Thrombin Receptor Signaling to Cytoskeleton Requires Hsp90*

Karnire S. Pai‡, Vinit B. Mahajan‡, Alice Lau, and Dennis D. Cunningham§

From the Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, California 92697-4025

Thrombin is a serine protease that evokes various cellular responses involved in injury and repair of the nervous system through the activation of protease-activated receptor-1 (PAR-1). Signals that modulate cell morphology precede most PAR-1 effects, but the initial signal transduction molecules are not known. Using the yeast two-hybrid system, we identified Hsp90, a chaperone with known signaling properties, as a binding partner of PAR-1. The interaction was confirmed by glutathione S-transferase pull-down, overlay, and co-immunoprecipitation assays. Morphological assays in mouse astrocytes were carried out to evaluate the importance of Hsp90 during cytoskeletal signaling. Reducing Hsp90 levels by antisense treatment or disruption of the Hsp90/PAR-1 complex by the Hsp90-specific drug geldanamycin attenuated thrombin-mediated astrocyte shape changes. Furthermore, overexpression of the PAR-1 cytoplasmic tail abrogated thrombin-induced cytoskeletal changes in neuronal cells. Treatment with geldanamycin specifically inhibited activation of RhoA without affecting thrombin-mediated intracellular calcium release, revealing the regulation of a distinct signaling pathway by Hsp90. Taken together, these studies demonstrate that Hsp90 may be essential for PAR-1-mediated signaling to the cytoskeleton.

The small GTP-binding protein RhoA and intracellular calcium mediate several thrombin responses (12–15). Activated GTP-bound RhoA translocates to the membrane, interacts with RhoA kinase, and mediates PAR-1-induced myosin light chain phosphorylation required for cytoskeletal reorganization (3). PAR-1 increases intracellular calcium levels by activating phospholipase C (9, 14). The PAR-1-mediated increase in intracellular calcium has been linked to coupling of the second intracellular loop of PAR-1 to Goς (14). On the other hand, PAR-1-mediated activation of RhoA and the subsequent morphological changes involve activation of Goς (3). Recently, we showed that the PAR-1 cytoplasmic tail (C-tail) may be important for the regulation of cytoskeletal changes (16). It is not known how PAR-1 transduces these diverse signals intracellularly.

Hsp90 is a chaperone molecule widely expressed in cells of various species that plays a key role in signal transduction under non-stress conditions and in protein renaturation and refolding under stress (17–19). By forming heterocomplexes with its substrates, Hsp90 has been shown to be important for various signaling events. In yeast and Drosophila, the Hsp90 counterparts Hsp82 and Hsp83 are required for steroid receptor, v-Src, and tyrosine kinase signaling events (20–22). In mammalian cells, Hsp90 association with Raf, MEK, and Src family proteins is required for signaling (19). The significance of these interactions was examined with the antitumor agent geldanamycin, which binds specifically to Hsp90 and disrupts interactions between the chaperone and associated proteins (19, 23). Hsp90 has also been shown to facilitate G protein-coupled pathways (24), but whether Hsp90 directly regulates G protein-coupled receptors is not known.

We used a yeast two-hybrid system to identify molecules that directly interact with the PAR-1 C-tail and regulate thrombin signaling. Hsp90 was found to interact specifically with the PAR-1 C-tail in the yeast system. The interaction was confirmed by various in vitro and in vivo assays. The physiological significance of the interaction was examined by carrying out morphological and biochemical studies in astrocytes and neuronal cells.

EXPERIMENTAL PROCEDURES

Materials—Thrombin was purchased from Calbiochem. Polyclonal antibody for PAR-1 was a gift from Marshael Runge (University of North Carolina, Chapel Hill, NC). A second anti-PAR-1 antibody was also kindly provided. Antibodies for RhoA and GST were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-Hsp90 antibody was obtained from Stressgen (Victoria, Canada). The GST/Rhoetkin-binding domain construct was a gift from Martin Schwartz (Scripps Research Institute, La Jolla, CA). Geldanamycin was provided by NCI (Rockville, MD).

Yeast Two-hybrid Assay—The polymerase chain reaction product of the human PAR-1 C-tail (residues 375–425) cloned into the NcoI and SalI sites of the Gal4 DNA-binding vector pAS2-1 was used as a bait vector. A human fetal brain cDNA library (CLONTECH) fused to the Gal4 activation domain in pGAD10 was used as a trap vector in yeast two-hybrid screening. The plasmids were transformed into yeast Y190
cells, and positive clones were selected on triple-minus plates (leu- trp his-). Assays for β-galactosidase activity were carried out on these clones using a filter lift method according to the manufacturer's instructions (CLONTECH). Interactions were confirmed by re-transforming the β-galactosidase-positive clones with either the bait vector or the original library (backbone) into yeast. Oligonucleotides for Hsp90 (10 mM HEPES-KOH (pH 7.5), 60 mM KCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol) for 5 min and then exposed for 10 min each to decreasing concentrations of guandine HCl (6, 3, 1.5, 0.75, 0.38, 0.19, 0.1, and 0 mM in incubation buffer B. Following incubation, the filter was washed three times with incubation buffer A. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-Hsp90 antibody.

Overlay Assay—A filter overlay assay was performed as described (25). Briefly, 1-2 μg of Hsp90 were fractionated by SDS-PAGE and blotted onto nitrocellulose. The filter was blocked in incubation buffer A (10 mM HEPES-KOH (pH 7.5), 50 mM KCl, 20 mM sodium molybdate), subjected to immunoblot analysis with anti-Hsp90 antibody, and treated with either 0.5 μg/ml GST or GST/C-tail overnight at 4 °C, and washed three times with incubation buffer A. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-Hsp90 antibody.

GST Pull-down Assay—The human PAR-1 C-tail was subcloned in-frame behind the GST construct pGEX4T-3 (Amersham Pharmacia Biotech). GST fusion constructs were transformed into Escherichia coli strain BL21(DE3), and expression was induced by addition of isopropyl-β-D-thiogalactopyranoside. Fusion proteins were purified using glutathione-Sepharose beads (Amersham Pharmacia Biotech). Purified Hsp90 (100 pmol; Stressgen) was added to 0.1 ml of incubation buffer A (10 mM Tris (pH 7.5), 50 mM KCl, 5 mM MgCl2, 20 mM sodium molybdate, 100 pmol; Stressgen) was added to 0.1 ml of incubation buffer A (10 mM Tris (pH 7.5), 50 mM KCl, 5 mM MgCl2, 20 mM sodium molybdate) in a fluorometric imaging plate reader (Molecular Devices, Inc.). Some wells received 1 μM U73122 (BIOMOL Research Labs Inc.) for 10 min prior to thrombin (20 nM) addition. Cells were lysed in radioimmune precipitation assay buffer (50 mM NaCl, 10 mM MgCl2, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each leupeptin and aprotinin). Cell lysates were centrifuged at 13000 g for 10 min at 4 °C, and the clear supernatants were incubated with GST/Rhotekin-binding domain (20-30 μg) for 2 h prior to thrombin (10 nM) addition. Control cells were incubated with GST alone or transformation of the Hsp90/Gal4 activation domain clone with the Gal4 DNA-binding vector alone or transformation of the Hsp90/Gal4 activation domain clone with the Gal4 DNA-binding vector alone or transformation of the Hsp90/Gal4 activation domain clone with the Gal4 DNA-binding vector alone.

Active RhoA levels were measured in primary rat astrocyte cultures as described (28). Astrocyte cultures were pretreated with galanin analog 2 (100 nM) for 2 h prior to thrombin (10 nM) addition. Cells were lysed in radiomimunoprecipitation assay buffer (50 mM NaCl, 10 mM MgCl2, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each leupeptin and aprotinin), and the bound proteins were immunopured with anti-RhoA antibody.

Intracellular Calcium Assay—Astrocytes were cultured in 96-well plates (Costar Corp.) and treated with 20 μM galanin analog 2 (100 nM) for 2 h prior to thrombin (10 nM) addition. Cells were washed three times with Tris buffer (50 mM Tris (pH 7.2), 500 mM NaCl, 10 mM MgCl2, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each leupeptin and aprotinin) and the bound proteins were immunopured with anti-RhoA antibody.

Morphological Assay—Primary astrocyte cultures were prepared from cell cultures of 1- or 2-day-old Sprague-Dawley rats. Cultured astrocytes were incubated overnight in serum-free Dulbecco’s modified Eagle’s medium and solubilized in immunoprecipitation buffer (50 mM Tris (pH 7.4), 1 mM EGTA, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μM apotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin) as described earlier (16). The pellet was resuspended with either the antibody (rabbit IgG) or anti-PAR-1 antibody generated against the SFLRN peptide (16) overnight at 4 °C in the presence of protein A-Sepharose. Immunoprecipitated PAR-1 was detected by Western blotting with a second antibody (BIODESIGN International). Proteins bound to the beads were washed three times with immunoprecipitation buffer, eluted with SDS sample buffer, resolved by 12% SDS-PAGE, and subjected to immunoblot analysis with anti-Hsp90 antibody.

Hsp90 Specifically Interacts with the PAR-1 C-tail in Yeast—To identify molecular components in PAR-1 signaling events, we used the 50-amino acid C-tail of human PAR-1 as bait in a yeast two-hybrid system (16). Of the 3.5 × 106 clones screened using a human fetal brain library, one was positive in a β-galactosidase filter lift assay. Sequencing and BLAST analysis revealed the cDNA clone to be identical to the 3′-region (amino acids 309–721) of Hsp90, a chaperone protein that plays a key role in signaling and protein renaturation and refolding (17–19). The interaction between Hsp90 and the PAR-1 C-tail was confirmed as specific since co-transformation of the isolated Hsp90/Gal4 activation domain clone with the Gal4 DNA-binding vector alone or transformation of the Hsp90/Gal4 activation domain clone alone in yeast did not activate the β-galactosidase reporter gene (Fig. 1). To determine if other intracellular regions of PAR-1 interact with Hsp90, CL1, CL2, and CL3 were co-transformed with the Hsp90/Gal4 activation domain clone into yeast. As shown in Fig. 1, CL1, CL2, and CL3 were positive in the β-galactosidase filter lift assay, indicating that Hsp90 specifically interacted in yeast with only the PAR-1 C-tail.

PAR-1 Interacts with Hsp90 Both in Vitro and in Vivo—The interaction between the PAR-1 C-tail and Hsp90 in yeast was confirmed by two separate in vitro assays. First, purified Hsp90 was incubated with bacterially expressed, partially purified...
Hsp90 and Thrombin Signaling

Fig. 1. Hsp90 interaction with PAR-1 C-tail in yeast. Shown are the results from the yeast two-hybrid analysis of various intracellular regions of PAR-1, Gal4 DNA-binding (DB) fusion protein constructs of CL1, CL2, and CL3 and the C-tail of PAR-1 were co-transformed into yeast along with the Hsp90 clone (obtained in the screen) in the Gal4 activation domain (AD) vector. Positive clones were selected on triplenumin plates and assayed for β-galactosidase activity using a filter lift assay. The results from a typical β-galactosidase filter lift assay with various controls are shown. A positive interaction was detected only between the PAR-1 C-tail and Hsp90.

GST or with a GST fusion of the PAR-1 C-tail bound to glutathione-Sepharose beads in a pull-down assay (16). Western blot analysis of the bound proteins using anti-Hsp90 antibody revealed that Hsp90 bound specifically to GST/C-tail fusion protein (Fig. 2A). A similar result was observed in a second in vitro overlay assay (25), wherein purified Hsp90 was subjected to SDS-PAGE, blotted, overlaid with either GST or GST/C-tail fusion protein, and probed with anti-GST antibody (Fig. 2B). These results demonstrate that the PAR-1 C-tail interacts with Hsp90 in a cell-free system.

To determine if full-length PAR-1 interacts with Hsp90 in vivo, PAR-1 was immunoprecipitated as described previously (16) from primary cultures of astrocytes, which are known to express PAR-1 (29). As shown in Fig. 2C, anti-PAR-1 antibody specifically co-immunoprecipitated Hsp90 from astrocyte cultures (second lane), whereas the control antibody (IgG) did not (first lane). Competition with the SFLRN peptide, against which the anti-PAR-1 antibody was developed, blocked co-immunoprecipitation of Hsp90 from astrocyte cultures (third lane). We also utilized geldanamycin, a small molecule that specifically binds the ATP-binding pocket in Hsp90 (but not other chaperones), alters the tertiary structure of Hsp90, and prevents Hsp90 interactions with target proteins (23). After treatment with geldanamycin (10 μM), PAR-1 no longer co-precipitated Hsp90 (fourth lane), indicating that the interaction between PAR-1 and Hsp90 was disrupted. These experiments suggest that PAR-1 exists in a complex with Hsp90 under physiological, non-stress conditions.

Hsp90 Is Essential for Thrombin-mediated Morphological Changes—To examine the role of Hsp90 in PAR-1 signaling, we decreased its expression by an antisense strategy as previously described (27). Primary astrocyte cultures were used for the antisense study since they express abundant PAR-1, and their morphology is regulated by thrombin (26, 30). Cultured astrocytes showed a stellate morphology with numerous processes extending from the cell soma, but upon addition of thrombin (10 pM), the processes retracted, and the cells bodies spread and appeared non-stellate (Fig. 3A) (13, 16). Consistent with the previous studies, transfection of Hsp90 antisense oligonucleotides into astrocyte cultures significantly decreased Hsp90 expression levels as measured by Western blotting (Fig. 3B, inset). When astrocyte cultures treated with Hsp90 antisense oligonucleotides were challenged with thrombin (10 pM), they no longer responded, and >75% of the cells showed a stellate morphology (Fig. 3, A and B). To determine the specificity of the antisense oligonucleotides, cells were treated with reverse oligonucleotides, and the thrombin effect was monitored. As shown in Fig. 3B, reverse oligonucleotides did not change the expression of Hsp90 and had no significant effect on morphological changes induced by thrombin compared with vehicle controls. These results suggest that Hsp90 may be required for thrombin-mediated morphological changes.

Since oligonucleotides may have nonspecific effects, an alter-
However, overexpression of CL2 of PAR-1, which was shown to be required for thrombin-induced intracellular calcium signaling (14), did not prevent thrombin-induced morphological changes, and the cells remained flat and non-refractile (Fig. 4G). Taken together, these results strongly suggest that the PAR-1 C-tail, but not CL2, is necessary for thrombin-induced morphological changes.

Hsp90 Is Required for RhoA Activation—Thrombin-induced changes in astrocyte morphology are mediated by RhoA, a small GTP-binding protein (3, 13). If Hsp90 were an essential component of initial PAR-1 signaling, we hypothesized that geldanamycin treatment would prevent thrombin-induced RhoA activation.

In this study, a GST fusion of the Rho-binding domain of Rhotekin, which binds activated RhoA, but not inactive RhoA (32), was used to specifically pull down GTP-RhoA as described (16). Addition of thrombin to astrocyte cultures resulted in a time-dependent increase in GTP-RhoA levels (Fig. 5A). Maximum RhoA activation was seen 20 min after thrombin addition. Pretreatment with geldanamycin (200 nM) for 2 h blocked thrombin-mediated RhoA activation (Fig. 5B). Control experiments using GST alone did not pull down activated GTP-RhoA (data not shown). These results indicate that Hsp90 regulates PAR-1-mediated cytoskeletal reorganization upstream of RhoA activation.

Thrombin-mediated Calcium Signaling Is Not Regulated by Hsp90—PAR-1 also mediates certain thrombin responses by activation of phospholipase C, which leads to calcium mobilization from intracellular stores (9, 14). Changes in intracellular calcium are not required for thrombin-mediated cytoskeletal reorganization (30, 33). Chimeric receptor studies further indicated that PAR-1 CL-2 (but not the C-tail) mediates calcium signals (14). We hypothesized that calcium signals may be independent of Hsp90 since the chaperone did not bind to CL2, and CL2 overexpression did not inhibit PAR-1 morphological responses in neuronal cells.

To evaluate the role of Hsp90 in the PAR-1-mediated increase in intracellular calcium levels, astrocytes pretreated with geldanamycin were loaded with Fluo-3, and calcium measurements were carried out with or without thrombin (10 pM) treatment. As shown in Fig. 6, thrombin elicited robust calcium
The structural determinants of PAR-1 required for PAR-1-mediated cytoskeletal changes are not clearly established. Our overexpression studies demonstrate that the PAR-1 C-tail is required for morphological changes, whereas CL2 is not. These results are consistent with previous findings that indicate that PAR-1 CL2 (but not the C-tail) is important for calcium signals (14, 16). The overexpression studies also suggest that proteins such as Hsp90 that interact with the PAR-1 C-tail might help regulate PAR-1-mediated morphological changes. Identifying and overexpressing the PAR-1 C-tail region necessary for Hsp90 binding in future studies would more clearly define the role of an Hsp90/PAR-1 interaction during thrombin signaling. The PAR-1 C-tail is also required for receptor recycling and down-regulation (40, 42), although the role of Hsp90 in these events remains to be explored. It is possible that the PAR-1

\[ n \leq 3, \quad \text{mean} \pm \text{S.D.,} \quad n = 3. \]

**FIG. 6.** Hsp90 is not required for thrombin-mediated intracellular calcium changes. Astrocytes were pretreated either with geldanamycin (GA; 20 \( \mu M \)) or phospholipase C inhibitor (1 \( \mu M \) U73122) prior to thrombin (T, 10 \( \mu M \)) treatment. Intracellular calcium levels were monitored with the fluorometric imaging plate reader detection system. The representative experiment displays the kinetics of intracellular calcium flux monitored after thrombin addition. Peak intracellular calcium was quantified and normalized to control values (mean \( \pm \) S.D., \( n = 3 \)).

signals within seconds that could be inhibited by the application of phospholipase C inhibitor (U73122) as described previously (34). Application of geldanamycin to astrocytes had no effect on the thrombin-induced increases in intracellular calcium. These results suggest that Hsp90 is required for regulation of the RhoA pathway, but not for the pathway affecting intracellular calcium levels.

**DISCUSSION**

Regulation of cell morphology is important for maintaining cellular architecture and normal functions. In neurons, for example, cell processes and morphology maintain synaptic connections and signaling. Astrocytes extend their processes to help form the blood-brain barrier with endothelial cells and provide a microenvironment for neuronal function (13, 35). During cerebrovascular injury, thrombin passes through the blood-brain barrier and extravasates into brain tissue (6, 36). In brain, thrombin mediates cell killing, gliosis, and proliferation, and shape change signals in neurons and astrocytes precede these events (6, 8). PAR-1 mediates thrombin-induced morphological changes, although the initial events in this receptor signaling are not known.

We identified Hsp90 as a possible binding partner of PAR-1. This is intriguing since Hsp90 has emerged as a chaperone with specific signaling activities under non-stress conditions. It has been implicated in membrane signaling events; but to our knowledge, no physical interactions with membrane receptors have been demonstrated (19, 24). The interaction between Hsp90 and the PAR-1 C-tail in yeast was found to be specific since Hsp90 did not bind to other intracellular regions of the receptor. Furthermore, the interaction was detected under various in vitro conditions. The interaction was also seen in vivo using co-immunoprecipitation and was blocked by geldanamycin, an Hsp90-specific drug. Incubation of PAR-1 immunoprecipitates with ATP released Hsp90, suggesting that the interaction between PAR-1 and Hsp90 is dynamic and supporting the findings suggesting that ATP binding and ATPase activity are necessary for Hsp90 function (37–39). Since Hsp90 is an abundant chaperone, it is difficult to study the interaction in intact cells using immunofluorescent co-localization techniques, as Hsp90 would appear to co-localize even without interacting proteins several microns away. The co-immunoprecipitation studies were carried out in unstimulated cells, indicating the pre-assembly of molecular components prior to the activation of the receptor. This is particularly important in the case of PAR-1 since thrombin-mediated cytoskeletal changes in a cell are initiated rapidly within seconds (30, 33). Co-immunoprecipitation experiments were also carried out to determine if thrombin stimulation enhances Hsp90 binding to PAR-1, and the results were inconclusive. This may be due to the down-regulation and degradation of PAR-1 that occur immediately after activation (40, 41).

Compared with other chaperone proteins, Hsp90 is distinguished by its specific interactions with various signaling proteins and its important role during signal transduction cascades under non-stress conditions (19). Our studies lend support to this premise since geldanamycin not only disrupted the interaction between PAR-1 and Hsp90, but also prevented the activation of RhoA and subsequent cytoskeletal reorganization without any effect on thrombin-mediated calcium signaling. The thrombin-induced morphological changes were also prevented by antisense inhibition of Hsp90. In some studies, disruption of the interaction between Hsp90 and its client proteins by geldanamycin resulted in down-regulation of the client proteins (19). This does not appear to be the mechanism behind the reduced PAR-1 signal since our studies (Fig. 2C, lower panel, fourth lane) indicated no significant degradation of PAR-1 by geldanamycin. Furthermore, geldanamycin treatment did not block intracellular calcium signals produced by thrombin, demonstrating that the receptor was intact and could generate full signals involving CL2. Together, these results make it less likely that geldanamycin has an effect on PAR-1 degradation, down-regulation, or membrane targeting.

The structural determinants of PAR-1 required for PAR-1-mediated cytoskeletal changes are not clearly established. Our overexpression studies demonstrate that the PAR-1 C-tail is required for morphological changes, whereas CL2 is not. These results are consistent with previous findings that indicate that PAR-1 CL2 (but not the C-tail) is important for calcium signals (14, 16). The overexpression studies also suggest that proteins such as Hsp90 that interact with the PAR-1 C-tail might help regulate PAR-1-mediated morphological changes. Identifying and overexpressing the PAR-1 C-tail region necessary for Hsp90 binding in future studies would more clearly define the role of an Hsp90/PAR-1 interaction during thrombin signaling. The PAR-1 C-tail is also required for receptor recycling and down-regulation (40, 42), although the role of Hsp90 in these events remains to be explored. It is possible that the PAR-1

\[^{2}\text{K. S. Pai, V. B. Mahajan, A. Lau, and D. D. Cunningham, unpublished data.}\]
C-tail may be able to carry out dual functions similar to the β-adrenergic receptor, which regulates both receptor down-regulation and signaling through its C-tail (43, 44).

PAR-1 regulates morphological changes through activation of RhoA (3). Recent evidence suggests that Go12/13 subunits act upstream of RhoA and are required for PAR-1-mediated cytoskeletal changes (3, 45). Our signaling studies indicate that Hsp90 acts upstream of RhoA since geldanamycin blocked activation of RhoA. It is not clear whether Hsp90 is essential for activation of Go12/13, but we speculate that Hsp90 might help couple Go12/13 to the PAR-1 C-tail. Increases in intracellular calcium are not required for PAR-1-mediated morphological changes and are thought to be regulated by PAR-1 CL2 coupling to Goq (14). Our binding and overexpression studies are consistent with previous observations (14, 16) and indicate that Hsp90 may not be required for calcium changes. Taken together, our results indicate that Hsp90 specifically regulates PAR-1-mediated morphological changes, but not PAR-1-mediated calcium changes.

How might Hsp90 regulate PAR-1 signaling? One hypothesis is that the binding of Hsp90 to PAR-1 may be necessary to maintain proper conformation of the C-tail for signal transduction, as shown for other proteins that interact with Hsp90 (19). A second hypothesis is that Hsp90 may act as a scaffold to orient proteins that are required for morphological changes onto the receptor. One such candidate might be creatine kinase B, an ATP-generating protein required in the cytoskeletal signaling pathway in astrocytes and neuronal cells (16). We are currently investigating this hypothesis.

PAR-1 also regulates fibroblast stress fiber formation, platelet aggregation, and endothelial permeability (5, 9). Changes in cytoskeletal reorganization and activation of the RhoA pathway precede these functional responses (6, 8, 46, 47). Hsp90 is also expressed in fibroblasts, platelets, and endothelial cells, and our preliminary experiments suggest a role for Hsp90 in the regulation of PAR-1-mediated morphological changes in these cells.

Acknowledgments—We thank P. Notthacker and O. Civelli for assistance with the calcium measurements, Marshel Runge for anti-PAR-1 antibody, and Martin Schwartz for the GST/Rhotekin-binding domain construct. We are grateful to David Toft and Wes Hatfield for critically reading the manuscript and helpful discussions. We also thank NCI for the generous gift of geldanamycin.

REFERENCES
1. Berndt, M., and Phillips, D. (1981) in Platelets in Biology and Pathology (Gordon, J., ed) pp. 43–74, Elsevier/North-Holland Biomedical Press, Amsterdam
2. Even-Ram, S., Uziel, B., Cohen, P., Grisaru-Granovsky, S., Mazo, M., Ginzhur, Y., Reich, R., Vladavsky, I., and Bar-Shavit, R. (1998) Nat. Med. 4, 909–914
3. Seasholtz, T. M., Majumdar, M., and Brown, J. H. (1999) Mol. Pharmacol. 55, 949–956
4. Striggow, F., Riek, M., Breder, J., Heinrich-Noack, P., Beymann, K. G., and Reiser, G. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2264–2269
5. Vaughan, P. J., Pike, C. J., Cotman, C. W., and Cunningham, D. D. (1995) J. Neurosci. 15, 5389–5401
6. Donovan, F. M., Pike, C. J., Cotman, C. W., and Cunningham, D. D. (1997) J. Neurosci. 17, 5316–5326
7. Donovan, F. M., and Cunningham, D. D. (1998) J. Biol. Chem. 273, 12746–12752
8. Turgur, V. L., Lloyd, E. D., Wang, S., Festoff, B. W., and Houenou, L. J. (1998) J. Neurosci. 18, 6892–6891
9. Grand, R. J., Turnell, A. S., and Grabham, P. W. (1996) Biochem. J. 313, 353–368
10. Vu, T. K., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Cell 64, 1057–1068
11. Coughlin, S. R. (1994) Semin. Hematol. 31, 270–277
12. Jalink, K., van Corven, E. J., Hengeveld, T., Monard, D., and Sessa, W. C. (1995) Science 268, 1303–1304
13. Pratt, W. B. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 297–326
14. Xu, Y., and Lindequist, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7074–7078
15. Cutforth, T., and Rubin, G. M. (1994) Cell 77, 1027–1036
16. Nathan, D. F., and Lindquist, S. (1995) Mol. Cell. Biol. 15, 3917–3925
17. Sieben, C. E., Ruo, A. A., Schneider, C., Rosen, N., Hartl, F. U., and Pavletich, N. P. (1997) Cell 89, 239–250
18. Garcia-Cardenas, G., Fan, R., Shah, V., Serrentino, R., Cirino, G., Papapetropoulos, A., and Sessa, W. C. (1998) Nature 392, 821–824
19. Li, M., Jan, Y. N., and Jan, L. Y. (1992) Science 257, 1225–1230
20. Grabham, P., and Cunningham, D. D. (1995) J. Neurochem. 64, 583–591
21. Conconi, M., Petropoulos, I., Emad, I., Turlin, E., Biville, F., and Friguet, B. (1998) Biochem. J. 333, 407–415
22. Ren, X. D., Kissoses, W. B., and Schwartz, M. A. (1999) EMBO J. 18, 578–585
23. Weinstein, J. R., Gold, S. J., Cunningham, D. D., and Gall, C. M. (1995) J. Neurosci. 15, 2906–2915
24. Suidan, H. S., Stone, S. R., Hemmings, B. A., and Monard, D. (1992) Neuron 8, 363–375
25. Luttrell, L. M., Ostrowski, J., Coteccchia, S., Kendall, H., and Lefkowitz, R. J. (1993) Science 259, 1453–1457
26. Reid, T., Furuyashiki, T., Izishaki, T., Watanabe, G., Watanabe, N., Fujisawa, K., Morii, N., Madaule, P., and Narumiya, S. (1996) J. Biol. Chem. 271, 13556–13560
27. Jalink, K., and Moenlaen, W. H. (1992) J. Cell Biol. 118, 411–419
28. Urban, J. L., and Reiser, G. (1997) Glia 21, 361–369
29. Tessier-Lavigne, M., and Goodman, C. S. (1996) Science 274, 1123–1133
30. Proctor, K., Gatling, J. H., and Lefkowitz, R. J. (1998) Trends Neurosci. 23, 399–407
31. Proctor, K., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1997) Cell 90, 65–75
32. Panaretou, B., Proctor, K., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1998) EMBO J. 17, 4829–4836
33. Proctor, K., O'Meara, F. L., and Ladbury, J. E., and Perl, M. (1998) J. Cell Biol. 143, 901–910
34. Shapiro, M. J., and Coughlin, S. R. (1999) J. Biol. Chem. 274, 29069–29014
35. Trejo, J., Hannes, S. R., and Coughlin, S. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13698–13702
36. Shapiro, M. J., Trejo, J., Zeng, D., and Coughlin, S. R. (1996) J. Biol. Chem. 271, 32874–32880
37. Hall, R. A., Kammun, T. R., Chow, C. W., Ploetz, J. T., Pitcher, J. A., Clain, A., Stoffel, R. H., Barak, L. S., Shendiak, S., Weinman, E. J., Grinstein, S., and Lefkowitz, R. J. (1997) Nature 392, 626–630
38. Hall, R. A., Ostedgaard, L. S., Prentin, R. T., Blitzer, J. T., Rahman, N., Welch, M. J., and Lefkowitz, R. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8496–8501
39. Offman, S., Luupu, K., Spicher, K., and Schulte, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 504–508
40. Esser, M., Amoo, M., Kruse, H. J., Kaibuchi, K., Weber, P. C., and Aepfelbacher, M. (1996) J. Biol. Chem. 271, 21867–21874
41. Klages, B., Brandt, U., Simon, M. I., Schultz, G., and Offman, S. (1999) J. Cell. Biol. 144, 745–754
