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Ligand and Protein Fishing with Heat Shock Protein 90 Coated Magnetic Beads

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Abstract

Heat shock protein 90α (Hsp90α) is a molecular chaperone that has been targeted for the development of new anticancer therapies. To date, co-immunoprecipitation (IP) has been primarily used to identify novel client proteins. We now report an alternative approach in which Hsp90α has been immobilized onto the surface of silica-based magnetic beads. The beads were used to isolate known Hsp90α ligands from a mixture containing ligands and nonligands. In addition, they were also used to isolated proteins from a mixture of proteins, as well as a cellular extract. The results indicate that the Hsp90α coated magnetic beads can be used to "fish" from complex chemical and biological mixtures for new lead drug candidates and client proteins.

Heat shock protein 90 (Hsp90) is a family of cellular proteins (Hsp90α, Hsp90*β*, Grp94, Trap1) that act as molecular chaperones, which guide the normal folding, intracellular disposition, and proteolytic turnover of many key regulators of cell growth and survival.[1] It is one of the most abundant molecular chaperones in eukaryotes[2] and is essential in the activation of over 100 client proteins, including receptors, protein kinases, and transcription factors.[1,3] Increased expression and activity of Hsp90 have been observed in human cancers, and this protein has become a therapeutic target for the development of new anticancer agents.[4] The intrinsic ATPase activity of Hsp90 is also a target in drug discovery as ATPase activity is a key component in the chaperone function.[5]

Although a large number of Hsp90 client proteins have been identified, it is likely that there are many as of yet unidentified client proteins whose function could help elucidate novel oncogenic signaling pathways. At the present time, co-immunoprecipitation (IP) is the primary method used to identify client proteins.[6] However, in this approach an antibody raised against the coprecipitating protein is required for identification by Western blot analysis. Thus, it is difficult to find new proteins without first knowing what you are looking for. Additional problems with this technique include antibody contamination, nonspecific binding interactions, and leaching contamination that can result from protein A or protein G immobilization on agarose.

We now report an alternative approach to the isolation of Hsp90 client proteins based upon the immobilization of Hsp90α on the surface of magnetic beads. In a previous study, we demonstrated that Hsp90 could be immobilized on a silica-based stationary phase through

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either the amino- or carboxy-terminus of the protein to produce Hsp90-NT (immobilization via N-terminus) and Hsp90α-(CT) (immobilization via C-terminus) columns and that the resulting column could be used in liquid chromatography experiments to identify small molecule Hsp90 ligands.[5] However, the Hsp90α-(NT) and Hsp90α-(CT) columns could not be readily adapted to the screening of complex biological matrixes, such as cellular extracts, for protein–protein interactions.

We have also recently demonstrated that human serum albumin (HSA) could be immobilized on the surface of magnetic beads and the resulting beads used for ligand fishing to isolate HSA ligands from a mixture of ligands and nonligands.[7] Using the same approach, we now demonstrate that the Hsp90 magnetic beads can be used to "fish" for Hsp90 small ligands and can be used to isolate and identify potential Hsp90 ligands in complex chemical and botanical mixtures. In addition, the data indicate that the immobilized Hsp90 retains its ability to bind client proteins, form multiple protein complexes, and maintain its ATP-induced conformational mobility. To our knowledge, this is the first demonstration that magnetic bead-based "fishing" with a target protein can be used to identify and isolate multiprotein complexes in complex matrixes and indicates that this technique can be used in proteomic studies.

MATERIALS AND METHODS

Materials

Coumermycin A_1 (CA1) was purchased from Biomol Int. (Plymouth Meeting, PA), geldanamycin (GM) was purchased from InvivoGen (San Diego, CA), 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), propranolol, and nicotine were purchased from Sigma Chemical Co. (St. Louis, MO); novobiocin (NOVO) and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from EMD-Calbiochem (Gibbstown, NJ). Recombinant human Hsp 90α, Hsp70, and p60HOP proteins were purchased from Stressgen-Assay Designs Inc. (Ann Arbor, MI). Recombinant bovine eNOS was from Cayman Chemical Co. (Ann Arbor, MI) Ammonium acetate, 1-ethyl-3-(3-methylaminopropyl)carbodiimide (EDC), glutaraldehyde, glycine, hydroxylamine hydrochloride, potassium phosphate dibasic, pyridine (99.8%), sodium azide, sodium cyanoborohydride, and sodium phosphate monobasic were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI). *N*-Hydroxysulfosuccinimide (Sulfo-NHS) was from Pierce (Rockford, IL). The water used in the study was prepared using a Milli-Q Water Purification System (Millipore Corporation, Bedford, MA). BcMag, amine terminated magnetic beads (50 mg/mL, 1 μm) were purchased from Bioclone Inc. (San Diego, CA). The manual magnetic separator Dynal MPC-S was from Invitrogen Corporation (Carlsbad, CA).

Methods

Immobilization of Hsp90α onto the Surface of Magnetic Beads (MB)

(a) Hsp90α-(NT)-MB: The Hsp90α protein was immobilized through reductive amination using a previously published method, with slight modifications.[5,7] Briefly, 25 mg of BcMag, amine coated magnetic beads were washed with 1 mL of pyridine buffer (10 mM, pH 6.0) in 2 mL microcentrifuge tube. After magnetic separation using the manual magnetic separator Dynal MPC-S, the supernatant was discarded and BcMag were suspended in 1 mL of 5% glutaraldehyde and shaken for 3 h. After magnetic separation, BcMag were washed 3× with 1 mL of pyridine buffer (10 mM, pH 6.0), and an additional 300 μL of buffer was added followed by 200 μg of recombinant human Hsp90α protein and 5 mg of sodium cyanoborohydride. The reaction was left for 7 days at 4 °C with gentle rotation. The supernatant was discarded and either 0.5 mL of glycine (1 M, pH 8) or 20 μL of 1 M hydroxylamine was added, the mixture shaken for 30 min at 4 °C, and the supernatant discarded. The Hsp90α (NT)-MB were rinsed three times with 1 mL of phosphate buffer (10 mM, pH 7.4) containing 150 mM NaCl and 0.02% sodium azide. The same buffer was used as storage buffer. The control glycine-coated

or hydroxylamine-coated (NT)-MB were made in the same manner but without the addition of Hsp90α protein.

(b) Hsp90α(CT)-MB: Amine groups on the BcMag and the Hsp90α protein were linked by slight modifications of a previously described method.[5] Briefly, 25 mg of BcMag in a 2 mL microcentrifuge tube were rinsed with 1 mL of MES (100 mM, pH 5.5) in 2 mL microcentrifuge tube. After magnetic separation, the supernatant was discarded, the BcMag suspended in 300 μL of MES, (100 mM, pH 5.5) and 200 μg of Hsp90α protein and 50 μL of a mixture of 10 mg of EDC and 15 mg of sulfo-NHS in 1 mL of water were added and the mixture was vortex-mixed for 5 min and left for 3 h at 4 °C with gentle rotation. This was followed by the addition of 20 μL of 1 M hydroxylamine, and the mixture was left for 30 min at 4 °C with gentle rotation. The supernatant was discarded and the Hsp90α (CT)-MB was rinsed three times with 1 mL of phosphate buffer (10 mM, pH 7.4) containing 150 mM NaCl and 0.02% sodium azide. The control (hydroxylamine-coated (CT)-MB) were made in this same way but without the addition of Hsp90α protein.

Ligand Fishing—An amount of 25 mg of Hsp90α(CT)-MB, Hsp90α (NT)-MB, or the respective control magnetic beads were suspended in a 2 mL microcentrifuge tube, to which 500 μL of 500 nM 17-AAG, 500 nM CA1, 500 nM GM, 10 nM nicotine, 250 nM NOVO, and 10 nM propranolol were added individually and as a mixture. The tube was vortex-mixed for 1.5 min and placed in the magnetic separator for 2 min. The supernatant (defined as nonadsorbed material) was collected, and the Hsp90α-MB were subsequently incubated with 500 μL of ammonium acetate buffer (10 mM, pH 7.4)/MeOH (80:20, v/v) for 1.5 min, and the eluate was collected and saved.

Both supernatant and eluate were analyzed by mass spectrometry using a system composed of an Agilent Technologies (Palo Alto, CA) 1100 LC/MSD composed of a vacuum degasser (G1379 A), a quarternary pump (1311A), a thermostatted autosampler (G1329 A), and a thermostatted column compartment (G1316A). The mass selective detector (MSD Quad SL, G1956B) was used with an electrospray ionization interface (ESI) and online nitrogen generation system (Parker). The data were acquired by Chem-Station software, Rev. A.10.02 [1757] (Agilent Technologies). The optimized conditions for compounds measurements were as follows: fragmentor voltage 70 V, gain 2, drying gas flow 6 L min, nebulizer pressure 60 psig, drying gas temperature 350 °C, vaporizer temperature 210 °C, and capillary voltage −4000. Target compounds were quantified in the negative-ion mode for single ion monitoring mode (SIM) at *m*/*z* 1108 (CA_1), *m*/*z* 611.2 (NOVO), *m*/*z* 363 (RAD) and in the positive-ion at *m*/*z* 608.2 (17-AAG), *m*/*z* 583.3 (GM), *m*/*z* 162.2 (nicotine), and *m*/*z* 260 (propranolol). The analyses were performed using a mobile phase composed of ammonium acetate [10 mM, pH 7.4]/acetonitrile (20: 80 v/v) delivered at a flow rate of 0.2 mL/min at 25 °C, 20 μL of each BcMag collected fraction was injected.

Protein Fishing—An amount of 1.25 mg of Hsp90α(CT)-MB or Hsp90α (NT)-MB (hydroxylamine-coated) or their respective control magnetic beads were suspended in a microcentrifuge tube, to which 25 pmol of eNOS, Hsp70, or p60 HOP in 50 μL of ammonium acetate buffer [10 mM, pH 7.4] were added individually or as a mixture. The mixture of proteins (100 μL) contains 12.5 pmol of each protein. The tube was vortex-mixed for 30 s and was placed in the magnetic separator for 30 s. The supernatant was collected, and the magnetic beads were subsequently incubated with 50 μL for individual proteins and 100 μL for the protein mixture with ammonium acetate buffer [10 mM, pH 10] for 15 min. The eluate was saved, and the beads were washed with ammonium acetate buffer [10 mM, pH 7.4] and stored in the cold room.

Effects of Small Molecules and Exogenous ATP on Protein Fishing—To determine the effects of ATP and small molecules, the Hsp90α (NT)-MBs were preincubated with 50 μL of 1 mM ATP and 1 mM $MgCl_2$ for 30 s or with 50 μL of 1 μM GM or 1 μM NOVO for 1 min prior to the protein fishing experiment.

Protein Fishing in Cellular Extract—Ten million KU-812 cells (basophils) were homogenized for 3 × 30 s at the setting of 11 on a model PT-2100 homogenizer (Kinematica AG, Luzern, Switzerland) in 1.5 mL of Tris buffer [50 mM, pH 7.5] containing 100 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM benzamidine, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 4 μM pepstatin, 100 μg/mL PMSF. The homogenate was centrifuged at 100 000*g* for 30 min at 4 °C. The supernatant was collected and partitioned into 100 μL aliquots.

Western Blot Analysis—Nonadsorbed material and eluates were concentrated by evaporated centrifugation using a speed vac concentrator. Lyophilized samples were resuspended in Laemmli sample buffer,[8] heated at 70 °C for 10 min, and then separated by 4–12% SDS-polyacrylamide gel electrophoresis before electrotransfer to a polyvinylidene difluoride membrane (Novex/Invitrogen). The membranes were soaked in blocking buffer [5% nonfat dry milk diluted in Tris-buffered saline-0.1% Tween-20 (TBS-T)] for 1 h at room temperature and incubated either for 1 h at room temperature or overnight at 4 °C with the indicated primary antibody [eNOS (1:500, Zymed); p60 HOP (1:500, Stressgen); Hsp70 (1:500, Santa Cruz Biotechnology)]. Blots were developed using a horseradish peroxidase-conjugated secondary antibody and a chemiluminescent detection system (GE Healthcare/Amersham Biosciences). For more details on Western blot analysis cf. to ref [9].

RESULTS

Ligand Fishing

The Hsp90α(CT)-MB and Hsp90α(NT)-MB were initially incubated with individual compound solutions of two C-terminus binders (CA1, NOVO), two N-terminus binders (17-AAG and GM), and two nonbinders (nicotine, propranolol). The magnetic beads were isolated using a Dynal Magnetic Separator, and the supernatant, which contains the unbound ligands, was then analyzed by MS. The bound material was subsequently eluted with a single incubation in ammonium acetate [10 mM, pH 7.4]/MeOH (80:20, v/v). The results collected with the Hsp90α(CT)-MB are consistent with previously observed chromatographic data obtained using the Hsp90α(CT)-columns.[5] N-terminal ligands 17-AAG and GM, bound the Hsp90α(CT)-MB at levels greater than 90%, while less than 30% of the nonbinders nicotine and propanolol, as well as the C-terminus binders CA1 and NOVO, were captured (Figure 1a).

The Hsp90α(NT)-MB (Figure 1a), in this case, captured the C-terminus binders at levels greater than 60%, while less than 35% of the nonbinders nicotine and propanolol and the N-terminus binders 17-AAG and GM were captured.

The beads were incubated in a sample mixture containing the six test compounds. After isolation of the magnetic beads, the supernatant (nonadsorbed material) was collected and the material bound to the beads was eluted with ammonium acetate [10 mM, pH 7.4/MeOH (80:20, v/v)]. The fractions were analyzed, and the data indicated that the Hsp90α(CT)-MB selectively bound the N-terminus ligands, 17-AAG and GM, >80% and >75%, respectively (Figure 1b), whereas the Hsp90α(NT)-MB selectively retained the C-terminus ligands CA1 and NOVO, >70% and >75%, respectively (Figure 1b).

Protein Fishing

In order to determine whether the Hsp90α-coated MBs retain their ability to form client–protein complexes, the beads were incubated with individual recombinant proteins eNOS, p60 HOP, and Hsp70 and in combination. Although both the Hsp90α-(NT)-MB and the Hsp90α-(CT)-MB were able to extract proteins from the recombinant protein mixture, Hsp90α-(NT)-MB consistently extracted more (in some cases up to 30% more) of p60 HOP, which is consistent with the fact that p60 HOP binds at the C-terminal end of Hsp90α. In the same manner, binding of the Hsp90 cochaperone p23 would be expected to be hindered on the Hsp90α-(NT)-MBs. Although data was obtained with both MBs, only data from experiments utilizing HSP90α (NT)-MB will be reported.

The Hsp90α(NT)-MB was incubated with solutions containing the individual proteins for 30 s, and both the supernatant (nonadsorbed material) and eluate were analyzed by Western blot analysis (Figure 2). Quantitative analysis done in triplicate showed that both eNOS and p60 HOP bound to the Hsp90α-(NT)-MB (Table 1), with only 8% and 14% remaining in the supernatant, respectively. On the other hand, Hsp70 was not efficiently retained by the Hsp90α-(NT)-MB with 70% remaining in the supernatant. The results are consistent with previous data showing that eNOS and p60 HOP bind directly to Hsp90, while Hsp70 binds indirectly through the cochaperone p60 HOP.[10]

Incubation of the Hsp90α-(NT)-MB with the combination of eNOS, p60 HOP, and Hsp70 resulted in retention of all three proteins on the surface of the MBs, with only 4%, 7%, and 9%, respectively, remaining in the supernatant (Table 2). The results indicate that the immobilized Hsp90α bound eNOS and p60 HOP in the presence of the other protein, either on the same or different Hsp90 molecules. However, the retention of Hsp70 demonstrates that a multiprotein complex containing at least p60 HOP and Hsp70 had been formed on the MB.

Effect of ATP on Protein Fishing

The effect of ATP on the protein complexes formed between the Hsp90α(NT)-MBs and the three recombinant proteins eNOS, p60 HOP, and Hsp70 was studied by preincubating the MBs with 1 mM ATP and 1 mM $MgCl_2$ prior to the protein fishing experiments. Preincubation of Hsp90α(NT)-MB reduced the binding of eNOS with only 7% being captured by the MBs versus 96% in the absence of ATP; similarly, the amount of p60 HOP binding to the beads was significantly lower, 36% in the presence of ATP versus 93% captured in the absence of ATP (Table 2). The reduction in the amount of p60 HOP being captured had a direct effect on the amount of Hsp70 captured by the beads, 17% in the presence of ATP versus 91% in the absence of ATP.

Effect of Geldanamycin on Protein Fishing

The effect of the addition of 0.5 μM GM on the client protein complex formed with the mixture of proteins was also tested on the Hsp90α(NT)-MBs (Table 2). Although, GM did not have an effect as pronounced as ATP, it did cause a significant loss in the retention of eNOS (44%), p60 HOP (44%), and Hsp70 (57%).

Effect of Novobiocin on Protein Fishing

The effect of the addition of 0.5 μM NOVO, a C-terminus binder, to the binding of proteins from the mixture was also tested (Table 2). For the Hsp90α-(NT)-MB, a significant reduction in proteins captured by the MBs was observed, only 15% of eNOS was captured in the presence of NOVO, while p60 HOP and Hsp70 also saw similar reductions in binding with only 10% and 33% remaining on the beads in the presence of NOVO.

DISCUSSION

In previous studies, we immobilized Hsp90α onto a silica-based stationary phase[5] and characterized the resulting column by determining the dissociation constant of a number of Hsp90α ligands. Here, Hsp90α was immobilized onto the surface of magnetic beads and used to fish out ligands and proteins individually and in sample mixtures. These results have demonstrated that the immobilized Hsp90α retained its ability to bind both the N-terminal and C-terminal ligands. As shown in Figure 1, differences in the supernatant were observed when comparing Hsp90α coated-MB and the uncoated-MB, which is indicative of specific interactions between the Hsp90α ligands and the immobilized protein.

The ability of the Hsp90α(CT)-column to bind compounds identified as N-terminus ligands and the Hsp90α(NT)-column to bind compounds identified as C-terminal ligands can be taken advantage of in drug development programs. The MBs can be used to rapidly differentiate between compounds that bind specifically at the ATP-binding motif at the N-terminus of Hsp90α and binding sites present in the carboxyl terminus of the chaperone. A single fishing experiment using the appropriate MB can be used to identify novel ligands that bind to either region of Hsp90.

Both Hsp90α(CT)-MB and the Hsp90α(NT)-MB were incubated with recombinant proteins. Initial results demonstrated that a significant amount of nonspecific binding was seen with the Hsp90α(NT)-MB that was absent with the Hsp90α(CT)-MB. The Hsp90α(NT)-MB was, therefore, endcapped with hydroxylamine in place of glycine, resulting in removal of the majority of observed nonspecific binding.

The ligand fishing protocol was optimized by studying the impact of incubation times and elution conditions. Multiple incubation times of the MBs with the proteins were tested, and it was determined that 30 s was optimal, as an increase in the incubation time resulted in an increase in observed nonspecific binding. The elution of the bound material was studied using various buffers: 1 mM ATP/1 mM $MgCl_2$; 0.5 M NaCl; 4 M urea; ammonium acetate buffer [10 mM] at multiple pHs (3.5, 7.4, 10.0); and ammonium acetate [10 mM, pH 7.4] supplemented with 15% or 20% MeOH. Of all these buffers, only ammonium acetate [10 mM, pH 10] efficiently eluted the bound material.

The three recombinant proteins that were tested were eNOS, p60 HOP, and Hsp70. eNOS is known to bind to the middle domain of the Hsp90,[11] p60 HOP binds to the C-terminal domain, while Hsp 70 binds to Hsp90 only through p60 HOP.[10] The results observed with the Hsp90α(NT)-MB demonstrate that the beads were efficient at fishing out eNOS and p60 HOP individually or in a mixture. A notable exception is Hsp70, which failed to bind to the immobilized Hsp90α when added alone but was able to interact upon its addition in a mixture containing p60 HOP. To our knowledge, this is the first time that a functionally relevant multiprotein complex has been formed on the surface of magnetic beads.

Hsp90α possesses an intrinsic ATPase activity that is necessary for its function as a chaperone.[1] The ATPase activity has been associated with the adenine nucleotide binding site on the N-terminus, although the C-terminus is also believed to play a role.[1,3] We have previously shown that the immobilization process onto a stationary phase did not affect Hsp90α ATPase activity.[5] The hydrolysis of ATP to ADP is believed to induce a conformational change in the Hsp90α, resulting in the release of the target protein[12] and that of p60 HOP and subsequently Hsp70. Although the ATPase activity was not measured on the Hsp90α(NT)-MBs, the addition of ATP to the protein mixture prevented the binding of all three recombinant proteins resulting from the ATP-mediated conformational change of the immobilized Hsp90α. After removal of the ATP, the Hsp90α reverted back to the open state and the protein was once again able to capture all three recombinant proteins.

Small molecule inhibitors of Hsp90α, NOVO, and GM were also tested to determine whether they were capable of inhibiting the formation of the protein complex. Although both GM and NOVO displayed inhibition of the protein complex formation on the Hsp90α(NT)-MB, NOVO had a much more pronounced effect, reducing the amount of protein captured to 15% (eNOS), 10%(p60 HOP), and 33%(Hsp70). This is consistent with what has been reported in literature on the effects of NOVO on Hsp90α.[13]

The initial results have demonstrated that protein–protein complexes can be formed and isolated on Hsp90α coated MBs. On the basis of these observations, a study was undertaken to ascertain whether the MBs can be used to isolate interacting proteins from crude cell lysates. Initial results using KU-812 cell lysates demonstrated that a detectable amount of p60 HOP was observed in the eluted fractions (Figure 3), indicating that the MBs were capable of protein fishing from a cellular matrix.

CONCLUSIONS

The results from this study indicate that Hsp90α can be immobilized onto the surface of MBs and used to fish out both small molecule ligands and protein–protein complexes. The MBs have been shown to be stable for up to 2 months with continuous use. Additionally the system can be fully automated using the Magtration System 12GC PSS Bio Instruments Inc., which can run up to 12 samples in parallel, as was previously shown using the HSA-coated MBs.[7] Another advantage of this system is that the protein-coated magnetic beads can isolate interacting partners from complex mixtures in less than 15 min, unlike IP which takes several hours to recover the protein of interest. The extension of this method to multiple complex cellular extracts and complex plant extracts is being investigated, and the results will be reported elsewhere.

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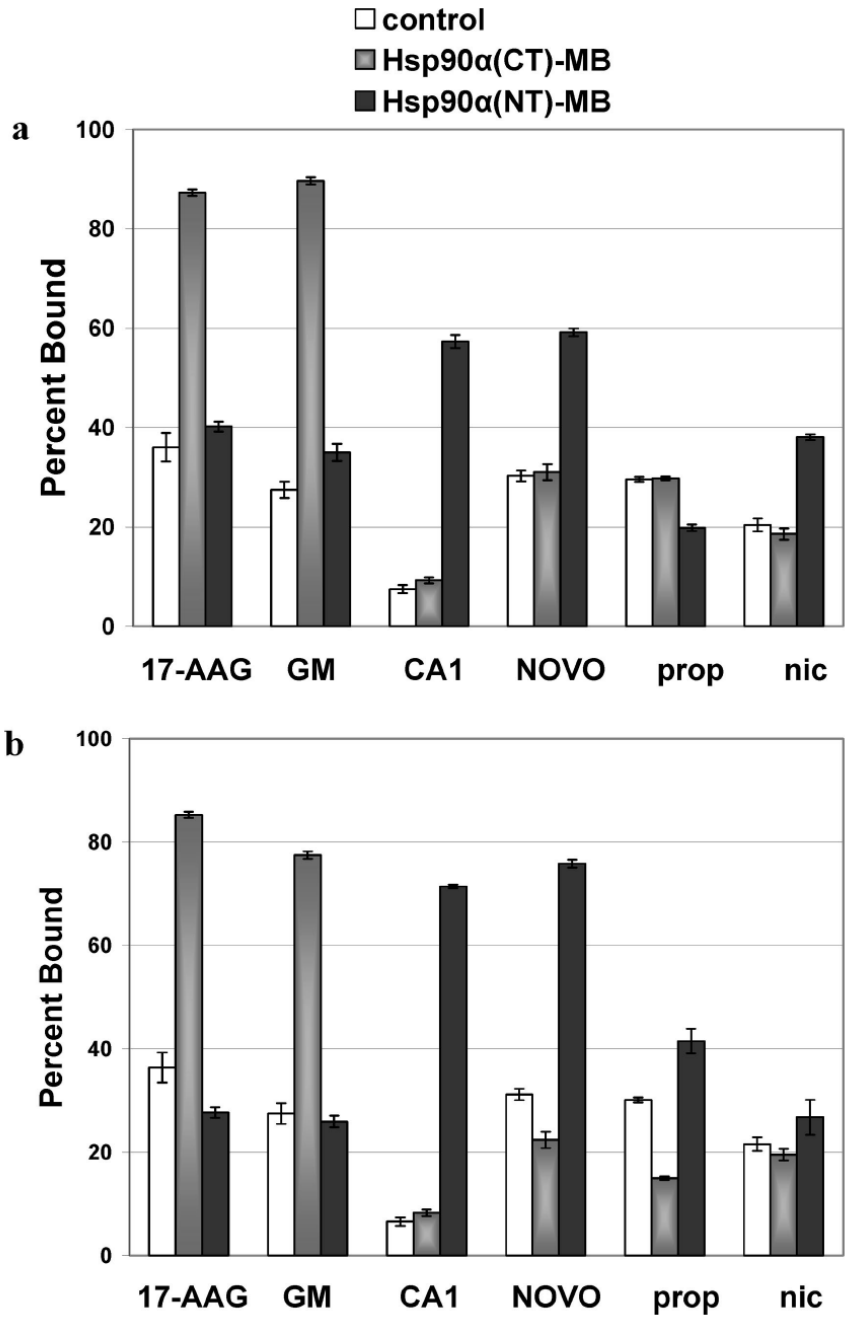

Figure 1.

Comparison of the (a) individual ligand fishing results for Hsp90α(CT)-MB and Hsp90α(NT)-MB and (b) mixture ligand fishing results for Hsp90α(CT)-MB and Hsp90α(NT)-MB. Two N-terminal binders (17-AAG, GM), two C-terminal binders (CA1, NOVO), and two nonbinders (nicotine, propanolol) were incubated with the beads for 1.5 min and eluted with ammonium acetate buffer [10 mM, pH 7.4] with 20% methanol. The histogram represents the percent of bound ligand to the Hsp90 coated MBs which was captured from the supernatant.

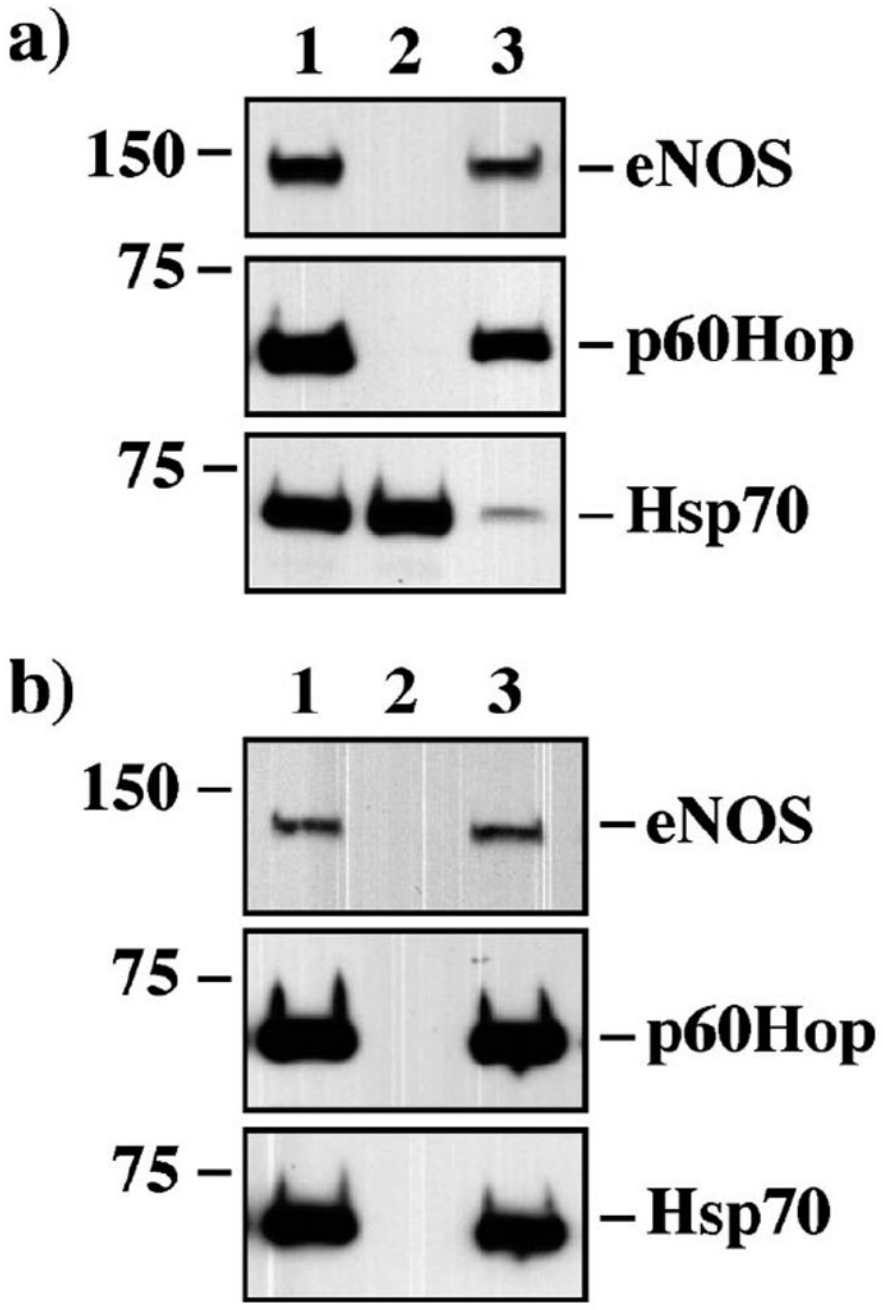

Figure 2.

Binding of recombinant proteins to Hsp90α immobilized on magnetic beads. Hsp90α (NT)-MBs were incubated with 25 pmol of recombinant eNOS, p60Hop, and Hsp70 protein added either individually (a) or 12.5 pmol of each protein as an admixture (b). The nonadsorbed material was collected, and the bound material was eluted with ammonium acetate buffer [10 mM, pH 10.0]. All samples were resuspended in sample loading buffer and resolved by SDS-polyacrylamide gel electrophoresis. Western blot analysis was carried out with an antibody raised against eNOS (top panels), p60Hop (middle panels), or Hsp70 (bottom panels). The positions of the molecular weight markers (in kilodaltons) are shown on the left. Lane 1, control; lane 2, Hsp90α-NT supernatant; lane 3, eluted material.

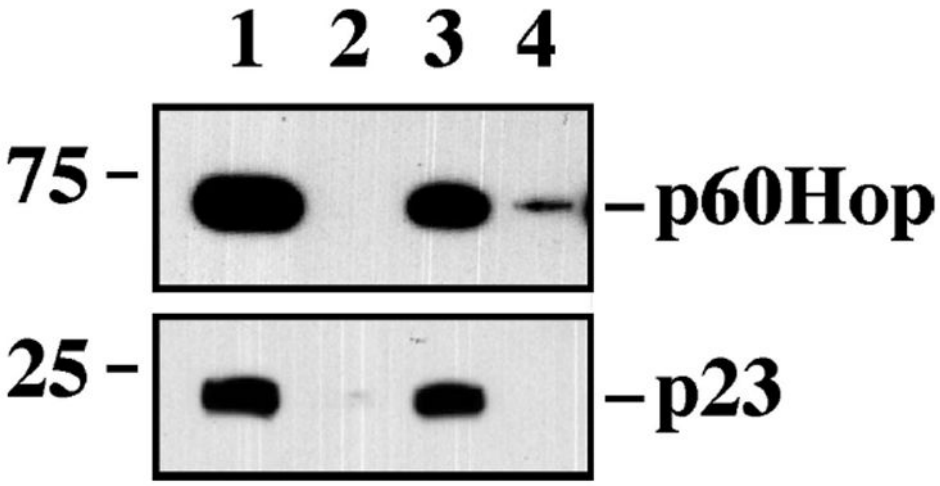

Figure 3.

Binding of cellular proteins to Hsp90α immobilized on magnetic beads. Hsp90α (NT)-MBs were incubated with lysates from KU-812 basophiles. The nonadsorbed material and the material eluted from the beads were analyzed by Western blot with antibodies against p60Hop (top panel) or the Hsp90 cochaperone, p23 (bottom panel). Lane 1, control; lane 2, eluted material from control; lane 3: Hsp90α-NT supernatant; lane 4, eluted material from Hsp90α-NT.

Table 1

Binding Profile of Recombinant Proteins Added Singly onto Hsp90α Immobilized on Magnetic Beads[a]

	control(NT)	Hsp90α(NT)-MBs	
	nonadsorbed	nonadsorbed	eluted
eNOS	100	8 ± 1	94 ± 44
p60Hop	100	14 ± 23	64 ± 22
Hsp70	100	70 ± 29	18 ± 19

[a] Control(NT) and Hsp90α(NT)-MBs were incubated with individual recombinant proteins as indicated in Materials and Methods. Nonadsorbed and eluted materials were resolved by SDS-PAGE and analyzed by Western blot. The protein bands were visualized with Hyperfilms and quantitated by densitometry. Values are means ± standard deviation from three to four independent experiments. The signal associated with nonadsorbed material from control(NT) beads was arbitrarily given the value of 100%.

Table 2

Binding Profile of an Admixture of Recombinant Proteins Added onto Hsp90α Immobilized on Magnetic Beads[a]

	pretreatment							
	none		ATP/$MgCl_2$		GM		NOVO	
	nonadsorbed	eluted	nonadsorbed	eluted	nonadsorbed	eluted	nonadsorbed	eluted
eNOS	7 ± 7	93 ± 71	96 ± 29	4 ± 6	86 ± 46	14 ± 14	90 ± 22	10 ± 14
p60Hop	11 ± 16	89 ± 6	74 ± 26	26 ± 13	67 ± 26	33 ± 36	80 ± 19	20 ± 19
Hsp70	7 ± 5	93 ± 7	55 ± 4	45 ± 2	44 ± 28	56 ± 3	72 ± 31	28 ± 30

[a] Hsp90α(NT) beads were incubated with an admixture of recombinant proteins in the absence or the presence of small Hsp90 inhibitors (0.5 μM GM or 0.5 μM NOVO) or ATP/Mg^{2+} to promote its intrinsic ATPase function. Nonadsorbed material and eluate were resolved by SDS-PAGE and analyzed by Western blot. The protein bands were visualized with Hyperfilms and quantitated by densitometry. Values are means ± standard deviation from three to four independent experiments. Values were tabulated such that the total of each set (NA + eluted) equal 100%.