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REVIEW

Structure, Properties, and Probable Physiological Role of Small Heat Shock Protein with Molecular Mass 20 kD (Hsp20, HspB6)

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Abstract—This review is devoted to critical analysis of data concerning the structure and functions of small heat shock proteins with apparent molecular mass 20 kD (Hsp20). We describe the structure of Hsp20, its phosphorylation by different protein kinases, interaction of Hsp20 with other small heat shock proteins, and chaperone activity of Hsp20. The distribution of Hsp20 in different animal tissues and the factors affecting expression of Hsp20 are also described. Data on the possible involvement of Hsp20 in regulation of platelet aggregation and glucose transport are presented and analyzed. Special attention is paid to literature data describing probable regulatory effect of Hsp20 on contraction of smooth muscle. Two hypotheses postulating direct effect of Hsp20 on actomyosin interaction or its effect on cytoskeleton are compared and analyzed. The most recent data on the effect of Hsp20 on apoptosis and contractile activity of cardiomyocytes are also presented.

Key words: small heat shock proteins, phosphorylation, chaperone activity, apoptosis, contractile activity of smooth and skeletal muscles

The family of small heat shock proteins (sHsp) combines a large group of proteins with molecular masses from 12 to 43 kD [18, 35]. These proteins are ubiquitous and have been detected in bacteria [31], plants [44], and in different animal and human tissues [18, 35]. The proteins of this family share a number of common properties. First, all these proteins contain highly conservative 80-100 residues long α-crystallin domain that was primarily detected in the structure of eye lens crystallin. Second, the members of the family of small heat shock proteins tend to form high molecular mass homo- or heterooligomers composed of identical or different subunits. Third, practically all small heat shock proteins undergo phosphorylation by different protein kinases, and phosphorylation might affect oligomeric structure or affect other important properties of these proteins. Finally, fourth, the small heat shock proteins possess chaperone activity and are able to prevent aggregation of partially denatured proteins. In spite of a large number of common properties, each of the members of this family has its own unique properties. This paper deals with the structure and properties of small heat shock proteins with apparent molecular mass 20 kD (Hsp20, HspB6).

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STRUCTURE, PHYSICOCHEMICAL PROPERTIES, AND DISTRIBUTION OF SMALL HEAT SHOCK PROTEIN WITH MOLECULAR MASS 20 kD

Hsp20 structure. Small heat shock protein with apparent molecular mass 20 kD is one of the 10 members of the family of small heat shock proteins expressed in different human tissues [23]. The primary structure of Hsp20 [14, 20, 23, 24] is highly homologous to that of αA- and αB-crystallin and to smaller extent to the primary structure of small heat shock protein with molecular mass 25/27 kD (Hsp25/27) [14, 23]. Recently published data indicate that the α-crystallin domain of Hsp20 is folded in a manner similar to that of the protein with molecular mass 23 kD that is co-chaperone of heat shock protein with molecular mass 90 kD (Hsp90) [16]. As in all members of the family of small heat shock proteins, the α-crystallin domain that is composed of sever-

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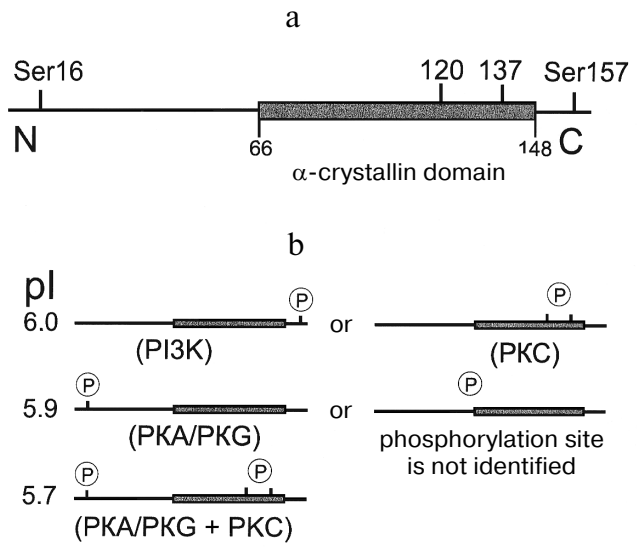

Fig. 1. a) Scheme of the structure of small heat shock protein with molecular mass 20 kD. Highly conservative α-crystallin domain and variable N- and C-terminal ends are marked. The position of Ser16 phosphorylated by cAMP- or cGMP-dependent protein kinases (PKA and PKG, respectively) and Ser157 phosphorylated by phosphatidylinositol 3-kinase (PI3K) or by protein kinases activated by this enzyme are marked by P. The peptide containing the sites phosphorylated by protein kinase C (PKC) or by protein kinases activated by protein kinase C is also indicated on the scheme. b) Scheme of possible location of the sites phosphorylated in Hsp20 having different p*I*. Protein kinases directly or indirectly involved in phosphorylation of specific sites are indicated in parenthesis.

al β-sheets is located in the C-terminal part of Hsp20 (Fig. 1). The less conservative N-terminal domain, as well as the variable C-terminal end contain small quantities of α-helices and do not have highly ordered structure. Hsp20 is phosphorylated by different protein kinases so that one site of phosphorylation is located in the very N-terminal part and the second (or other) sites of phosphorylation are located in the central or C-terminal parts of Hsp20 (Fig. 1).

Already in the earliest studies dealing with Hsp20 [24], it was found that Hsp20 is co-purified with αB-crystallin and Hsp27 and forms high molecular mass oligomers with these proteins. According to the literature, isolated Hsp20 forms low (43-67 kD) and high (200-470 kD) molecular mass complexes [24, 48]. Our investigations have shown that the recombinant human Hsp20 predominantly forms dimers [10]. Partial proteolysis of the C-terminal end or the drop of pH to 5.5 lead to Hsp20 aggregation and formation of unordered aggregates with high molecular mass. Mutation mimicking phosphorylation of Ser16 has no significant effect on the oligomeric state of Hsp20 [10].

Phosphorylation of Hsp20. At the very beginning of Hsp20 investigation, it has been shown that this protein can be phosphorylated *in vivo* [1, 5, 38]. The p*I* value of the unphosphorylated protein is close to 6.3 [41]. In addition to this form, different tissues contain Hsp20 forms with p*I* 6.0, 5.9, and 5.7 [2, 55]. Comparing these data, one can suppose that the forms with p*I* 6.0 and 5.9 represent singly phosphorylated Hsp20 containing phosphorylated sites in the different parts of the protein molecule, whereas a form with p*I* 5.7 represents doubly phosphorylated protein. Special attempts have been undertaken to determine the sites of Hsp20 phosphorylation.

In rat skeletal muscle, a form with p*I* 6.0 represents Hsp20 phosphorylated at Ser157. This site seems to be phosphorylated by phosphatidylinositol 3-kinase (or protein kinases activated by this kinase) [54]. Surprisingly, human and bovine Hsp20 lack Ser157 (in these proteins Ser157 is replaced by Pro). However, bovine tissues contain a phosphorylated form of Hsp20 with p*I* 6.0 [1, 5]. In this case, the form with p*I* 6.0 contained the single phosphorylated site that was located in the peptide restricted by residues 120-137 (probably Thr133, Ser134, or Ser137, the numbering corresponding to human Hsp20) [2]. This site is phosphorylated in response to stimulation by phorbol esters that activate protein kinase C. However, this peptide does not contain the consensus sequence recognized by protein kinase C itself. Therefore, it seems probable that this site is phosphorylated not by protein kinase C itself, but by protein kinases that are activated by protein kinase C.

The form with p*I* 5.9 contains Ser16 phosphorylated by cyclic nucleotide dependent protein kinases or probably another still undetermined site [2]. Hsp20 with p*I* 5.7 also contained phosphorylated Ser16 and another phosphorylated site inside the peptide restricted by residues 120-137 (probably Thr133, Ser134, Ser137, following numbering of human Hsp20) [2]. Thus, Hsp20 can be phosphorylated at different sites (see Fig. 1b). It was suggested [41] that under normal conditions Hsp20 is either unphosphorylated or contains a single phosphorylated site in the central or C-terminal part of the molecule (Ser157 of rat Hsp20 or peptide restricted by residues 120-137 in bovine tissues). Upon activation of cyclic-nucleotide dependent protein kinases Ser16 becomes also phosphorylated. Phosphorylation affects the interaction of Hsp20 with other proteins [5], as well as the functions performed by this protein in the cell (see below).

Interaction of Hsp20 with other small heat shock proteins. Many indirect data of the literature indicate that Hsp20 is able to form heterooligomeric complexes with αB-crystallin and Hsp27 and that phosphorylation affects their ability to form these complexes [8, 24, 43]. Mixing of human Hsp20 and Hsp27 results in formation of heterooligomeric complexes with apparent molecular mass 100 and 300 kD containing roughly equal quantities of each small heat shock protein [10]. Mutation mimicking phosphorylation of three serine residues (Ser15, Ser78, and Ser82) of Hsp27 increased the rate of formation of heterooligomeric complexes that under these conditions

had apparent molecular mass equal to 100 kD [10]. Taking into account that skeletal and smooth muscles contain a rather high quantity of Hsp20, Hsp27, and αB-crystallin [20, 24], we can conclude that the probability of formation of heterooligomeric complexes in these tissues is rather high.

If these complexes are really formed, then it will be rather difficult to determine the exact location of isolated Hsp20 inside the cell and protein partners involved in direct interaction with Hsp20. For example, many investigations indicate that Hsp20 is localized on or close to actin filaments [12, 36, 42]. However, this conclusion is questionable. Indeed, it is known that both Hsp27 and αB-crystallin are also co-localized with actin filaments [43]. Therefore it is difficult to decide which of the small heat shock proteins is responsible for direct interaction with actin and what heterooligomeric complexes take part in stabilization of actin filaments.

Chaperone activity of Hsp20. For a long time it was considered that Hsp20 has a very low chaperone activity *in vitro* [48]. Recently published data contradict this assumption. It was shown that the recombinant human Hsp20 has chaperone activity that is comparable with that of commercial α-crystallin [10]. These data agree with earlier published data of van de Klundert et al. [49]. These authors have shown that overexpression of either Hsp20 or αB-crystallin confer thermal resistance in Chinese hamster ovary cells. However, αB-crystallin enhances recovery of co-expressed firefly luciferase, whereas Hsp20 was ineffective [49]. This fact may indicate that the molecular mechanisms of protective action of Hsp20 might be different from that of αB-crystallin.

Tissue distribution of Hsp20. Changes of Hsp20 expression during ontogenesis and under different experimental conditions. In the very beginning of Hsp20 investigation, it has been shown that this protein is expressed in practically all organs and tissues. The Hsp20 content is especially high in slow skeletal muscle (*m. soleus*), diaphragm, heart, and smooth muscles where its quantity was 1,200-13,000 ng/mg cell protein. The lowest Hsp20 content was detected in liver and different regions of the brain where its content was 3-6 ng/mg cell proteins [24]. It is worthwhile to mention that Hsp20 content is different in different blood vessels. For instance, smooth muscle cells of the left interior mammary artery contained the largest quantity of small heat shock proteins (αB-crystallin, Hsp20, Hsp27), whereas the smooth muscle cells from the umbilical vein contained the lowest quantity of these proteins [32]. This conclusion was confirmed in later publications where it has been shown that in rats immediately after birth the level of Hsp20 in *m. soleus* is increased from 100 up to 8,000 ng/mg, whereas during the same period of time in extensor *digitorum longus* the Hsp20 content increased from 150 up to 700-900 ng/mg [20]. A similar tendency was observed in different organs of pigs during their ontogenesis. As a rule, during ontogenesis the Hsp20 content is increased both in heart and skeletal muscle [51].

Different experimental conditions strongly affect the expression of Hsp20. Indeed it has been shown that spinal cord transection [19] and hind limb suspension induced atrophy [21] or denervation [20] are accompanied by significant decrease in Hsp20 content in slow muscles or in muscle with antigravity function. At the same time spinal cord transection or denervation have small effect on Hsp20 content in flexor muscles [19, 20]. It was concluded that Hsp20 is predominantly expressed in muscle fibers expressing slow isoforms of myosin heavy chains [19]. Several attempts were undertaken in order to localize Hsp20 inside different muscles. Using immunofluorescence microscopy Pipkin et al. [36] have shown that Hsp20 is co-localized with actin filaments. Moreover, anti-Hsp20 antibodies stained cell membranes, probably at the sites of actin filament fixation (dense bodies). Confocal microscopy of pig carotid artery indicates that Hsp20 is present throughout the cytoplasm, although some focal regions of the cytoplasm were found to contain more Hsp20 than the remaining cytoplasm [42]. Thus, at present there is no straightforward data indicating that Hsp20 is directly bound to the contractile apparatus. However, many authors believe that Hsp20 is somehow bound to the muscle contractile apparatus. We suppose that this viewpoint is superficial. Under rest, the largest part of small heat shock proteins is diffusely distributed in the cell. In response to different unfavorable conditions (i.e. myofibril damage induced by long contraction) the small heat shock protein are translocated to the contractile apparatus and participate in its protection and/or reparation [26]. Such translocation was detected for αB-crystallin and small heat shock protein with molecular mass 27 kD (Hsp27). However, stimulation of smooth muscle by different agonists [5, 42] or poisoning of smooth muscle by arsenite [8] was not accompanied by translocation of Hsp20 from cytoplasm to contractile apparatus. At the same time, proteasomal inhibition in cardiac myoblast cell line H9C2 was accompanied by translocation of Hsp20 to stress fibers [50] and cyclic nucleotide dependent phosphorylation of Hsp20 seems to increase its affinity to actin filaments in smooth muscles and cardiomyocytes [12, 38]. Thus, at present the exact intracellular location of Hsp20 remains ambiguous.

Finishing this section, it is worthwhile to mention that damage of carotid artery endothelium is followed by significant decrease in Hsp20 content inside of the cell with concomitant increase in Hsp20 level in the blood. Similar increase in Hsp20 level in the blood was observed in cardiomyopathic hamsters [27]. It is probable that the damage of cardiomyocytes or vessels results in liberation of Hsp20 into the blood where this small heat shock protein may participate in regulation of platelet aggregation (see below).

FUNCTIONAL ROLE OF SMALL HEAT SHOCK PROTEIN WITH MOLECULAR MASS 20 kD

Participation of Hsp20 in regulation of platelet aggregation. In the end of 1990s it was found that at low concentration (0.01-0.10 mg/ml) Hsp20 inhibits thrombin-induced, but does not affect ADP-induced aggregation of platelets [29]. Further investigations revealed that Hsp20 blocks thrombin-induced entrance of calcium from outside of the cell but does not affect calcium release from intracellular stores. In addition, increased level of Hsp20 was detected in the blood of patients with cardiovascular disorders [33]. It was postulated that Hsp20 inhibits thrombin-induced activation of phospholipase C that hydrolyzes phosphatidylinositols and leads to accumulation of diacylglycerol that activates protein kinase C [27]. Endothelial injury results in liberation of Hsp20 into the blood stream and in this way may prevent platelet aggregation [27]. Recent investigations have shown that short peptides restricted by residues 9-17 of αB-crystallin (WIRRPFFPF) or by analogous residues of Hsp20 (WIRRASAPI) effectively interact with platelets and prevent aggregation induced by thrombin, botrocetin, or ristocitin (stimulator of the platelet glycoprotein Ib/V/IX) [28]. Taking into account that these peptides block both adhesion and platelet activation one can conclude that the short 5-9 member peptides of α-crystallin and Hsp20 might be very effective in therapy of thrombosis and cardiovascular diseases.

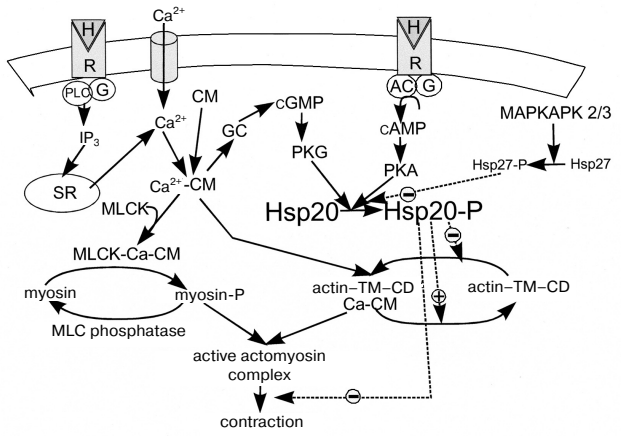

Fig. 2. Possible participation of Hsp20 in regulation of smooth muscle contraction. Interaction of hormones (H) with receptors (R) can through G-proteins (G) activate calcium influx and activate cAMP and/or inositol-3-phosphate (IP_3) synthesis. Calcium entering from outside or IP_3 induce calcium release from endoplasmic reticulum (SR). Increase in calcium concentration activates protein kinase C that (itself or by downstream protein kinases) phosphorylates Hsp20. Increase in intracellular calcium concentration is accompanied by formation of calcium–calmodulin (Ca–CM) complex that activates myosin light chain kinase (MLCK), reverses inhibitory action of caldesmon (CD) on actin filaments containing tropomyosin (TM), and activates soluble guanylate cyclase (GC). Phosphorylated myosin and unblocked actin form active actomyosin complex that generates contraction. cAMP- or cGMP-dependent protein kinases (PKA and PKG) phosphorylate Ser16 of Hsp20. This phosphorylation can be inhibited by small heat shock protein with molecular mass 27 kD (Hsp27). Hsp20 phosphorylated at Ser16 either prevents "switching-on" of actin filaments or affects fixation of actin filaments to cytoskeleton and by this means prevents contraction and induces relaxation.

Probable participation of Hsp20 in metabolic regulation of skeletal and smooth muscle by insulin. In the beginning of 1990s, it was found that addition of insulin to strips of extensor *digitorum longus* or to *m. soleus* induced Hsp20 phosphorylation [53, 54]. Addition of insulin was accompanied by accumulation of Hsp20 with p*I* 6.0 that contained phosphorylated Ser157 [54]. Wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase, blocked Hsp20 phosphorylation. These findings indicate that insulin through a number of different intermediates activates phosphatidylinositol 3-kinase that itself or through downstream protein kinases participates in Hsp20 phosphorylation [54]. It is worthwhile to mention that epinephrine decreased insulin-induced phosphorylation of Hsp20. Continuing their investigations, the New Zealand scientists found that in rat skeletal muscles (*m. soleus*) epinephrine (or forskolin) simultaneously prevents insulin-induced phosphorylation of Ser157 and activates phosphorylation of Ser16 of Hsp20 [53]. Surprisingly, in smooth muscles insulin does not affect phosphorylation of Ser157 and increases phosphorylation of Ser16 of Hsp20. This effect of insulin might result in vasorelaxation and provide better transportation of glucose and insulin to skeletal muscles and more efficient glucose utilization [53]. A hypothesis on probable participation of Hsp20 in glucose transportation to peripheral tissues was supported experimentally [55]. Indeed, overexpression of Hsp20 is accompanied by significant changes in glucose transportation in cultured muscle cells. Although the detailed molecular mechanism of Hsp20 action remains enigmatic, it is concluded that Hsp20 is a very promising marker of experimental diabetes [55]. However, certain restrictions should be taken into account in realization of this suggestion since human Hsp20 lacks residue homologous to Ser157 of the rat. Nevertheless, all experiments indicating participation of Hsp20 in glucose transportation are of great interest and should be continued.

Possible participation of Hsp20 in regulation of contractile activity of smooth muscle. Starting from the earliest publications from the laboratory of Brophy [1], it is well accepted that Hsp20 can be phosphorylated by cAMP- or cGMP-dependent protein kinases and that this phosphorylation somehow promotes smooth muscle relaxation. Before analyzing the literature on the role of Hsp20, let us overview the overall scheme of regulation of smooth muscle contraction (Fig. 2).

Electrical stimulation or specific agonists may regulate calcium entrance through specific channels or increase the intracellular calcium concentration by stimulation of inositol-3-phosphate (IP_3)-dependent channels of endoplasmic reticulum. Increase in the intracellular calcium concentration leads to saturation of calcium-binding sites of calmodulin (CM). The Ca–calmodulin complex (Ca–CM) activates many intracellular enzymes. First, Ca–CM activates myosin light chain kinase (MLCK) that phosphorylates myosin light chains and in this way promotes formation of active actomyosin complex, which is able to generate contraction. Second, Ca–CM reverses the inhibitory action of caldesmon on actin. Thus, actin filaments become switched-on and able to interact with phosphorylated myosin with formation of active actomyosin complex. Third, Ca–CM is able to activate membrane adenylate cyclase that synthesizes cAMP. Finally, under certain conditions Ca–CM can activate soluble NO-synthase. This enzyme using arginine as a substrate produces NO that activates guanylate cyclase synthesizing cGMP. Increase in cAMP and/or cGMP level activates cyclic-nucleotide dependent protein kinases that phosphorylate many intracellular substrates and among them the small heat shock protein with molecular mass 20 kD (Hsp20) (Fig. 2). Due to the lack of space, we will not analyze either effects of phosphorylation and dephosphorylation of myosin light chains or the role of caldesmon (or calponin) in regulation of smooth muscle contraction. These problems are thoroughly described in a number of recently published reviews [17, 25, 30, 37, 52]. Let us analyze only the effect of Hsp20 on the regulation of smooth muscle contraction.

The experimental results concerning this problem can be divided in two groups. The largest share of publications derived from Brophy's laboratory. Other publications derived from independent laboratories sometimes contradict the data of Brophy's laboratory. In early investigations [1, 2, 56], it was shown that *in vivo* Hsp20 can be phosphorylated by protein kinase C and cyclic-nucleotide dependent protein kinases. cAMP- and cGMP-dependent protein kinases phosphorylate Ser16 of Hsp20 and this event is accompanied by relaxation of carotid artery that was contracted by depolarization or by addition of serotonin. This relaxation was not related to dephosphorylation of myosin light chains. It is worthwhile mentioning that in the umbilical artery activation of cyclic nucleotide-dependent protein kinase phosphorylation (by simultaneous addition of forskolin and 3-isobutyl-1-methylxanthine) was accompanied by insignificant increase in Hsp20 phosphorylation and did not induce relaxation. It was concluded that phosphorylation of Hsp20 is one of the important mechanisms providing smooth muscle relaxation.

According to Woodrum et al. [56], relaxation induced by Hsp20 phosphorylation was not accompanied by decrease in oxygen consumption or by any significant change in the level of myosin light chain phosphorylation. Therefore, the authors concluded that phosphorylation of Hsp20 does not affect formation of active actomyosin complex, but somehow disturbs interaction of actomyosin complex with the structural elements providing its fixation to the cell membrane. Recently published data to some extent correlate with this hypothesis. According to the data of Brophy's laboratory [7, 47], Hsp20 co-immunoprecipitates with actin and α-actinin and phosphorylation of Ser16 decreases interaction of Hsp20 with actin and α-actinin. According to Brophy, during contraction unphosphorylated Hsp20 is bound to actin filaments and α-actinin. This stabilizes actin filaments and their fixation to dense bodies interacting with the cell membrane. After phosphorylation, Hsp20 dissociate from polymerized actin and α-actinin. This somehow prevents transduction of force generation and results in relaxation.

Special investigations deal with the role of NO in smooth muscle relaxation. It is well known that under certain conditions mechanical stress of endothelia induces activation of NO-synthase. Entering the medium, NO activates guanylate cyclase and increases the intracellular level of cGMP that activates cGMP-dependent protein kinase. This enzyme phosphorylates Hsp20, thus inducing vasorelaxation. This chain of events was followed in a number of publications [6, 22, 45, 46]. It is interesting to mention that serotonin increases the intracellular calcium concentration in smooth muscles of vessels. On one side this leads to activation of contraction and on the other side by activating Ca–calmodulin-dependent NO-synthase results in increase in cGMP level, phosphorylation of Hsp20, and vasorelaxation [6].

Continuing analysis of the papers from Brophy's laboratory, it is worthwhile to mention the interaction of different small heat shock proteins. In elegant experiments performed on borderline hypertensive rats Fuchs et al. [15] found that behavioral stress is accompanied by significant increase in expression of small heat shock protein with molecular masses 27 (Hsp27) and 20 (Hsp20) kD. At the same time, behavioral stress was accompanied by increase in Hsp27 phosphorylation and simultaneous decrease in Hsp20 phosphorylation. Moreover, under *in vitro* conditions phosphorylated Hsp27 inhibited phosphorylation of Ser16 of Hsp20 catalyzed by cAMP-dependent protein kinase [15]. A hypothesis was put forward according to which Hsp27 and Hsp20 have opposite effects on smooth muscle contraction. Phosphorylation of Hsp20 somehow promotes relaxation of smooth muscle, whereas phosphorylation of Hsp27 either prevents phosphorylation of Hsp20 (and in this way inhibits relaxation) or directly activates smooth muscle contraction [3, 4].

Development of new methods has provided new data on the role of Hsp20 in regulation of smooth muscle contraction. Synthetic peptide consisting of 24 residues was designed in Brophy's laboratory. The first 11 residues (so-

called protein transduction domain) provide penetration of this peptide through the membrane, whereas the last 13 residues contain Hsp20 sequence surrounding Ser16 [13, 46]. This peptide easily penetrates culturing and vascular cells. Addition of this peptide containing phosphorylated Ser16 results in relaxation of pig carotid artery contracted by serotonin [13]. Scrambled peptide was ineffective in induction of relaxation. Peptide containing phosphorylated Ser16 residue prevented contraction of human saphenous vein, inhibited migration, and had no effect on proliferation of human smooth muscle cells [46]. All these properties of this peptide are very important for transplantation. Grafts of saphenous vein often underwent spastic contraction and intimal hyperplasia. Addition of penetrating peptide containing phosphorylated peptide of Hsp20 prevents both vasospasm and intimal hyperplasia [46]. Recently new data on transfection of intact Hsp20 gene to rat mesangial cells appeared in the literature [57]. In the course of these experiments, the contractile activity of mesangial cells transfected with green fluorescence protein or with construct containing green fluorescence protein and Hsp20 were compared. Overexpression of Hsp20 decreased the contractile activity of mesangial cells. An attempt to determine the intracellular localization of Hsp20 was not very successful. However, overexpression of Hsp20 results in disappearance of actin stress-fibers in the central part of the cell [57]. Trying to explain the mechanism of Hsp20 functioning the authors again suggest that Hsp20 somehow affects different actin-binding proteins (α-actinin [47] or cofilin [46]) or actin polymerization and in this way prevents the development of contraction.

A principally different viewpoint prevails in publications from other laboratories. Thus, according to Rembold et al. [38] phosphorylation of Ser16 of Hsp20 indeed induces relaxation of vessel smooth muscle. However, according to these authors phosphorylation of Hsp20 is accompanied by decrease in oxygen consumption, thus indicating that the small heat shock protein somehow affects formation of active actomyosin complex (Fig. 2). Moreover, these authors suggest that phosphorylation increases affinity of Hsp20 to actin filaments. It is postulated that Hsp20 contains a site with primary structure similar to that of inhibitory peptide of troponin I. Thus, according to a hypothesis of Rembold et al. [38] cyclic nucleotide-dependent phosphorylation of Hsp20 leads to translocation of Hsp20 from cytoplasm to actin filaments and to inhibition of normal functioning of myosin cross-bridges. Continuing their experiments, Rembold and O'Connor found that relaxation of pig carotid artery is indeed accompanied by phosphorylation of Hsp20, whereas relaxation induced by high magnesium concentration does not depend on the level of Hsp20 phosphorylation [40]. More detailed analysis of relaxation induced by forskolin and nitroglycerine results in detection of two phases. The first rapid phase of relaxation was accompanied by dephosphorylation of myosin light chains. In the later stages of relaxation phosphorylation of myosin light chains returned practically to its high level, but the muscle remained relaxed. This unusual behavior is probably due to phosphorylation of Hsp20 that somehow is able to switch-off actin filaments and in this way prevents formation of active actomyosin complex (Fig. 2) [41]. The heat shock (heating of coronary artery rings up to 43-46°C for 4 h) is accompanied by increase in Hsp20 expression and by significant increase in its Ser16 phosphorylation [34, 39]. This increase in Ser16 phosphorylation was not correlated with increase in intracellular level of cyclic nucleotides [39]. Therefore, it was supposed that increase in Hsp20 phosphorylation is due not to the activation of cyclic nucleotide dependent protein kinases but to inhibition of different protein phosphatases. As expected, heat induced increase in Hsp20 phosphorylation correlates with decreased contraction induced by histamine [34, 39].

Thus, literature unequivocally indicates that phosphorylation of Hsp20 by cyclic nucleotide dependent protein kinases correlates with smooth muscle relaxation. However, detailed molecular mechanism of this process remains enigmatic. All authors agree that relaxation correlating with Hsp20 phosphorylation is based on the ability of Hsp20 to affect actin filaments. However, two alternative hypotheses are put forward. According to Brophy and coauthors, Hsp20 has no effect on switching-on actin filaments, but affects fixing of actin filaments to the cell membranes and cytoskeleton. However, according to Rembold et al. [38], under certain conditions Hsp20 is tightly bound to actin and similar to troponin I is switching off actin filaments. Anyhow, both these hypotheses postulate that Hsp20 is an actin-binding protein. According to Brophy, phosphorylated Hsp20 predominantly interacts with monomeric, whereas unphosphorylated Hsp20 predominantly interacts with polymeric actin [7]. In contrast to this viewpoint, Rembold et al. [38] postulate that phosphorylated Hsp20 interacts more tightly with polymeric actin and (unlike unphosphorylated Hsp20) is able to switch-off actin filaments. We have a feeling that up to now there is no direct evidence unequivocally indicating that Hsp20 is indeed an actin-binding protein.

Let us analyze the data favoring the suggestion that Hsp20 is an actin-binding protein. The first paper postulating that Hsp20 is an actin-binding protein was published by Brophy et al. in 1999 [7]. It is very surprising that after ultracentrifugation a very large portion of polymerized actin remained in the supernatant, whereas highly soluble Hsp20 was predominantly detected in the pellet (see Fig. 4 of [7]). This strange distribution of both proteins makes questionable the main conclusion on the interaction of Hsp20 with actin. Experimental data with co-immunoprecipitation of Hsp20 with both actin [7] and α-actinin [47] are also contradictory and not com-

pletely convincing. Unfortunately, the data of Rembold et al. [38] on co-precipitation of Hsp20 with actin filaments (see Fig. 5 of the above mentioned paper) are also not very convincing. We analyzed the interaction of recombinant human Hsp20 and its mutant mimicking phosphorylation (S16D) with isolated F-actin or F-actin containing smooth muscle tropomyosin, α-actinin, or calponin [9]. Under all conditions used, Hsp20 was not bound either to isolated actin or to actin containing different actin-binding proteins. Thus, isolated Hsp20 (or its mutant mimicking phosphorylation) has low affinity to polymerized actin.

If Hsp20 were indeed a real actin-binding protein, then under certain conditions these two proteins would be strictly co-localized in the cell. Unfortunately, the cytochemical papers are very contradictory and their authors, as a rule, find diffuse distribution of Hsp20 in the cell, although there is a limited number of reports indicating co-localization of Hsp20 with actin (or actin-binding proteins) [36, 42]. Thus, at present there is no direct evidence that Hsp20 is able to interact either with monomeric or polymeric actin. If Hsp20 does not interact with actin, then how might phosphorylation of Hsp20 effect smooth muscle relaxation? There is a feeling that Hsp20 interacts with certain soluble proteins that somehow affect either the structure or fixation of actin filaments to cytoskeleton. In this respect it is interesting to mention that if whole cell extract was subjected to the size-exclusion chromatography, then increase in intracellular concentration of cyclic nucleotides leads to decrease in apparent molecular mass of the protein fraction containing Hsp20 [5]. Unfortunately, at present it is difficult to determine what the protein target of Hsp20 is. Thus despite prominent success in investigation of the physiological role of Hsp20 in regulation of smooth muscle contraction and despite significant importance of this protein in transplantation and cardiovascular surgery, the detailed mechanism of Hsp20 action remains enigmatic.

Possible participation of Hsp20 in regulation of contractile activity and apoptosis of cardiomyocytes. New data on the possible role of Hsp20 in cardiomyocytes appeared recently in the literature. The mechanism of regulation of contractile activity of heart is different from that of smooth muscle. Indeed, activation of dihydropyridine receptor induces liberation of calcium from sarcoplasmic reticulum. In addition, liberation of calcium from sarcoplasmic reticulum can be initiated by calcium entering cardiomyocytes from outside (see Fig. 3). Intracellular calcium saturates Ca-specific sites of troponin C. Ca-induced conformational changes of troponin C are transmitted to two other troponin components (troponin I and troponin T) as well as to tropomyosin. As a result of all these processes actin filaments become switched-on, interacting with myosin forming active actomyosin complex and developing contraction (see Fig. 3). Activation of β-adrenergic receptors leads to activation of adenylate cyclase (AC) and increase in cAMP level. Activated cAMP-dependent protein kinase phosphorylates a number of intracellular protein targets. Phosphorylation of troponin I leads to decrease in affinity of troponin C to calcium ions and therefore increases the rate of relaxation. cAMP-dependent protein kinase phosphorylates phospholamban, a specific protein of sarcoplasmic reticulum, and in this way increases the rate of calcium uptake by Ca-ATPase (Fig. 3). This process will also increase the rate of cardiac relaxation. Finally, cAMP-dependent protein kinase can participate in phosphorylation of calcium channels in the outer membrane. This will increase the inward current of calcium and increase the amplitude of cardiac contraction. Thus, regulation of cardiac contraction is significantly different from that of smooth muscle. However, in both cases calcium ions play a crucial role in initiation of contraction and cAMP-dependent phosphorylation provides additional levels of regulation.

According to the recently published data from laboratory of Kranias [11, 12], stimulation of cardiomyocytes by isoproterenol results in phosphorylation of many intracellular proteins. In the early stages of stimulation of cardiomyocytes by isoproterenol (5 min stimulation) troponin I and succinyl-CoA synthetase become phosphorylated. In the later stages of stimulation (30 min), Hsp20

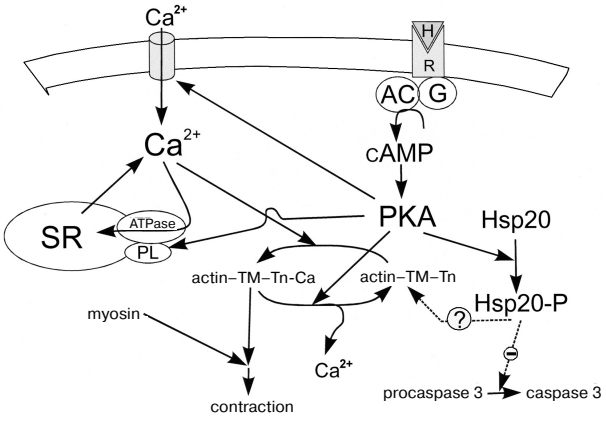

Fig. 3. Possible participation of Hsp20 in regulation of contraction and apoptosis of cardiomyocytes (symbols are the same as in Fig. 2). Electrical or hormonal stimulation results in increase in intracellular calcium concentration. Calcium is bound to troponin complex (Tn) and switches on actin filaments. Cyclic nucleotide dependent protein kinases phosphorylate troponin I and in this way decrease affinity of troponin complex to calcium and promote relaxation. cAMP-dependent protein kinase (PKA) phosphorylates phospholamban (PL) and in this way increases the rate of uptake of calcium by sarcoplasmic reticulum. cAMP-dependent protein kinase can phosphorylate Ca-channels and increase calcium influx. cAMP-dependent protein kinase phosphorylates Hsp20 that being phosphorylated blocks conversion of procaspase 3 and in this way prevents apoptosis. In addition, phosphorylated Hsp20 somehow stabilizes cytoskeleton and might affect the amplitude of contraction.

also becomes phosphorylated [11]. Infection of cardiomyocytes by recombinant adenovirus encoding Hsp20 results in overexpression of this protein. These cardiomyocytes had higher contractility and the amplitude of their intracellular calcium transients was much larger than in the control cells [11]. In contrast to smooth muscles, overexpression of Hsp20 was not accompanied by increase in the rate of cardiomyocyte relaxation [11].

Prolonged exposure to isoproterenol induces apoptosis of cardiomyocytes [12]. Apoptosis induced by isoproterenol and related to increased intracellular level of cAMP can be retarded or completely prevented if cardiomyocytes were transfected with adenovirus carrying Hsp20 cDNA. It is important to mention that antiapoptotic activity of Hsp20 mutant mimicking phosphorylation of Ser16 (S16D) was higher than that of the wild type protein. At the same time, Hsp20 mutant lacking the site of phosphorylation (S16A mutant) was ineffective in preventing apoptosis. It was supposed that phosphorylated Hsp20 or its mutant mimicking phosphorylation interacts with procaspase 3 and in this way prevents its proteolytic activation [12]. In addition, it was found that upon phosphorylation Hsp20 is translocated from cytosol to the contractile apparatus and to different elements of cytoskeleton. The authors supposed that phosphorylated Hsp20 binds to actin filaments and protects them from injuries induced by apoptosis [12]. At the same time, the authors could not exclude that actin filaments interact not with isolated Hsp20, but with Hsp20 inside of heterooligomeric complex consisting of αB-crystallin (and/or Hsp27) and Hsp20. It is also impossible to exclude the possibility that Hsp20 interacts and stabilizes intermediate filaments. Thus, again the detailed mechanism of Hsp20 functioning in heart is far of being absolutely clear.

Summing up, we conclude that Hsp20 has a number of unique properties that are of great interest to both physiologists and physicians. Since the detailed mechanism of Hsp20 functioning remains unclear, extended biochemical investigations are needed to understand how this protein can affect so many diverse processes.

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Notes added in proof. Recently published data of Dreiza, C. M., Brophy, C. M., Komalavilas, P., Furnish, E. J., Joshi, L., Pallero, M. A., Murphy-Ullrich, J. A., von Rechenberg, M., Ho, Y-S. J., Richardson, B., Xu, N., Zhen, Y., Peltier, J. M., and Panitch, A. (2005) *FASEB J.* **19**, 261-263) indicate that a short peptide containing phosphorylated Ser16 of Hsp20 interacts with 14-3-3 protein. This leads to displacement of phosphorylated cofilin from its complex with 14-3-3. Liberated cofilin after dephosphorylation induces actin depolymerization and in this way might affect cytoskeleton. This very interesting hypothesis postulating indirect effect of phosphorylated Hsp20 on cytoskeleton (and probably muscle contraction) is awaiting further confirmation both on the level of isolated proteins and on the level of cell biochemistry.

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