Disruption of the Raf-1-Hsp90 Molecular Complex Results in Destabilization of Raf-1 and Loss of Raf-1-Ras Association*

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Cytosolic Raf-1 exists in a high molecular weight complex with the heat shock protein Hsp90, the purpose of which is unknown. The benzoquinone ansamycin, geldanamycin, specifically binds to Hsp90 and disrupts certain multimeric complexes containing this protein. Using this drug, we are able to demonstrate rapid dissociation of both Raf-1-Hsp90 and Raf-1-Ras multimeric complexes, concomitant with a markedly decreased half-life of the Raf-1 protein. Continued disruption of the Raf-1-Hsp90 complex results in apparent loss of Raf-1 protein from the cell, although Raf-1 synthesis is actually increased. Prevention of Raf-1-Hsp90 complex formation interferes with trafficking of newly synthesized Raf-1 from cytosol to plasma membrane. These data indicate that association with Hsp90 is essential for both Raf-1 protein stability and its proper localization in the cell.

Raf-1, a serine/threonine kinase, is part of a highly conserved kinase cascade that mediates signaling by extracellular growth factors and leads to the stimulation of mitogen-activated protein kinases (1, 2). Raf-1 functions downstream of Ras, which in its active, GTP-bound state binds directly to the amino-terminal regulatory domain of Raf-1 (3). This interaction is transient and apparently serves to recruit Raf-1 to the cell membrane (4, 5), a step that is necessary for Raf-1 activation. The requirement for Ras can be bypassed by coupling a plasma membrane targeting signal to Raf-1 (6). Following its recruitment by Ras, Raf-1 associates with cytoskeletal components via an unknown mechanism (7).

Although activated Raf-1 is plasma membrane-associated, this kinase is primarily cytosolic in location and exists in a native heterocomplex with the heat shock proteins Hsp90 and p50 (8). Hsp90 is an ubiquitously expressed molecular chaperone that has been found in complexes with a variety of proteins including steroid hormone receptors, dioxin receptor, actin, v-src, and other kinases (9–13). Raf-1 binds to Hsp90 via its COOH-terminal catalytic domain (8) and remains complexed to Hsp90 and p50 even when bound to Ras at the plasma membrane (14). It is not clear why native Raf-1 associates with Hsp90, although it has been proposed that this heat shock protein is involved in Raf-1 transport to the cell membrane (15).

The benzoquinone ansamycin, geldanamycin (GA), has been shown to bind specifically and directly to Hsp90 and to disrupt the Hsp90-p60
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complex, leading to destabilization of p60
g (16). Although originally described as tyrosine kinase inhibitors, benzoquinone ansamycins have been shown to be inactive when added directly to purified tyrosine kinases at concentrations >1500 times their effective in vivo dose (17, 18). Additionally, several attempts to demonstrate direct association of ansamycins with tyrosine kinases in vivo and in vitro have been unsuccessful (16, 19). This class of drug is now thought to exert kinase inhibitory activity indirectly by somehow destabilizing these proteins (18, 20, 21). Consistent with this hypothesis, binding to Hsp90 and destabilization of the Hsp90-p50
complex occurs both in vivo and in vitro at nanomolar concentrations of ansamycin (16). These drug levels are very similar to the concentration of ansamycin previously reported to produce decrements in cellular lck, v-src, and epidermal growth factor receptor protein level and activity (18, 20, 21), leading to the hypothesis that benzoquinone ansamycins are tyrosine kinase inhibitors because they disrupt Hsp90-kinase heterocomplexes (16).

We now report that GA also disrupts the association between Hsp90 and the serine/threonine kinase Raf-1. The purpose of this study was to use GA to analyze the function of the Raf-1-Hsp90 complex.

**EXPERIMENTAL PROCEDURES**

**Materials**—GA was obtained from the Developmental Therapeutics Program, NCI. Culture media were purchased from Biofluids, Inc., [35S]methionine was obtained from ICN Biomedicals, Inc., and protein A-Sepharose beads were purchased from Pharmacia Biotech Inc. Raf-1 antibody (clone C-12) was purchased from Santa Cruz Biotechnology, Hsp90 antibody (SPA-830) was purchased from Stressgen, and Ras antibody (pan-ras Ab-3) was purchased from Oncogene Science. A horseradish peroxidase-conjugated secondary antibody to rabbit (Raf-1) or mouse (Hsp90, Ras) IgG was purchased from Amersham Corp. and used in conjunction with Western blot chemiluminescence reagent (Ren millennium, Du Pont). Nitrocellulose membrane was obtained from Schleicher & Schuell. All other chemicals were of highest available commercial grade.

**Tissue Culture**—MCF7 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle medium containing 10% bovine calf serum and 10 mM HEPES. CHP100 cells were obtained from Dr. A. Evans (Children's Hospital of Philadelphia) and grown in RPMI 1640 medium with 10% bovine calf serum and 10 mM HEPES. For labeling proteins with [35S]methionine, cells were incubated in methionine-free media for 30 min. Then, 100 μCi/ml [35S]methionine were added for 2 h. If the experiment required a chase, the cells were subsequently washed with phosphate-buffered saline and kept in methionine-free media for 1–4 h.

**Raf-1 Immunoprecipitation**—Cells growing in log phase were lysed with TENS buffer (50 mM Tris·HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate, 1% Nonidet P-40) containing proteinase inhibitors (aprotinin 20 μg/ml, leupeptin 20 μg/ml, 200 μM phenylmethylsulfonyl fluoride). 1800 Ci/ml [35S]methionine were added for 2 h. If the experiment required a chase, the cells were subsequently washed with phosphate-buffered saline and kept in medium containing nonradioactive methionine for 3–4 h.

**Raf-1 Immunoprecipitation**—Cells growing in log phase were lysed with TENS buffer (50 mM Tris·HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate, 1% Nonidet P-40) containing proteinase inhibitors (aprotinin 20 μg/ml, leupeptin 20 μg/ml, 200 μM phenylmethylsulfonyl fluoride). 1800 μCi/ml total protein were immunoadsorbed with 1 μg of Raf-1 antibody followed by protein A-Sepharose. The immunoadsorbed pellets were washed 5 times with TENS buffer and finally resuspended in SDS sample buffer (3% SDS, 100 mM dithiothreitol, 80 mM Tris·HCl, pH 6.8, 10% glycerol).

Gd Electroforetics and Western Blotting—Cell lysates or immuno-
adsorbed protein. A-Sepharose pellets were heated at 90°C in SDS sample buffer for 5 min, chilled on ice, and electrophoresed through 8 or 10% SDS-polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked for 2 h with a solution containing 5% nonfat dry milk, 10 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, pH 8, 50 mM NaCl, and 0.05% Tween 20 and then probed with the primary antibodies mentioned above diluted in blocking solution. After six washes with washing buffer (10 mM Tris-HCl, 2.5 mM EDTA, pH 8, 50 mM NaCl, 0.05% Tween 20, the membranes were exposed to horseradish peroxidase-labeled secondary antibody diluted in blocking solution. After an additional six washes, the proteins were visualized with Western blot chemiluminescence reagent following manufacturer’s instructions.

[35S]Methionine-labeled proteins were immunoprecipitated and electrophoresed as described above. The SDS-PAGE gel was fixed with a solution of 10% acetic acid and 50% methanol, washed copiously in water, and enhanced with Enlightening solution (DuPont NEN) prior to gel drying and autoradiography. Films of either chemiluminescent or radioactive blots were scanned into a Macintosh computer using a Fuji/Eclipse Gel Analysis system (Fuji/En), and band intensities were quantified using Collage Analysis software (Fotodyne). Raf-1 half-life was determined by regression analysis of log transformed Raf-1-specific band intensities.

Fractionation of Cytoplasmic and Membrane Components—Cells were homogenized with a Dounce homogenizer in TESV buffer (TENSV buffer without detergent) with protease inhibitors and further disrupted by sonication. After ultracentrifugation at 100,000 × g for 60 min at 4°C, the supernatant fraction, representing cytosolic components, was set aside, and the pellet, representing the Nonidet P-40-insoluble membrane fraction, was solubilized with 6% Nonidet P-40. The remaining pellet, representing the Nonidet P-40-soluble membrane fraction, was set aside, and the supernatant, representing the Nonidet P-40-soluble membrane fraction, was solubilized with 6% SDS. Protein concentrations of each fraction were determined using the manufacturer’s instructions.

RESULTS AND DISCUSSION

GA Destabilization of Raf-1 Protein—We chose to study in detail two cell lines in which Raf-1 is abundantly expressed: the breast cancer cell line MCF7 and the neuroepithelioma cell line CHP100. MCF7 cells were grown in log phase and lysed with TENSV buffer after treatment with and without GA for 4 h. 1800 μg of total protein were immunoprecipitated with Raf-1 antibody (lanes 1 and 2) and immunoblotted. We were able to demonstrate coprecipitation of both Hsp90 and Ras with Raf-1 (Fig. 1, lane 1). Coprecipitation of Hsp90 and Ras with Raf-1 disappeared after brief treatment with GA (Fig. 1, lane 2). Disruption of these heterocomplexes occurred in the absence of detectable changes in either cytosolic Hsp90 or Ras protein levels as assayed by Western blotting of 50 μg of total protein (Fig. 1, lanes 3 and 4).

Although cytosolic Raf-1 protein was reduced by 55% in GA-treated cells (compare Raf signal in lanes 3 and 4, Fig. 1), Raf-1-specific immunoprecipitation from 1.8 mg of total protein resulted in apparent antibody saturation, because drug treatment only minimally reduced the amount of Raf-1 recoverable by immunoprecipitation (compare Raf signal in lanes 1 and 2, Fig. 1). Because native Raf-1-Hsp90 heterocomplexes are unstable compared with the p6050°-src-Hsp90 complex (8), the data shown in Fig. 1 do not represent a stoichiometric coprecipitation. However, this technique yields qualitatively useful data demonstrating the disruption of existing Raf-1-Hsp90 and Raf-1-Ras complexes. Because Hsp90 is a very abundant protein and serves as a chaperone for a variety of other proteins, only a minor fraction of it is associated with Raf-1, although at least all cytosolic Raf-1 appears to occur in a complex with heat shock proteins (14). The amount of GTP-Ras bound to Raf-1 depends on the activation status of the cell.
immunoblotting of extracts prepared from the three subcellular fractions revealed that Raf-1 disappeared equally from the three fractions upon treatment with GA (fractions revealed that Raf-1 disappeared equally from the immunoblotting of extracts prepared from the three subcellular complexes. Although a model in which enzymatically active cytoskeleton-bound Raf-1 preclude recovery of protein hetero-

double membrane fraction are also affected by GA (Fig. 3, is associated with Ras, depends on the presence of Hsp90. Furthermore, Raf-1 stability, even when the protein remains associated with the cytosol (Fig. 3, A and D). Loss of Raf-1 from this fraction occurred too quickly to be due solely to inability to recruit new Raf-1 from the cytosol. Immunoprecipitation of Raf-1 no longer co-precipitated Ras at a time when significant amounts of Raf-1 were still found in the detergent-soluble membrane fraction (compare Fig. 3A, lane 6 with Fig. 1, lane 2), suggesting that Ras association with Raf-1 requires the continued participation of Hsp90. Furthermore, Raf-1 stability, even when the protein is associated with Ras, depends on the presence of Hsp90.

Finally, the kinetics of Raf-1 turnover in the detergent-insoluble membrane fraction are also affected by GA (Fig. 3, A and D). Whether this means that anchorage of Raf-1 to cytoskeletal elements requires participation of Hsp90 remains to be determined, because the harsh conditions necessary to solubilize cytoskeleton-bound Raf-1 preclude recovery of protein heterocomplexes. Although a model in which enzymatically active Raf-1 is no longer associated with Hsp90 would be appealing in that such a model would be strikingly similar to that proposed for association of Hsp90 with pp60jun (22), such a model is not consistent with our current data. Together with destabilization, Raf-1-Hsp90 complex disruption might also affect the ability of cytosolic Raf-1 to be recruited by Ras. To address this question, we pulsed MCF7 cells, which had been exposed to GA for 16 h, with [35S]methionine and followed the labeling with a 4 h chase (Fig. 3C). As described above (see Fig. 2A), Raf-1 synthesis in drug-treated cells was elevated (approximately 3-fold). However, in two separate experiments, the chase period was sufficient to allow 68% of newly synthesized Raf-1 (61 and 76%, respectively) to appear in the Nonidet P-40-soluble membrane fraction of untreated cells, whereas 32% of the labeled protein (24 and 39%, respectively) was recovered from the cytosol. In contrast, in GA-treated cells only 30% of radiolabeled Raf-1 (25 and 35%, respectively) was recovered from the cytosol. These data are consistent with a model in which disruption of Raf-1-Hsp90 cytosolic complexes not only destabilizes Raf-1 but also interferes with its proper intracellular trafficking and recruitment to the membrane by Ras.

Destabilization of Raf-1 by GA Occurs in Various Cell Lines—Because Raf-1 is a ubiquitously expressed and highly conserved protein (2, 23, 24), its destabilization by GA should be a general phenomenon. To test this hypothesis, we chose a diverse panel of cell lines representing nonmalignant mouse fibroblasts (NIH 3T3) as well as sarcomatous (CHP100), carcinomatous (HeLa, MCF7, and DU145), lymphomatous (Raji), and leukemic (CEM) human cell lines. Although these cell lines differed in terms of Raf-1 levels, they all showed a marked reduction in the steady-state level of Raf-1 protein after GA treatment (Fig. 4).
Raf-1 Destabilization by Disruption of Raf-1-Hsp90 Complex

FIG. 4. GA depletes Raf-1 from both transformed and untransformed cell lines. Cells were grown in log phase culture for 16 h without (lanes 1, 3, 5, 7, 9, 11, and 13) or with GA (2 μM) for 4 h (lanes 2, 6, and 10), 16 h (lanes 3, 7, and 11), or 40 h (lanes 4, 8, and 12). The cells were lysed, and preparations of cytosol (lanes 1–4) and Nonidet P-40-soluble (lanes 5–8) and -insoluble (lanes 9–12) membrane fractions were obtained. 15 μg of total protein/lane were electrophoresed through 10% SDS-PAGE minigels. Western blotting was performed for Raf-1. B, Western blotting for Ras was performed using the fractions obtained from untreated and 16-h GA-treated samples. Lanes 1 and 2 represent cytosol from untreated and GA-treated samples, respectively; lanes 3 and 4 represent Nonidet P-40-soluble membrane preparations from untreated and GA-treated samples, respectively; and lanes 5 and 6 represent Nonidet P-40-insoluble membrane preparations from untreated and GA-treated samples, respectively. C, MCF7 cells were pulsed with [35S]methionine after pretreatment without (lanes 1 and 2) or with (lanes 3 and 4) GA (2 μM) and then chased for 4 h with nonradioactive media. Cytosol and Nonidet P-40-soluble membrane preparations were obtained, and equal amounts of total protein were immunoprecipitated with Ras antibody, electrophoresed on an 8% SDS-PAGE gel, and visualized by autoradiography. Cytosolic preparations were represented in lanes 1 and 3; Nonidet P-40-soluble membrane fractions are represented in lanes 2 and 4. This experiment was performed twice. Densitometric analysis of the Raf-1 bands in untreated cells was determined by densitometric analysis. A graphical representation of the cumulative results is depicted. Data are displayed as the fraction of the value obtained in untreated cells ± S.D.

REFERENCES
1. Magnuson, N. S., Beck, T., Vahidi, H., Hahn, H., Smola, U., and Rapp, U. R. (1994) Semin. Cancer Biol. 5, 247–253
2. Errede, B., and Levin, D. E. (1993) Curr. Opin. Cell Biol. 5, 254–260
3. Zhang, X. F., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993) Nature 364, 308–313
4. Traverse, S., Cohen, P., Paterson, H., Marshall, C., Rapp, U. R., and Grand, R. J., A. (1993) Oncogene, 3175–3181
5. Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M., and Hancock, J. F. (1994) Science 264, 1463–1467
6. Lewis, S. J., Paterson, H. F., and Marshall, C. J. (1994) Nature 369, 411–414
7. Hall, A. (1994) Science 264, 1413–1414
8. Stancato, L. F., Chow, Y. H., Hutchison, K. A., Perdue, G. H., Jove, R., and Pratt, W. B. (1993) J. Biol. Chem. 268, 21711–21716
9. Sanchez, E. R., Toft, D. O., Schiesinger, M. J., and Pratt, W. B. (1985) J. Biol. Chem. 260, 12398–12401
10. Perdue, G. H. (1988) J. Biol. Chem. 263, 13802–13805
11. Miyata, Y., and Yahara, I. (1991) J. Biol. Chem. 266, 8779–8783
12. Xu, Y., and Lindquist, S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7074–7078
13. Miyata, Y., and Yahara, I. (1992) J. Biol. Chem. 267, 7042–7047
14. Wartmann, M., and Davis, R. J. (1994) J. Biol. Chem. 269, 6695–6701
15. Pratt, W. B. (1993) J. Biol. Chem. 268, 21455–21458
16. Whitesell, L., Minnaugh, E. G., De Costa, B., Myers, C. E., and Neelk, L. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8324–8328
17. Whitesell, L., Shifrin, S. D., Schwab, G., and Neelk, L. M. (1992) Cancer Res. 52, 1721–1728
18. June, C. H., Fletcher, M. C., Ledbetter, J. A., Schieve, G. L., Siegel, N. J., Phillips, A. F., and Samelson, L. E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7722–7726
19. Miller, P., Schnur, R. C., Barbacci, E., Moyer, M. P., and Moyer, J. D. (1994) Biochem. Biophys. Res. Commun. 201, 1313–1319
20. Uehara, Y., Murakami, Y., Sugimoto, Y., and Mizuno, S. (1989) Cancer Res. 49, 780–785
21. Murakami, Y., Mizuno, S., and Uehara, Y. (1994) Biochem. J. 301, 63–68
22. Brugg, J. S. (1986) Curr. Top. Microbiol. Immunol. 223, 1–22
23. Dickson, B., Sprenger, F. M., Morrison, D., and Hafen, E. (1992) Nature 360, 590–593
24. Han, M., Golden, A., Han, Y., and Sternberg, P. W. (1993) Nature 363, 133–140

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