ATP and ADP Modulate a Cation Channel Formed by Hsc70 in Acidic Phospholipid Membranes

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Heat shock proteins are molecular chaperones that participate in different cellular processes, particularly the folding and translocation of polypeptides across membranes. In this regard, members of the Hsp70 family of heat shock proteins have been observed in close proximity to cellular membranes. In this study, the direct interaction between Hsc70, which is constitutively expressed in cells, and lipid membranes was investigated. Recombinant Hsc70 was incorporated into artificial lipid bilayers, and a transmembrane ion flow was detected, suggesting the incorporation of an ion pathway. This ion flow was very stable and occurred in well defined, multilevel discrete electrical current events, indicating the formation of a multiconductance ion channel. The Hsc70 channel activity is ATP-dependent and is reversibly blocked by ADP. This channel has cationic selectivity. Thus, Hsc70 can directly interact with lipid membranes to create functionally stable ATP-dependent cationic pathways.

Heat shock proteins (hsp) are molecular chaperones that participate in different normal cellular processes, including the folding of newly synthesized proteins and their import into subcellular compartments such as the endoplasmic reticulum and mitochondria, the oligomeric assembly of proteins, and protein degradation (1–3). hsp also play an important role in the recovery of cells from a variety of stresses. In this process, they participate in the resolubilization of proteins that become denatured as a consequence of the stress, as well as the stabilization of cellular pathways such as transcription and translation (3). hsp are grouped into families according to size and sequence homology. This most studied of the hsp is the Hsp70 family, which is composed of four members: Hsc70, Hsp70, BIP, and Mtp70. All members of the Hsp70 family contain three structural and functional domains. The domain at the N terminus of the molecule (44 kDa) binds and hydrolyzes ATP. The subsequent region (18 kDa) participates in the interaction with target proteins (peptide binding domain). The C terminus of the molecule (10 kDa) seems to be involved in the association with co-chaperone molecules such as DnaJ (2, 4). The interaction of Hsp70s with peptides is modulated by the presence and hydrolysis of ATP. Thus, ATP is necessary for the recognition of the peptide, whereas hydrolysis of ATP to ADP increases the affinity for the peptide (5). Proteins in the Hsp70 family are located in different subcellular compartments. Hsc70 and Hsp70 are found in the cytosol, whereas BIP and Mtp70 are located in the endoplasmic reticulum and mitochondrial matrix, respectively. This subcellular localization is necessary for the coordinated action of these proteins in the translocation of polypeptides across membranes. Consequently, it is not surprising to observe these proteins in close proximity to cellular membranes (6, 7). The interaction of Hsp70s with membranes may be necessary for the translocation of polypeptides across these lipid barriers. In the present study, the direct interaction of Hsc70 with membrane lipid moieties and the effects of ATP and ADP were analyzed using artificial lipids in a planar lipid bilayer system. This technique allows the study of direct protein-lipid interaction using purified components. When insertion across the lipid membrane occurs, the protein may create a transmembrane pathway that permits the flow of ions.

MATERIALS AND METHODS

Preparation of Proteoliposomes—Liposomes were prepared by hydration of air-dried palmitoyloleoyl phosphatidylethanolamine (10 mg) with 1 mM potassium aspartate, pH 7.0 (1 ml), followed by sonication for 5 min. The liposome suspension (50 μl) was combined with recombinant Hsc70 (5 μg; Stressgene), followed by sonication for an additional period of 2 min. An aliquot (5 μl) of the proteoliposome suspension was added to the solution in the cis side of the planar lipid bilayer chamber and stirred.

Planar Lipid Bilayer Methodology—Planar lipid bilayers were made as described (6). Briefly, a suspension of palmitoyloleoyl phosphatidylethanolamine and palmitoyloleoyl phosphatidylethanolamine, 1:1, in n-decane was prepared. This suspension was applied to an orifice of about 100–120 μm in diameter with a Teflon™ film separating two compartments. The ionic solutions in the compartments contained either symmetrical concentrations of KCl (200/200 cis/200 trans mM) or asymmetric concentrations (200 cis/50 trans mM) and 5 mM K-HEPES, pH 7, 1 mM MgCl2, 0.5 mM CaCl2. Unless otherwise specified, these solutions all contained ATP (2 mM). The two ionic compartments were electrically connected via agar bridges and Ag/AgCl pellet electrodes to the input of a voltage clamp amplifier. Current was recorded using a patch clamp amplifier (Axopatch-1D equipped with a low noise (CV-4B) headstage; Axon Instruments, Foster City, CA), and data were stored on a PCM/VCR digital system (Toshiba) with a frequency response in the range from direct current to 25,000 Hz. Off-line analysis of the channel activity was carried out using the software package Pclamp 6 and Axoscope 7 (Axon Instruments).

RESULTS

Hsc70 Forms a Stable Ion Pathway in Planar Lipid Bilayers—To study the interaction of Hsc70 with a planar lipid bilayer, the protein was incorporated into liposomes, which were then allowed to fuse with the lipid bilayer. Proteoliposomes were prepared by sonication of a suspension of palmitoyloleoyl phosphatidylethanolamine and pure recombinant Hsc70 protein (Stressgene). Small aliquots of this proteoliposome suspension were added to one of the two ionic solutions.
separated by the artificial lipid bilayer. The incorporation of Hsc70 into the lipid bilayer occurred by spontaneous fusion of the proteoliposome with the artificial membrane. The pattern of current activity was uniformly sustained for very long periods of time as illustrated by 13 min of continuous recording. This long recording also confirms the stability of the Hsc70 ion pathway.

A detailed analysis of the electrical activity revealed that the ion flow occurred in well defined multilevel current events, indicating that the ion pathway gated between several open and closed states. Because the level of the electrical potential across the membrane was kept constant during the experiment, the ion channel formed by the incorporation of Hsc70 into the lipid membrane seems to have a multiconductance nature (9). To further characterize this channel, the ion pathway activity was recorded in symmetrical KCl concentration solutions and an electrical potential gradient of 100 mV. The channel displayed a clear multiconductance activity by either completely closing or switching between different open levels (A). The current records at expanded amplitude scale illustrate the well defined levels that characterize the lower conductance range (B). A current amplitude histogram was constructed from plotting each current value from the electrical recordings shown in B. Marquardt least square fitting of this histogram indicates that the current values distributed mainly into six gaussians (C).
Hsc70 Cation Channel

CONTROL

ATP (1 mM)

peak in the histogram are: 5 ± 0.037, 9 ± 0.07, 12 ± 0.148, 20 ± 0.136, 24 ± 0.12, and 36 ± 0.14 pS.

**Hsc70 Channel Opens in the Presence of ATP**—Ion channel activity was also observed after spontaneous insertion of Hsc70 into a lipid bilayer. An example in which Hsc70 was added directly into one of the two ionic solutions separated by the lipid bilayer is presented in Fig. 3. The appearance of occasional current events indicated that Hsc70 directly incorporated into the lipid membrane and formed an ion channel (Fig. 3A). The moderate frequency of electrical events was dramatically changed after the addition of ATP (1 mM) (Fig. 3B). The Hsc70 channel immediately became highly active and displayed the same characteristic current pattern described in Fig. 1. The ionic current in the presence of ATP is more consistent and higher in amplitude than the one observed in the absence of ATP (Fig. 3A). These results demonstrate that Hsc70 spontaneously incorporates into lipid membranes and requires the presence of ATP for consistent channel activity.

**Hsc70 Channel Has Cationic Selectivity**—To determine the selectivity and the effect of electrical potential on the ion conductance of the Hsc70 channel, the protein was incorporated via proteoliposomes into a planar lipid membrane separating asymmetric KCl concentration (200\textsubscript{cis}/50\textsubscript{trans} mM) and in the presence of 2 mM ATP. After detection of a stable ion channel activity, the membrane was subjected to a variety of electrical potentials between plus and minus 100 mV. The results obtained for seven different membrane potential levels are summarized in Fig. 4. Notice that when the electrical potentials in the cis side of the membrane (where the ion concentration was higher) was maintained at positive levels, larger current events were generated as compared with the amplitude of the current events generated by negative potentials of the same absolute value. Because of the existing chemical gradient, downward going current events were observed at zero membrane potential (bottom record). This result indicates that when the channel was opened, charges were still moving from the cis to the trans compartment. The relationship between the mean amplitude of unitary current events and the membrane voltage in the cis compartment was plotted and analyzed by linear regression (Fig. 4B). A straight line was obtained, illustrating that the conductance of the channel is linear between the studied voltage range. The slope of the line indicates that the channel has a conductance of 90 pS. The intersection on the negative side of the cis voltage axis indicates that the incorporated Hsc70 channel preferentially permits the flow of cations. The fraction of time that the active Hsc70 channel was conducting was also studied at a 200-mV range between positive and negative transmembrane voltages (Fig. 4C). There was a high variability in this parameter within each potential. This analysis indicates that the open time probability of the channel shows no dependence on the sign or amplitude of the membrane voltage.

**Hsc70 Channel Activity Is Modulated by ATP and ADP**—The chaperone activity of Hsc70 is modulated by binding and hydrolysis of ATP (5). We have found that the coincident presence of ATP is necessary to observe stable Hsc70 ion channel activity. To further confirm this finding and to extend this study to a similarly structured nucleotide, the effect of ADP was also investigated. Hsc70 channel activity was first measured in the presence of ATP (2 mM) in asymmetrical ionic conditions (200\textsubscript{cis}/50\textsubscript{trans} mM KCl) and zero electrical potential (Fig. 5A). The resulting ion channel activity displayed the same regular pattern as described in Figs. 1 and 3B. The channel was consistently opened and showed a maintained conductance level of 148 pS, as calculated from the slope of instantaneous current voltage plots (not shown). The subsequent addition of ADP (2 mM), still in the presence of ATP, did not affect the channel activity in terms of conductance or gating kinetics (Fig. 5A, middle record). A further increase in the ADP concentration to 3 mM over 2 mM ATP produced a series of fluctuations in the channel gating activity (Fig. 5A, bottom records). These fluctuations eventually led to a complete closure of the ion pathway, rendering the membrane electrically silent. The channel activity remained blocked as long as the ADP excess over ATP was maintained. In the conditions of excess ADP concentrations over ATP, application of electrical potentials to the membrane within a range between positive and negative 40 mV was ineffective in opening the Hsc70 channel (Fig. 5B). Removal of the nucleotides by washing the chamber with a nucleotide-free ionic solution reestablished a moderate current channel activity (Fig. 5C), which was reminiscent of the one observed in the absence of ATP (Fig. 3A). The channel activity recovered to the amplitude observed in the control conditions when the concentration of ATP was restored to 2 mM (Fig. 5D). In order to follow the instantaneous changes in the activity generated by the
addition and removal of the two nucleotides, the open time probability of the Hsc70 channel was monitored at 1-s time intervals during the duration of this experiment. The results of this type of analysis are shown in Fig. 5E. The open time probability oscillated between 85 and 100% during the control condition (in the presence of 2 mM ATP). The open time probability was not altered dramatically during the time period that the channel was in the simultaneous presence of ADP and ATP.
at equal concentrations (2 mM), although the range of fluctuation was wider, between 70 and 100%. When the ADP concentration (3 mM) was in excess with respect to ATP (2 mM), the open time probability underwent even larger fluctuations until it became zero. Reinstating the original ATP concentration brought the channel back to a fully activated state. The open time probability increased back to fluctuate around 100%, as seen in the control conditions. These results indicate that the activity of the ion pathway created by Hsc70 in lipid membranes is modulated by the presence of ATP and ADP.

**DISCUSSION**

The finding that Hsc70 can form a well defined and stable cationic selective channel in artificial lipid bilayers is intriguing. Although Hsc70 is predominately a cytosolic protein, the presence of Hsp70s in association with or in close proximity to cellular membranes has been previously reported (6, 7, 10). The capacity of Hsc70 to form channels is a function that cannot be predicted a priori from the structure and chaperone role of this protein. Perhaps the interaction of Hsc70 with lipids is important in the processes of translocation and folding of membrane proteins. The activity of Hsc70 ion pathways was also found to be regulated by the presence of ATP and ADP, which is consistent with the role these nucleotides play in the other functions of Hsc70. Binding of ATP produces a change of conformation in Hsc70 that modulates its interaction with target polypeptides (5). The effect of ATP and ADP on the Hsc70 ion pathway may be similar. A conformational change of Hsc70 may be necessary for the opening of the channel. Previous observations have also suggested a possible interaction between Hsp70s and lipids. Alder et al. (11) showed that Hsp70s were capable of opening pores into liposomes, producing a leakage of the liposome content. In addition, they observed Hsp70-induced currents in artificial lipid bilayers. In this early work, the Hsp70-induced currents were not characterized. The addition of exogenous Hsp70 to patch-clamped membranes was reported to activate potassium channels (12). Recently, we reported that Hsc70 is also capable of inducing liposome aggregation in a nucleotide regulated process (13).

All members of the Hsp70 family contain two major structural and functional domains. The N terminus of the molecule binds and hydrolyzes ATP, and the C terminus participates in the interaction with target proteins (peptide binding domain) (2). Members of the Hsp70 family self-associate to form oligomers of low order in the absence of peptide targets or denatured proteins, also in an ATP-dependent manner (14–17). The C terminus of Hsc70 has been shown to be essential for self-association (18). It is quite likely that the actual Hsc70 channel may be formed by oligomers of this protein. The characteristic multiconductance nature and the various patterns of channel activity observed after incorporation of Hsc70 into the lipid bilayer may be the result of the incorporation of different order oligomers. However, we cannot discard the possibility that multiconductance is due to conformational changes of Hsc70. These oligomers may be inserted into the lipid bilayer by the C terminus of the molecule. This assumption is based on the observation that channel activity is reversibly regulated by ATP and ADP. Thus, the ATP binding domain (N-terminal) should be exposed to the aqueous phase. We have recently observed that Hsp70-mediated liposome aggregation is also modulated by ATP and ADP. This observation is consistent with the assumption that the nucleotide binding site is accessible to the nucleotides in the aqueous solution after the insertion of Hsc70 into the liposome membrane (13). The three-dimensional structure of fragments containing the N-terminal (ATP binding site) and C-terminal (peptide binding site) of Hsc70 have been resolved by x-ray crystallography (19, 20) and NMR spectroscopy (21). This region contains a cluster of β-sheets and a long α-helix. These regions may associate directly with other Hsc70 molecules to create a complex that has the proper configuration to spontaneously insert into the lipid bilayer. The peptide binding domain of Hsc70 is over 50 Å in length, large enough to span a biological membrane. There are several examples of molecules with clusters of β-sheet structures that interact with membranes to form ion channels, such as some members of the annexin family (22, 23), Alzheimer’s β-amyloid (8), and human amylin (24). In addition, hydrophobic patches, which are thought to be likely regions for interaction with membrane lipids, have been observed in the C-terminal of the ATP binding domain (19) and in the N-terminal of the peptide binding site (21), which may be involved in the interaction with the lipid bilayer.

The preceding observations demonstrate that Hsc70 can stably interact with lipids. The biological significance of this observation remains to be elucidated. A possibility is that ion pathways generated by Hsc70 may create the proper ionic environment necessary for the process of polypeptide translocation across membranes. Echoing this assumption, O’Brien and McKay (25) have shown that the ATPase activity of Hsc70 is modulated by potassium ions. In addition, a potassium ion requirement is necessary for the dissociation of denatured proteins from bacterial Hsp70 (26). Given these observations, the fact that Hsc70 can form channels with selectivity for cations opens new and exciting possibilities for the biological role of this chaperone protein.

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