Degradation of Heterotrimeric Gαo Subunits via the Proteasome Pathway Is Induced by the hsp90-specific Compound Geldanamycin*

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One mechanism utilized by cells to maintain signaling pathways is to regulate the levels of specific signal transduction proteins. The compound geldanamycin (GA) specifically interacts with heat shock protein 90 (hsp90) complexes and has been widely utilized to study the role of hsp90 in modulating the function of signaling proteins. In this study, we used GA to demonstrate that levels of heterotrimeric Gαo subunits can be regulated through interactions with hsp90. In a dose-dependent manner, GA significantly reduced the steady state levels of endogenous Gαo expression in two cell lines (PC12 and GH3) and had a similar effect on Gαo transiently expressed in COS cells. Gαo synthesis and degradation was studied in PC12 cells and in transiently transfected COS cells. 35S labeling followed by immunoprecipitation demonstrated no effect of GA on the rate of Gαo synthesis, but GA accelerated degradation of Gαo, in both PC12 cells and COS cells. The use of inhibitors, including lactacystin (a proteasome-specific inhibitor), suggests that Gαo is predominantly degraded through the proteasome pathway. In vitro translated 35S-labeled Gαo could be detected in hsp90 immunoprecipitates, and this interaction did not require N-terminal myristoylation. Taken together, these results suggest that heterotrimeric Gαo subunits are protected from degradation by interaction with hsp90 and that the interaction of Gαo subunits with heat shock proteins may be a general mechanism for regulating Gαo levels in the cell.

Cells respond to a wide range of physical, chemical, and hormonal stimuli through cell surface receptors that are coupled to heterotrimeric G proteins. G proteins are composed of Gα and Gβγ subunits and are attached to the plasma membrane through lipid modifications on Gα and Gγ subunits (reviewed in Ref. 1). Activated receptors induce a conformational change in Gα that leads to GDP release and GTP binding. GTP-bound Gα dissociates from Gβγ, and both subunits can interact with a variety of intracellular effectors until the intrinsic GTPase activity of Gα hydrolyzes GTP to GDP. Many types of G protein-coupled receptors and G proteins are expressed within the same cell, and the mechanisms that generate specific cellular responses are not well understood. Some specificity lies at the interface of receptor-G protein and G protein-effector, but in reconstituted systems multiple G proteins can couple to the same sets of receptors and effectors (reviewed in Ref. 2). Furthermore, in a single cell type, a single Gα subunit can couple to at least three different effector pathways (3). In the cell, multiple mechanisms are likely to be important for maintaining signaling specificity, and these include regulation by modulatory proteins such as receptor kinases (reviewed in Ref. 4) and RGS (regulators of G protein signaling) proteins (reviewed in Ref. 5).

An additional cellular mechanism to regulate signaling pathways is to control the degradation of individual signaling molecules. Lipid modifications on the N terminus of Gα subunits are important for targeting and attachment to the plasma membrane, but the interaction of Gα subunits with cytosolic proteins prior to plasma membrane association have not been defined. The 90-kDa heat shock protein (hsp90) is a highly conserved protein chaperon representing up to 5% of total cell protein under non-stress conditions. Interestingly, hsp90 interacts with a diverse group of proteins involved in cellular signaling that include several families of tyrosine kinases and steroid hormone receptors. The functional implications for the interaction of signaling molecules with hsp90 depends upon the protein. Steroid hormone receptors are stabilized by interaction with hsp90, and this interaction is necessary for high-affinity ligand binding (7). In contrast, the membrane-associated tyrosine kinase pp60src interacts transiently with hsp90 following synthesis until the nascent protein is inserted in the membrane (8). The role of hsp90 in regulating the functions of some cellular proteins has been facilitated by the recognition that quinone ansamycin antibiotics, such as geldanamycin (GA), are highly specific inhibitors of hsp90-protein complexes (9). The GA-hsp90 complex has been crystallized and reveals GA binding to a highly conserved pocket of hsp90 (residues 9–232) (10). Because hsp90 interactions are important for the proper function of a variety of signaling molecules, we asked whether G protein α subunits could also interact with hsp90. We used the hsp90-specific compound geldanamycin to address this question, and we found that GA induced a decline in the level of endogenous Gαo in PC12 cells, GH3 cells, and transiently transfected COS cells without affecting the level of other cellular proteins. The enhanced degradation of Gαo occurred pre-

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The abbreviations used are: G protein, guanine nucleotide-binding protein; hsp90, 90-kDa heat shock protein; GA, geldanamycin; PAGE, polyacrylamide gel electrophoresis; Z-LLL, benzylxoycarbonyl-leucinyl-leucinyl-leucinal-H; LCN, lactacystin; N-Ac-LLL, N-acetyl-leucinyl-leucinyl-methionyl-H; HA, hemagglutinin.
dominantly through the proteasome pathway. Furthermore, immunoprecipitates of hsp90 from in vitro translated of Goa, coprecipitated Goξ, and this interaction was independent of N-terminal myristoylation.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—GA was kindly provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute.

Hemagglutinin (HA) antibody (clone SCP-12CA5-J) was purchased from Berkeley Antibody Co., and anti-heat shock protein 90 (mouse monoclonal, IgM) antibody was purchased from StressGen (Colleville, PA). Protease inhibitors N-Ac-LLM, LCN, and Z-LLL were from Biomol Research Laboratories (Plymouth Meeting, PA). Chemicals were from Sigma, and chemiluminescence reagents were from Pierce.

**Buffers**—The buffers used were: A, 50 mM Tris-HCl, pH 7.6, 6 mM MgCl2, 75 mM sucrose, 1 mM dithiothreitol, 1 mM EDTA, 3 mM benzamidine, 1 μg/ml leupeptin, soy and lima bean trypsin inhibitors; B, 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate; C, 1% Triton X-100, 20 mM sodium molybdate.

**Cell Culture and Transient Transfections**—PC12 cells were kindly provided by Dr. Eva Neer (Brigham and Women’s Hospital, Boston, MA) and cultured as described previously (11). Goa, cDNA in Bluescript (12) was amplified by polymerase chain reaction to generate a blunt C-terminal end that was then cloned into a modified Bluescript vector containing the HA epitope. The sequence was confirmed by dideoxynucleotide sequencing. HA Goa was cloned into the eukaryotic expression vector pCDNA3 (Invitrogen) using the XbaI and Apal sites. COS cells were cultured and transfected as described previously using LipofectAMINE (Life Technologies, Inc.) (13), and 24–48 h after transfection cells were treated with GA dissolved in dimethyl sulfoxide or with dimethyl sulfoxide alone under various conditions.

**Electrophoresis and Immunoblotting**—Levels of Goa expression were determined 48–72 h after transfection of COS cells or from confluent monolayers of PC12 cells and GH3 cells after treatment with GA for specified conditions. Cells were washed with phosphate-buffered saline, suspended in 300 μl of cold Buffer A, frozen and thawed 3 times in liquid nitrogen, and passed 15 times through a 27-gauge needle. The homogenates were cleared by centrifuging at 1,500 rpm for 5 min at 4 °C. Total protein levels were determined by the Bradford method, and SDS-PAGE sample buffer was added to the supernatant. Equivalent amounts of total protein were analyzed by SDS-PAGE, and the level of Goa expression was determined by Western blotting using the anti-Goa monoclonal antibody at a dilution of 1:1000 or an anti-Gβo antibody (R4) at a dilution of 1:2000 (courtesy of E. Neer (14)). Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies were used at a dilution of 1:10,000, and bands were visualized by chemiluminescence.

**Metabolic Labeling Studies and Immunoprecipitations**—PC12 cells and transfected COS cells (at 48 h) were treated with 2 μM GA for 18 h (COS cells) or 24 h (PC12 cells) and then incubated for 30 min in a methionine- and cysteine-free medium. Tran35S-label (100 Ci/mmol, ICN Radiochemicals) was added to a final concentration of 200 μCi/ml for 60–90 min and chased with medium containing cold methionine/cysteine for various lengths of time. For synthesis studies, cells were labeled at t = 0 and immunoprecipitated at 30-min intervals. Cells were washed and scraped, and homogenates were prepared as described above. 35S-Labeled proteins were immunoprecipitated from homogenates in Buffer B after clearing with protein A-Sepharose. 12CA5 antibody (1/250 μl) for HA Goa, transiently expressed in COS cells (or R4 (1/100 μl) for PC12 cells) was added for 1–4 h at 4 °C followed by protein A-Sepharose for 1 h. Samples were centrifuged, and the pellets were washed three times with Buffer A. The immunoprecipitates were eluted with SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by autoradiography.

**In Vitro Translation**—cDNAs for wild-type Goa (not HA-tagged) and myristoylation-minus mutant (G1A) were in vitro translated with [35S]methionine in a single-step rabbit reticulocyte lysate (Promega) as described previously (13). The in vitro translation was mixed 1:1 with 2× Buffer C and then divided in half. One tube was incubated with the anti-hsp90 antibody coupled to protein A-Sepharose (15), and the other was a control of nonspecific IgM antibody similarly coupled to protein A-Sepharose. The mixtures were rocked at 4 °C for 1 h, centrifuged, and washed three times with cold Buffer C. Samples were eluted with SDS-PAGE sample buffer and analyzed as described above.

**RESULTS AND DISCUSSION**

Regulating the levels of signaling proteins is an important mechanism contributing to the accurate development and maintenance of signal transduction pathways. hsp90 interacts with several families of signal transduction proteins including receptor and non-receptor tyrosine kinases, serine threonine kinases, and mutated p53 (reviewed in Ref. 16). The compound GA destabilizes the interaction between hsp90 and its associated kinases, and mutated p53 (reviewed in Ref. 16). The compound GA destabilizes the interaction between hsp90 and its associated kinases, and mutated p53 (reviewed in Ref. 16). The compound GA destabilizes the interaction between hsp90 and its associated kinases, and mutated p53 (reviewed in Ref. 16). The compound GA destabilizes the interaction between hsp90 and its associated kinases, and mutated p53 (reviewed in Ref. 16). The compound GA destabilizes the interaction between hsp90 and its associated kinases, and mutated p53 (reviewed in Ref. 16). The compound GA destabilizes the interaction between hsp90 and its associated kinases, and mutated p53 (reviewed in Ref. 16).
and time-dependent decreases in steady state Gaα levels for all three cell lines. Interestingly, 2 nM GA treatment caused a noticeable increase in steady state Gaα levels (Fig. 1) in all three cell lines. The explanation for this observation is not immediately apparent but may relate to compensatory changes in synthesis and/or degradation at low dose exposure to GA. C-terminal hemagglutinin epitope-tagged Gaα was used in the COS cell studies to facilitate subsequent experiments requiring immunoprecipitations. Transient expression of Gaα in COS cells results in over 75% of the protein separating into the particulate fraction (13, 18), an amount consistent with results in bovine brain (19). In addition, placing the epitope on the C terminus does not affect separation into soluble and particulate fractions (not shown), and HA Gaα exchanges guanine nucleotides as determined by a tryptic conformation assay (not shown). Furthermore, the HA epitope on the C terminus of Gpa1, the yeast Ga subunit, did not affect the stability of the protein (6). The closed arrows (Fig. 1) indicate Gaα; the open arrowhead indicates a nonspecific protein present in vector-transfected (PC) control cells (first lane) and in HA Gaα-transfected cells. The intensity of the nonspecific protein does not change with increasing GA doses, but there is a dose-dependent decrease in steady state Gaα levels (Fig. 1, closed arrows). The time-dependent effects of 2 μM GA were not apparent until after 3 h, and maximal effects were consistently seen with 18–24 h of exposure (not shown). Steady state Gaα levels were reduced by 78 ± 2% (n = 7) in COS cells and to a similar degree in PC12 and GH3 cells.

The lower steady state protein levels of Gaα in the presence of GA could arise from effects on the rate of Gaα synthesis and/or degradation. To distinguish among these possibilities, the rate of Gaα synthesis in PC12 and COS cells was determined by comparing the amount of [35S]methionine/[35S]cysteine incorporated into Gaα at various times after labeling, and the Gaα degradation rate was measured by pulse-chase experiments. Fig. 2A shows that the amount of label incorporated at 30, 60, 90, and 120 min was indistinguishable in PC12 cells in the presence or absence of GA. Similar results were obtained in transiently transfected COS cells (not shown). The rate of degradation was significantly faster in the presence of GA for endogenous Gaα in PC12 cells and in transfected COS cells (Fig. 2B). Not surprisingly, the half-life of endogenous Gaα in PC12 cells is significantly longer (>24 h) than the half-life of Gaα transiently expressed in COS cells (~6 h). Nevertheless, the effect of GA was similar in the two systems: a significant increase in the amount of degradation was evident at nearly every time point. Taken together, these results indicate that the predominant effect of GA on steady state Gaα levels is through accelerated degradation.

Most intracellular protein degradation is catalyzed by calpains, lysosomal proteases, or by the ubiquitin-proteasome system (reviewed in Ref. 20). To test which of these pathways was responsible for the accelerated degradation of Gaα, a series of well characterized peptide aldehyde protease inhibitors were utilized (21). Two of these peptides, Z-LLL and N-Ac-LLL, exhibit similar inhibitory activity against calpains and lysosomal cathepsins, but Z-LLL is a more potent proteosome inhibitor than N-Ac-LLL (21, 22). Fig. 3 shows that when COS cells expressing Gaα are treated with GA plus an inhibitor of proteolysis (lanes 2–4) there are different effects on the levels of Gaα. In comparison with no inhibitor, treatment with N-Ac-LLL has no significant effect on Gaα levels (lane 3), and this is consistent with little degradation occurring through the calpain and lysosomal pathways. Z-LLL also blocks the proteosome pathway, and this inhibitor was partially capable of blocking degradation of Gaα induced by GA (lane 2). This finding suggests that degradation of Gaα occurs through the proteosome pathway. To confirm this, we used lactacystin (LCN), the most specific inhibitor of the proteosome (inhibits all five proteolytic activities) (23, 24). As is seen in Fig. 3, lane 4, lactacystin significantly blocks degradation of Gaα (closed arrow). When the Western blots of total cellular extracts are exposed for increasing lengths of time, higher molecular mass species gradually become apparent (Fig. 3, bottom, lanes 2–4). The open arrows

![Fig. 2. GA effects on rates of synthesis and degradation of Gaα. (A) rate of synthesis. Confluent PC12 cells were pretreated with or without GA (2 μM) for 24 h and exposed to a Tran35S-label pulse (200 μCi/ml of methionine/cysteine-free medium) at time 0. At 30, 60, 90, and 120 min after labeling, cells were scraped and lysed in Buffer B, and 400 μg of total protein was immunoprecipitated with R4 anti-Gaα antibody in Buffer B, separated on 13% SDS-PAGE, and visualized by autoradiography. A representative autoradiogram is shown in the inset, and mean densitometry units (± the range) are plotted over time. ○, absence of GA; ●, presence of GA, B, rate of degradation. PC12 cells or Gaα-transfected COS cells (analyzed at 48 h) were cultured in the absence and presence of GA (2 μM, 18 h COS, 24 h PC12) and then incubated in methionine/cysteine-free medium for 1 h followed by labeling with Tran35S-label medium containing medium for different periods of time. Immunoprecipitations were done as described under "Experimental Procedures." Gels were exposed for 12–48 h, and bands were analyzed as described in Fig. 1. For PC12 cells, the mean of three independent experiments (± S.E.) is plotted over time, and for COS cells a representative experiment (n = 2) is shown. For both cell lines, immunoprecipitated Gaα is shown below.

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highlight the appearance of new bands initially between G\(\alpha\) and hsp90 although other control proteins were not detected (not shown). The control immunoprecipitations with nonspecific antibody contain almost no background (Fig. 4). G\(\alpha\) was consistently detected in hsp90 immunoprecipitates (\(n = 4\)), but the fraction precipitated was low (<10% of starting material). The requirements for detergent and the low-affinity nature of the interaction between hsp90 and G\(\alpha\) are the most likely explanations for this result. The hsp90 antibody can precipitate hsp90 alone or in a complex with other proteins, so we cannot exclude the possibility that the interaction of G\(\alpha\) with hsp90 is indirect. The N terminus of G\(\alpha\) is important for interactions with G protein \(G\beta\gamma\) subunits and for interactions with the plasma membrane (12, 13, 18). Myristoylation of the N-terminal glycine (first amino acid after cleavage of methionine) and palmitoylation on cysteine 2 are also important to these functions. However, mutation of N-terminal glycine to alanine (G1A) did not disturb the interaction with hsp90 (Fig. 4), and likewise, this mutation in Gpa1 did not affect its degradation through the proteosome pathway (6). Similar results were obtained with other G\(\alpha\) mutants previously characterized (13, 18), which are deleted up to 20 amino acids from the N terminus (not shown). These results indicate that myristoylation and an N-terminal amino acid sequence are not necessary for association of G\(\alpha\) with the hsp90 complex.

The identity of signaling molecules and their levels expressed in the membrane play an important role in signal transduction specificity. Critical to determining these levels are the mechanisms through which G protein \(\alpha\) subunits are degraded. The results described above, and studies in yeast (6), are consistent with degradation of G\(\alpha\) subunits through the ubiquitin-proteosome pathway. Several studies have demonstrated that activated G\(\alpha\) subunits (through cholera toxin (G\(\alpha\)), by activating mutation, or by receptor stimulation) are more rapidly degraded, but the mechanisms of degradation are not addressed (25–27). Furthermore, G\(\alpha\) subunits have different half-lives depending upon the cell type. In GH4 cells the half-life of G\(\alpha\) is about 28 h, but is greater than 72 h in cardiac myocytes (28). These different half-lives occur, in part, because of differences in rates of protein degradation (28). Our results suggest that steady state G\(\alpha\) protein levels are regulated by interaction with an hsp90 complex that prevents degradation through the proteosome pathway and that N-terminal myristoylation is not required for this interaction. Although the time course and dose responses of GA treatment will vary among cell lines, the observation that this mechanism appears to be preserved in a transient expression system will make more detailed study of these mechanisms feasible. Other cytosolic pro-

![Fig. 2. Effects of protease inhibitors on GA-enhanced degradation. Top, transfected COS cells expressing G\(\alpha\) were pretreated with GA (2 \(\mu\)g/ml) alone (lane 1) or in combination with protease inhibitors for 18 h (Z-LLL (100 \(\mu\)M, lane 2), LLL, N-Ac-LLL (100 \(\mu\)M, lane 3), LLM), and lactacystin (10 \(\mu\)M, lane 4). The arrow marks migration of G\(\alpha\). Bottom, G\(\alpha\)-expressing cells treated with GA and LCN as described above (lane 1) and Western blots overexposed for increasing lengths of time (lanes 2–4). Vector-transfected control cells from the same experiment and gel (lane 1) are included to demonstrate background bands that are not affected by GA and LCN. The open arrows mark the appearance of higher molecular mass bands. New bands are initially detected between G\(\alpha\) (39 kDa) and the 50-kDa background band (lanes 2, 3), and with longer exposures new bands appear between 90 and 110 kDa (lane 4).]

![Fig. 4. hsp90 immunoprecipitates of in vitro translated G\(\alpha\) and GIA. Wildtype G\(\alpha\) (not epitope-tagged) in Bluescript and glycine to alanine point mutant of first amino acid (GIA) were [\(^{35}\)S]methionine-labeled by in vitro translation as described under “Experimental Procedures.” The starting amounts of in vitro translated G\(\alpha\) and GIA were similar. Translates (50 \(\mu\)l) were divided in half and mixed with an equal volume of 2% Triton, 20 mM sodium molybdate. Lanes were incubated with 17 \(\mu\)l of a 20% suspension of hsp90 antibody prebound to protein A-Sepharose. A similar amount of coated protein A-Sepharose coupled to a nonspecific antibody was used as a control. Pellets were washed and analyzed by SDS-PAGE followed by autoradiography (exposure time = 36 h).]
teins are likely to participate in this process and together provide an important mechanism for regulating Gα levels and function.

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