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Heat-shock protein 90 complexes in resting and thrombin-activated platelets☆

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Abstract

Heat-shock protein 90 (hsp90) is a chaperone important for the function of many signaling proteins. In this study, we show that hsp90 exists in resting platelets as a complex with the heat-shock cognate protein 70 (hsc70), the α- and β-subunits of protein kinase CK2, and other unidentified phosphoproteins. Platelet activation by thrombin caused the rapid dissociation of hsc70 and CK2α from the hsp90 complex, the ex vivo phosphorylation of many protein components, and the stimulation of protein kinase(s) associated with the hsp90 complex. These results suggest that the hsp90 complex, with its associated protein kinase(s), which may include CK2, and their substrates, is involved in thrombin-induced platelet activation.

Keywords: Platelets; Hsp90; Hsc70; CK2; Thrombin; Aggregation; Signaling complex

Blood platelets are essential for hemostasis, responding within seconds to agonists such as thrombin, ADP, and collagen [1,2], which activate signal-transduction pathways and changes in the phosphorylation state of many platelet proteins. Understanding the regulation of these pathways represents a significant issue in platelet research, with special interest in the balance between protein kinase and phosphatase activities [3].

Heat-shock protein 90 (hsp90) is a major molecular chaperone with roles in protein stabilization, translocation, and degradation, as well as in signal-transduction pathways [4,5]. A number of proteins, including transcription factors and protein kinases, can associate with hsp90 as 'substrates' [6]. In addition, hsp90 can interact in vitro with protein kinase CK2 (formally known as casein kinase 2), helping to stabilize and prevent CK2 from aggregation [7]. However, the physiological consequences of these interactions are not yet clear. CK2 consists of two catalytic subunits (α_2, α'_2, or $\alpha\alpha'$) and two regulatory subunits (β_2) [8,9], and has been shown to be important for many biological processes and able to phosphorylate at least 160 proteins [10]. Therefore, the activity and specificity of CK2 must be tightly controlled; however, its regulation is not well understood and, apparently, is not affected by known second messengers [11].

Hsp90 complexes in human platelets were first reported by Polanowska-Grabowska et al. [12,13], showing that platelet adhesion to collagen induces rapid changes in the complex, both in phosphorylation state and the association of proteins including heat-shock cognate protein 70 (hsc70) and protein phosphatase 1 (PP1), suggesting a role of the complex. In addition, an hsp90 inhibitor geldanamycin (GA) inhibits platelet aggregation and causes disruption of platelet membranes [14], also supporting roles for hsp90 in platelet structure and function. In the present study, we have analyzed the involvement of hsp90 complexes in thrombin-induced platelet aggregation. We have found that hsp90 existed in resting platelets as a complex containing hsc70, CK2α, CK2β, as well as other phosphoproteins. Exposure of platelets to thrombin caused hyperphosphorylation of complex components, increased kinase activity associated with the complex, and caused the dissociation of hsc70 and CK2α from the complex.

☆ *Abbreviations:* Hsp90, heat-shock protein 90; Hsc70, heat-shock cognate protein 70; CK2, casein kinase 2; IP, immunoprecipitate.

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Materials and methods

Materials. Antibodies against hsp90 and hsc70 were obtained from StressGen (British Columbia, Canada). Antibodies against CK2 (α, α′, and β) were a gift from Dr. David Litchfield (Department of Biochemistry, Faculty of Medicine, University of Western Ontario, Canada) and Dr. Ken Lerea (New York Medical College, Valhalla, NY) and were also purchased from StressGen (British Columbia, Canada) and Transduction Laboratory (Franklin Lakes, NJ), respectively. Protein G-immobilized beads and other chemicals were from Sigma (St. Louis, MO).

Preparation of platelets. Human venous blood was collected and anticoagulated with acid-citrate dextrose (ACD) for a final citrate concentration of 11.5 mM [1]. Washed platelets were prepared, as described earlier [15], and suspended in an Eagle's/Hepes buffer, but omitting albumin [16]. Final platelet concentrations determined by resistive-particle counting were adjusted to 1×10^9 platelets/mL. For [^{32}P]-labeling, platelets were incubated with [^{32}P]orthophosphate (0.2 mCi/ 3 mL platelets) as described [14] and resuspended in an Eagle's/Hepes buffer, but omitting albumin [16].

Immunoprecipitation. Washed platelets (5×10^8 platelets) were incubated at 37 °C for 15 min. To prepare a resting platelet lysate, preincubated platelets were mixed with lysis buffer (1×: 2 mM EGTA, 2 mM EDTA, 1 mM DTT, 10 mM Na_2HPO_4, 20 mM NaF, 0.1 mM vanadate, 20 mM molybdate, and 1% Triton X-100) and protease inhibitors (1 mM PMSF, 10 mM benzamidine, 20 μg/mL aprotinin, and 10 μg/mL leupeptin). For thrombin-activated platelet lysates, preincubated platelets were reacted with thrombin (1 U/mL) for 10 s in a test tube under stirring conditions (1000 rpm) and then mixed with lysis buffer. Lysates were then dispersed with a pellet pestle motor (Kimble/Kontes, Vineland, NJ), incubated on ice for 30 min, and centrifuged at 3000 rpm for 3 min to remove undissolved materials. Specific antibodies against hsp90 (AC88) or hsc70 (1B5) or non-immune IgGs were then added to the supernatants, followed by incubation overnight at 4 °C with gentle inversion mixing. The lysates were incubated with protein G-immobilized beads and the beads were washed twice with lysis buffer. For SDS–PAGE analyses, the beads were subjected to two additional washes with PBS, mixed with Laemmli's loading buffer, boiled for 5 min, and then centrifuged and the supernatants were collected for further analysis.

In vitro kinase assay. Immunoprecipitates were prepared as described earlier, and after washing with the lysis buffer, the protein G-immobilized beads were washed one more time with the kinase buffer (50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 0.1 mM ATP, and 1 mM DTT). Then, the beads were incubated with the kinase buffer containing 10 μCi [^{32}P]ATP at 30 °C for 10 min to allow phosphorylation to occur. The reactions were stopped with ice-cold kinase buffer and the beads were washed twice with kinase buffer and twice with PBS. The samples were subsequently prepared for SDS–PAGE.

Gel electrophoresis and Western blotting. Samples were loaded onto 10% polyacrylamide gels and subjected to electrophoresis at 20 mA. For autoradiography, the gels were dried and exposed to autoradiography film (X-Omat Blue XB-1 from Kodak, Rochester, NY) at −70 °C overnight. For Western blotting after gel electrophoresis, separated proteins were transferred to nitrocellulose membranes at 110 V for 90 min and the membranes were blocked overnight at 4 °C with TTBS buffer (Tween 20-containing Tris buffer saline with 5% albumin). Then, the blots were washed in TTBS, incubated with primary antibody for 90 min, washed with TTBS, and incubated with secondary antibodies conjugated with alkaline phosphatase (Bio-Rad, Hercules, CA) or horseradish peroxidase (Amersham, Piscataway, NJ) for 45 min. The blots were developed by using an Immun-Star AP Substrate (Bio-Rad) and ECL detection reagents (Amersham), respectively, followed by exposure to film.

Results

Hsp90 and hsc70 exist as complexes consisting of many phosphoprotein components

Since the hsp90/hsc70 complex may be involved in platelet adhesion to collagen [12] and inhibition of hsp90 by GA has adverse effects on platelets [14], the complex was investigated for potential roles in platelet aggregation. Phosphoproteins present in the hsp90 complex isolated by IP from ^{32}P-labeled platelets were analyzed by autoradiography of the solubilized proteins separated by gel electrophoresis. In resting platelets, the hsp90 immunoprecipitates (IPs) contained a number of phosphoproteins: evident by strong autoradiography signals at 220, 188, 164, 133, and 102 kDa, as well as by weaker signals at 85, 75, 62, 43, and 37 kDa (Fig. 1). Reverse immunoprecipitation with hsc70, a component of hsp90 complex in

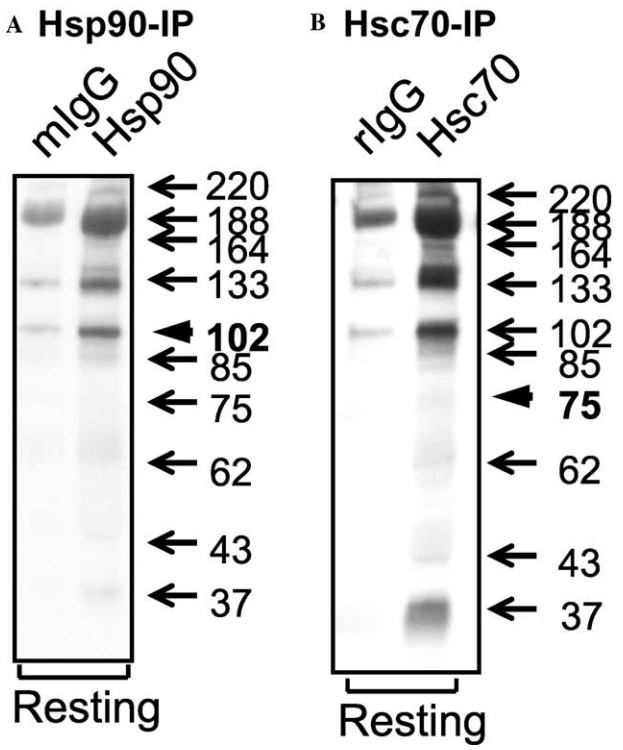

Fig. 1. The presence of phosphoproteins in hsp90 and hsc70 complexes in resting platelets. ^{32}P-labeled, washed platelets (5×10^8) were subjected to immunoprecipitation (IP) with control non-immune IgGs or antibodies against hsp90 (AC88) or hsc70 (1B5), as described in Materials and methods. The samples were then analyzed by 10% SDS–PAGE and autoradiography. (A) and (B) are autoradiograms showing phosphoprotein components in hsp90 (A) and hsc70 (B) immunoprecipitates prepared from resting platelets. Control non-specific IPs using non-immune mouse IgG (A) or rat IgG (B) are shown as 'mIgG' and 'rIgG,' respectively. Arrows on the right represent calculated molecular weights of protein bands. The arrowheads indicate protein bands containing hsp90 (A) and hsc70 (B). The autoradiograms are representative of three different experiments.

resting platelets and other cell types, showed a similar pattern (Fig. 1) and to what has been reported previously [12].

Thrombin induces increased phosphorylation in the hsp90–IP complex, but decreases it in the hsc70–IP complex

Since platelet agonists including thrombin induce phosphorylation of many proteins [3,17,18], the hsp90 complex from thrombin-induced platelets was also studied. Upon activation by thrombin, phosphorylation of many proteins occurred (Fig. 2A), including p47-pleckstrin and the p20-myosin-light chain (MLC, not shown). Thrombin also increased the phosphorylation of many proteins in the hsp90–IP, including 220-, 188-, 133-, 102-, and 85-kDa protein bands (Fig. 2B). There were lesser increases in the phosphorylation of 75-, 62-, 42-, and 37-kDa protein bands. In contrast, thrombin did not increase the phosphorylation of proteins present in hsc70–IPs (Fig. 2C). Rather there was major dephosphorylation, or the associated proteins were dissociated from hsc70 following thrombin activation.

Thrombin causes the dissociation of hsc70 from the hsp90 complex

The hsp90 and hsc70 complexes isolated from resting platelets were similar in terms of phosphorylated protein components (Fig. 1). However, thrombin elicited very different changes in these complexes as described above. Since hsp90 and hsc70 are found in the same complex(es) in many cell types including platelets [12,19], we analyzed the association status of hsp90 with hsc70 following thrombin stimulation. As shown by previously [12], hsc70 is present in the hsp90 complex prepared from resting platelets, but upon activation by thrombin, the hsc70 protein content was dramatically diminished in the hsp90 complex (Fig. 3). Reverse immunoprecipitations using an antibody against hsc70 also revealed a similar result (data not shown).

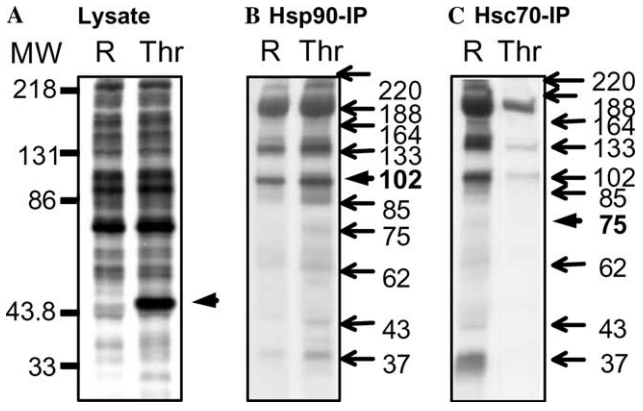

Fig. 2. Effects of thrombin on phosphoproteins present in hsp90 and hsc70 complexes. ^{32}P-labeled, washed platelets were mixed with saline (R) or thrombin (Thr, 1 U/mL) for 10 s and subjected to immunoprecipitation (IP) as described in Fig. 1 and Materials and methods. The samples were then analyzed by 10% SDS–PAGE and autoradiography. (A) Autoradiogram of ^{32}P-labeled platelet lysates prepared from resting and thrombin-activated cells is shown. The arrowhead represents phosphorylation of p47-pleckstrin in thrombin-activated platelets. Autoradiograms in (B) and (C) demonstrate phosphoprotein components in hsp90 and hsc70 immunoprecipitates, respectively. 'mIgG' and 'rIgG' represent control non-specific IPs using non-immune mouse IgG (B) or rat IgG (C), respectively. Molecular weight standards are shown in the left in kilodaltons and arrows on the right represent calculated molecular weights of protein bands. The arrowheads indicate protein bands containing hsp90 (B) and hsc70 (C). The autoradiograms are representative of results from three experiments.

Protein kinase activity in the hsp90 complexes isolated from resting and thrombin-activated platelets

Previous studies have shown that protein kinases such as v-Src and Raf-1 can be associated with hsp90 [6]. Therefore, the presence of protein kinase(s) in the platelet hsp90 complex was examined by using IP/In vitro kinase assays. We found that the hsp90 complex prepared from resting platelets contained significant protein kinase activity, reflected by strong phosphorylation of high-molecular weight protein bands at 220, 197, and 133 kDa, with weaker signals at 102, 75, and 60 kDa (Fig. 4). Similar patterns were observed for the hsc70 complexes from resting platelets (data not shown). When platelets were stimulated by thrombin, kinase activity in the hsp90 complex was strongly enhanced (220, 197, 133, and 102 kDa protein bands, Fig. 4). In addition, new protein bands at 85, 75, 60/64/68 triplet, and 37 kDa were observed in the hsp90 complex prepared from thrombin-activated platelets (Fig. 4).

CK2α and CK2β are present in platelet hsp90 complexes

The above results strongly support the presence of protein kinase activity in the hsp90 and hsc70 complexes. Therefore, candidate protein kinase(s) present in

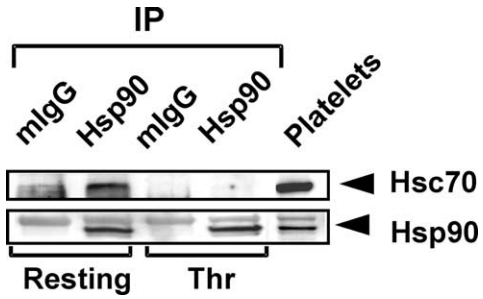

Fig. 3. Thrombin induces dissociation of hsp90 from hsc70. Hsp90 immunoprecipitates were prepared from 'resting' and thrombin-activated (Thr) platelets, as described in Fig. 1, and subjected to SDS–PAGE and Western blotting with antibodies against hsc70 (upper panel) and hsp90 (lower panel). 'mIgG' represent non-specific IP controls and the lower panels represent a control blot for the amounts of hsp90 present in the immunoprecipitates. Platelet lysates (platelets) were run as protein controls. The blots are representative of three experiments.

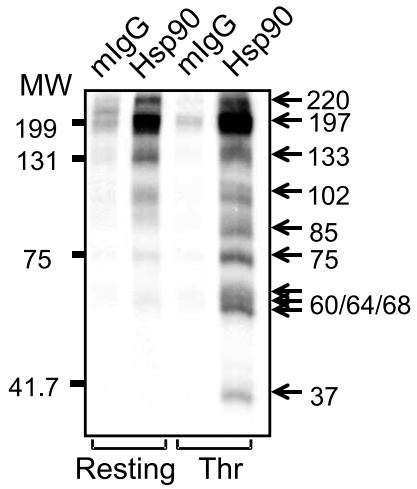

Fig. 4. Kinase activity in the hsp90 complex. Hsp90 immunoprecipitates were prepared from 5×10^8 resting or thrombin-activated (Thr) platelets and subjected to In vitro kinase assay in the presence of [^{32}P]ATP. Non-immune mouse IgG (mIgG) was used as non-specific controls. Kinase activity, reflected by phosphorylation of proteins present in the complexes, was then assessed by autoradiography. Molecular weight standards are present on the left and calculated molecular weights of protein bands are shown on the right.

hsp90 complex were assessed. The first candidate was Raf-1, a substrate of hsp90 in other cell types [20]. Even though platelets contained Raf-1, it was not detected in the hsp90–IP (data not shown). The next candidate was CK2 which can interact with and phosphorylate hsp90 at Ser residues in vitro [7,21] and is present in platelets [22]. The presence of the catalytic (CK2α) and regulatory (CK2β) subunits of CK2 in the hsp90 complex was investigated, and both CK2α and CK2β were detected in the hsp90 complex of resting platelets (Fig. 5). The

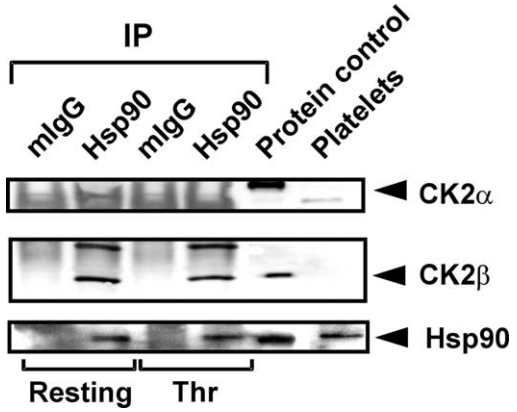

Fig. 5. CK2α and CK2β are present in the hsp90 complex. Hsp90 immunoprecipitates were prepared from resting and thrombin-activated (Thr) platelets and analyzed by Western blotting with antibodies against CK2α (top panel) and CK2β (middle panel). The lower panel represents control blots of hsp90. 'mIgG' represents non-specific IP controls. Platelet lysates (platelets) and appropriate purified proteins (protein control) were run as controls.

associations of CK2α and/or CK2β with the hsp90 complexes were also examined in platelets stimulated by thrombin (Fig. 5). We found that thrombin caused the dissociation of CK2α from the hsp90 complex, but did not change the interaction with CK2β (middle panel, Fig. 5). Similar amounts of hsp90 were detected by Western blots in the control and thrombin-activated situations (lower panel, Fig. 5).

Discussion

In the present study, we have investigated the potential involvement of hsp90 complexes during thrombin-induced platelet aggregation. Hsp90 was associated with a phosphoprotein complex in resting platelets, together with hsc70 as well as CK2α and CK2β. The association between hsc70 and hsp90 in resting platelets has previously been reported [12,13]. We now show that thrombin induced major changes in the hsp90 and hsc70 complexes, causing increases in both the phosphorylation of protein components and protein kinase activity. In contrast, thrombin caused a major reduction of phosphoprotein content in the hsc70 complex (Fig. 2), mostly due to the dissociation of hsc70 from hsp90 (Fig. 3). Thrombin also induced the dissociation of the CK2α catalytic subunit from the hsp90 complex, while the CK2β regulatory subunit remained associated with the complex.

When platelets were activated by thrombin, increases occurred in the phosphorylation of a number of protein bands present in the hsp90 complex (Fig. 2). Some of these phosphoprotein bands may correspond to those phosphorylated by thrombin-activated protein kinase(s) associated with the complex (Fig. 4). Therefore, it is possible that a protein kinase or kinases and their physiological substrates are associated with the complex, enabling the hsp90 complex to serve as a scaffold for a signaling complex with relevant enzymes and substrates [23,24]. The net result of the close proximity of enzymes and substrate proteins is to enable rapid and efficient reactions, critical for rapid platelet function under arterial-flow conditions [2]. However, it is also possible that new partner protein(s) become associated with the hsp90 complex during thrombin activation, including protein kinases, phosphatases, their substrates as well as other proteins involved in the regulation of protein phosphorylation.

CK2 has previously been shown to be present in platelets [22]; however, its involvement in platelet function is not clear. Even though a number of CK2 substrates as well as roles of CK2 in biological processes have been described [10], the regulation of CK2 is not well understood. The intracellular localization of the kinase is believed to be an important factor controlling CK2 activity [11,25]. We have now demonstrated that CK2α- and CK2β-subunits were associated with the hsp90 complex in resting platelets and that thrombin caused the dissoci-

ation of CK2 catalytic subunit from the complex. Therefore, the activated protein kinase(s) in the hsp90 complex isolated from thrombin-activated platelets (Fig. 4) are unlikely to include CK2.

Hsp90 complexes have been suggested to be involved in the intracellular translocation of many proteins, and platelet adhesion to collagen induces the subcellular translocation of hsp90 from cytosolic to membrane- and cyto-skeletal fractions [12]. Therefore, it is possible that a function of CK2, whose regulation is thought to be related to its subcellular localization [25], may be modulated by its association with the hsp90 complex.

In conclusion, we have shown the association of hsp90, hsc70, CK2α, CK2β, and other phosphoproteins as a complex. Platelet activation by thrombin stimulated protein kinase activity and the phosphorylation of a number of proteins present in the hsp90 complex. In addition, thrombin caused the rapid dissociation of hsc70 and CK2α from the hsp90 complex (<10 s), supporting dynamic functional roles for hsp90 complex(es). These data are consistent with hsp90 serving as a scaffolding protein for efficient signal transduction and enabling the extremely rapid induction of platelet function required for hemostasis [1–3].

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