The Role of Hsp90N, a New Member of the Hsp90 Family, in Signal Transduction and Neoplastic Transformation*

Nicholas Grammatikakis‡§, Adina Vultur‡§, Chilakamarti V. Ramana‡, Aliki Siganou‡, Clifford W. Schweinfest**, Dennis K. Watson**, and Leda Raptis‡ ‡‡

From the Departments of Microbiology and Immunology, Queen’s University, Kingston, Ontario K7L 3N6, Canada, the Department of Molecular Biology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195, and the Laboratory of Cancer Genomics, Medical University of South Carolina, Charleston, South Carolina 29425

The 90-kDa heat shock protein (Hsp90), the target of the ansamycin class of anti-cancer drugs, is required for the conformational activation of a specific group of signal transducers, including Raf-1. In this report we have identified a 75-kDa Raf-associated protein as Hsp90N, a novel member of the Hsp90 family. Intriguingly, the ansamycin-binding domain is replaced in Hsp90N by a much shorter, hydrophobic sequence, preceded by a putative myristylation signal. We demonstrate that, although much less abundant, Hsp90N binds Raf with a higher affinity than Hsp90. In sharp contrast to Hsp90, Hsp90N does not associate with p50 Bcl2, the Hsp90 kinase cofactor. Hsp90N was found to activate Raf in transiently transfected cells, while Raf F11 fibroblasts stably transfected with Hsp90N exhibited elevated activity of the Raf and downstream ERK kinases. This may be due to Raf binding to myristylated Hsp90N, followed by Raf translocation to the membrane. To examine whether Hsp90N could therefore substitute for Ras in Raf recruitment to the cell membrane, Hsp90N was transfected in c-Ras-deficient, 10T1/2-derived preadipocytes. Our results indicate that, as shown before for activated Ras or Raf, the introduction of even low levels of Hsp90N through transfection in c-Ras-deficient preadipocytes causes a dramatic block of differentiation. Higher levels of Hsp90N expression resulted in neoplastic transformation, including interruption of gap junctional, intercellular communication, and anchorage-independent proliferation. These results indicate that the observed activation of Raf by Hsp90N has a profound biological effect, which is largely c-Ras-independent. With the recent finding that p50 Bcl2 is tumorigenic in transgenic mice, these results reinforce the intriguing observation that the family of heat shock proteins represents a novel class of molecules with oncogenic potential.

The classical mitogen-activated protein kinase/ERK1 kinase cascade, composed of c-RafI (Raf), MEK, and ERK itself, relays proliferative signals from the plasma membrane to the transcriptional and cell cycle progression machinery (1–3). Several lines of experimental evidence have illustrated the central importance of the Raf kinase in mammalian cell signaling. Surprisingly, despite the fact that Raf has been one of the most well studied kinases, its mode of regulation remains largely unsolved (1, 3). Extensive genetic and biochemical data indicate that, following growth factor receptor stimulation, Rap1-GTP tethers Raf to the plasma membrane for further activation by phosphorylation (reviewed in Ref. 1). In fact, recruitment of Raf to the membrane was found to be both necessary and sufficient for activation, as demonstrated in experiments where Raf was artificially targeted to the membrane through fusion to a myristylation signal sequence. Such membrane-bound Raf was constitutively active, even in the absence of Ras-GTP (4, 5).

In addition to membrane targeting, regulation of Raf function requires the interaction with a number of partners. In fact, extensive genetic, biochemical, and pharmacological evidence indicates that the chaperone Hsp90 is an important Raf activator (6–9). Hsp90 is an essential and abundant heat-shock protein in all eukaryotes studied, and is highly conserved in evolution. Unlike other, promiscuous heat shock proteins, Hsp90 exhibits specificity for its clients; moreover, in contrast to other chaperones, Hsp90 binds substrates which have already reached their correct folded state post-translationally (reviewed in Refs. 10 and 11). Due to the fact that a large proportion of its clientele is composed of proteins involved in proliferation and cell cycle progression, Hsp90 has emerged as the cardinal chaperone for signal transducers, tightly connected to the function of kinases, as well as ligand-induced transcriptional factors, the latter mainly of the steroid hormone receptor family (10, 11). Hsp90 consists of two highly conserved domains, of 25 and 50 kDa, located at the N terminus and C terminus, respectively, connected with a highly charged linker (10). A structural hallmark of Hsp90 is an ATP/ADP-binding site embedded within its N-terminal 25-kDa domain. A class of ATP-mimetic drugs, including geldanamycin (GA), bind this nucleotide-pocket region and inhibit Hsp90 activity (10). According to the current model, GA competitively displaces ATP and locks Hsp90 into its inactive conformation. GA binding to Hsp90 results in Hsp90s dissociation from its targets, with the latter ultimately degraded upon prolonged in vivo GA

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‡ To whom correspondence may be addressed: Dept. of Microbiology and Immunology, Botterell Hall, Queen’s University, Kingston, Ontario K7L 3N6, Canada. Tel.: 613-533-6000 (ext. 75491); Fax: 613-533-6796; E-mail: ngrammat@earthlink.net.

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** To whom correspondence may be addressed: Dept. of Microbiology and Immunology, Botterell Hall, Queen’s University, Kingston, Ontario K7L 3N6, Canada. Tel.: 613-533-2462; Fax: 613-533-6796; E-mail: Rapitls@post.queensu.ca.

The abbreviations used are: ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase; GA, geldanamycin; Hsp90, 90-kDa heat shock protein; GST, glutathione S-transferase; KD, kinase-defective.
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treatment (10, 11). In addition to the above N-terminal located chaperone site and ATP/ADP pocket, the existence of a second nucleotide site and an independent substrate-binding site residing at the C-terminal 50-kDa domain of Hsp90 has been recently suggested (12–14). The importance of the C-terminal 50-kDa domain of Hsp90 is also underlined by the fact that it is also the region known to mediate the formation of Hsp90 dimers, the presumed active conformation of this chaperone (10, 11).

Activation of the Hsp90 client proteins is known to require the synergism of co-chaperones. According to one of the best studied in vivo models of Hsp90-regulated kinases, Hsp90, in synergy with p50cdc37, its kinase-specific cofactor and oncogene, regulates Raf function (7, 9). We have previously shown that, acting as a Hsp90-specific target modulator, p50cdc37 binds Hsp90 via its C-terminal half and to the catalytic domain of Raf through its N-terminal region (9). A ternary kinase-p50cdc37-Hsp90 complex is then formed, which is essential for catalytic activity (7, 9, 15). Treatment of mammalian cells with drugs which specifically inhibit Hsp90 results in the inability of Raf to become activated and, upon prolonged drug treatment, in the proteasomal degradation of the kinase (8). Finally, exclusion of Hsp90 from the Raf heterocomplex, through expression of dominant negative (DN) p50cdc37 mutants, inhibits Raf catalytic activity (9, 16). Notably, the above Raf population which is bound to DN p50cdc37 and almost entirely devoid of Hsp90, has a normal half-life, and is not degraded (9), underscoring the fact that the requirement for Hsp90 in Raf function (6, 8, 9, 16) may be at a level other than merely supporting the inherently unstable conformation of the kinase, as originally proposed (18, 19).

Hsp90N (originally named Hsp90αΔN), encoding a protein of ~75 kDa, is a novel Hsp90 isoform, originally isolated as a cDNA overexpressed in pancreatic carcinomas (23). Hsp90N shares the 509-amino acid C-terminal region with Hsp90; however, possibly due to differential promoter utilization or splicing, it differs from its abundant 90-kDa counterpart in that in place of the 223-amino acid N-terminal domain containing the nucleotide/ansamycin drug-binding site, it possesses a much shorter, 30-amino acid hydrophobic sequence (23). In the present article, we have identified and cloned Hsp90N as a novel component of the native Raf complex which regulates the catalytic activity of Raf and functions as a potent oncogene in established cell culture systems.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Culture Techniques, and Stable Gene Expression**—The rat F11 fibroblasts have been used extensively in the study of the effect of a variety of oncogenes upon the cellular phenotype because they display a very low rate of spontaneous transformation (20). The mouse 10T1/2 line has been previously described (21). Hsp90N was expressed in these cells through transfection with a pSG5-based plasmid (Stratagene) together with the py3 plasmid and hygromycin resistance selection (21). For the production of c-Ras-deficient cells, 10T1/2 fibroblasts were transfected with a retrovirus containing the Hsp90N insert were labeled at 48 h post-transfection with [3H]myristate and for 20 additional hours in fresh medium containing [3H]myristate (7, 9, 16). For expression of Hsp90N by in vitro translation or in mammalian cells, constructs containing the complete open reading frame in pSG5 in combination with the T7 RNA polymerase system (Promega) or subcloned in the pcMV7 and pSG5 vectors were used as described (23). For expression of c-Raf (Hsp90) by in vitro translation, a construct involving full-length Hsp90 in pGEM was generously provided by Dr. D. Toft (Mayo Clinic, Rochester, MN). Antibodies directed against Hsp90 (SPA-830 and SPA-771) and Hsp70 (SPA-812) were obtained from Stressgen. Antibodies against Raf (C-12), MEK, ERK-2, or GST (Z-5) epitope tag were obtained from Santa Cruz. Monoclonal antibodies against ERK1/2 were purchased from Transduction Laboratories, and anti-ERK antibody from Oncogene Science (pan-Ras Ab2), and the anti-FLAG antibody from Kodak. Anti-active polyclonal ERK1/2 antibodies were a gift of Dr. Erik Schaefer (QB/BIOSOURCE International).

**Transfection, Immunoprecipitation, and Immunoblotting**—CV-1 or COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and transfected with a total of 2.5 μg DNA using LipofectAMINE (Invitrogen). 24 h later, cells were incubated in fresh complete medium or serum starved, depending on the experiment. Cells were then lysed in extraction buffer consisting of 20 mM Hepes, pH 7.5, 0.1 mM NaCl, 2 mM EGTA, 0.5% Nonidet P-40, 10% glycerol, 50 mM glycylglycine, 2 mM dithiothreitol plus protease and phosphatase inhibitors (2 mM sodium vanadate, 1 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and soybean trypsin inhibitor), followed by detergent treatment under stringent conditions, the extraction buffer was supplemented with 2.5 M urea. For immunoprecipitation, immunoprecipitation was performed for 2 h at 4 °C, using the indicated antibodies and immune complexes recovered by binding to protein A/G-Sepharose (Sigma). Alternatively, GST fusion proteins were purified using pre-equilibrated GSH-Sepharose (9). After three washes with 50 volumes of extraction buffer, GSH-Sepharose-bound proteins and immunocomplexes were eluted with Laemmli SDS-loading buffer (25 mM Tris, pH 6.8, 1% SDS, 2.5% β-mercaptoethanol, 0.5 mg/ml bromophenol blue, 5% glycerol), separated by SDS-PAGE and transferred to a Hybond-ECL membrane (Amersham Biosciences Inc.). Immunoblot detection was performed with the antibodies specified in each experiment performed on 5% dried skim milk in phosphate-buffered saline and developed using alkaline phosphatase-coupled secondary antibodies reblotted to nitrocellulose membrane and proteins were visualized by autoradiography.

**In Vitro Synthesis of Radiolabeled Proteins and in Vivo Metabolic Labeling**—Hsp90 or Hsp90N full-length proteins were transcribed and translated in vitro from the pGEM or the pSG5 expression constructs in the presence of 20 μCi of [3H]methionine (Amersham Biosciences, Inc.), using the coupled rabbit reticulocyte lysate and SP6 or T7 RNA polymerase system, respectively (Promega). To examine protein myristylation in vitro, cells transfected with the empty vector (pSG5) or the vector containing the Hsp90N insert were labeled at 48 h post-transfection and for 20 additional hours in fresh medium containing [3H]myristate (9, 10–11) and for 10 min at ambient temperature.

For cell fractionation, following washing with ice-cold phosphate-buffered saline, cells were solubilized by sonication and resuspended in a hypotonic lysis buffer consisting of 25 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 10 mM NaF, 1 mM EGTA, 150 mM Na+, 1 mM dithiothreitol, plus phosphatase and protease inhibitors as described above. After 20 min on ice, cells were disrupted with 40 strokes in a Dounce homogenizer and centrifuged twice at 1,500 g for 10 min. The postnuclear supernatants were centrifuged at 100,000 × g for 30 min. Pellets (P100) were resuspended in the above buffer supplemented with 1% Nonidet P-40, sonicated for 3 min at 4 °C and centrifuged at 100,000 × g for 30 min. The supernatant, containing membrane proteins, was collected, protein concentration determined and either subjected to immunoprecipitation with anti-Raf antibodies or analyzed directly by SDS-PAGE followed by Western blotting as described above.

**Protein Purification and in Vitro Association Assays**—To identify Raf-associated proteins, Raf was transiently expressed in COS-1 cells as a GST fusion protein and bound proteins analyzed using glutathione-linked Sepharose pull-down assays. The 75-kDa GST-Raf-associated polypeptide was visualized by Ponceau S staining and cut out from the nitrocellulose membrane. It was then incubated in 100 mM Tris-HCl, pH 7.8, 1% Triton X-100, and 10% acetonitrile at 37 °C for 90 min, and
digested with trypsin for 16 h at 37 °C. The fragments were then microsequenced as described (24).

Procracyotically expressed GST fusion proteins were purified by GSH-Sepharose affinity chromatography in NETN buffer (20 mM Tris, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40) supplemented with protease and phosphatase inhibitor (9). Kinase-defective (KD) bacterial (His)_6-MEK-1 (K97M) was similarly prepared by affinity chromatography on a Ni²⁺ column (Qiagen). For studying in vitro associations, GSH-Sepharose-bound GST fusion proteins were directly incubated with in vitro translated proteins in NETN buffer for 2 h at 4 °C. Bound complexes were subsequently washed 3 times in NETN buffer and subjected to SDS-PAGE analysis. They were either immunoblotted or for [35S]methionine-labeled proteins, directly analyzed by fluorography.

**Protein Kinase Assays**—For kinase reactions, immunocomplexes were washed in 50 volumes of kinase buffer (25 mM Hepes, pH 7.5, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM dithiothreitol; 20 μM of [γ-32P]ATP and 0.5 μg of (His)₆-MEK-1 (K97M) protein substrate (9) were subsequently added to washed immunoprecipitates. After a 30-min incubation at room temperature, assays were terminated by the addition of Laemmli SDS loading buffer, the boiled samples resolved by SDS-PAGE and phosphorylated substrate proteins quantitated by autoradiography. As kinase assay controls, catalytically active Raf prepared by co-infection of insect SF9 cells with wild type Raf and v-Src, or inactive Raf (K375M) were used. A virally infected culture of SF9 cells was performed essentially as previously described (9, 25). 48 h post-infection, Raf was immunoprecipitated, quantitated, and tested for kinase activity as above.

**Anchorage-independent Growth Assays**—Approximately 10⁴ cells were suspended in 2 ml of 0.3% agarose (Sigma) in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum, on a feeder layer of the same medium containing 9.7% agarose, in 6-cm Petri dishes. Growth was recorded and photographs taken 20 days later under brightfield illumination. For anchorage-independent growth quantitation, cells were plated in Methocel (Sigma) in Dulbecco’s modified Eagle’s medium with 15% fetal calf serum and 20 μCi/ml ³Hthymidine (20). Twenty days later, cells were washed from the water-soluble Methocel and trichloroacetic acid-precipitable radioactivity determined.

**Induction and Quantitation of Adipocytic Differentiation**—Standard procedures described before were followed (22, 26). Briefly, ~10⁵ cells were seeded into each well of a 24-well Linbro plate (Corning) in 10% fetal calf serum. At confluence, cultures were treated with differentiating medium containing 10% fetal calf serum. This medium was changed 48 h later to medium from a marine mussel, according to the manufacturer’s instructions (Collaborative Research, Inc.).

**Examination of Gap Junctional, Intercellular Communication**—It was performed essentially as described (20, 27). Briefly, cells were grown on a glass slide, half of which was coated with electrically conductive, optically transparent, indium-tin oxide (Ask Science Products, Kingston, Canada). The fluorescent dye, Lucifer yellow (5 mg/ml from a marine mussel, according to the manufacturer) was added to the culture medium containing 10% fetal calf serum. This medium was changed 48 h later to medium containing 10 μg/ml insulin and 10% fetal calf serum. Lipid droplets were washed in 50 volumes of prechilled NETN buffer and after SDS-PAGE analysis they were either immunoblotted or, for [35S]methionine-labeled proteins, directly analyzed by fluorography.

**RESULTS**

**Identification of Hsp90N as a Distinct Hsp90 Variant Which Associates with Raf in Vivo and in Vitro**—Our search for Raf-associated proteins led us to a polypeptide migrating at the 70–75-kDa range on SDS-PAGE. Microsequencing revealed that this protein is identical to a previously identified fragment of the chaperone protein Hsp90, whose mRNA was found to be elevated in cDNA libraries from pancreatic cancer tissues (23). To examine the properties of the new Hsp90 isoform, its full-length cDNA (23) was transcribed and translated in vitro, in parallel with Hsp90. As expected, in vitro translated Hsp90N migrated as a 75-kDa polypeptide on SDS-PAGE, a size distinct from the one of Hsp90, the latter migrating at the 90-kDa range (Fig. 1A). To facilitate the functional characterization of Hsp90N, we raised a monoclonal antibody specifically recognizing the newly cloned protein, but not Hsp90. Indeed, as shown in Fig. 1B, the anti-Hsp90 antibody reacts with a 75-kDa polypeptide on Western blots of total cell extracts from CV-1 cells as expected, while an antibody specific for Hsp90 recognizes a 90-kDa protein. To confirm that the cloned protein is identical to the 75-kDa Raf-associated protein that is recognized by the anti-Hsp90 antibody, Hsp90N was overexpressed in CV-1 cells and its ability to bind Raf examined. As expected, anti-Raf immunoprecipitates contained a protein recognized by the anti-Hsp90 antibody and at the molecular weight range expected for the 75 kDa, Raf-associated protein (Fig. 1C, compare levels in lanes 1 and 2). Therefore, the cloned protein is able to associate with Raf upon transfection, supporting the conclusion that Hsp90N is the same Raf-bound 75-kDa protein we had observed in vivo. To test whether Raf and Hsp90N are able to also form a complex in vitro, Hsp90N was expressed by in vitro translation and allowed to bind to GST-Raf which had been produced in bacteria (Fig. 1D). As expected for proteins that interact directly, GST-Raf, but not the GST propeptide alone, was found to associate with [35S]methionine-labeled Hsp90N (Fig. 1D, compare lane 2 with lanes 1 and 3).

**Specificity of the Raf-Hsp90N Interaction**—Since MEK-1 and p50cdc37 are well known partners of Raf, we tested whether their native immunoprecipitates also contain Hsp90N. Endogenous p50cdc37 MEK, or Raf were immunoprecipitated from CV-1 cell extracts and the Western blot probed for Hsp90N. As shown in Fig. 2A, neither of the above two proteins were able to interact with Hsp90N (lanes 2 and 3). 14-3-3, another well characterized abundant component of the Raf1 signalsome was also found unable to bind Hsp90N; furthermore, in contrast to Raf, p50cdc37 MEK, or 14-3-3 were unable to associate with Hsp90N in in vitro binding assays (not shown). Interestingly, as the experiment in Fig. 2A also shows, anti-MEK1 immunoprecipitates were found to contain very low amounts of the abundant isoform, Hsp90, detectable only upon prolonged exposure (Fig. 2, middle panel, lane 3). Strikingly, p50cdc37, a kinase cofactor well known for its ability to associate in almost stoichiometrical amounts with Hsp90 (9), although as expected it bound strongly to this chaperone, it failed to interact with Hsp90N, a finding which underlines the specificity of the observed associations. Therefore, as shown in Fig. 2A (lane 1), Raf is able to bind roughly equal amounts of Hsp90N and Hsp90 in vivo. Since the levels of expression of Hsp90 are much greater than Hsp90N (Fig. 2A, left panel), it appears that the Hsp90N isoform binds Raf preferentially (Fig. 2A, compare total Western and immunoprecipitation panels).

To further confirm the above conclusions derived from examining the endogenous proteins, we tested the association of endogenous Hsp90N with transfected GST-tagged MEK-1, GST-p50cdc37, or GST-Raf in CV-1 cells (Fig. 2B). In a parallel assay we tested BXB-Raf, a widely used Raf construct devoid of the N-terminal regulatory domain, and hence constitutively active (amino acids 1–25308–648) (28). The results obtained confirm the conclusions drawn from the experiment described in Fig. 2A above. To further examine the specificity of interactions, we analyzed the association of ERK-2 and Hsp90, established partners of MEK-1, with p50cdc37 and Raf, respectively. As shown in Fig. 2B, ERK2 bound MEK and to a lesser extent p50cdc37, but not Raf. As an additional control, we also blotted against Hsp70, another chaperone which has been previously observed in complexes with inactive steroid hormone receptors and certain kinases. As shown in Fig. 2B, lane 3, there is a strong association between Hsp70 and Raf; this association has...
Fig. 1. Identification of Hsp90N as a distinct Hsp90 variant which associates with Raf in vivo and in vitro. A, the pSG5-Hsp90N and pGEM-Hsp90 plasmids were transcribed and translated in vitro using T7 RNA polymerase and a reticulocyte lysate system. 7.5 μl of each reaction alone (lanes 2 and 3) or mixed together (lane 4) were loaded on SDS-PAGE and visualized by fluorography. The sizes of molecular weight marker proteins are indicated at the right (in kDa). In lane 1, control reaction, where the pGEM-Hsp90 plasmid was transcribed and translated in the antisense orientation. B, total cell extracts from CV-1 cells were probed with the anti-Hsp90 (lane 1) or the anti-Hsp90N (lane 2)-specific antibodies, or a mixture of both (lane 3). C, Raf was immunoprecipitated from CV-1 cells transfected with the pSG5 vector alone (lane 1) or pSG5-Hsp90N (lanes 2 and 3). Immunoprecipitated protein complexes were examined by Western blot/ECL for the presence of Raf or the associated Hsp90N using antibodies against Raf (C-12) or Hsp90N, respectively, as indicated. In lane 3, control Raf immunoprecipitation using stringent conditions (see “Experimental Procedures”). D, the pSG5-Hsp90N plasmid was transcribed and translated in vitro using T7 RNA polymerase and a reticulocyte lysate system. 10 μl of each reaction were either analyzed directly (bottom panel) or assayed in vitro for binding to bacterially produced GST-Raf or GST propeptide alone (middle panel), as indicated. Hsp90N and the immobilized GST-Raf or GST proteins were visualized by fluorography or by anti-GST blotting, respectively. Lane 3 represents a control reaction, where the Hsp90N plasmid was transcribed and translated in the antisense orientation.

not been previously reported. In addition, the constitutively active Raf mutant BXB-Raf exhibits a weaker association with Hsp70. In contrast, BXB-Raf binds avidly to Hsp90N, even more strongly than its full-length Raf counterpart (Fig. 2B, compare lanes 3 and 4). The above results taken together lead us to conclude that Hsp90N is a novel, highly specific partner of Raf which binds the catalytic domain of the kinase with higher affinity than Hsp90.

Hsp90N activates Raf upon transient overexpression and associates with membrane Hsp90N—Previously reported genetic and biochemical data demonstrated that Hsp90, the abundant isoform of Hsp90N, regulates the activity of Raf, among other kinases (7, 9, 16, 18). Given the structural homologies and the demonstrated strong association of Hsp90N and Raf, we examined whether Hsp90N might also affect Raf activity. To this effect, Raf together with Hsp90N or control vector plasmid was transiently transfected in CV-1 cells. Cultures were serum-starved, Raf-immunoprecipitated, and examined for its in vitro kinase activity toward a recombinant, kinase-inactive (KD) MEK1 substrate, produced in bacteria. As shown in Fig. 3A (lanes 3 and 4), phosphorylation of the MEK substrate is increased, indicating that Hsp90N transfection stimulates Raf catalytic activity.

As mentioned above, an intriguing feature of Hsp90N is its unique N terminus; in place of the 25-kDa ATP/GA binding N-terminal domain present in Hsp90, Hsp90N possesses a unique 30-amino acid N-terminal domain: MGKASYCNCN-CFCVSKVECSFLYFLYWSYP. This domain is composed of mostly hydrophobic amino acids, preceded by a putative myristylation signal (GEKAS). Following this sequence there is a PKCβ zinc finger-like motif (CNCNFCSVKVEC) and a block of aromatic amino acids which resembles a caveolin-binding motif (FLYFLYWSYP) (29). The presence of these structural features implicates Hsp90N as possibly operating at the plasma membrane. Given that Raf activation is known to take place at the plasma membrane (1, 30), the strong Hsp90N-Raf interaction we have observed might be very significant for Raf regulation. It follows that if Raf is brought to the plasma membrane by the myristylated-Hsp90N for constitutive activation, this would provide a mechanistic explanation for the observed stimulatory effect of Hsp90N upon the Raf kinase, an event which occurs even in the absence of growth factor stimulation (Fig. 3A). To approach this question, we tested whether endogenous Raf normally associates with myristylated-Hsp90N at the plasma membrane and whether this association is increased in CV-1 cells upon transfection of Hsp90N, an event which stimulates the catalytic activity of the kinase (Fig. 3A).

Hsp90N or control vector were transfected in CV-1 cells and Raf immunoprecipitated from [3H]myristate-labeled cell extracts (Fig. 3B). As a control, Hsp90N was precipitated from the same extracts in parallel. As expected, a polypeptide co-immunoprecipitated with Raf at the size expected for Hsp90N (Fig. 3B, top panel). In addition, the amount of Hsp90N bound to Raf increased upon Hsp90N transfection (Fig. 3B, compare lanes 2 and 3). This band was indistinguishable from the one directly immunoprecipitated with anti-Hsp90N antibodies in terms of both size and immunoreactivity (Fig. 3B, bottom panel, lane 5). In a parallel assay, the above immunoprecipitates were examined by autoradiography for the presence of [3H]myristate-labeled polypeptides. The data shown in Fig. 3B, bottom panel, suggest that Hsp90N is a potential myristylated protein which associates with Raf in vitro. To examine whether an increase in Hsp90N expression results in an increased association with endogenous Raf at the plasma membrane, we repeated the experiment using cell fractionation. Endogenous Raf was immunoprecipitated from the membrane fraction of CV-1 cells that had been transfected with Hsp90N or control vector plasmids. As shown in Fig. 3C (compare lanes 1 and 2), upon Hsp90N transfection both the amount of plasma membrane-localized Raf and the Raf-Hsp90N association are in-
creased. The above experimental data provide evidence that Hsp90N is a novel Raf regulator which possibly functions by targeting Raf to the membrane for activation.

**Hsp90N Transfection in Rat F111 Fibroblasts Leads to Raf Activation and Neoplastic Transformation—**The Ras/Raf/MEK/ERK pathway has been shown to be crucial in mammalian cell signaling (2). To examine the biological consequences of Raf activation by Hsp90N, rat F111 fibroblasts were stably transfected with a plasmid containing the full-length Hsp90N gene under control of the SV40 promoter. A panel of 53 independent clones expressing different levels of Hsp90N were obtained by hygromycin resistance selection and subsequently tested for Hsp90N levels by immunoblotting of cell lysates (see “Experimental Procedures”). As shown in Fig. 4A, there is an approximately 5-fold increase in Hsp90N protein levels in line F111-Hsp90N-1a, compared with the background of control cells transfected with the pSG5 vector alone. Examination of Raf in vitro kinase activity toward MEK1 (KD) as substrate indicated a dramatic increase in Hsp90N-transfected, F111 cells compared with the parental line (Fig. 4B). These results indicate that, although Hsp90N is present in normal fibroblasts, its overexpression augments Raf activity.

To test whether Hsp90N expression also leads to activation of effectors downstream of Raf, we tested the same clones for ERK1/2 activity levels by immunoblotting with an anti-active ERK1/2 antibody. As shown in Fig. 4C (lanes 1–3), the observed increase in Hsp90N protein leads to a substantial increase in ERK1/2 activity which is proportional to the Hsp90N levels present.

To investigate the consequences of Hsp90N overexpression upon the cellular phenotype, cellular morphology of rat F111 cells stably expressing Hsp90N was microscopically examined. As shown in Fig. 5A, the parental F111 cells have a fibroblast-like, elongated shape when grown on plastic, while after Hsp90N overexpression (e.g. line F111-Hsp90N-1a) there is a dramatic change to a distinctly transformed morphology. When their ability to grow under anchorage-independent conditions was tested, these cells were found to be able to grow in agar (Fig. 5C, b), in a manner dependent upon levels of Hsp90N expression (26), although not as efficiently as F111 cells transformed by other oncogenes, such as the simian virus 40 large tumor antigen or v-Ras (Table 1).
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FIG. 3. Myristylated Hsp90N associates with Raf and targets it to the plasma membrane for activation. A, Raf together with Hsp90N or control vector plasmid was transiently transfected in CV-1 cells as indicated. Both cultures were serum-starved and extracts prepared as described under "Experimental Procedures." Raf in vitro kinase activity was measured toward recombinant inactive MEK1 substrate, following immunoprecipitation with anti-Raf antibodies (top panel). As a control (lane 1), an equal amount of lysate from the culture involving transfected Hsp90N was immunoprecipitated using non-immune serum. Lane 2 shows a similar immunoprecipitation as lane 4, except that the MEK1 substrate was omitted. Bottom panel, anti-Raf Western (WB)/ECL was performed to ensure that equal levels of Raf protein were used in the in vitro kinase reactions. B, Raf co-precipitates with a 75-kDa [3H]myristate-labeled protein which is recognized by the anti-Hsp90N antibody and comigrates with Hsp90N. Hsp90N or control vector plasmid were transiently transfected in CV-1 cells as indicated. 24 h later cells were further incubated with [3H]myristate for 20 h. Labeled cells were solubilized as described under "Experimental Procedures," and endogenous Raf immunoprecipitated from the Hsp90N or from the vector-transfected cultures (lanes 1–4). Among the latter, the culture transiently transfected with the Hsp90N construct was additionally subjected to Hsp90N immunoprecipitation (lanes 5 and 6). To detect the Raf-associated or directly immunoprecipitated [3H]myristate-labeled Hsp90N polypeptide, the membranes were subjected to autoradiography (bottom panel). For size comparisons, [3H]methionine-labeled Hsp90N from the in vitro translation reaction in Fig. 1A were run in lane 7. Note the presence of co-precipitated Hsp90N and Raf as weakly stained bands in lanes 3 and 5, top and bottom panels, respectively. Lanes 1, 4, and 6 involve parallel control immunoprecipitations using stringent conditions. C, endogenous Raf was immunoprecipitated from the membrane fraction of CV-1 cells which had been transiently transfected with Hsp90N or control vector plasmid. Both precipitates and control total cell extracts were examined for Raf, Hsp90N, or control epidermal growth factor receptor levels using the appropriate antibodies as indicated. The position and sizes of molecular weight markers are shown at the right of each panel.

scribed for F111 cells above (Fig. 4, A and B). Results from representative clones are shown in Fig. 4A, lanes 6–10. Line 25B8-Hsp90N-7a expressed ~6 times the Hsp90N levels of the control 25B8 transfected with the empty pSG5 vector (lanes 10 versus 6). As shown in Fig. 4B (lanes 7 versus 9), this led to an increase in Raf kinase activity in Hsp90N-transfected 25B8 cells, indicating that Raf activation by Hsp90N can occur even in the face of very low c-Ras levels. In addition, comparison of Raf kinase levels between serum-starved (lanes 11 and 13) and serum-stimulated (lanes 12 and 14) cultures of 25B8 and 25B8-Hsp90N-7a cells revealed an increase in both, indicating that Hsp90N transfected cells remain responsive to growth factor addition. In keeping with the results from the F111 system, examination of ERK1/2 activity levels showed a substantial increase in Hsp90N-expressing cells, indicating that this chaperone variant is able to activate ERK1/2 despite a c-Ras deficiency (Fig. 4C, lanes 4 versus 6).

To examine whether Hsp90N requires c-Ras activity to induce neoplastic transformation, we tested the transformation-related parameters of the Hsp90N-expressing, c-Ras-deficient, 25B8 cells. Due to c-Ras down-regulation, the growth rate of these cells is greatly reduced, compared with their 107½ counterparts with normal c-Ras levels (22). It was previously shown that their growth defect cannot be overcome through expression of oncogenes upstream of c-Ras, such as Src or the middle tumor antigen of polyoma virus, while these cells can be transformed by activated Ras or Raf (22). In fact, microscopic observation indicated a transformed morphology, increased growth rate, and cell density at confluence upon Hsp90N expression. In addition, examination of gap junctional communication using the in situ electroporation technique revealed a substantial reduction in dye transfer upon Hsp90N expression, compared with the parental cells (not shown). As shown in Fig. 6F, 25B8-Hsp90N-7a cells were found to be able to grow in an anchorage-independent manner, indicating that Hsp90N expression can lead to neoplastic transformation, even in a c-Ras-deficient cellular background.

Previous results demonstrated that the c-Ras-deficient, 25B8 cells are able to differentiate into adipocytes under conditions of confluence in high serum, as revealed by their extensive lipid
targets Raf to the plasma membrane for activation. Furthermore, possibly as a myristylated protein, Hsp90N preferentially with the isolated catalytic domain of the molecule. Lanes 7–25B8 transfected with the pSG5 vector lacking the Hsp90N insert and selected for hygromycin resistance. In fact, as shown in Fig. 6, even a 2-fold increase of Hsp90N expression in 25B8 cells (Fig. 6, lane 5; K375M, lane 6) led to an almost complete block in kinase activity is increased after Hsp90N expression. Raf-1 was immunoprecipitated from detergent cell extracts from the lines indicated at the top (lanes 2–4 and 7–9), and tested for in vitro kinase activity using kinase-dead, recombinant MEK1 as substrate (see “Experimental Procedures”). As controls, catalytically active (lane 5) or inactive (K375M, lane 6) Raf produced in Sf9 cells and a reaction lacking the MEK substrate (lane 10) were included. In addition, for the 25B8-control and 25B8-Hsp90N-7a lines, kinase assays were performed from serum-starved and serum-stimulated cultures (lanes 11 and 12, and 12 and 14, respectively). C, Hsp90N increases ERK1/2 activity. Extracts from the lines indicated at the top (see A above) were separated by electrophoresis and transferred to a nitrocellulose membrane which was probed with an antibody against the phosphorylated form of ERK1/2.

**FIG. 4.** Hsp90N expression leads to activation of Raf and ERK1/2. A, quantitation of Hsp90N protein levels by immunoblotting of cell lysates. Total proteins in detergent cell extracts from the lanes indicated at the top were resolved by electrophoresis and transferred to a nitrocellulose membrane which was probed with the antibody against Hsp90N. Lane 1, control F111 parental line transfected with the pSG5 vector lacking the Hsp90N insert and selected for hygromycin resistance. Lanes 2–5, Hsp90N-transfected F111 cells. Lane 6, control, c-Ras-deficient line, 25B8 transfected with the pSG5 vector lacking the Hsp90N insert and selected for hygromycin resistance. Lanes 7–10, Hsp90N-transfected 25B8 clones. F111-Hsp90N-1m and 25B8-Hsp90N-7m are mixtures of a number of transfected clones. Lower panel, blots of the above F111 and c-Ras-deficient 25B8 cell extracts were probed with an antibody to Ras. Horizontal bars point to the position of molecular weight standards. B, Raf kinase activity is increased after Hsp90N expression. Raf-1 was immunoprecipitated from detergent cell extracts from the lines indicated at the top (lanes 2–4 and 7–9), and tested for in vitro kinase activity using kinase-dead, recombinant MEK1 as substrate (see “Experimental Procedures”). As controls, catalytically active (lane 5) or inactive (K375M, lane 6) Raf produced in Sf9 cells and a reaction lacking the MEK substrate (lane 10) were included. In addition, for the 25B8-control and 25B8-Hsp90N-7a lines, kinase assays were performed from serum-starved and serum-stimulated cultures (lanes 11 and 12, and 12 and 14, respectively). C, Hsp90N increases ERK1/2 activity. Extracts from the lines indicated at the top (see A above) were separated by electrophoresis and transferred to a nitrocellulose membrane which was probed with an antibody against the phosphorylated form of ERK1/2.

accumulation which is microscopically visible under phase contrast or after Oil Red-O staining, even in the absence of insulin and dexamethasone (22). In fact, as shown in Fig. 6, even a 2-fold increase of Hsp90N expression in 25B8 cells (e.g. line 25B8-Hsp90N-3a, Table I) led to an almost complete block in their ability to differentiate into adipocytes; cells were unable to accumulate lipid, in the presence or absence of insulin and dexamethasone (Fig. 6, B and C). The above results taken together indicate that, consistent with the notion that Hsp90N is able to bring Raf to the membrane for activation, Hsp90N can exert its biological effect of transformation and differentiation block even in the face of very low c-Ras activity levels.

**DISCUSSION**

A rapidly growing body of literature has established the 90-kDa heat shock protein Hsp90 as a potential target of anticancer therapy (11). This has been based mainly on two realizations: first, the majority of its known substrates are signaling intermediates of clinical importance, including kinases (10, 11), which require Hsp90 for proper conformation and activity. Second, Hsp90 can be specifically inhibited by ansamycins and anticancer drugs which bind the nucleotide-binding pocket and inhibit the ATPase activity of the molecule. In this article we have identified for the first time a second isoform of Hsp90, in complex with a key signal transducer, the Raf kinase. Hsp90N associates with Raf in vitro and in vivo and preferentially with the isolated catalytic domain of the molecule. Furthermore, possibly as a myristylated protein, Hsp90N targets Raf to the plasma membrane for activation.

Hsp90N-mediated Raf Activation Is Independent of p50cdc37

Action—Previous results showed that, following growth factor receptor stimulation, Hsp90 is required for Raf activation, in concert with p50cdc37 (9). In fact, p50cdc37 was previously found to be absolutely essential for Hsp90 action onto a given client kinase in all cases studied (9, 15), and that the two complex with each other with high avidity (see Ref. 9, and also Fig. 2, B and C). Binding of p50cdc37 and Hsp90 was reported to be mediated through the C-terminal half of p50cdc37 (9, 15). As a result, C-terminal mutants of p50cdc37, although still competent to bind to the kinase (by binding through the intact N terminus), were unable to bind to and accumulate Hsp90 to the complex (7, 9, 15). The “dominant-negative”-like action of these mutants resulted in the formation of aberrant complexes with the kinase in which the kinase was not catalytically active (7, 9, 15). In addition, previous work revealed that once Hsp90 was pharmacologically blocked with geldanamycin, p50cdc37 was by itself unable to bind to and activate Raf, even in combination with activated tyrosine kinases (9, 15). Contrary to Hsp90, however, we failed to detect an association between Hsp90N and p50cdc37 (Fig. 3, B and C). Therefore, it appears that the 25-kDa N-terminal domain of Hsp90, which is absent in Hsp90N, might be critical for the Hsp90-p50cdc37 association. In addition, p50cdc37 coexpression by transient transfection was unable to either enhance the Raf-Hsp90N association or to further increase the Hsp90N-stimulated Raf activity (not shown). Thus, it appears that Hsp90N, by lacking the N-terminal ATPase activity of Hsp90 which is a crucial determinant...
for p50<sup>cdc73</sup> entering the complex and regulating Raf, fails to accumulate p50<sup>cdc73</sup> onto Raf. The above results taken together indicate that Hsp90N might bind to and regulate Raf on its own right and independently of p50<sup>cdc73</sup>. In support of such a concept, work from the Buchner and Hartl (13, 14) laboratories has demonstrated that the isolated C-terminal domain of Hsp90 possesses a chaperone activity of its own which is independent from the one residing at the N terminus. In addition, binding and release of this mutant to protein substrates in vitro was unaffected by Hsp90 cofactors (13). Since the above C-terminal chaperone site is apparently conserved in Hsp90N, it is reasonable to hypothesize that Hsp90N also possesses the capacity to bind to and chaperone its substrate(s) in a direct manner. How exactly Hsp90 and Hsp90N each act on Raf, once they make contact with the kinase, remains to be addressed in the future through direct experimentation, possibly involving a crystallographic approach. Nevertheless, our results show that Hsp90N binds Raf and positively affects its activity directly. Our present discovery of a natural N-terminal truncation mutant of Hsp90 which maintains the C-terminal chaperone site provides a physiological setting to test the above in vitro findings. In fact, our present observation that Hsp90N is able to bind to and activate Raf in vivo supports and extends the results from the Buchner and Hartl laboratories (13, 14). Finally, by lacking the GA-binding site, Hsp90N might be immune to the action of drugs which normally inhibit Hsp90. Given that these drugs are currently considered to have anticancer value, being under evaluation in NIH-sponsored clinical trials (11), a future task will be to carefully study the implications of Hsp90N overexpression in carcinogenesis.

Hsp90 Expression Induces Neoplastic Transformation and Blocks Differentiation in a c-Ras-independent Manner—Our results demonstrate that introduction of Hsp90N in rat F111 fibroblasts through transfection can activate Raf and induce neoplastic transformation; Hsp90N-expressing cells displayed a transformed morphology and were able to grow in the absence of anchorage to a solid surface. In addition, in agreement with previous data indicating that interruption of gap junctional, intercellular communication requires lower levels of oncogene expression than anchorage independent proliferation in a number of systems (27, 32), Hsp90N expression caused a dramatic reduction in junctional permeability in F111 fibroblasts. The observed phenotypic effects were found to clearly correlate with the levels of Hsp90N expression and Raf activation. Our results further show that Hsp90N can activate Raf and its downstream target, ERK1/2 under conditions of c-Ras deficiency (Fig. 4), indicating that Raf activation by Hsp90N may be to a large extent independent of c-Ras. It is tempting to speculate that Hsp90N, due to its ability to become myristylated and bind Raf, it directs Raf to the plasma membrane for further activation through phosphorylation. Nevertheless, Hsp90N-mediated Raf activation is further enhanced by serum treatment in c-Ras-deficient, Hsp90N-transfected 25B8 cells, indicating that Raf is regulated by additional mechanism(s), which depend on upstream growth factor signaling.

Previous results demonstrated a reciprocal regulation of differentiation and several transformation-associated properties in response to graded levels of v-ras or v-raf gene expression in 25B8 cells, with the loss of differentiative capacity, morphological changes and anchorage-independent proliferation requiring increasing levels of these oncogenes (22, 26). In addition, it was found that even very low levels of expression of oncogenes such as v-ras or v-raf, insufficient to restore the growth rate of the cells to normal, are enough to block differentiation (22). This makes these cells into a very sensitive test system to examine the neoplastic potential of even weakly transforming oncogenes, and its dependence upon Ras expression. The present data regarding Hsp90N, confirm and reinforce the above findings.

**Fig. 5. Hsp90N induces neoplastic transformation in rat F111 fibroblasts.** A, Hsp90N induces morphological transformation in rat F111 fibroblasts. Control F111 cells (a) are compared with their Hsp90N-transfected, F111-Hsp90N-1a counterparts (b). Bar corresponds to 100 μm. B, Hsp90N expression results in a dramatic reduction in gap junctional intercellular communication. The fluorescent dye, Lucifer yellow was introduced by electroporation into control F111 (a) or the Hsp90N-expressing F111-Hsp90N-1a (c) cells growing on partly conductive slides and cells photographed 5 min later under fluorescence illumination (see “Experimental Procedures”). b and d, same frames as in a and c, phase-contrast illumination. Bar corresponds to 100 μm. C, Hsp90N induces anchorage-independent cell growth. The control F111 line (a) or its Hsp90N-expressing F111-Hsp90N-1a counterpart (b) were placed in agar and photographed 20 days later under brightfield illumination. Bar corresponds to 100 μm.

**Table I**

| Cell line | F111 control<sup>a</sup> | F111-Hsp90N-7d | F111-Hsp90N-1a | FSV1a<sup>b</sup> | 25B8 control<sup>c</sup> | 25B8-Hsp90N-3a | 25B8-Hsp90N-7a | 25B8-vRas<sup>d</sup> |
|-----------|--------------------------|----------------|----------------|------------------|------------------------|----------------|----------------------|---------------------|
| Hsp90N levels<sup>e</sup> | 1 | 3.5 | 5 | 1 | 1 | 1 | 2 | 6 | 1 |
| %<sup>f</sup>[<sup>3</sup>H]T-cpm<sup>g</sup> | 2 | 2 | 53 | 100 | 100 | 1 | 1 | 40 | 80 |

<sup>a</sup> Control F111 and 25B8 lines, expressing the pSG5 vector lacking the Hsp90N insert.

<sup>b</sup> F111 cells expressing the large tumor antigen of simian virus 40.

<sup>c</sup> F111 cells expressing Ras<sup>Leu<sub>61</sub></sup>.

<sup>d</sup> Ras-deficient, 25B8 cells expressing Ras<sup>Leu<sub>61</sub></sup>.

<sup>e</sup> Hsp90N levels in transfected cells were measured by Western blotting and fluorimager analysis (Fig. 4A). Numbers refer to fold increase relative to the parental lines.

<sup>f</sup> 10<sup>4</sup> cells were plated in Methocel containing 20 μCi/ml [<sup>3</sup>H]thymidine for 20 days, then washed and trichloroacetic acid precipitable radioactivity determined. Incorporation is shown relative to that in SVLT-expressing F111 cells (clone FSV1a), which is taken as 100%. Numbers are averages of three independent experiments.

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observations; even low levels of Hsp90N in c-Ras-deficient preadipocytes can block differentiation of c-Ras-deficient preadipocytes, indicating that the observed activation of Raf by this chaperone variant has a profound biological effect, which is c-Ras-independent. Higher levels of Hsp90N expression brought about a fully transformed phenotype, including interruption of gap junctional communication and anchorage-independent proliferation.

In summary, we have identified Hsp90N as a novel component of the Raf signalosome. Our results show that Hsp90N, possibly as a myristylated protein, associates with Raf and targets it to the plasma membrane for activation, thus substituting for c-Ras in this role. However, since Hsp90 has been found to activate a number of kinases besides Raf, we cannot exclude the possibility that Hsp90N might have additional targets. We further show that, perhaps due to Raf activation (22, 26), cellular differentiation programs are exquisitely sensitive to changes in Hsp90N intracellular levels. The overexpression of Hsp90N in tumor tissues (23) points to the possibility that Hsp90N might be causally related to tumor formation. These results clearly demonstrate that a new class of cellular proteins, the chaperone family, which were heretofore thought to be involved mostly in protein folding, can function as oncoproteins, and in a largely c-Ras-independent manner.

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The Role of Hsp90N, a New Member of the Hsp90 Family, in Signal Transduction and Neoplastic Transformation

Nicholas Grammatikakis, Adina Vultur, Chilakamarti V. Ramana, Aliki Siganou, Clifford W. Schweinfest, Dennis K. Watson and Leda Raptis

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