant that was tested. Similarly, the overexpression of NF-IL6 enhanced the response to IL-6 in the transfected cells but once again the IL-6 response was virtually abolished at the highest level of the dominant negative STAT-3 mutant that was transfected. These results indicate therefore that only a very small IL-6-mediated stimulation of hsp90-promoter activity can be obtained when only one of the two signalling pathways is active, even when the transcription factor that is mediating activation by this pathway is greatly over-expressed. Hence the hsp90 promoter appears to be unique in that it requires both the IL-6-stimulated signalling pathways for significant gene induction.

Inspection of the short region containing both an NF-IL6-binding site and two binding sites for STAT-3 (Figure 1) also reveals that it contains the characteristic tripartite motif which mediates binding by the HSF [22]. The presence of binding sites for the HSF in this region is in agreement with the importance of the region of the hsp90β gene promoter from −1044 to −300 bp in its heat inducibility [15]. In view of the close proximity of an HSF-binding site to those for IL-6 and STAT-3, we therefore investigated the effect of co-transfecting an HSF expression vector together with either the NF-IL6 expression vector or that encoding STAT-3.

In these experiments (Figure 6) NF-IL6 or STAT-3 were able to activate the promoter as expected. Interestingly, however, whereas co-transfection of NF-IL6 and HSF resulted in a strong activation of the promoter greater than that observed with either factor alone, similar co-transfection of STAT-3 and HSF resulted
Regulation of the hsp90β gene

Figure 6  Assay of CAT activity in HuH7 cells transfected with the hsp90β gene reporter construct with expression vectors encoding HSF, NF-IL6 and STAT-3

Expression vectors (5 µg) encoding HSF (H), NF-IL6 (N) or STAT-3 (S) were used, either alone or in combination. Cells were not treated with IL-6 in this experiment. The Figure illustrates a typical result of this experiment which was repeated three times with similar results.

In a reduced activation of the promoter which was lower than that observed with STAT-3 alone. These results were observed both in the HuH7 hepatoma cells (Figure 6) and in ND7 neuronal cells (results not shown), indicating that they are not cell-type specific. Hence, whereas HSF and NF-IL6 appear to co-operate to activate the hsp90 promoter, the effect of STAT-3 and HSF appears to be antagonistic. A similar synergy between HSF/NF-IL6 and antagonism between HSF/STAT-3 was also observed on the pBLCAT2 construct containing the –643 to –623 bp region of the promoter (results not shown), confirming that these effects could occur with this isolated region upstream of a heterologous promoter.

Having established that NF-IL6 and STAT-3 interact differently with HSF, we wished to determine whether this differential interaction also occurred with a heat-shock stimulus. To do this, cells were transfected with the hsp90 promoter construct together with either NF-IL6 or STAT-3 expression vectors or with empty expression vector and were then treated with IL-6 with or without a heat shock. Smaller amounts of the expression vectors were used in this experiment compared with earlier experiments to maximize the effect of the heat shock. For this reason, only minimal activation of the promoter by the expression vector encoding NF-IL6 was observed at 37 °C in IL-6-treated cells (Figure 7). However, clear activation was observed with this vector in heat-shocked cells treated with IL-6. In contrast, when a heat shock was given together with IL-6, transfection of STAT-3 reduced the activity of the promoter below that observed with empty expression vector. Similarly, cells transfected with STAT-3 and given a heat shock together with IL-6 showed lower promoter activity than cells transfected with STAT-3 and exposed to IL-6 alone. Hence, the presence of STAT-3 and IL-6 abolishes the stimulatory effect of heat shock on the hsp90 promoter whereas the effect of NF-IL6 and IL-6 is enhanced by heat shock. Hence NF-IL6 and STAT-3 do indeed have opposite effects on the activation of the hsp90 promoter by heat shock.

We wished to determine which of these opposite interactions of IL-6-stimulated transcription factors with HSF predominated when cells were exposed to both heat shock and IL-6 in the absence of any transfected transcription factors. Cells were therefore transfected with the hsp90 promoter–reporter construct and exposed to either heat shock or IL-6 alone or both in combination. As shown in Figure 8 both heat shock and IL-6 individually activated the hsp90 promoter, as expected. When both stimuli were applied together a much weaker increase in promoter activity was observed compared with that seen for either stimulus alone. Hence the synergistic interaction between HSF and NF-IL6 that we observed in transfection experiments appears to be overcome by the antagonistic interaction of HSF
and STAT-3 when the transcription factors are activated by the appropriate stimuli rather than by overexpression. In agreement with this idea, IL-1, which activates only the NF-IL6 and not the STAT-3 pathway [4,8], was able to synergize with heat shock and produce strong activation of the hsp90 promoter (Figure 8).

**DISCUSSION**

Although the mechanisms mediating the induction of the heat-shock-protein genes by heat shock have been intensively studied (for review see [22]) much less attention has been paid to their regulation by other stimuli, even though hsp gene expression is extensively modulated by many stimuli or differentiation processes such as protein-kinase-C activators [23], T-cell activation [24] and monocytic macrophage differentiation [25]. Here we have extended our previous study documenting the induction of hsp90β activation by IL-6 [14] to show that the promoter of this gene is stimulated by both IL-6-activated transcription factors: NF-IL6 and STAT-3. Moreover, experiments with dominant negative mutants of these factors show that the effect of IL-6 itself on the hsp90β gene promoter is strongly dependent on the synergistic interaction of NF-IL6 and STAT-3. Although the effect of STAT-3 on the hsp90 promoter could be indirect and mediated via the activation of other genes by STAT-3, our finding of an element in the hsp90β promoter that binds STAT-3 and which can confer the effect on a heterologous promoter suggest that the effect is a direct one.

Hence the hsp90β promoter appears to have a novel pattern of inducibility which is dependent upon both the IL-6-activated pathways involving the threonine phosphorylation of NF-IL6 by MAP (mitogen-activated protein) kinase [26] and the tyrosine phosphorylation of STAT-3 by Jak-family kinases [27]. This finding renders this promoter distinct from those of the liver acute-phase protein genes that appear to fall into two separate classes which are predominantly regulated either by the NF-IL6 pathway or the STAT-3 pathway [4,8,9]. It is possible that this difference may reflect the tissue-specific expression of the acute-phase protein genes that appear to fall into two separate classes which are predominantly regulated either by the NF-IL6 pathway or the STAT-3 pathway [4,8,9].

It is unlikely, however, that this difference is responsible for the unique response of the hsp90β gene promoter, since we observed identical responses of the promoter in both HuH7 liver cells, which express the acute-phase protein genes, and ND7 neuronal cells, which do not. It is more likely therefore that these results reflect the need for the different stimuli which regulate the hsp90β gene promoter to interact with one another so that the correct level of hsp90 protein is produced when cells are exposed to multiple stimuli.

Thus we have shown that whereas NF-IL6 and HSF synergistically regulate the hsp90 promoter, STAT-3 and HSF antagonize one another. The synergy between NF-IL6 and HSF is likely to reflect an ability to bind simultaneously to their adjacent sites within the promoter and either interact with one another or, with different components of the basal transcriptional complex, to synergistically stimulate transcription. In contrast, the antagonism between STAT-3 and HSF may reflect either a protein–protein interaction between the two factors which produces a non-DNA-complex or competition for overlapping binding sites on the DNA which prevents both factors binding simultaneously. Alternatively, it is possible that the two factors can bind simultaneously but that such binding produces a reduced ability to stimulate transcription either because of a direct protein–protein interaction or altered folding of each factor (so as to, for example, mask the activation domain) when the other factor is bound at an adjacent site.

Although further experiments will be required to investigate these possibilities, it is clear that the antagonism between STAT-3 and HSF can have functional effects on the response to specific stimuli. Thus, when cells are exposed to IL-6 with an accompanying heat shock, transfection of NF-IL6 results in increased levels of promoter activity, whereas transfection of STAT-3 reduces the promoter response. When cells are exposed to both heat shock and IL-6 in the absence of any transfected transcription factors, however, an antagonistic effect is observed suggesting that the stimulatory interaction of NF-IL6 and HSF is overcome by the inhibitory interaction of STAT-3 and HSF when both stimuli are given together.

It is clear therefore that the response to a heat-shock stimulus can be influenced by the relative activation levels of NF-IL6 and STAT-3. Hence if the heat shock is given at different times after IL-6 treatment, it might be expected that different levels of promoter activation would be observed depending on the relative rate at which the activated state of NF-IL6 and STAT-3 decays after withdrawal of IL-6. In addition, whereas IL-6 activates both the NF-IL6 and STAT-3 pathways, other stimuli activate one or other of these pathways specifically. Thus, for example, both IL-1 and tumour necrosis factor-α activate the MAP kinase pathway, leading to the activation of NF-IL6/NF-IL6β, and do not activate the Jak/STAT pathway [8], whereas interferon-α and IL-9 stimulate only the Jak/STAT pathway [10,18,29]. Indeed we observed a synergistic effect on the hsp90 promoter when IL-1 and heat shock were given together compared with the effect observed with each stimulus alone. Hence the interaction of heat shock with other activating stimuli will vary depending on whether either or both of the pathways is activated by these stimuli.

It is clear therefore that the hsp90β gene shows a novel pattern of IL-6-mediated activation which is dependent upon both the NF-IL6 and STAT-3 pathways. Moreover, these pathways show opposite interactions with the well-characterized heat-shock-activated pathway. These results therefore open up a new aspect of hsp90-gene regulation which is additional to and interacts with the heat-shock-activated pathway.

We thank N. Rebbe for the hsp90 promoter constructs and J. Wisniewski and C. Wu for the HSF1 expression vector. This work was supported by the Arthritis and Rheumatism Council.

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Received 21 August 1997/3 October 1997; accepted 29 October 1997