The Heat Shock Protein 90-CDC37 Chaperone Complex Is Required for Signaling by Types I and II Interferons

Received for publication, September 8, 2005, and in revised form, October 25, 2005. Published, JBC Papers in Press, November 9, 2005, DOI 10.1074/jbc.M509901200

Limin Shang and Thomas B. Tomasi

From the Laboratory of Molecular Medicine, Department of Immunology, Roswell Park Cancer Institute, Buffalo, New York 14263 and Departments of Medicine and Microbiology and Immunology, School of Medicine and Biomedical Sciences, State University of New York, Buffalo, New York 14214

Interferon signaling pathways are critical to both innate and adaptive immunity. We have demonstrated here that the inhibition of heat shock protein 90 (Hsp90) functions by small interfering RNAs or chemical inhibitors blocking interferon-induced gene expression. Hsp90 was required for signal transducers and activators of transcription 1 phosphorylation, and in its absence, Janus kinase (JAK) 1/2 were degraded by the proteosome. JAK1 interacts with Hsp90 and the CDC37 co-chaperone, and both interactions are destabilized by Hsp90 inhibitors. The biological consequences were suggested by experiments showing that T cell activation by interferon-γ primed macrophages and the antiviral response of interferons required Hsp90. We conclude that JAK1/2 are client proteins of Hsp90 and that Hsp90 and CDC37 play a critical role in types I and II interferon pathways.

Interferons were originally described as antiviral agents and have broad functions in immunity to infectious agents, tumors, and autoimmune diseases (1–7). Most interferon-inducible genes are activated through the JAK/STAT2 pathway (8, 9). STAT1 and STAT2, following phosphorylation by JAKs, form a heterodimer and associate with the interferon-responsive factor (IRF)-9 to produce the interferon-stimulated gene-factor-3 complex. This complex induces transcription of IFN-α/β target genes having an interferon-stimulated response element site in their promoter (8). IFN-γ, on the other hand, activates JAK1 and JAK2, which in turn phosphorylate STAT1 on Tyr-701. Phospho-STAT1 forms a homodimer and enters the nucleus to activate target IFN-γ-inducible genes (9). During this process, STAT1 is also phosphorylated on Ser-727, which is required for maximal transcriptional activity (10, 11).

Heat shock protein 90 (Hsp90) is an abundant, highly conserved cellular chaperone that functions as a key component of a multiprotein chaperone complex, which includes CDC37 and several other proteins that regulate folding, maturation, stabilization, and renaturation of a select group of client proteins (12, 13). CDC37 is important in the context of this work, because it is thought to specifically strengthen the interaction between Hsp90 and kinase clients (14, 15). Many Hsp90 client proteins are involved in tumorigenesis, and interruption of Hsp90-managed pathways has a broad effect on cell growth and susceptibility to apoptosis (12, 13), which has made Hsp90 a prospective target in cancer treatment. Geldanamycin (GA), a benzoquinone ansamycins, specifically inhibits Hsp90 by binding to its ATP-binding pocket, and a derivative of GA, 17-AAG, designed to treat cancer, is now in clinical trials (16).

Following IFN-γ signaling, Hsp90 protein levels are increased, and STAT1 has been shown to interact with HSF1 and activate the Hsp90β promoter (17). However, it is not clear whether Hsp90 plays a role in interferon pathways and JAK/STAT signaling. Here we have demonstrated that STAT1 phosphorylation induced by IFN-γ is inhibited when the function of Hsp90 or its co-chaperone CDC37 is blocked by siRNA or by chemical inhibitors. In addition, we have demonstrated that the IFN-α pathway is dependent on Hsp90 and CDC37. Further study has established that JAK1 interacts with Hsp90 as well as CDC37, and both interactions are destabilized by the inhibition of Hsp90. Ex vivo experiments showed that Hsp90 was required for macrophage activation and T cell proliferation induced in a mixed lymphocyte reaction and for IFN-α- and -γ-mediated antiviral effects directed against the vesicular stomatitis virus (VSV). Our results have demonstrated an important role for Hsp90 in JAK/STAT signaling and interferon-induced gene expression and potentially in immune surveillance pathways involving types I and II interferons.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Human cervical carcinoma cell line HeLa was from American Type Culture Collection (ATCC) (Manassas, VA) and cultured according to ATCC’s instruction. JAK1-deficient HeLa-derivative cell line E2A4 was provided by Dr. Naveen Bangia and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 µg/ml amphotericin B, 4% fetal bovine serum. For PECs, mice were pretreated with thioglycolate for 3–5 days and adherent cells (>80% macrophages) recovered. For mouse BMDC, bone marrow was harvested from mouse femurs and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 µg/ml amphotericin B, 10 ng/ml granulocyte/macrophage colony-stimulating factor, and antibiotics. After culturing the cells for 5 days, spontaneously released non-adherent cells were collected and adherent cells (>80% macrophages) recovered. For mouse BMDC, bone marrow was harvested from mouse femurs and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 µg/ml amphotericin B, 10 ng/ml granulocyte/macrophage colony-stimulating factor, and antibiotics. After culturing the cells for 5 days, spontaneously released non-adherent cells were collected for further analysis. Human IFN-α and IFN-γ were obtained from R & D Systems (Minneapolis, MN). GA and radicicol were from Sigma. GA concentrations were selected that did not alter cell viability according to trypan blue staining. Because the sensitivities of different cell lines to GA varied greatly, GA concentrations used in this study varied from 50 nM in HeLa cells up to 5 µM in...
A20 cells. 100 units/ml IFN-γ and 500 units/ml IFN-α were used throughout, unless otherwise specified.

Construction of Plasmids and Transient Transfections—The original JAK1 expression plasmid was kindly provided by Dr. Heinz Baumann. The sequence for the FLAG tag was inserted at the 5′-end of a JAK1 expression sequence using the QuickChange II XL site-directed mutagenesis kit from Stratagene (La Jolla, CA). The correct sequences were verified by DNA sequencing. Plasmids were transfected into E2A4 cells using Effectene from Qiagen (Valencia, CA). For GA treatment, cells were first transfected for 24 h and then treated with 1 μM GA for 1 h.

Reporter Gene Assay—PIV-CIITA-luciferase construct was described previously (20). 24 h after transfection, the cells were either pretreated with GA for 3 h or left untreated. IFN-γ was then added, and the cells were incubated for 24 h. Luciferase and β-galactosidase activities were determined, and Luciferase was normalized with the β-galactosidase activity of the same sample. Results were expressed as the fold induction calculated from the ratio between Luciferase activities of the treated and untreated samples.

Transient Transfection with siRNA—Hsp90 and CDC37 stealth siRNA were designed using the BLOCK-iT™RNAi Designer and synthesized by Invitrogen. The target sequences for the siRNAs were: Hsp90α, 5′-CCCAG UUGAU GUCAU GUAUC AUCAA-3′; Hsp90β, 5′-GGCAG AGGAA GAGAA AGGUG AGAAA-3′; and CDC37, 5′-ACAGA UCAAG CACUU UGGCA UGCCU-3′. β-actin siRNA and scrambled siRNA were from Ambion (Austin, TX). All siRNAs were transfected into HeLa cells using Lipofectamine 2000.

Flow Cytometry, RT-PCR, and Real-time PCR—Flow cytometric analysis of cell surface markers was previously described (50). RT-PCR and real-time PCR analysis were performed according to a previous report (50). Primers and probes for other RT-PCR and real-time PCR are shown in the supplementary methods. For real-time PCR data analysis, the ΔΔCt method was employed as described previously (50).

Co-immunoprecipitation and Western Blot Analysis—Cells were scraped from the plates and lysed on ice for 30 min in lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 0.5% deoxycholate, 20 mM sodium molybdate) supplemented with protease mixture (from Sigma), 1 mM NaF, and 2 mM sodium orthovanadate. For immunoprecipitation, 500 μg of cell extract was incubated with relevant antibody at 4 °C on an end-to-end rotator. The supernatant was centrifuged at 10,000 × g for 10 min, and protein G beads (Upstate Biotechnology, Lake Placid, NY) were added. The mixture was rotated at 4 °C for 3 h, and the immunocomplexes were washed with lysis buffer three times. SDS sample buffer was added to the beads, and the beads were incubated at 95 °C for 5 min and the supernatant subjected to Western blot analysis. The Hsp90 antibody was provided by Dr. David Toft. Other antibodies used in this study were anti-p5727-STAT1 (United States Biological, Swampscott, MA), anti-pY701-STAT1 (Cell Signaling, Beverly, MA), anti-STAT1, anti-STAT2, anti-CD37, anti-JAK1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-pY689-STAT2 (Upstate Biotechnology), anti-JAK2 (Biosource, Camarillo, CA), goat anti-rabbit IgG-horseradish peroxidase and goat anti-mouse IgG-horseradish peroxidase (Promega, Madison WI), and mouse IgG True-Blot (eBioscience, San Diego, CA).

T Cell Alloactivation—Adherent cells isolated from BALB/c PECs were treated with 200 or 500 nm GA for 3 h and then IFN-γ was added for 24 h. After treatment, adherent PECs were washed and treated with 30 gray of γ-irradiation to inhibit proliferation. Nylon wool-purified T cells (4 × 10^5 cells/well) from C57BL/6 mouse spleens were mixed with the PECs (1 × 10^7 cells/well) and incubated for 72 h at 37 °C, and 3H-thymidine incorporation was measured by liquid scintillation.

Cytotoxic Effect Assay—HeLa cells were plated into 96-well plates with 1 × 10^3 cells/well and either treated with 100 nM GA or left untreated for 12 h. The cells were then either treated with 250 units/ml IFN-α, 500 units/ml IFN-γ, or left untreated for another 12 h, infected with different amounts of VSV Indiana strain (ATCC) for 24 h, and stained with crystal violet.

RESULTS

Hsp90 Is Required for IFN-γ-induced MHC Classes I, II, and CD40 Expression—During an investigation of the antitumor effects of Hsp90 inhibitor GA in vitro, we noted that the drug affected the expression of several genes critical to immunity. GA in non-apoptotic concentrations (supplemental Table I) essentially eliminated IFN-γ-induced MHC classes I and II mRNA and cell surface protein expression on the human cervical carcinoma cell line HeLa (Fig. 1, A–C). However, the constitutive expression of MHC class I in HeLa was not altered by GA (Fig. 1, A and C). The GA derivative 17-AAG used in clinical trials gave a quantitatively similar inhibitory effect as GA on HeLa cells. Two other IFN-γ-inducible cells, SK-N-SH (human) and Colon26 (murine), showed results similar to HeLa (data not shown). The inhibitory effects of GA were also found in normal cells, and GA substantially suppressed MHC class II expression induced by IFN-γ in mouse primary kidney cells (supplemental Fig. SLA) and mouse peritoneal exudate cells (PECs) (Fig. 1, D and E). CD40, an important co-stimulatory factor was induced by IFN-γ in mouse PECs, and the induction was suppressed by GA (Fig. 1F). However the constitutive expression of MHC class II in both B cells (A20) and mouse bone marrow dendritic cells (mBMDC) was not altered by GA treatment (Fig. 1, G and H). The use of non-apoptotic concentrations, the preservation of MHC classes I and II constitutive expression, and the stable levels of the housekeeping gene GAPDH demonstrate that the effects of GA are not due to a general inhibition of gene expression. In addition, both IFN-γ and histone deacetylase (HDAC) inhibitor trichostatin A were shown to up-regulate MHC class II expression in the mouse pre-B cell line NFS-5, and GA specifically inhibited IFN-γ-induced MHC class II expression but had no effect on trichostatin A-induced MHC class II expression (supplemental Fig. S1B). This again illustrates the specificity of the inhibitory effects of GA on the IFN-γ pathway. The effect of GA on IFN-γ-induced MHC class II is likely because of its well established and highly specific inhibitory effect on Hsp90 chaperone activity. In support of this, radicicol, another Hsp90 inhibitor structurally unrelated to GA, also suppressed IFN-γ-induced class II expression (supplemental Fig. S1C).

The class II transactivator (CIITA) is the “master regulator” of MHC class II, and three cell-type-specific forms (PI, PIII, PIV) have been identified (18). Promoter regulation of the B cell CIITA (PII-CIITA) is quite different from the classical IFN-γ-inducible type (PIV-CIITA), in that the PII proximal promoter does not contain STAT1 or IRF-1 binding motifs (19, 20). In HeLa cells, IFN-γ-induced PII- and PIV-CIITA were both inhibited by GA (Fig. 2A). The IFN-γ-induced PIII-CIITA in HeLa cells is most likely regulated by the upstream IFN-γ regulatory site previously described (21). Consistent with its lack of effect on constitutive MHC class II expression in B cells, GA did not significantly alter the high levels of constitutive PIII-CIITA in mouse A20 cells (Fig. 2B). In macrophage lineage cells, PI-CIITA was stimulated by IFN-γ. PI-CIITA induction by IFN-γ in a mouse macrophage cell line IC-21 and in PECs were both inhibited by GA (data not shown and Fig. 2C), indicating that PI-CIITA induction by IFN-γ also requires Hsp90. It is not clear how PI-CIITA expression is controlled by the IFN-γ pathway, because an...
Hsp90 Inhibition Blocks IFN-γ-stimulated MHC Classes I and II and CD40 Expression.

Shown are RT-PCR and flow cytometric analyses of CIITA, MHC II, MHC I, or CD40 in cells pretreated with varying concentrations of GA and subsequently stimulated with GA plus 100 units of IFN-γ for 24 h. A, RT-PCR of Hela cells pretreated with 100 nM GA followed by GA plus IFN-γ. B, flow analysis for MHC II on Hela cells treated as in A. C, flow analysis of MHC I on cells treated as in A. D, RT-PCR of MHC II in mouse PECs pretreated with 500 nM GA and stimulated with GA plus IFN-γ. E, flow analysis of MHC II on PECs pretreated with 500 nM GA and stimulated with GA plus IFN-γ. F, same as E, except analyzed for CD40. G and H, real-time PCR analysis of MHC II and its independence of GA treatment in the mouse A20 cell line (G) and mouse bone marrow dendritic cells (mBMDC) (H).
IFN-γ activation site/IRF-1 site has not been identified in the PI-CIITA proximal promoter (18). The constitutive level of PI-CIITA in mBMDC was not suppressed by GA (Fig. 2D). Thus, IFN-γ induction of all three CIITA promoters requires functional Hsp90, whereas the constitutive PIII-CIITA in B cells and constitutive PI-CIITA in dendritic cells are independent of Hsp90.

GA Affects IFN-γ-induced Gene Expression by Inhibiting STAT1 Phosphorylation—We next explored whether the effects of GA were specific for certain immune genes or more general to the IFN-γ-signaling pathway. Initial microarray studies using HeLa cells and human genome gene set U133 plus 2.0 suggested a general inhibition by GA of multiple IFN-γ-induced genes, including those known to regulate MHC expression, whereas GA showed little effect on the basal expression of most of these genes (data not shown). Results on selected classical IFN-γ-induced genes (GBP1, IRF-1, STAT1, and TAP1) were confirmed by RT-PCR (Fig. 3A). The Hsp90 inhibitor radicicol also suppressed multiple IFN-γ-induced genes (data not shown). Because many IFN-γ-induced genes are activated through JAK/STAT signaling, we studied the effect of Hsp90 inhibitors on this pathway. GA pretreatment caused a dose-dependent decrease of IFN-γ-induced STAT1 phosphorylation on Tyr-701 and Ser-727 (Fig. 3B). Tyr-701 phosphorylation precedes STAT1 dimerization, and Ser-727 phosphorylation is required for maximum transcriptional activation (9–11). Consistent with these effects, IFN-γ-induced expression of a luciferase gene driven by the PIIV-CIITA promoter, a known STAT1- and IRF-1-dependent promoter was blocked by GA in a transient transfection experiment (supplemental Fig. S1D).

Hsp90 Maintains JAK1/2 and Prevents Their Proteosome-mediated Degradation—Hsp90 has been reported to interact with STAT3, and the interaction is required for STAT3 phosphorylation (22). It seemed reasonable therefore to hypothesize that STAT1 might be a client of Hsp90 based on the structural similarity between STAT1 and STAT3. However, GA treatment did not show an effect on constitutive STAT1 levels (Fig. 3B), whereas most Hsp90 clients were degraded by GA treatment. In addition, GA did not alter STAT1 dephosphorylation when added after IFN-γ was withdrawn (supplemental Fig. S2A). This observation makes it unlikely that Hsp90 stabilizes a phospho-STAT1 structure or protects STAT1 from dephosphorylation. GA also showed no effect on IFN-γ-induced STAT1 phosphorylation when it was added immediately before IFN-γ, even at high concentrations (10 μM) (supplemental Fig. S2B), suggesting that Hsp90 is probably not required to maintain STAT1 in a structure that is accessible to JAK1-mediated phosphorylation. Furthermore, IFN-γ induction of STAT1 gene expression, which is independent of STAT1 phosphorylation (23), was also blocked by GA (Fig. 3A). These observations suggest that an event upstream of STAT1 phosphorylation is responsible for Hsp90 inhibition.

Because JAK1 and JAK2 are the protein kinases responsible for Tyr-701-STAT1 phosphorylation, the effect of GA on JAK1 and JAK2 was investigated. Decreased levels of JAK1 became apparent in HeLa cells
after a 2.5-h treatment with 1 μM GA, and thereafter, the level progressively decreased (Fig. 3C). Prolonged treatment (8 h) essentially eliminated JAK1 protein expression. JAK2 followed a similar course as JAK1, and decreased levels became apparent after a 4-h GA treatment and decreased steadily with treatment. Although there were some variations in the kinetics of JAK1 and JAK2 degradation, 1 μM GA for 8 h showed a substantial effect on both JAK1 and JAK2 protein levels in HeLa cells. The degradation of JAK1 and JAK2 proteins were mediated by the proteosome, because co-treatment with lactacystin, a specific proteosome inhibitor, restored JAK1 and JAK2 levels in the GA-treated cells (Fig. 3D). MG-132, another proteosome inhibitor, showed similar effect as lactacystin (data not shown). The role of the proteosome has been shown for other Hsp90 clients (16), but JAK1 and 2 have not, to our knowledge, been reported to be clients of Hsp90. Because type I interferon signaling also requires JAK1, it would be expected that GA would block this pathway. Consistent with this are experiments showing that GA substantially suppressed IFN-α-inducible genes and their suppression by GA in HeLa cells.

**FIGURE 3.** GA inhibits IFN-γ and IFN-α pathways by down-regulating JAK1 and JAK2 expression and STAT1 phosphorylation. A, RT-PCR of representative IFN-γ-inducible genes and their suppression by GA in HeLa cells. B, Western analysis of IFN-γ-stimulated STAT1 phosphorylation and its inhibition by GA. C, total JAK1 and JAK2 protein levels in HeLa cells were determined by Western blots after treatment with GA for the specified lengths of time (in hours). D, the proteosome inhibitor lactacystin reverted the effect of GA on JAK1 and JAK2. E, Western analysis of STAT1 and STAT2 phosphorylation after GA and IFN-α treatment in HeLa cells. F, RT-PCR of representative IFN-α-inducible genes and their suppression by GA in HeLa cells.

**FIGURE 4.** Hsp90 siRNA synergizes with GA in blocking the IFN-γ pathway. A, Hsp90 siRNA knocked down Hsp90 protein levels by immunoblot. β-Actin was used as a loading control. B, Hsp90 siRNA suppressed STAT1 phosphorylation synergistically with GA treatment. For GA treatment, cells were first transfected with 20 nM each of Hsp90a and Hsp90β siRNAs for 56 h and then treated with 10 or 20 nM GA. After 16 h of incubation, the cells were subjected to IFN-γ treatment or left alone for 30 min. Scrambled siRNA (40 nM) was used as a negative control. Shown are RT-PCR (C) and real-time PCR (D) analyses of the effects of Hsp90 siRNA and GA on IFN-γ-induced CITTA. For real-time PCR analysis, samples were standardized with GAPDH values of each sample and error bars represent the S.D. of three independent measurements.
siRNA knockdown, because the protein is present in high concentrations in cells and occurs in two biologically active isoforms. We prepared stealth siRNA to both the Hsp90 α and β isoforms and demonstrated that, when combined, they substantially, but not completely, reduced Hsp90 levels in HeLa (Fig. 4A). However, this treatment did not block STAT1 phosphorylation, presumably because of residual functional Hsp90. Nevertheless, siRNA to Hsp90 α and β synergized with low concentrations of GA to significantly inhibit STAT1 phosphorylation and the IFN-γ activation of CIITA (Fig. 4, B and C).

CDC37, unlike Hsp90, does not have multiple isoforms and is present in low concentration, potentially making it a preferable target for siRNA knockdown. SiRNA to CDC37 was designed and its efficiency verified by Western analysis (Fig. 5A). CDC37 siRNA alone inhibited IFN-γ-induced STAT1 phosphorylation and synergized with suboptimal concentrations of GA (Fig. 5B).

The effect of CDC37 siRNA on the IFN-γ pathway was also manifest by inhibition of the IFN-γ target gene (CIITA) and by blocking of a reporter construct driven by the PIV-CIITA promoter (Fig. 5, C and D). Consistent with the ability to inhibit STAT1 phosphorylation, CDC37 siRNA also repressed IFN-α-induced gene expression (Fig. 5E). These experiments suggest that both Hsp90 and CDC37 play an important role in interferon-mediated gene expression pathways.

**Hsp90 and CDC37 Bind JAK1 in a Complex That Is Destabilized by GA**—To further explore the interactions of Hsp90 and CDC37 with JAK1, we determined whether JAK1 binds to Hsp90 by co-immunoprecipitation. FLAG-tagged JAK1 was constructed and shown to be competent in restoring STAT1 phosphorylation in transient transfection of E2A4, a JAK1-deficient derivative of HeLa (supplemental Fig. S2C). The FLAG antibody was able to co-precipitate Hsp90 in FLAG-JAK1-transfected E2A4 cells but not in FLAG control vector-transfected E2A4 cells (Fig. 6A). CDC37 was also found to interact with JAK1 by co-immunoprecipitation. The interaction of JAK1 with both Hsp90 and CDC37 was decreased following a 1-h treatment with 1 μM GA (Fig. 6A), consistent with previous reports that inhibitors of Hsp90 block the interaction between Hsp90 and its client proteins (14, 24, 25). Hsp70 has also been reported to be a component of the Hsp90 chaperone complex (13), and it also co-precipitated with FLAG-JAK1 (Fig. 6A). GA treatment for 1 h did not alter the level of Hsp70 in HeLa cells (data not shown). However, the interaction between JAK1 and Hsp70 was enhanced by GA treatment (Fig. 6A), suggesting that Hsp90 client proteins are shifted to an Hsp70 complex when Hsp90 function is inhibited (26). The co-immunoprecipitation between JAK1 and Hsp90, as well as between JAK1 and CDC37, was also shown to be blocked by GA when Hsp90 and CDC37 antibodies were used for the initial precipitation (Fig. 6, B and C). Furthermore, endogenous JAK1 was shown to co-immunoprecipitate with Hsp90, because Hsp90 was detected in the immune complex when JAK1 antibody was used to precipitate HeLa cell extract, whereas IRF-1 antibody did not precipitate Hsp90 (Fig. 6D). These data suggest that Hsp90 and CDC37 are components of a complex that interacts with and stabilizes JAK1.

**GA Suppresses IFN-γ-stimulated T Cell Alloactivation**—Because IFN-γ activates macrophage cells and enhances their capacity to induce T cell proliferation, we evaluated the effects of Hsp90 inhibition on T cell activation in an ex vivo alloassay. Adherent cells from PECs of BALB/c (H-2b) mice were treated with IFN-γ alone or together with different concentrations of GA. The cells were then γ-irradiated and incubated with isolated T cells from spleens of allogeneic C57BL/6 (H-2b) mice. Untreated allogeneic PECs elicited a low level of T cell proliferation, which was greatly enhanced by prior treatment of PECs with IFN-γ (Fig. 7A). GA suppressed macrophage activation by IFN-γ in...
Hsp90 Is Required for IFN Types I and II Pathways

In this study, we demonstrated for the first time that JAK1 and likely JAK2 are client proteins of Hsp90 and that the inhibition of Hsp90 function by chemical inhibitors or siRNA blocks types I and II interferon pathways. Mechanistic studies show that JAK1 and -2 are degraded by the proteasome in the absence of functional Hsp90, leading to a defect in STAT1 phosphorylation. Hsp90 and CDC37 bind to JAK1 and are required to maintain types I and II interferon-induced gene expression. Ex vivo experiments showed that Hsp90 is required for T cell activation by IFN-γ-stimulated macrophages in an allogeneic lymphocyte reaction and in interferon-α- and -γ-mediated protection against the cytopathic effects of VSV.

Hsp90 has been proposed to be a remnant of an ancient antigen presentation system, and evidence has been presented for its involvement in MHC class I peptide processing and cross-presentation (27–29). Our results showed that Hsp90 is also required for IFN-γ-induced MHC classes I and II expression. With the exception of professional antigen-presenting cells, most cells do not express MHC class II constitutively, and IFN-γ is a major inducer of MHC class II in these cell types (30, 31). Regulation of MHC class II expression occurs mainly at the transcriptional level, and both constitutive and inducible MHC class II expression are controlled by CIITA, which in turn is tightly regulated transcriptionally by three cell type-specific promoters (31). PIV was originally designated as the IFN-γ-inducible promoter, and although it is the major promoter type activated by IFN-γ in many cells including epithelial cells, recent studies have demonstrated that all three promoters are inducible by IFN-γ (18–21, 32, 33). The studies reported here demonstrate that Hsp90 is required in the IFN-γ-induced transcription of P1, PII, and PIV-CIITA, and as such, Hsp90 is an important “manager” of CIITA, the master regulator of MHC class II transcription. The Hsp90 inhibitor studies clearly illustrate that PI- and PIII-CIITA utilize unique and separable mechanisms for constitutive and inducible expression. This confirms previous work showing the distinct regulation of constitutive expression of these two CIITA types (18, 33) and the identification of an upstream IFN-γ regulatory site for IFN-γ induction of PIII-CIITA (21). It will be of interest in future studies to determine whether the PI-CIITA promoter has a unique IFN-γ-regulated site out-

**DISCUSSION**

Inhibition of Hsp90 Compromises the Antiviral Effects of IFN-α and IFN-γ—Biological and clinical studies have shown the antiviral effects of both types of interferons (2, 4). To determine whether the inhibition of interferon pathways by GA would affect their antiviral activity, we pretreated HeLa cells with either IFN-α alone or IFN-α with GA. The cells were then infected with different amounts of VSV. The cytopathic effect assays demonstrated that IFN-α induced >50-fold protection against VSV in HeLa cells and that the protection was markedly impaired by GA (Fig. 7B). Similarly, IFN-γ showed a weaker but definite (5–10-fold) protection against VSV infection of HeLa cells, and the protection was compromised by GA pretreatment (Fig. 7C).

In this study, we demonstrated for the first time that JAK1 and likely JAK2 are client proteins of Hsp90 and the inhibition of Hsp90 function by chemical inhibitors or siRNA blocks types I and II interferon pathways. Mechanistic studies show that JAK1 and -2 are degraded by the proteasome in the absence of functional Hsp90, leading to a defect in STAT1 phosphorylation. Hsp90 and CDC37 bind to JAK1 and are required to maintain types I and II interferon-induced gene expression.

**FIGURE 6. JAK1 binds to Hsp90 and CDC37 in a complex that is disrupted by GA. A–C, co-immunoprecipitation of Hsp90 and CDC37 with FLAG-JAK1 and their inhibition by GA in JAK1-deficient E2A4 cells. Lanes 1, control vector; lanes 2, FLAG-JAK1; lanes 3, FLAG-JAK1 treated with 1 μM GA for 1 h; lanes 4, lysate from FLAG-JAK1-transfected E2A4 cells without immunoprecipitation. In A, anti-FLAG antibody was used for immunoprecipitation (IP). Hsp90 antibody was used for immunoprecipitation in B and CDC37 antibody in C. D, interaction between Hsp90 and endogenous JAK1 in HeLa cells. HeLa cell lysates were immunoprecipitated and blotted with the indicated antibodies. Lane 3 is the HeLa cell lysate without immunoprecipitation as a positive control for Hsp90.**

**FIGURE 7. T cell activation by IFN-γ-primed macrophage as well as the antiviral effects of IFN-α and IFN-γ were suppressed by inhibition of Hsp90. A, GA inhibits IFN-γ-stimulated macrophage activation of T cells in an allogeneic lymphocyte reaction. Adherent PECs from BALB/c (H-2d) mice were used to stimulate T cells from C57BL/6 (H-2b) mice. IFN-γ-primed macrophage as well as the antiviral effects of IFN-α and IFN-γ were suppressed by inhibition of Hsp90. A, GA inhibits IFN-γ-stimulated macrophage activation of T cells in an allogeneic lymphocyte reaction. Adherent PECs from BALB/c (H-2d) mice were used to stimulate T cells from C57BL/6 (H-2b) mice. Shown also are cytopathic effect assays of the antiviral effects of IFN-α (B) and IFN-γ (C) and their inhibition by GA.**
side of the proximal promoter similar to that of PIII-CIITA. It is notable that the differences in inducibility of the cell type-specific CIITAs are not related to global defects in the IFN-γ signaling, as shown by the failure of IFN-γ to stimulate PI-CIITA in HeLa, a cell that has a robust response to IFN-γ, which includes PIII- and PIV-CIITA. This suggests the presence of an as yet undefined cell type-specific factor(s) and/or co-factor(s) involved in PI-CIITA transcription in Mφ/dendritic cells. The enhanced expression of MHC class I after IFN-γ treatment is also repressed by GA, likely via the inhibition of CIITA, which is known to regulate MHC class I as well as class II (31).

Our previous work has demonstrated that the HDAC inhibitor trichostatin A can induce MHC class II in certain cells by a CIITA-independent pathway (34). This was shown here for a pre-B cell line, and additionally, although GA inhibited the IFN-γ induction of class II, it did not affect the up-regulation of class II by trichostatin A. This is not only confirmation of the difference between IFN-γ and HDAC inhibitor pathways of the MHC class II gene induction but also further confirms that the effects of GA show specificity.

The effects of Hsp90 inhibitors are not restricted to CIITA and MHC genes but are a manifestation of a more general effect on the interferon pathways. Hsp90 and CDC37 siRNAs and chemical inhibitors block interferon signaling by abrogating STAT1 phosphorylation. However, the STAT1 protein does not appear to be the primary target of Hsp90, because inhibition of Hsp90 function shows no immediate effect on STAT1 phosphorylation, and prolonged treatment with Hsp90 inhibitors does not significantly alter endogenous STAT1 protein levels. On the other hand, JAK1 and JAK2 proteins were shown to be down-regulated by GA treatment, and this inhibition is likely mediated by the proteasome, because proteosome inhibitors were able to restore JAK levels. Further study showed that JAK1 binds to both Hsp90 and its kinase-specific co-chaperone CDC37 and the interactions were impaired by GA treatment. The interaction between Hsp70 and JAK1 is not, however, inhibited by GA treatment. It has been shown that, when the interaction of the Hsp90-CDC37 complex with its client proteins is destabilized by GA treatment, the dissociated clients are transferred to an Hsp70 containing complex before degradation (26). Our results are consistent with this model and strongly suggest that JAK1, and likely JAK2, are client proteins of Hsp90. Inhibitors of HDACs have been shown to block the IFN-γ pathway by a mechanism involving STAT1 that has not been completely defined (23). Because HDAC inhibitors enhance Hsp90 acetylation, which impairs Hsp90 function (35, 36), one possibility suggested by our work is that HDAC inhibitors block the IFN-γ pathway by inducing Hsp90 acetylation and JAK degradation. Our results also suggest that HDAC inhibitors might repress the IFN-α pathway by affecting STAT phosphorylation in addition to its known effects downstream of the interferon-stimulated gene factor-3 complex (37).

Of note in this study is the finding that CDC37 is required for STAT1 signaling and that CIITA induction by IFN-γ is inhibited by CDC37 siRNA. CDC37 was identified as a factor required for G1 cell cycle progression and usually acts in concert with Hsp90, perhaps as a kinase-targeting co-factor, although CDC37 has been reported to have an inherent chaperone activity independent of Hsp90 (39). CDC37 plays a critical role in v-Src oncogenes and is required, together with its partner Hsp90, in maintaining multiple protein kinase pathways implicated in apoptosis induction (14, 15). Here we have shown that CDC37 inhibition is sufficient to repress both types I and II interferon pathways. Thus, CDC37 could also be a target for therapeutic interventions, either alone or in combination with Hsp90. Because the Hsp90 chaperone mechanisms are complex with multiple co-chaperones (13), it seems likely that regulation of immune genes via the Hsp90 pathway may involve additional factors than those reported here. Studies along these lines are currently in progress.

The interferons were discovered as agents having antiviral activity and have numerous direct effects on viral replication, activation of cellular antiviral effector mechanisms, and on the development of innate and adaptive immunity to viruses (2–4, 39). Viral infections also induce the production of type I interferons, which activate multiple genes that are involved in viral resistance. Some of these genes can have a direct effect on viral replication and survival, such as the 2′,5′ OAS5 gene, whose expression was shown to require Hsp90 and CDC37. In the intact animal, IFN-α/β mediates protective effects by inducing MHC class I, stimulating dendritic cell maturation and enhancing IFN-γ production by CD4 T cells (40–42). These activities, at least in part, may be regulated by Hsp90. In this study, we have shown that Hsp90 is required for the IFN-α-mediated antiviral response to VSV. We did not evaluate other pathways, such as the recently described IFN-As, which have potent antiviral effects mediated via class II cytokine receptors (43). These receptors are distinct from those of the IFN-α system but also signal via the Jak/STAT pathway and may therefore be dependent on Hsp90. IFN-γ is particularly important in the activation of macrophages involved in the resistance to a variety of infectious agents and is also a critical factor in tumor immunity (2, 5). We found that IFN-γ markedly enhances the ability of murine macrophages to activate T cells in an allospecific lymphocyte reaction and that macrophage activation is blocked in a dose-dependent manner by Hsp90 inhibitors. The requirement for Hsp90 in macrophage activation, together with its effect on viral infection, suggests its potential biological relevance in host resistance. In addition to its prominent role in interferon signaling, JAK1 is also required for cytokines, which signal through GP130 (interleukin-6, and -11), oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor, and granulocyte colony-stimulated factor) as well as those signaling through the common γ chain (interleukin-2, -4, -7, -9, -15, and -21) (44). Because of the broad and important role these cytokines play in immunity and hematopoiesis, further studies of the effects of Hsp90 and CDC37 on signaling by these cytokines are indicated to appreciate the full spectrum of Hsp90 influence.

In normal cells, Hsp90 is primarily in an uncomplexed, low affinity state (45). However, when under stress (such as during chemotherapy, concomitant infections, autoimmune attack, etc.), we speculate that Hsp90 in normal cells may shift to a complexed (with co-chaperones), high affinity state, similar to that which has been described in tumor cells (46). Stressed normal cells and potentially autoimmune cells could therefore become more sensitive to GA. In this regard, both IFN-γ and particularly IFN-α have been implicated in the development of autoimmunity (7). Disseminated lupus would be a particularly relevant model to investigate the role of Hsp90, because both IFN-α and IFN-γ pathways are thought to be etiological factors in this disease (7, 47, 48).

Finally, it has been suggested that Hsp90 is a capacitor during evolution of the species and is responsible for canalization, a process by which the stability of the phenotype is maintained during development and macroevolution (49). It has been proposed that, during evolution, the complexity of signaling pathways increase, and this contributes to the stabilization of the phenotype against environmental changes (50). Hsp90 is a candidate-buffering factor, which by virtue of its ability to maintain the function of key kinase signaling pathways enhances stability during evolution. We suggest that Hsp90 also normally functions as an immune capacitor and maintains critical immune network components of the IFN-γ and IFN-α/β pathways and possibly others yet to be identified.
Hsp90 Is Required for IFN Types I and II Pathways

Acknowledgments—We acknowledge and thank Dr. David Toft for the Hsp90 antibody, Dr. Naveen Bangia for the E2A4, Dr. Heinz Baumann for the JAK1 expression plasmid, and utilization of the core facilities at Roswell Park Cancer Institute supported by a National Cancer Institute Grant CA16056. We also thank Drs. David Toft, Heinz Baumann, and Elizabeth Repasky for reviewing this manuscript.

REFERENCES

1. Levy, D. E., and Darnell, J. E., Jr. (2002) Nat. Rev. Mol. Cell Biol. 3, 651–662
2. Schroder, K., Hertzog, P. J., Ravasi, T., and Hume, D. A. (2004) J. Immunol. 172, 1723–1728
3. Biron, C. A. (2001) Immunity 15, 39–50
4. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., and Schreiber, R. D. (2002) Nat. Immunol. 3, 95–101
5. Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M. F., Fritz, L. C., and Burrows, R. I., and Lindquist, S. (1997) Cell 91, 9578–9583
6. Luft, T., Pang, K. C., Thomas, E., Hertzog, P., Hart, D. N., Trapani, J., and Cebon, J. (1998) J. Immunol. 161, 1947–1953
7. Nguyen, K. B., Watford, W. T., Salomon, R., Hofmann, S. R., Perez, C., and Lindermann, J. (1957) Proc. Natl. Acad. Sci. U. S. A. 43, 425–431
8. Yamaoka, K., Saharinen, P., Pesu, M., Holt, V. E., III, Silvennoinen, O., and O'Shea, J. J. (2004) J. Exp. Med. 199, 101, 9578–9583
9. Decker, T. (2003) Annu. Rev. Immunol. 21, 419–440
10. Issacs, A., and Lindermann, J. (1957) Proc. Soc. Exp. Biol. Med. 94, 253–256
11. Muhlethaler-Mottet, A., Otten, L. A., Steimle, V., and Mach, B. (1997) EMBO J. 16, 2851–2860
12. Langer, J. A., Sheikh, F., Dickensheets, H., and Donnelly, R. P. (2003) Nat. Immunol. Today 1, 286–292
13. Rafique, S. A., and Behrens, T. W. (2004) Curr. Opin. Immunol. 16, 801–807
14. Arlander, S. J., Eapen, A. K., Vroman, B. T., McDonald, R. J., Toft, D. O., and Karnitz, L. M. (2003) J. Biol. Chem. 278, 52572–52577
15. Lehtinen, L., Minnaugh, E. G., De Costa, B., Myers, C. E., and Neckers, L. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8324–8328
16. Xu W., Minnaugh, E., Rosser, M. F., Nicchitta, C., Marcu, M., Yarden, Y., and Neckers, L. M. (2001) J. Biol. Chem. 276, 3702–3708
17. Triantafillou, K., Triantafillou, M., and Dedrick, R. L. (2001) Nat. Immunol. 2, 338–345
18. Mathias, S., Murata, S., Shimbara, N., Tanaka, N., Chiba, T., Tanaka, K., Yui, K., and Udono, H. (2002) J. Exp. Med. 196, 185–196
19. Wrana, J. L., and Srivastava, P. K. (2005) Nat. Immunol. 6, 593–599
20. Klemm, D., Trowsdale, J., and Toft, D. O. (2003) J. Exp. Med. 199, 393–406
21. Arlander, S. J., Eapen, A. K., Vroman, B. T., McDonald, R. J., Toft, D. O., and Karnitz, L. M. (2003) J. Biol. Chem. 278, 52572–52577
22. Whitesell, L., Minnaugh, E. G., De Costa, B., Myers, C. E., and Neckers, L. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8324–8328
23. Xu W., Minnaugh, E., Rosser, M. F., Nicchitta, C., Marcu, M., Yarden, Y., and Neckers, L. M. (2001) J. Biol. Chem. 276, 3702–3708
24. Muhlethaler-Mottet, A., Otten, L. A., Steimle, V., and Mach, B. (1997) J. Biol. Chem. 272, 233–240
25. Shah, M., Patel, K., Fried, V. A., and Sehgal, P. B. (2002) J. Biol. Chem. 277, 45662–45669
26. Klampfer, L., Huang, J., Swaby, L. A., and Augenlicht, L. (2004) J. Biol. Chem. 279, 30358–30368