We have expressed the mitogenic signaling proteins Src, Ras, Raf-1, Mek (MAP kinase kinase), and Erk (MAP kinase) in baculovirus-infected Sf9 insect cells in order to study a potential role for the chaperone hsp90 in formation of multiprotein complexes. One such complex obtained by immunoadsorption with anti-Ras antibody of cytosol prepared from cells simultaneously expressing Ras, Raf, Mek, and Erk contained Ras, Raf, and Erk. To detect directly the protein-protein interactions involved in forming multiprotein complexes, we combined cytosols from single infections in vitro in all possible combinations of protein pairs. We detected complexes between Ras-Raf, Ras-Src, Raf-Mek, and Raf-Src, but no complex containing Erk was obtained by mixing cytosols. Thus, cellular factors appear to be required for assembly of the Erk-containing multiprotein complex. One cellular factor thought to be involved in signaling protein complex formation is the chaperone hsp90, and we show that Src, Raf, and Mek are each complexed with insect hsp90. Treatment of Sf9 cells with geldanamycin, a benzoquinone ansamycin that binds to hsp90 and disrupts its function, did not decrease coadsorption of either Raf or Erk with Ras, although it did decrease the level of cytosolic Raf. To study geldanamycin action, we treated rat 3Y1 fibroblasts expressing v-Raf and showed that the antibody blocked assembly of Raf-hsp90 complexes at an intermediate stage of assembly where Raf is still bound to the p60 and hsp70 components of the assembly mechanism. As in Sf9 cells, Raf levels decline with geldanamycin treatment of 3Y1 cells. To determine if geldanamycin affects mitogenic response, we treated HeLa cells with epidermal growth factor (EGF) and showed that geldanamycin treatment decreased EGF signaling and decreased the level of Raf protein without affecting the EGF-mediated increase in Raf kinase activity. We conclude that hsp90 is not required for forming complexes between the mitogenic signaling proteins or for Raf kinase activity and that EGF signaling is decreased indirectly by geldanamycin because the anti-biotic increases degradation of Raf and perhaps other components of the signaling pathway.

Several receptors for polypeptide ligands, including those for insulin, epidermal growth factor, platelet-derived growth factor, and nerve growth factor, transduce signals by activating the mitogen-activated protein (MAP) family of serine/threonine kinases (also called Erks for extracellular signal-regulated kinases) (see Refs. 1 and 2, for review). The receptors themselves are tyrosine kinases that undergo ligand-induced autophosphorylation leading to the recruitment of the Grb2 adaptor and its associated Ras activator protein Sos. Subsequent Ras binding to the Raf-1 serine/threonine kinase leads to phosphorylation by Raf-1 of another kinase called Mek (also called MAP kinase kinase), which in turn, phosphorylates and activates Erk. Erk is a terminal effector of this signal transduction pathway in that it can directly phosphorylate transcription factors that regulate gene expression. The receptor-mediated signaling system can be short circuited in the sense that the oncoprotein Src can activate Raf-1 (3–5).

A question that has not been resolved is the extent to which the proteins Ras, Src, Raf, Mek, and Erk exist in a multiprotein complex(es) with each other, an association that might greatly enhance the efficiency of signal transduction. Using either in vitro binding assays or the two-hybrid system, it has been shown that Ras interacts directly with Raf (6–9). Direct interactions between Raf and Mek (8, 10) and between Raf and Src (11) have also been demonstrated. In addition to these binary interactions, a native ternary Ras-Raf-Mek complex has been demonstrated using the two-hybrid system (8) and a similar complex has been formed in vitro by incubating immobilized Ras with brain cytosol (12). Importantly, in the latter case, immobilized Ras-GMP-PNP was incubated with cytosol and the washed sedimented pellet was shown to phosphorylate myelin basic protein and to release soluble MAP kinase (12). These observations are consistent with the possibility of a quaternary Ras-Raf-Mek-Erk signaling complex.

In addition to associations with other components of the MAP kinase signal transduction pathway, Src and Raf exist in native complexes with components of a heat shock protein (hsp) chaperone system. As soon as it is translated, Src becomes associated with hsp90 and a 50-kDa protein (p50) of unknown function (Refs. 13–15; see Ref. 16, for review). The Src-hsp90-p50 heterocomplex can be formed in vitro using a multicompo-
nent protein folding system present in reticulocyte lysate (17, 18). Similarly, Raf exists in a native heterocomplex with hsp90 and p50 (19, 20) that can be reconstituted by the protein folding system of reticulocyte lysate (19). As with the steroid receptors, the Raf/hsp90 heterocomplexes may also contain an immunophilin chaperone of the FK506 binding class (21). The protein folding system that forms these heterocomplexes itself exists in multiprotein complexes consisting of hsp90 bound to various amounts of hsp70, a 60-kDa stress-related protein, and a conserved and widely distributed 23-kDa protein (for review, see Refs. 22 and 23). The hsp90 and its associated proteins act as a self-sufficient protein folding machine (24, 25) that has recently been reconstituted from purified components (26).

To date, it is not known whether reversible protein-protein interactions between the components of the signaling system are sufficient to form the binary complexes and the higher order ternary and quaternary complexes that have been detected, or whether cellular factors like the hsp90 chaperone system participate in complex formation. Experiments with the antibiotic geldanamycin that were recently reported by Schulte et al. (27) suggest that hsp90 is required for Raf-Ras association. Geldanamycin is a benzoquinone ansamycin that was found to revert transformation induced in cultured cells by Src and some other oncogenic tyrosine kinases (28, 29). Geldanamycin does not inhibit kinase activity (29), but it binds in a pharmacologically specific manner to hsp90 and inhibits Src-hsp90 heterocomplex formation in reticulocyte lysate (30). Schulte et al. (27) found that a 4-h treatment of human MCF7 breast cancer cells with 2 μM geldanamycin disrupted multiprotein complexes containing Raf, Ras, and hsp90. This was accompanied by a marked decrease in the half-life of the Raf protein through an increased rate of its degradation (27). It was concluded that hsp90 was required for maintenance of the Raf-Ras complex and for protecting Raf from degradation. Although a negative impact on Raf function was predicted, Raf function itself was not assayed.

In this work we have expressed Src, Ras, Raf, Mek, and Erk singly and in combination in baculovirus-infected Sf9 insect cells. When expressed singly, Mek, like Src and Raf, is in a native complex with hsp90. Cytosols from single infections were combined in vitro in all possible combinations of protein pairs and immunoadsorbed with antibodies against each member of the pair. By this communoadsorption protocol we detect the Ras-Raf, Raf-Mek, and Raf-Src complexes referred to above as well as an unanticipated interaction between Ras and Src. When Sf9 cells are simultaneously infected with baculoviruses encoding Ras, Raf, Mek, and Erk, Ras-Raf-Mek-Erk complexes are immunoadsorbed with anti-Ras antibody. No complex containing Erk can be obtained by mixing any combination of cytosols from single infections, suggesting that cellular factors are required to produce a multiprotein complex containing Erk. Although treatment of Sf9 cells for 4 h with 10 μM geldanamycin causes a decrease in the amount of cytosolic Raf, it does not decrease recovery of Ras-Raf-Erk complexes. The mechanism of the geldanamycin effect is further explored in rat 3Y1 cells stably expressing v-Raf where we show the antibiotic blocks assembly of mature Raf/hsp90 complexes and decreases Raf levels. We also show that geldanamycin inhibits signaling through the EGF receptor in HeLa cells, but the inhibition can be accounted for by the ability of the antibiotic to decrease the level of Raf and perhaps the levels of other components of the signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**

125I-Conjugated goat anti-mouse and anti-rabbit IgG's were from DuPont NEN. Nuclease-treated rabbit reticulocyte lysate was from Promega. Protein A-Sepharose and goat anti-mouse and anti-rabbit IgG horseradish peroxidase conjugates were from Sigma. The rabbit anti-serum against hsp70 and hsp90 (31) was a generous gift from Dr. Ettore Appella (National Cancer Institute). The AC58 monoclonal Ig against hsp90 and the antibody 60-kDa monoclonal were from Stressgen, Victoria, BC, Canada. The LA-069 anti-Ras and N2-17 anti-Src monoclonal IgGs were from Quality Biotech (Rockville, MD). The C-12 rabbit anti-Raf serum was from Santa Cruz Biotechnology (Santa Cruz, CA). The T7-Tag monoclonal IgG used to immunoadsort polyhistidine-tagged Mek or Erk was from Novagen (Madison, WI). The rabbit antisera raised against a GST-Erk fusion protein used for immunoblotting Erk and the baculovirus encoding murine erk1 were kindly provided by Dr. Stuart Decker (Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI). The anti-Raf rabbit antisera was prepared against a peptide corresponding to the carboxyl-terminal 12 amino acids of human Raf-1 (32). The DS14F5 anti-p60 monoclonal IgG was a kind gift from Dr. David Smith (University of Nebraska, Omaha, NE). Human recombinant EGF was purchased from PeproTech (Rocky Hill, NJ). Purified recombinant Mek1 (33) and purified Erk K52R mutant were kindly provided by Dr. Thomas Sturgill (University of Virginia, Charlottesville, VA). Geldanamycin was obtained from the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program, National Cancer Institute.

**Methods**

**Cell Culture and Infection—**Recombinant baculoviruses containing the complete coding sequences of chicken c-src (34), murine v-ras (3), human c-raf-1 (5), murine mek1 (35), or murine erk1 cDNAs were used to infect Sf9 insect cells as described elsewhere (34). Cytosol was prepared from infected cells by rupturing them with a Dounce homogenizer in a low salt HE buffer (10 mM HEPES, pH 7.5, 2 mM EDTA) containing 20 mM sodium molybdate. 3Y1 rat fibroblasts stably transfected with DNA encoding v-Raf were cultured in Dulbecco's modified Eagle's medium plus 10% bovine calf serum, and HeLa cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum.

**Immunoadsorption—**Aliquots (100 μl) of infected Sf9 cytosol were immunoadsorbed with the indicated antibody prebound to 8 μl of protein A-Sepharose. To isolate native signaling protein heterocomplexes from cytosol prepared from Sf9 cells infected with several recombinant baculoviruses, the immunoadsorbed pellets were washed by suspension and centrifugation three times in 1 ml of TEG buffer (10 mM TES, 50 mM NaCl, 4 mM EGTA, 10% glycerol, pH 7.6) plus 0.1% Triton X-100. To isolate native signaling protein-hsp90 heterocomplexes, the TEG buffer contained 2 mM sodium molybdate and Triton X-100 was excluded. In the set of experiments shown in Fig. 2, 50–100-μl aliquots of cytosol prepared from Sf9 cells infected with a single recombinant baculovirus were mixed together for 20 min at 0°C, the proteins were immunoadsorbed to protein A-Sepharose and washed with TEG buffer plus 0.1% Triton X-100.

**Reconstitution of Signaling Protein-hsp90 Heterocomplexes—**Immunoadsorbed protein A-Sepharose pellets were first stripped of any signaling protein-associated insect hsp90 by suspending the pellets in 1 ml of TEG buffer containing 0.5 mM NaCl (but without molybdate) for 1 h at 0°C followed by two washes with 1 ml of TEG buffer. The stripped immune pellets were then mixed with 100 μl of rabbit reticulocyte lysate, and the mixture was suspended by trituration and incubated 20 min at 30°C. Following the incubation in reticulocyte lysate, all pellets were washed 3 times with 1 ml of TEG buffer containing 20 mM sodium molybdate.

**Gel Electrophoresis and Western Blotting—**The immunoadsorbed protein A-Sepharose pellets were heated in SDS sample buffer, and proteins were resolved on 7 or 12% (for Ras) SDS-polyacrylamide gels and transferred to Immobilon-P membranes (17). Membranes were probed with 0.02% rabbit antisera against hsp70 and hsp90 to detect heat shock proteins, 0.1% anti-Raf serum to detect Raf, 1 μg/ml LA-069 to detect Ras, 1 μg/ml N2-17 to detect Src, or 0.5 μg/ml TT-Tag antibody to detect polyhistidine-tagged Mek, or 0.1% of rabbit antisera against GST-Erk to detect Erk. To visualize immunoreactive bands, the immunoblots were incubated a second time with the appropriate horseradish peroxidase-labeled counterantibody and color was developed. The blots were then incubated with the appropriate 125I-labeled counterantibody and exposed to film for autoradiography.

**Assay of Raf Kinase Activity—**Prior to treatment, 100-mm dishes containing confluent HeLa cells were washed once with sterile phosphate-buffered saline and then incubated in serum-free Dulbecco's modified Eagle's medium. After 16 h of serum starvation, cells were pretreated for 4 h with 2 μM geldanamycin or Me2SO vehicle and then for
ATP (20 μM) was incubated for 10 min at 30°C. To each incubation was added human Mek1 were added to each Raf immune pellet, and the mixture was terminated by addition of 5 mM EDTA, 1% Triton X-100. Lysates were clarified by centrifuging for 10 min in a precooled Microfuge. Replicate aliquots of HeLa lystate were rotated for 1 h at 4°C with 20 μl of C-12 anti-Raf serum plus 20 μl of a 50% slurry of protein A-Sepharose. The immune pellets were washed twice with 1 ml of lysis buffer and all liquid was aspirated from the pellet without allowing the pellet to dry. Thirty-five μl of Raf kinase assay mixture (0.2 mM nonradioactive ATP, 30 mM MgCl₂, 2 mM MnCl₂, 40 mM sodium β-glycerophosphate, 0.2 mM sodium orthovanadate, 2 mM okadaic acid, 0.2% β-mercaptoethanol) and 1 μg of purified recombinant human Mek1 were added to each Raf immune pellet, and the mixture was incubated for 10 min at 30°C. To each incubation was added [³²P]ATP (20 μCi) followed by 1 μg of purified Erk K52R mutant (kinase dead), and the incubation was continued for 2 min at 30°C. The reaction was terminated by addition of 5 × sample buffer and boiling, and proteins were resolved by electrophoresis on 10% SDS-polyacrylamide gels and transferred to an Immobilon-P membrane. The membrane was cut and ³²P-labeled Erk was detected with a PhosphorImager. The other half of the membrane was Western blotted for Raf using a monoclonal antibody from Transduction Labs, and bands were developed by counter blotting with ⁵¹I-labeled goat anti-mouse secondary antibody. This portion of the immunoblot was also exposed to the PhosphorImager screen to normalize the Erk radioactivity to the amount of Raf immunoadsorbed in order to generate the data of the bar graph in Fig. 7A.

Luciferase Expression Assay—For each data point, HeLa cells growing in a 100-mm dish at 75% of confluency were transfected by electroporation with 5 μg of an interferon-stimulated response element derived from the interferon-responsive gene ISG15 and linked 5’ to a thymidine kinase minimal promoter-luciferase reporter (36). The cells were then incubated 4 h in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, and g’damycin or vehicle was added. One hour later, 50 ng/ml EGF was added and cells were incubated for an additional 5 h before preparation of cell lysates and assay of luciferase activity by luminometer. EGF can activate transcription through the signal transducers and activators of transcription proteins, which interact with interferon-stimulated response elements. Phosphorylation of signal transducers and activators of transcription serine residues (37) and MAP kinase (Erk) activity (38) are required for this activation.

RESULTS

Protein-Protein Interactions between Signaling Proteins—Sf9 cells were infected with wild-type baculovirus or with recombinant baculoviruses containing the cDNA for Ras, Src, Raf, Mek, or Erk and aliquots of cytosol were immunoblotted with antibody directed against the expressed protein (Fig. 1). To determine the complexes between signaling proteins that can be accounted for by direct protein-protein interactions, cytosols prepared from Sf9 cells expressing a single signaling protein were mixed in all possible combinations of protein pairs and immunoadsorbed with antibodies against each member of the pair. The immunopellets were Western blotted for each member of the pair to determine by coimmunoadsorption the existence of protein-protein complexes that formed when solutions (cytosols) containing the signaling proteins were mixed. The circles above each set of immunoblots in Fig. 2 summarize the results.

Immunoadsorption of a Raf/Mek mixture with antibody against either component yielded coimmunoadsorption of the other component (indicated by the solid interface between the two circles). Evidence for Ras/Src, Ras/Raf, and Src/Raf protein complexes is based upon coimmunoadsorption being detected with antibody against one member of the pair but not with the other (indicated by the hatched interface between the two circles). For all other signaling protein mixtures, no evidence for a complex was obtained by coimmunoadsorption with antibody against either member of the pair. We have available baculoviruses containing cDNA for either polyhistidine-tagged or untagged Erk but only for polyhistidine-tagged Mek, and the only way we can immunoadsorb Erk is by immunoadsorbing the...
His tagged Erk with the T7-Tag monoclonal antibody, which also immunoadsorbs the His tagged Mek. Thus, the possibility of a Mek-Erk interaction could only be tested with one immunoadsorbing antibody and, as indicated in Fig. 2, coimmunoadsorption was not observed. As a Src-Ras interaction has not been previously reported, we were surprised to find coimmunoadsorption of Src with the anti-Ras monoclonal antibody. It should be emphasized that we do not know whether this coimmunoadsorption reflects a direct protein-protein interaction between Src and Ras or an indirect interaction through one or more Sf9 cell proteins.

**Recovery of Multiprotein Complexes from Sf9 Cells Expressing Four Signaling Proteins**—To determine whether multiprotein complexes were formed between signal proteins, Sf9 cells were co-infected simultaneously with baculoviruses containing cDNAs for Ras, Raf, Mek, and Erk. Cytosols prepared from cells expressing all four proteins were then immunoadsorbed to protein A-Sepharose pellets prebound with nonimmune IgG (lane 1), anti-T7 Tag monoclonal IgG to immunoadsorb Mek (lane 2), anti-Ras monoclonal IgG (lane 3), preimmune rabbit serum (lane 4), and rabbit anti-Raf serum (lane 5). Samples were resolved by SDS-PAGE and immunoblotted for Ras, Raf, Erk, and Ras as indicated on the left of the Fig. The symbols lc and lc on the right indicate antibody heavy chain and light chain, respectively.

Comparing the cytosol mixing results of Fig. 2 with the data of Fig. 3 derived from immunoadsorption of cytosol of cells simultaneously expressing Ras, Raf, Mek, and Erk, we can account for the Raf-Mek interactions seen in lanes 2 and 5 of Fig. 3 on the basis of the direct protein-protein binding occurring upon mixture of the two proteins in the experiment of Fig. 2. We can also account for a Ras-Raf interaction but not for the coadsorption of Erk in Fig. 3 (lane 3), either as a Ras-Erk binary complex or as a Ras-Raf-Erk ternary complex. Indeed, in Fig. 2, we see no interaction of Erk with any of the signaling proteins using the pair binding format.

**Sf9 Cellular Factors May Be Required for the Presence of Erk in a Multiprotein Complex**—It is possible that Erk is only bound when multiple proteins are present. Thus in the experiment of Fig. 4A, we have compared the signaling protein complex detected by immunoadsorption with anti-Ras antibody after mixture of cytosols containing singly-expressed Ras, Raf, Mek, and Erk with the complex obtained after immunoadsorption of cytosol from Sf9 cells expressing all four proteins simultaneously. As shown in lane 2 of Fig. 4A, mixture of the four proteins yields coimmunoadsorption of Raf with Ras. Neither Mek nor Erk are present in the immunopellet. As in lane 3 of Fig. 3, however, immunoadsorption of Ras from cytosol of Sf9 cells expressing all four proteins yields coimmunoadsorption of both Raf and Erk (Fig. 4A, lane 4).

Although Raf and Mek associate with each other to form a stable complex (Fig. 2 and Refs. 8 and 10), Mek is not immunoadsorbed in the complex with Ras, Raf, and Erk from cytosol of cells expressing all four proteins. Nevertheless, we examined if the simultaneous expression of Mek was required for formation of a ternary complex containing Erk. In the experiment of Fig. 4B, Ras was immunoadsorbed from cytosol prepared from Sf9 cells simultaneously expressing Ras, Raf, and Erk and Erk was coimmunoadsorbed (lane 2), showing that expression of Mek is not required for Erk coimmunoadsorption. In this experiment we also immunoadsorbed Ras from cytosol prepared...
from Sf9 cells simultaneously expressing only Ras and Erk (Fig. 4B, lane 4) and Erk was communoadsorbed with Ras. If only Erk is expressed, no Erk is communoadsorbed by the anti-Ras antibody. This indicates that Raf is not required for the presence of Erk in the complex and that Ras may be the primary site for interaction with Erk.

Because mixing of cytosols containing singly-expressed proteins does not yield a complex containing Erk, while communoadsorption of Ras from cytosol from Sf9 cells simultaneously expressing multiple proteins yields communoadsorption of Erk, it is reasonable to suggest that Sf9 cellular factors are required to assemble a complex containing Erk. Schulte et al. (27) found that treatment of cells with geldanamycin disrupted both Raf-hsp90 and Raf-Ras complexes, and concluded that association with hsp90 is required for proper localization of Raf in the cell and formation of Ras-Raf complexes. Thus, we wanted to determine whether the hsp90-based chaperone system constituted the Sf9 cellular factors required for the formation of signaling complexes containing Erk.

Association of Src, Raf, and Mek with hsp90—We first wanted to examine what signaling proteins were associated with hsp90. In Fig. 5, the proteins, expressed singly in Sf9 cells, were communoadsorbed to protein A-Sepharose and the immune pellets were immunoblotted for both the expressed protein and insect hsp90. The cytosols were prepared by rupturing cells in a low salt buffer without detergent but containing sodium molybdate to stabilize the complex with hsp90. These are the conditions we have used previously to communoadsorb the Raf-hsp90 complex (19). As shown in lane 3 of Fig. 5, panels A-C, respectively, communoadsorption of Src, Raf, or Mek yielded communoadsorption of hsp90. The communoadsorption is specific for the presence of the signaling protein because communoadsorption of the same cytosol with nonimmune antibody (lane 1) or communoadsorption of cytosol from wild-type baculovirus-infected cells with antibody directed against the signaling protein (lane 2) did not yield hsp90. In contrast to Src,

![Image](image_url)
is likely that geldanamycin produces the same effect on normal

the catalytic domain (19), which is shared with c-Raf-1. Thus, it

of geldanamycin has been tested here on 3Y1 cells ex-

control (Fig. 7A), the amount of Raf in each immune pellet. The values are the

presence of Raf and Raf-associated hsp70 and p60 coimmunoabsorbed with Raf.

hsp90 complexes in COS cells. The Raf and hsp90

with murine sarcoma virus 3611 DNA encoding v-Raf. In this

cell line, we have previously demonstrated native Raf:hsP90 hetero-plexes using the monoclonal AC88 antibody to de-

velop hsp90 (19). As shown in Fig. 7A, a 4-h treatment of 3Y1
cells with geldanamycin causes a loss of about 50% of the
cytosolic and membrane fractions. As shown in Fig. 7B, 4 h of
geldanamycin treatment caused a loss of some Raf-associated
hsP90 and the appearance of Raf-associated hsP70 and p60. The same observation was recently reported by Smith et al. (39) for the effect of geldanamycin on progesterone receptor-hsP90 complexes in COS cells. The Raf and hsP90 bands were cut out of 6 immunoblots such as that of Fig. 7B to assay the relative amount of bound 125I-labeled counteranti-

body. The mean hsP90:Raf ratio in cells treated with geldana-

mycin for 4 h was 0.65 (± 0.08 S.E.) of that in vehicle-treated
cells ($p = 0.003$). It should be noted that, although the amount of Raf is reduced about 50% by 4 h of geldanamycin treatment

(Fig. 7A), the amount of Raf immunoprecipitated is the same as

control (Fig. 7B) because of antibody saturation. Although the
effect of geldanamycin has been tested here on 3Y1 cells ex-

cessing v-Raf-1, we have previously shown that hsP90 binds to the
catalytic domain (19), which is shared with c-Raf-1. Thus, it

is likely that geldanamycin produces the same effect on normal

cellular Raf.

Effect of Geldanamycin on EGF Response in HeLa Cells—To
determine the effect of geldanamycin on the functioning of a Raf-dependent signal transduction pathway, we examined the
effect of the antibiotic on EGF stimulation of Raf kinase activity and expression from a reporter gene in HeLa cells. In Fig.

8A, HeLa cells were treated for 4 h with 2 μM geldanamycin, which were the conditions employed by Schulte et al. (27) in
their study of Raf interaction with hsP90 in MCF7 cells. The

HeLa cells were then briefly treated with EGF, Raf was immuno-

absorbed from the cell lysates and the Raf kinase activity of the

immune pellet was assayed by the method of Dent et al. (33). In this assay, Raf first phosphorylates purified recombinant human Mek and the activated Mek then phosphorylates purified, kinase-inactive Erk. It can be seen in Fig. 8A that EGF treatment increases ERK phosphorylation about 2.5-fold

(lane 2) and that this stimulation is not inhibited by geldana-

mycin (lane 3). Aliquots of cytosol from both the control and the
geldanamycin-treated HeLa cells were immunoblotted for Raf as in Fig. 7A and the 125I-labeled bands were excised and
counted for radioactivity. Geldanamycin decreased the level of
cytosolic Raf by an average of 50% in the two experiments of

Fig. 8A (data not shown).

Again, it should be emphasized that the anti-Raf antibody is

saturated and geldanamycin treatment could decrease cellular

Raf levels without affecting basal or EGF-stimulated Raf ki-

nase activity in the assay of Fig. 8A. Thus, we examined the

effect of geldanamycin on the activity of the complete signal transduction pathway by assaying EGF induction of luciferase expression from a reporter gene. As shown in Fig. 8B, EGF treatment increased luciferase activity (lane 2) and geldana-

mycin pretreatment blunted this increase (lanes 3 and 4).

FIG. 7. Effect of geldanamycin treatment of 3Y1 cells on the amount of Raf, hsP90, and Raf-associated proteins. A, geldana-

mycin treatment decreases the amount of Raf but not hsP90 present in cytosol. Cytosols were prepared from 3Y1 cells treated for 3, 6, and 9 h

with 10 μM geldanamycin or for 9 h with 0.1% Me2SO vehicle (0 h control). Proteins from 5 μl of cytosol were resolved by SDS-PAGE and Western blotting for Raf and hsP90. The Western blots were then

probed with 125I-labeled counterantibody, and the bands were excised and counted to determine the relative amount of Raf (●) and hsP90 (○).

The autoradiogram above the graph presents the cytosolic Raf and hsp90 (lanes 3 and 4) and immunoabsorbed with preimmune rabbit antiserum (lanes 1 and 3) or with anti-Raf serum (lanes 2 and 4) and the proteins were resolved.

FIG. 8. Geldanamycin treatment of HeLa cells impairs EGF signaling. A, geldanamycin does not affect EGF stimulation of Raf kinase activity. HeLa cells were pretreated for 4 h with 2 μM geldana-

mycin and then treated with EGF for 5 min. Raf was immunoadsorbed from cell lysates and Raf kinase activity was assayed by incubation with purified human recombinant Mek1 and subsequent phosphorylation of the Erk K52R mutant as described under “Methods.” The images of

32P-labeled Erk and the relative amount of Raf in each sample determined by Western blotting with a 125I-labeled counterantibody are shown at the top. The bar graph shows the relative phosphorylation of

Erk corrected for the amount of Raf in each immune pellet. The bars present the average and range of values from two experiments. Condi-
tions are: 1, untreated cells; 2, cells treated with EGF alone; 3, EGF plus geldanamycin; 4, geldanamycin alone. B, geldanamycin inhibits EGF-mediated induction of luciferase expression. HeLa cells trans-

fected with a luciferase reporter plasmid under the control of an inter-

feron-stimulated response element were treated 6 h with 2 or 10 μM geldanamycin, with EGF being present for the last 5 h. Luciferase

activity was assayed as described under “Methods.” The values are the

means from three experiments with the standard error indicated by the

vertical line in the bar. Conditions are: 1, untreated cells; 2, cells incubated with EGF alone; 3, EGF plus 2 μM geldanamycin; 4, EGF plus

10 μM geldanamycin. Condition 2 is different from condition 3 at $p < 0.05$ and from condition 4 at $p < 0.03$. 
The concept that components of the MAP kinase cascade may be preassociated with each other in a multiprotein complex is attractive, in that preassociation of the components would be expected to markedly increase the speed of phosphorylation events leading to the ultimate activation of Erk. A model in which such a signalosome complex exists prior to signal reception is quite different and perhaps inherently more efficient than a model in which sequential events somehow “recruit” the individual proteins of the cascade to the membrane. How such a complex might be formed is not known. But, as we describe in the Introduction, evidence for its existence is accumulating in several cell types. Baculovirus-infected Sf9 cells coexpressing multiple mitogenic signaling proteins constitute a useful system for studying protein-protein interactions of potential importance in the formation of such a complex. It is important to note that Agarwal et al. (40) have used this coexpression system to reconstitute signal transduction from the membrane to the nucleus in Sf9 cells. Thus, it is entirely possible that the signaling protein complexes we have identified in this Sf9 system relate to the overall signal transduction process.

In a screen for simple protein-protein interactions between components, we were unable to detect association of Erk with Ras, Raf, Src, or Mek (Fig. 2), and we were unable to create an Erk-containing complex simply by mixing Ras, Raf, Mek, and Erk in solution (Fig. 4A). Yet, from Sf9 cells simultaneously expressing Ras, Raf, Mek, and Erk, we can isolate what seems very likely to be a ternary complex containing Ras, Raf, and Erk (Figs. 3 and 4A). Since Raf and Mek form a stable complex with each other (Figs. 2 and 3 and Refs. 8 and 10), the absence of Mek from a ternary Erk-containing complex may suggest that the presence of Erk decreases the affinity of the Raf-Mek interaction such that Mek dissociates during the immunoadsorption and washing procedure, leaving us with the Ras-Raf-Erk complex. Expression of Mek is not required for formation of the Erk-containing ternary complex (Fig. 4B). Because Erk is immunoadsorbed with Ras from cytosol of Sf9 cells simultaneously expressing only Ras and Erk (Fig. 4B), Ras appears to be necessary for the presence of Erk in any higher order complex.

Because the Sf9 cell can make an Erk-containing complex that we do not form by mixing the components in vitro, it would seem that a cellular process is required to form a complex containing Erk. Our detection of native complexes of insect hsp90 with Raf, Mek, and possibly Erk (Fig. 5), suggested that the hsp90 chaperone system might be involved in the assembly of an Erk-containing multiprotein complex. The observation of Schulte et al. (27) that treatment of cells with the hsp90-binding antibiotic geldanamycin causes dissociation of both Raf-hsp90 and Raf-Ras multimolecular complexes led us to examine the effect of geldanamycin on the coadsorption of Raf and Erk when Ras was immunoadsorbed from cytosol of Sf9 cells coexpressing multiple mitogenic signaling proteins. Geldanamycin did not affect recovery of the Ras-Raf-Erk complex (Fig. 6A), but as reported by Schulte et al. (27), the amount of cytosolic Raf was decreased with geldanamycin treatment (Fig. 6, A and B). Schulte et al. (27) found that geldanamycin treatment eliminated the Raf association with hsp90. We did not see a change in Raf binding to hsp90 in Sf9 cells treated with geldanamycin (Fig. 6B), but the rather poor antibody reaction with hsp90 makes the insect cell an inadequate system in which to look for anything less than total elimination of the Raf-hsp90 complex.

When 3Y1 cells were treated with geldanamycin, the amount of hsp90 coimmunoadsorbed with Raf was moderately decreased (35%), but importantly, the Raf heterocomplex was different in that it contained substantial amounts of p60 and hsp70 as well. In a study of the time course of protein association with the progesterone receptor during heterocomplex assembly in reticulocyte lysate, Smith (41) showed that this complex represents an intermediate stage in the receptor-hsp90 heterocomplex assembly process. Smith et al. (39) have now shown that addition of geldanamycin to the reticulocyte lysate assembly system or geldanamycin treatment of intact cells results in a receptor complex with increased hsp70 and p60 and with somewhat decreased hsp90. Proper hsp90 chaperoning is required for the progesterone receptor to bind steroid (41), and both in the reticulocyte lysate and in the intact cell, geldanamycin treatment rapidly inactivated the steroid binding capacity of the receptor (39). Thus, although the receptor complex that is formed in the presence of geldanamycin contains hsp90, it is not the functional (i.e. steroid binding) heterocomplex. As we show in Fig. 7B, treatment of 3Y1 cells with geldanamycin blocks Raf-hsp90 heterocomplex assembly at the same intermediate stage at which it blocks steroid receptor-hsp90 heterocomplex assembly.

Schulte et al. (27) demonstrated that the rate of Raf degradation is increased in MCF7 cells treated with geldanamycin. The levels of both cytoplasmic and membrane-bound Raf decreased at the same rate, with the half-time for Raf disappearance from the cell being about 4 h (27). In 3Y1 cells, geldanamycin treatment also results in the disappearance of Raf with a half-time of about 4 h (Fig. 7A). One interpretation of these data is that Raf-hsp90 complexes are in a dynamic state of assembly and disassembly in the cell, much as has been reported for steroid receptors (41). Binding of geldanamycin to hsp90 allows hsp90 to bind to Raf (or receptors) in the presence of the required p60 and hsp70 components of the heterocomplex assembly system, but the complex cannot proceed from this intermediate state to the mature form, which does not contain p60. When it is in the mature complex with hsp90, the turnover of Raf is much slower than when it is in the geldanamycin-blocked complex, and in geldanamycin-treated cells, Raf is rapidly degraded.

In contrast to Schulte et al. (27) who found that geldanamycin treatment resulted in dissociation of Ras from Raf, we find no effect of the antibiotic on the coimmunoadsorption of Raf or Erk with Ras (Fig. 6A), suggesting that hsp90 is not required for forming complexes between the mitogenic signaling proteins. In addition, we find that geldanamycin does not affect basal or EGF-stimulated Raf kinase activity in HeLa cells (Fig. 8A), but it does reduce overall EGF-mediated signaling as assayed by induction of luciferase expression from an appropriate reporter gene (Fig. 8B). In the experiments of Fig. 8, the Raf level is decreased by 50% in the geldanamycin-treated HeLa cells, and this effect on turnover of Raf and possibly Mek and other components of the signaling pathway might explain the inhibition of EGF signaling by the antibiotic.

Because the three mitogenic signaling proteins Src, Raf, and Mek are bound to hsp90 (Fig. 5), it is important to determine how the chaperone affects their cellular activity. Several observations lead to the conclusion that formation of the Src-hsp90 heterocomplex is critical for Src function. In yeast, expression of v-Src produces growth arrest, and Xu and Lindquist (42) showed that lowering the level of hsp90 expression relieved cell cycle arrest and rescued cell growth in yeast cells expressing v-Src. Also, point mutations in hsp90 have been shown to affect v-Src activity in yeast (43), and a mutation in the yeast DnaJ homolog, Ydj1, was found to rescue v-Src lethality (44). Similar experiments have not been performed for Raf and Mek, but by analogy with Src, hsp90 may be required for their cellular function as well. At this time, it is not known if hsp90 chaper-
oning plays a role in signaling protein function in the cell beyond stabilization of the proteins to degradation, as observed for Raf (Ref. 27 and Fig. 7A). We would predict that heterocomplex formation with hsp90 is not required for signaling proteins to form complexes with each other or for their intrinsic kinase activity. However, other roles (e.g. in trafficking of signaling proteins) are possible, and both the genetic observations cited above and the results of geldanamycin experiments reported by Schulte et al. (27) and in this paper support the notion that hsp90 chaperoning is required for this signal transduction pathway to operate in the cell.

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REFERENCES
1. Egan, S. W., and Weinberg, R. A. (1993) Nature 365, 781–783
2. Crews, C. M., and Erikson, R. L. (1993) Cell 74, 215–217
3. Williams, N. G., Roberts, T. M., and Li, P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2922–2926
4. Faham, J. R., Vojtek, A. B., Cooper, J. A., and Morrison, D. K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5982–5986
5. Pumiglia, K., Chow, Y-H., Faham, J., Morrison, D., Decker, S., and Jove, R. (1995) Mol. Cell. Biol. 15, 398–406
6. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214
7. Warne, P. H., Viciana, P. R., and Downward, J. (1993) Nature 364, 352–355
8. Van Aelst, L., Barr, M., Marcus, S., Polverino, A., and Wiger, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6213–6217
9. Hallberg, B., Rayter, S. I., and Downward, J. (1994) J. Biol. Chem. 269, 3913–3916
10. Huang, W., Alessandrini, A., Crews, C. M., and Erikson, R. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10947–10951
11. Clegon, V., and Morrison, D. K. (1994) J. Biol. Chem. 269, 17749–17755
12. Moodie, S. A., Williamson, B. M., Weber, M. J., and Wolfman, A. (1993) Science 260, 1658–1661
13. Brugge, J. S., Erikson, E., and Erikson, R. L. (1981) Cell 25, 363–372
14. Courtneidge, S. A., and Bishop, J. M. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 7117–7121
15. Brugge, J. S., Yonemoto, W., and Darrow, D. (1983) Mol. Cell. Biol. 3, 9–19
16. Brugge, J. S. (1986) Curr. Top. Microbiol. Immunol. 123, 1–22
17. Hutchison, K. A., Brott, B. K., De Leon, J. H., Perdew, G. H., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 2902–2906
18. Hutchison, K. A., Stancato, L. F., Jove, R., and Pratt, W. B. (1992) J. Biol. Chem. 267, 13952–13957
19. Stancato, L. F., Chow, Y-H., Hutchinson, K. A., Perdew, G. H., Jove, R., and Pratt, W. B. (1993) J. Biol. Chem. 268, 21711–21716
20. Wartmann, M., and Davis, R. J. (1994) J. Biol. Chem. 269, 6695–6701
21. Stancato, L. F., Chow Y-H., Owens-Grillo, J. K., Yem, A. W., Deibel, M. R., Jove, R., and Pratt, W. B. (1994) J. Biol. Chem. 269, 22157–22161
22. Pratt, W. B. (1995) J. Biol. Chem. 268, 21455–21458
23. Smith, D. F., and Toth, D. O. (1993) Mol. Endocrinol. 7, 4–11
24. Hutchison, K. A., Dittmar, K. D., and Pratt, W. B. (1994) J. Biol. Chem. 269, 27894–27899
25. Schumacher, R. J., Hurst, R., Sullivan, W. P., McMahon, N. J., Toth, D. O., and Matts, R. L. (1994) J. Biol. Chem. 269, 9493–9499
26. Dittmar, K. D., Hutchison, K. A., Owens-Grillo, J. K., and Pratt, W. B. (1996) J. Biol. Chem. 271, 12883–12889
27. Schulte, T. W., Blagosklonny, M. V., Ingui, C., and Neckers, L. (1995) J. Biol. Chem. 270, 24585–24588
28. Uehara, Y., Murakami, Y., Mizuno, S., and Kawai, S. (1988) Virology 164, 294–296
29. Whitesell, L., Shifrin, S. D., Schwah, G., and Neckers, L. M. (1992) Cancer Res. 52, 1721–1728
30. 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
31. Erhart, J. C., Duthu, A., Ullrich, S., Appella, E., and May, P. (1988) Oncogene 3, 595–603
32. Chow Y-H., Pumiglia, K. M., Jun, T. H., Dent, P., Sturgill, T. W., and Jove, R. (1995) J. Biol. Chem. 270, 14105–14106
33. Dent, P., Reardon, D. B., Morrison, D. K., and Sturgill, T. W. (1995) Mol. Cell. Biol. 15, 4125–4135
34. Park, S., Marshall, M. S., Gibbs, J. B., and Jove, R. (1992) J. Biol. Chem. 267, 3917–3925
35. Dent, P., Chow, Y-H., Wu, J., Morrison, D. K., Jove, R., and Sturgill, T. W. (1994) Biochem. J. 303, 105–112
36. pine, R., Canova, A., and Schindler, C. (1994) EMBO J. 13, 158–167
37. Wen, Z., Zhong, Z., and Darnell, J. E. (1995) Cell 82, 241–250
38. David, M., Petricoin, E., Benjamin, C., pine, R., Weber, M. J., and Larner, A. C. (1995) Science 269, 1721–1723
39. Smith, D. F., Whitesell, L., Nair, S. C., Chen, S., Prapapanich, V., and Rimerman, R. A. (1995) Mol. Cell. Biol. 15, 6804–6812
40. Agarwal, S., Corbley, M. J., and Roberts, T. M. (1995) Oncogene 11, 427–438
41. Smith, D. F. (1993) Mol. Endocrinol. 7, 1418–1429
42. Xu, Y., and Lindquist, S. (1993) Science 261, 1362–1365
43. Bianco, K. A., and Lindquist, S. (1995) Mol. Cell. Biol. 15, 3917–3925
44. Kimura, Y., Yahara, I., and Lindquist, S. (1995) Science 268, 1362–1365