Signal Transducer and Activator of Transcription-1 and Heat Shock Factor-1 Interact and Activate the Transcription of the Hsp-70 and Hsp-90β Gene Promoters*

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Anastasis Stephanou‡, David A. Isenberg§, Koichi Nakajima*, and David S. Latchman

From the Departments of Molecular Pathology and $Medicine, Windyer Institute of Medical Sciences, University College London, 46 Cleveland Street, London W1P 6DB, United Kingdom and the ¶Department of Molecular Oncology, Biomedical Research Center, Osaka University Medical School, Osaka 565, Japan

We have previously demonstrated that interleukin-6 (IL-6) increases the levels of the heat shock protein 90 (Hsp-90) and activates the Hsp-90β promoter via the IL-6-activated transcription factors NF-IL6 and signal transducer and activator of transcription-3 (STAT-3). Here, we show that interferon-γ (IFN-γ) treatment increases the levels of Hsp-70 and Hsp-90 and also enhances the activity of the Hsp-70 and Hsp-90β promoters with these effects being dependent on activation of the STAT-1 transcription factor by IFN-γ. These effects were not seen in a STAT-1-deficient cell line, indicating that IFN-γ modulates Hsp induction via a STAT-1-dependent pathway. The effect of IFN-γ/STAT-1 was mediated via a short region of the Hsp-70/Hsp-90 promoters, which also mediates the effects of NF-IL6 and STAT-3 and can bind STAT-1. This region also contains a binding site for the stress-activated transcription factor HSF-1. We show that STAT-1 and HSF-1 interact with one another via a protein-protein interaction and produce a strong activation of transcription, which is in contrast to our previous finding that STAT-3 and HSF-1 antagonize one another. To our knowledge this is the first report of HSF-1 interacting directly via a protein-protein interaction with another transcription factor. Such protein-protein interactions and the binding of a number of different stress and cytokine-activated transcription factors to a short region of the Hsp-90 and Hsp-70 gene promoters are likely to play a very important role in Hsp gene activation by non-stressful stimuli and the integration of these responses with the stress response of these genes.

The heat shock proteins (Hsps) are a group of proteins that were originally identified on the basis of their increased synthesis in cells exposed to elevated temperatures and subsequently were shown to be similarly induced by exposure of cells to a variety of stresses (1, 2). The induction of Hsps in response to various stresses is dependent on the activation of a specific transcription factor, the heat shock factor (HSF-1), which binds to the heat shock element (HSE) in the promoters of Hsp genes (3). In addition, many Hsps are also expressed in unstressed cells, and their levels are regulated in response to a wide variety of biological processes such as T lymphocyte activation (4) and monocyte to macrophage differentiation (5). In general, however, the stimuli that induce such alteration in Hsp gene expression under non-stress conditions have been poorly characterized, and the mechanisms by which they act are unclear.

We have recently shown, however, that treatment of human peripheral blood lymphocytes with interleukin-6 (IL-6) results in enhanced expression of the Hsp-90β gene (6). IL-6 is a multifunctional cytokine with pleiotropic activities on a variety of cell types (7). This property of IL-6 is dependent on the IL-6 receptor, which includes the glycoprotein 130 subunit that is shared among the other cytokine receptors belonging to the IL-6 receptor superfamily (leukemia inhibitory factor, IL-11, oncostatin M, and cardiotrophin-1) as well as a receptor chain, which is unique to the IL-6 receptor (8). Binding of IL-6 to its receptor is known to stimulate two distinct signal transduction pathways, resulting in the activation of two distinct transcription factors NF-IL6 (C/EBPβ) and STAT-3 (9). Both these factors have been shown to activate the Hsp-90β promoter and have a strong synergistic effect on its transcription resulting in its observed activation by IL-6. Interestingly, activation of the Hsp promoters by these factors is mediated via NF-IL6 and STAT-like binding sites that are located close to the HSE (10). Moreover, the two transcription factors interact differently with HSF-1 and heat shock stress (10). Thus activation of STAT-3 reduced the stimulatory effect of HSF-1 or heat stress, whereas activation of NF-IL6 enhanced it. These results are of interest because they demonstrate that a specific stimulus such as IL-6 can enhance the expression of the Hsp in non-heat-stressed cells in an HSF-1-independent manner and also indicate that others can functionally interact with HSF-1 and play a role in Hsp gene regulation.

In view of the effect of the STAT-3 transcription factor on the Hsp-90β promoter, we wished to investigate whether the closely related STAT-1 factor would also have an effect. To do this we tested the effect of IFN-γ on Hsp gene expression. Like IL-6, IFN-γ is also a multifunctional cytokine that is known to have antiviral and antitumor properties by inducing specific IFN-γ-responsive genes (11, 12). In contrast to IL-6, however, IFN-γ specifically activates the STAT-1 signaling pathway. In the present study, we report that IFN-γ treatment induced the expression of Hsp-70 and Hsp-90 in a STAT-1-dependent manner. In addition, overexpression of STAT-1 enhanced the activities of the Hsp-70 and Hsp-90β promoters. Interestingly, STAT-1 and HSF-1 were shown to have an additive effect in activating the Hsp-70 and Hsp-90β promoters and to directly interact via a protein-protein interaction. These studies have identified a composite response element that integrates the

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† To whom correspondence should be addressed.
‡ The abbreviations used are: HSF, heat shock factor; IL, interleukin; IFN, interferon; HSE, heat shock element; CAT, chloramphenicol acetyltransferase; STAT, signal transducer and activator of transcription.
HSF-mediated heat shock response with IL-6 and IFN-γ signaling to mediate the differential regulation of Hsps.

MATERIALS AND METHODS

Cell Culture and Reagents—HepG2 hepatoma cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in 10% Dulbecco's modified Eagle's medium, U3A and U3A-ST1 cells were kindly provided by Ian Kerr (Imperial Cancer Research Fund, London, UK). Recombinant IFN-γ was purchased from Autogen Bioclear, Wiltshire, UK. Antibodies to STAT-1 were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, and antibodies for Hsp-70, Hsp-90, and HSF-1 were purchased from StressGen (Victoria, BC, Canada).

Purification of DNA and Nuclear Proteins—The 5′ Hsp-90β CAT reporter constructs A (−1044 to +36) and C (−299 to +36) were kindly provided by Neil Rebbe (Washington University School of Medicine, St. Louis, MO). The Hsp-70 CAT reporter constructs LSN (−188 to +1) and LSNP (−100 to +1) were kindly provided by Richard Morimoto (Northwestern University, Evanston, IL). The HSE/STAT-1 CAT was constructed by ligation of the −643 to −623 fragment (5′-GCTTGAAACTGCTGTGAAT-3′) in the heterologous reporter construct pBLCAT2. The expression vector for HSF-1 was kindly provided by Carl Wu (NIH, Bethesda, MD). The expression plasmid-encoding STAT-1 was in pcAGSNeo-HASAT-1.

Transfection of Reporter Constructs was performed by the calcium phosphate method by using 10 μg of the reporter plasmid and 5 μg of the STAT-1 or the HSF-1 expression vectors. To assess and normalize for transfection efficiency, a high affinity SIEm67 (5′-GATCCGGCAGAAACCTGGATATTTCCCCGACCT-3′) DNA probe. A 23-base pair change from wild-type to mutant-type) DNA probe. A 23-base pair high affinity SIEm67 (5′-GATCTTGAAACTGCTGTGAAT-3′) DNA probe that serves as a binding site for STAT-1 was used in competition studies. For supershift assays, nuclear extracts were incubated with anti-STAT-1 for 30 min prior to incubation with the DNA probe. Complexes were separated by 4% SDS-polyacrylamide gel electrophoresis and exposed to autoradiography.

Immunoprecipitation—HepG2 cells (5 × 10⁶) were lysed in radioimmune precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) and incubated with either anti-STAT-1 or anti-HSF-1 for 1 h at 4 °C on a shaking platform. Protein A-Sepharose was added to each tube and incubated further for 3 min. Samples were then electrophoresed on an 8% SDS-polyacrylamide gel, transferred onto nitrocellulose filters, and subjected to Western blotting with either anti-STAT-1 or anti-HSF-1 antibodies.

RESULTS

To test whether IFN-γ could stimulate the levels of Hsp in the IFN-γ-responsive HepG2 cell line, we treated these cells with IFN-γ and measured the Hsp-70 and Hsp-90 levels by Western blotting. As shown in Fig. 1, both Hsp-70 and Hsp-90 levels were increased by IFN-γ in a dose-dependent manner. 50 ng/ml IFN-γ resulted in an 8- and 6-fold induction in the levels of Hsp-70 and Hsp-90, respectively.

To test whether this effect was mediated via a direct effect on the Hsp gene promoters, we transfected HepG2 cells with Hsp-70 and Hsp-90β promoter reporter constructs and treated the cells again with IFN-γ. As indicated in Fig. 2, IFN-γ stimulated the activities of the Hsp-70 and Hsp-90β promoters. These results suggest that IFN-γ is acting directly to increase Hsp-70 and Hsp-90 levels by activating the corresponding gene promoters.

In other IFN-γ-responsive cell types, the effects of IFN-γ are mediated by the activation and phosphorylation of the STAT-1 pathway. We therefore determined whether overexpression of STAT-1 in HepG2 cells could also activate the Hsp-70 and Hsp-90β promoters. As illustrated in Fig. 2, co-transfection of an expression vector for STAT-1 resulted in enhanced stimulation of both Hsp-70 and Hsp-90β promoters upon addition of IFN-γ. In contrast, overexpression of STAT-1 alone caused only a modest stimulation of either promoter, suggesting that STAT-1 requires Janus kinase-STAT-1 activation and phosphorylation via IFN-γ for maximal Hsp promoter activation.

To confirm that the effect of IFN-γ on the Hsp genes was mediated via STAT-1, we repeated the above experiments in the STAT-1-deficient cell line U3A. As shown in Fig. 3, INF-γ was unable to significantly enhance the levels of Hsp-70 and Hsp-90 in U3A cells. However, when STAT-1 was re-introduced into the STAT-1-deficient cells (U3AST1), INF-γ treatment enhanced the levels of both Hsp-70 and Hsp-90 (Fig. 3). Similar results were also obtained by transfection studies with the Hsp-70 and Hsp-90β promoters positively responding to IFN-γ in U3AST1 cells but not in the U3A cells (data not shown). Hence, the induction of Hsp-70 and Hsp-90 expression by IFN-γ is dependent upon STAT-1.

As described previously, STAT-3-like binding sites are present on the Hsp-90β promoter in close proximity to the HSE (−643 to −623 base pairs) (10). In addition, analysis of the Hsp-70 promoter also indicated a similar STAT-3-like binding site close to the HSE (−110 to −90 base pairs) (Fig. 4). Because STAT-1 and -3 proteins recognize very similar binding sites, we investigated whether the IFN-γ signaling effect observed above is mediated by this same element by analyzing various Hsp-70 and Hsp-90β reporter constructs. As illustrated in Fig. 5 and Table I, by deleting the region of the Hsp-90β promoter from −1044 (construct A) down to −299 base pairs (construct C) we abolished the activation of the promoter by IFN-γ and STAT-1. In addition, a Hsp-70 construct that was deleted from −188...
FIG. 3. Western blot showing the protein levels of Hsp-70 and Hsp-90 in untreated (C), IFN-γ-treated (50 ng/ml) STAT-1-deficient U3A cells, or U3A-ST1 cells in which a STAT-1 gene was reintroduced. The actin level was also measured to assess for protein loading.

FIG. 4. Sequences of the 70- and 90-HSE/STAT region of the Hsp-70 and Hsp-90β promoter. The homologies for the consensus binding sites for HSF-1 (19) and STAT are shown (12, 20).

FIG. 5. Functional CAT activity of the full-length and truncated Hsp-70 and Hsp-90 reporter constructs, respectively, containing or lacking the STAT/HSE region in response to either IFN-γ or STAT-1 overexpression in HepG2 cells. CAT activity was assessed from a total of three experiments (mean ± S.D.).

(construct LSN) to −100 (construct LSNP), resulting in the loss of the HSE-containing region, was unresponsive to IFN-γ and STAT-1 (Fig. 5 and Table I). Hence, in both promoters, stimulation by IFN-γ is eliminated by deletions that remove the region of the promoter containing the HSE/STAT-like element, suggesting that this element is necessary in both the Hsp-90β and Hsp-70 gene promoters for them to respond to activated STAT-1 produced by IFN-γ.

To address this point further, oligonucleotides containing the HSE/STAT site in the Hsp-70 and Hsp-90β promoters were coupled to the heterologous thymidine kinase promoter and assessed for responsiveness to IFN-γ and STAT-1 activation. As shown in Table I, both HSE/STAT-Hsp-70 and HSE/STAT-Hsp-90β constructs conferred responsiveness to STAT-1 activation by IFN-γ to the heterologous promoter with similar induction being observed to that seen with the intact Hsp promoters. Hence this short region of either promoter can render a heterologous promoter inducible by IFN-γ and STAT-1.

To demonstrate that the effect of STAT-1 on gene transcription via the HSE/STAT region was mediated via binding of STAT-1 to this region of both the Hsp-90β and Hsp-70 promoter, we examined the ability of IFN-γ to induce DNA binding of STAT-1 to the labeled 70- and 90-HSE/STAT oligonucleotides in band shift assays. The IFN-γ-activated site (GAS) m67SIE oligo probe containing STAT-1 binding sites was used as a positive control. As shown in Fig. 6, a low mobility-retarded band was obtained with both the 70- and 90-HSE/STAT probe, and the intensity of this band was enhanced in response to IFN-γ or heat shock. The binding observed was sequence-specific because it was competed with excess unlabeled probe but not with a nonspecific probe. In addition the retarded band was also competed with the unlabeled m67SIE oligonucleotide containing a consensus STAT-1 binding site, suggesting that the band was likely to contain STAT-1. This was confirmed by showing that the band was abolished by an addition of anti-STAT-1 antibody but not by an anti-STAT-3 antibody. Moreover, this band was also abolished by the addition of an antibody to HSF-1, indicating that it also contains HSF-1. Hence exposure to IFN-γ and/or heat shock results in the detection of a complex containing both STAT-1 and HSF-1, which binds to the HSE/STAT element in the Hsp-70 and Hsp-90 gene promoters. This complex was virtually abolished by mutation of the STAT site in the 90-HSE/STAT element (Fig. 6C), indicating that the DNA binding of the complex containing the two transcription factors is strongly reduced by mutating the binding site for one of the two factors.

In view of the finding that the response to IFN-γ/STAT-1 was mediated via binding sites adjacent to the HSF-1 region and formation of a DNA binding complex containing both STAT-1 and HSF-1, we wished to investigate whether the HSF-1 and HSF-1 pathways interacted with one another. To do this, we performed co-transfection studies in the U3A cell line (lacking endogenous STAT-1) with the Hsp reporter constructs together with expression vectors for STAT-1 or HSF-1 alone or in combination. Interestingly, STAT-1 and HSF-1 had an additive effect in activating both the Hsp-70 and Hsp-90β constructs compared with the effect of either factor alone (Fig. 7, A and B). We also investigated whether a similar effect could be observed by exposing the cells to a heat shock stress, which activates HSF-1, alone or in combination with IFN-γ, which activates STAT-1. As shown in Fig. 8, A and B, an additive effect was observed on both the Hsp-70 and Hsp-90 promoters when both HSF-1- and STAT-1-activating stimuli were given together compared with the effect with either stimulus alone. This suggests that although both factors are bound to the promoter following exposure to either stimulus alone, maximal activa-

| Construct | CAT activity |
|-----------|--------------|
| IFN-γ     | +IFN-γ       |
| Hsp-90 (A) (−1044) | 1.1 ± 0.22 | 4.9 ± 0.72 |
| Hsp-90 (C) (−288)  | 1.8 ± 0.37 | 1.77 ± 0.33 |
| 90-HSE/STAT (−643 to −623) | 1.2 ± 0.33 | 5.5 ± 0.69 |
| Hsp-70 (LSN)         | 0.44 ± 0.18 | 3.4 ± 0.41 |
| Hsp-70 (LSNP)       | 0.72 ± 0.30 | 0.82 ± 0.29 |
| 70-HSE/STAT (−132 to −105) | 1.5 ± 0.21 | 5.2 ± 0.81 |
| pBLCAT2          | 1.2 ± 0.25 | 1.4 ± 0.27 |
tion is only observed when both stimuli are present, presumably resulting in the post-translational activation of both factors, for example by phosphorylation.

The finding that the binding sites for HSF-1 and STAT-1 are close together and the formation of a DNA binding complex containing both factors suggest the possibility of a direct physical protein-protein interaction between HSF-1 and STAT-1. We therefore performed co-immunoprecipitation experiments by immunoprecipitating with anti-STAT-1 and then immunoblotting the precipitate with a specific antibody to HSF-1. As illustrated in Fig. 9, a visible HSF-1-specific band was observed when cell extracts were immunoprecipitated with an anti-STAT-1 antibody, and the immunoprecipitate was then probed by Western blotting with anti-HSF-1. No HSF-1 band was observed by Western blotting after immunoprecipitating with an anti-STAT-3 antibody, although STAT-3 itself was readily detectable in the immunoprecipitate. Hence HSF-1 interacts specifically with STAT-1 but not with STAT-3.

DISCUSSION

Although the mechanisms mediating the induction of Hsp genes by heat stress have been intensively studied (3, 14, 16) and shown to predominantly involve the HSF-1 transcription factor, much less attention has been paid to elucidating the mechanism mediating Hsp gene expression modulated by non-stressful stimuli such as immune activation. Our previous reports (6, 10) together with our present study have analyzed the molecular mechanisms regulating Hsp gene expression by the cytokines IL-6 and IFN-γ. Thus, IL-6 has been demonstrated to induce the expression of Hsp-90 via signaling pathways activating the transcription factors STAT-3 and NF-IL6 (10). Here we have shown that IFN-γ is also able to activate the promoters for Hsp-70 and Hsp-90β via the STAT-1 signaling pathway.
that is able to bind to the HSEs on Hsp promoters. HSF-1 has also been recently demonstrated to regulate the IL-1 promoter under non-heat stress conditions (18). Our present results have also shown that HSF-1 interacts with other transcription factors to modulate Hsp promoter activity under non-heat stress conditions. These studies suggest that HSF-1 is able to modulate promoter activity in the absence of physiological stress. A number of studies have demonstrated that transcriptional activity is attained by protein-protein interaction of transcription factors on DNA response elements. Recently HSF-3 and c-Myb have been reported to interact physically, and such interaction may be important in the regulation of Hsp gene expression during cellular proliferation (19).

Our results are the first demonstration of HSF-1 forming a direct protein-protein interaction with another transcription factor, STAT-1, and suggest that the effect of HSF-1 and STAT-1 on the Hsp promoters may require such physical interaction. Thus, in contrast to the additive effect of STAT-1 and HSF-1, STAT-3 and HSF-1 appear to have a mutually antagonistic effect. Although STAT-3 and HSF-1 can individually activate the Hsp-90β promoter, such activation is greatly reduced in the presence of both factors together (10). Interestingly, unlike STAT-1 and HSF-1, STAT-3 and HSF-1 do not interact with one another in the same conditions where a STAT-1/HSF-1 interaction can be demonstrated (Fig. 8). It is possible therefore that HSF-1 and STAT-3 compete for binding to the short Hsp promoter region that contains both of these binding sites resulting in an antagonistic effect. In contrast, the protein-protein interaction between STAT-1 and HSF-1 may facilitate binding of both factors to the promoter leading to enhanced activation of transcription. Although the sequence of the STAT-like binding site located around the HSE region of both the Hsp-70 and Hsp-90β promoter does not fully match the previously characterized STAT-1 binding site ATT-NNNNAAT (12), there is evidence that the consensus STAT binding site on other genes characterized recently may not match fully, for example CTGGNAA, which is found in the promoter of the CD14 binding protein gene (20). The ability of STAT-1 to bind to the sequence in the Hsp-70 and Hsp-90 promoters has been directly confirmed in our experiments where the protein binding to this element in IFN-γ-treated cells was identified as STAT-1 on the basis of its reactivity with anti-STAT-1 antibody and its removal with competitor oligonucleotide containing a consensus STAT-1 binding site.

From our previous and present data, it is clear therefore that the Hsp-70 and Hsp-90β promoters contain a composite response element that is able to integrate multiple regulatory signals and allow different transcription factors to interact with each other to have either a stimulatory or inhibitory effect on transcriptional activation. Preliminary analysis of other Hsp promoters in different species shows similar sequences with potential binding sites for STATs and NF-IL6 around the HSE and suggests that such a region has been conserved during evolution. This conserved element may thus be of critical importance in regulating the activation of Hsp genes by non-stressful stimuli and integrate the responses with their responses to stressful stimuli.

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