Role of Hsp90 in Biogenesis of the β-Cell ATP-sensitive Potassium Channel Complex

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INTRODUCTION

ATP-sensitive potassium (KATP) channels in pancreatic β-cells, by virtue of their sensitivities to intracellular nucleotides ATP and ADP, serve as molecular linkers between cell metabolism and cell excitability, thus mediating glucose-regulated insulin secretion (Aguilar-Bryan and Bryan, 1999; Ashcroft, 2005; Flanagan et al., 2009). Studies characterizing disease-associated KATP channel mutations have indicated that many mutations, especially those identified in congenital hyperinsulinism and diabetes (Aguilar-Bryan and Bryan, 1999; Ashcroft, 2005; Flanagan et al., 2009). Studies characterizing disease-associated KATP channel mutations have indicated that many mutations, especially those identified in congenital hyperinsulinism, cause channel dysfunction by disrupting folding or assembly of channel proteins in the endoplasmic reticulum (ER) and thereby subsequent trafficking to the plasma membrane (Cartier et al., 2001; Taschenberger et al., 2002; Ashcroft, 2005; Lin et al., 2006; Yan et al., 2007). Despite the importance of channel biogenesis and surface expression in β-cell function, little is known about how these processes are governed at the molecular level.

Generation of KATP channels in the ER requires correct folding and coassembly of two different channel subunits with distinct structures. Whereas Kir6.2 is a relatively simple protein with only two-transmembrane helices and cytosolic N- and C-terminal domains, SUR1 contains 17 transmembrane helices divided into three transmembrane domains TMD0, TMD1, and TMD2, and two cytoplasmic nucleotide binding domains, NBD1 and NBD2 (Neagoe and Schwappach, 2005). We have shown previously that normal biogenesis of KATP channels is an inefficient process; only ~20% newly synthesized channel subunits mature into functional KATP channels via SUR1, thereby affecting functional expression of the channel in β-cell membrane.
translated proteins. Recent studies suggest that manipulation of molecular chaperones could be used to treat human disease caused by protein misfolding (Welch, 2004; Balch et al., 2008; Powers et al., 2009; Yang et al., 2009). A prominent example is the cystic fibrosis transmembrane conductance regulator (CFTR) mutant ΔF508, which accounts for most of the cystic fibrosis cases (Rowe et al., 2005). CFTR is structurally homologous to SUR1, both proteins belonging to the ATP-binding cassette (ABC) transporter protein family (Higgins, 1995). The molecular chaperone network monitoring CFTR biogenesis has been extensively studied and shown to involve the ER luminal chaperone calnexin and the cytosolic heat-shock protein (Hsp)40/70/90 chaperone complex (Loo et al., 1998; Wang et al., 2006). By contrast, virtually nothing is known about the molecular chaperones that participate in folding and degradation of KATP channels. In addition, there is a lack of understanding of how biogenesis of heteromeric protein complexes such as KATP channels is regulated. This lack of knowledge hinders progress on exploiting molecular chaperones for treatment of disease caused by misfolding of KATP channels.

To address these issues, we have taken a proteomics approach in which epitope-tagged SUR1 and Kir6.2 are expressed in the insulinoma cell line INS-1. Proteins associated with the channel subunits were isolated by affinity purification and identified by tandem mass spectrometry. Here, we show that KATP channel subunits are associated with components of the Hsp90 chaperone complex in the ER, including Hsp40, heat-shock complex (Hsc)70, and Hsp90. The Hsp90 chaperone network has been reported to affect the biogenesis/maturaiton efficiency of several membrane proteins, including the aforementioned ABC transporter CFTR and the voltage-gated potassium channel human ether-a-go-go-related gene (hERG; Kv11.1). However, other membrane proteins with similar complex topology such as the P-glycoprotein and the multidrug resistance-associated protein (MRP), also ABC transporters, and other Kv channels, including Kv1.5 and Kv2.1, are not affected by perturbation of Hsp90 function (Loo et al., 1998; Ficker et al., 2003). We therefore sought to determine whether the Hsp90 chaperone complex plays a role in KATP channel processing and maturation. We demonstrate that inhibition of Hsp90 reduces KATP channel expression at the cell surface, whereas overexpression of Hsp90 increases the number of KATP channels in the plasma membrane. Importantly, we found that the Hsp90 complex interacts preferentially with the SUR1 subunit. Moreover, although perturbation of Hsp90 significantly alters cell surface levels of a SUR1 expressed alone, it has little effect on those of a Kir6.2 expressed alone. Our results indicate that the Hsp90 chaperone regulates biogenesis efficiency of KATP channels by targeting the SUR1 but not the Kir6.2 subunit.

MATERIALS AND METHODS

Expression Constructs

Wild-type hamster SUR1 or FLAG epitope (DYKDDDDK)-tagged SUR1 (referred to as SUR1) cDNAs were cloned in the pCDNA3.1(−) vector (Invitrogen, Carlsbad, CA). Kir6.2 or HA epitope (YPYDVPDYA)-tagged Kir6.2 cDNAs were cloned into pcDNA3, as described previously (Cartier et al., 2001). Construction of adenovirus carrying wild-type (WT) ISUR1 or WT Kir6.2 or hemagglutinin (HA)-tagged WT Kir6.2 cDNA was as described previously (Lin et al., 2008). The FLAG-tag for SUR1 was placed at the extracellular N terminus of the protein. The HA-tag for Kir6.2 was inserted between amino acid 100 and 101 in the extracellular domain; an extra nine amino acids, DLYAYMEKG, was added between amino acid 98 and 99 to aid accessibility of the epitope. We have shown in previous studies that these epitope tags do not affect the trafficking or function of the channels (Cartier et al., 2001; Lin et al., 2005). The ISUR1 recombinant adenovirus was made using a modified pShuttle plasmid (AdEasy kit; Stratagene, La Jolla, CA) containing a tetracycline-inducible promoter. Recombinant viruses were amplified in human embryonic kidney 293 cells and purified according to the manufacturer’s instructions.

Virus Infection

INS-1 cells clone 832/13 (kindly provided by Dr. Christopher Newgard, Duke University, Durham, NC) were plated in 10-cm plates and cultured for 24 h in RPMI 1640 medium with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol (Hohmeier et al., 1997). For Kir6.2 expression, recombinant adenoviruses containing Kir6.2 or HA-Kir6.2 with desired titters were used as described previously (Lin et al., 2008). For ISUR1 expression, cells were coinfected with equal amounts of two recombinant adenoviruses, one adenovirus encoding a tetracycline-inhibited transactssor (ITA) and the other adenovirus a ITA-regulated gene expressing ISUR1 (Lin et al., 2008). At ~70% confluency were washed once with phosphate-buffered saline and incubated for 1.5 h at 37° C in Opti-MEM containing low bovine serum and a mixture of viruses. The multiplicity of infection for each virus was determined empirically. After 90 min, 2x growth medium was added, and the cells were incubated at 37°C until reaching appropriate density for the various experiments.

Affinity Purification of KATP Channel Complexes

INS-1 (832/13) cells were transduced with adenoviruses carrying ISUR1, HA-Kir6.2, or ISUR1 and Kir6.2 as described above. Twenty-four-hour after infection, the cells were lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, and 1% Triton X-100), with Complete protease inhibitors [Roche Diagnostics, Indianapolis, IN] on ice for 30 min. Cell lysates were centrifuged at 16,000 × g for 5 min at 4°C, and the supernatant was used for affinity purification by addition of 100 μl of FLAG- or HA-antibody conjugated agarose beads (Sigma-Aldrich, St. Louis, MO) overnight at 4°C. After washing three times with the lysis buffer, bound proteins were eluted by incubation with FLAG peptide (250 μl/ml for ISUR1 sample) or HA peptide (10 μg/ml, for HA-Kir6.2 sample) at room temperature for 30 min.

Proteomics and Mass Spectrometry Analysis

Affinity-purified samples were concentrated to a final volume of 20 μl, mixed with Laemmli sample buffer, and electrophoresed briefly into 10% Bis-Tris gels (Invitrogen) at 100 V in 3(N-morpholino)propanesulfonic acid buffer for 5 min. The gel was stained with Imperial Coomassie (Pierce Chemical, Rockville, IL), placed on a clean glass plate, and each sample was excised by cutting just below the dye front and removing the entire top of the lane including the bottom of the well. The excised gel pieces were then cut into 1- to 2-mm sections, washed twice by shaking in 0.5 ml of water for 15 min, washed twice again by shaking in 0.5 ml of 1:1 solution of acetonitrile-100 mM ammonium bicarbonate for 30 min, and then dried by vacuum centrifugation. After reduction and alkylation of proteins in the excised gel slices by incubation with dithiothreitol (10 mM in 100 mM ammonium bicarbonate) and then in iodoacetamide (25 mM in 100 mM ammonium bicarbonate), proteins were digested by rehydrating the gel slice on ice in trypsin digestion solution containing 100 mM ammonium bicarbonate, and 10 μg/ml proteases grade trypsin (Sigma-Aldrich). The digests were incubated with sufficient 100 mM ammonium bicarbonate was added to cover the gel slices; samples were incubated overnight at 37°C. The solution was then removed from the gel slices, additional peptides were extracted, the volume of the collected digestes was decreased by vacuum centrifugation, and samples were transferred directly to autosampler vials for analysis.

The peptide mixture was injected onto a 1 mm x 8 mm trap column (Michrom BioResources, Auburn, CA) at 20 μl/min in a mobile phase consisting of 0.1% formic acid. The trap cartridge was then placed in-line with a 0.5×250-mm column containing 5-μm Zorbax SB-C18 stationary phase (Agilent Technologies, Santa Clara, CA), and peptides were separated by a 2-30% acetonitrile gradient over 95 min at 10 μl/min by using a 1100 series capillary high-performance liquid chromatography (Agilent Technologies) equipped with a diode array detector. Peptides were analyzed using a LTQ linear ion trap fitted with an ion Max Source and 34-gauge metal needle kit (Thermo Fisher Scientific, Waltham, MA). Survey mass spectrometry (MS) scans were alternated with three data-dependent tandem MS (MS/MS) scans by using the dynamic exclusion feature of the software to increase the number of unique peptides analyzed. Peptide identification was performed by comparing observed MS/MS spectra to theoretical fragmentation spectra of peptides generated from a protein database using Sequest (Thermo Fisher). The protein database created in April 2008 containing hamster, mouse, and rat sequences was searched using Sequest, version 27, revision 12 (Thermo Fisher Scientific). A Swiss-Prot database created in April 2008 containing hamster, mouse, and rat sequences was searched using Sequest, version 27, revision 12 (Thermo Fisher Scientific). A Swiss-Prot database was used (Swiss Institute of Bioinformatics, Geneva, Switzerland). Scaffold software (Proteome Software, Portland, OR) was used to validate MS/MS-based identifications. Peptide identifications were accepted if they could be established at >95% and 95% probability, respectively (Keller et al., 2002; Nesvizhskii et al., 2003) and contained at least two identified peptides matched per protein entry.
Western Blotting and Immunoprecipitation Assay

INS-1 (832/13) cells were infected with SUR1 and Kir6.2 as described above. Twenty-four hours after infection, the cells were lysed in 1 ml of lysis buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl, and 1% Triton X-100, with Complete protease inhibitors) on ice for 30 min. The cell lysate was centrifuged at 16,000 ρ × g for 30 min, and the supernatant was used for Western blot or immunoprecipitation. Immunoprecipitation was performed as described under Affinity Purification. Eluted proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. The membrane was probed with appropriate primary antibodies including anti-FLAG (Sigma-Aldrich), anti-Hsp90α/β (Santa Cruz Biotechnology), anti-Hsp40 (Abcam, Cambridge, MA), and anti-Hsc70 (Abcam), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), and visualized by enhanced chemiluminescence (Super Signal West Femto; Pierce Chemical).

Chemiluminescence Assay for Surface Expression

COS6 or INS-1 cells grown in 35-mm dishes were transfected with 2% poraformaldehyde for 20 min at room temperature 48 h after transfection or infection. Fixed cells were preblocked in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) for 1 h, incubated in M2 anti-FLAG antibody (10 µg/ml) for 1 h, washed 4 × 30 min in PBS with 0.1% BSA, incubated in horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) for 20 min, washed again 4 × 30 min in PBS, and 2 × 5 min in PBS. Chemiluminescence signal was read in a TD-20/20 luminoimeter (Turner Designs, Sunnyvale, CA) after 10-s incubation in Power Signal ELISA luminescent solution (Pierce Chemical). The results of each experiment are the average of two dishes. Signals observed in untreated transfectants were normalized to corresponding untreated controls.

Metabolic Labeling and Immunoprecipitation

COS6 cells or INS-1 cells in 35-mm dishes were transfected with ISUR1 and Kir6.2 for 24 h. The cells were incubated in methionine/cysteine-free DMEM supplemented with 5% dialyzed fetal bovine serum for 30 min before labeling with L-[35S]methionine (Tran 35S-Label, 150–250 Ci/ml; MP Biomedicals, Solon, OH) for 60 min at 37°C. Labeled cultures were chased in regular medium supplemented with 10 mM methionine at 37°C. At the end of the chase, the cells were lysed in 500 µl of the lysis buffer described above. For immunoprecipitation, 500 µl of cell lysate was incubated with 100 µl of FLAG-antibody–conjugated agarose beads overnight at 4°C. The precipitate was washed three times in the lysis buffer, and the eluted proteins were eluted with FLAG-peptide. The eluted proteins were separated by 8% SDS-PAGE, and the dried gels were analyzed using a Storm PhosphorImager (GE Healthcare).

Results

Identification of Molecular Chaperones Associated with K_{ATP} Channel Proteins

To identify proteins that interact with K_{ATP} channels in the early secretory pathway in β-cells, we overexpressed ISUR1 or HA-Kir6.2 in INS-1 cells (clone 823/13, a model β-cell line from rat; Hohmeier et al., 1997) by using recombinant adenoviruses (Lin et al., 2005, 2008). The rationale for the overexpression approach is that it will enhance the probability of capturing molecular chaperones that are associated with each subunit during biosynthesis and folding by increasing fraction of folding intermediates present. The epitope-tagged channel subunits were used to facilitate affinity purification, and we have shown previously that the FLAG- or HA-epitope on SUR1 or Kir6.2, respectively, do not affect channel expression or function (Cartier et al., 2001; Lin et al., 2005). Note that viral transduction was used because INS-1 cells exhibit low transfection efficiency using nonviral transfection methods (Lin et al., 2005, 2006; Yan et al., 2007). Proteins that interact with the channel subunits were captured using an affinity purification approach by incubation of the lysate with agarose beads conjugated with anti-FLAG or anti-HA antibody followed by elution using FLAG- or HA-peptide. The eluted proteins were identified by tandem mass spectrometry as described under Materials and Methods.

In total, 10 experiments were conducted: three in INS-1 cells transfected with viruses for ISUR1 overexpression only (ISUR1 virus plus IATA virus; see Materials and Methods), two in INS-1 cells transfected with the virus for HA-Kir6.2 only, and five in cells transfected with viruses for overexpression of ISUR1 and untagged Kir6.2. In all experiments, uninfected INS-1 cells were subjected to the same experimental procedure to serve as a negative control. Results of three representative experiments from the three different conditions are shown in Table 1. The chaperone proteins consistently co-purified with the channel subunits are Hsp40, Hsc70, and Hsp90α/β. Calnexin and Hsp organizing protein (HOP) were also present in a subset of experiments in which ISUR1 was used as a bait (Table 1). In yet another proteomics experiment using COS cells transfected with ISUR1 and Kir6.2 cDNAs, Hsp40, Hsc70, and Hsp90α/β were also detected in the ISUR1 immunoprecipitate (data not shown).

Differential Association of K_{ATP} Channel Subunits with the Hsp90 Chaperone Complex

To confirm the interaction between channel subunits and chaperone proteins identified by mass spectrometry, Hsp40, Hsc70, and Hsp90α/β were coimmunoprecipitated with ISUR1 or HA-Kir6.2 from INS-1 cells transfected with the ISUR1 or the HA-Kir6.2 virus, respectively, as described under Materials and Methods. Results revealed that all three chaperones interact with ISUR1 (Figure 1A, left). Similarly, all three chaperones were detected in the HA-Kir6.2 immunoprecipitate (Figure 1A, right). Note that overexpression of individual channel subunits in INS-1 cells did not significantly alter the abundance of the various heat shock proteins examined (input).

Because INS-1 cells express endogenous K_{ATP} channel proteins albeit at a lower level than the exogenously expressed ISUR1 or HA-Kir6.2 (Lin et al., 2006), it is possible that association with ISUR1 or HA-Kir6.2 was mediated by endogenous channel subunits interacting with the epitope-tagged exogenous channel subunits. We therefore expressed ISUR1 or HA-Kir6.2 individually in COS cells that do not have endogenous SUR1 or Kir6.2 and performed similar
coimmunoprecipitation experiments. As shown in Figure 1B, Hsp90, Hsc70, and Hsp40 still coimmunoprecipitated with fSUR1, but they were not detected in the Kir6.2 immunoprecipitate. Although the lack of signal for these chaperone proteins in the Kir6.2 immunoprecipitate could be caused by factors such as the cell type used or the expression level of Kir6.2, the simplest interpretation of the results is that these chaperone proteins might interact preferentially with SUR1 subunit in the KATP channel complex.

Inhibition of Hsp90 Reduces KATP Channel Surface Expression

To determine the role of Hsp90 complexes in channel biogenesis, we examined the effects of 17-AGG, a geldanamycin derivative that inhibits the ATPase activity and client binding properties of Hsp90 (Kamal et al., 2003), on KATP channel biogenesis efficiency and surface expression in COS cells. SUR1 has two N-linked glycosylation sites. As the SUR1/Kir6.2 complex traffics through the Golgi network, SUR1 becomes complex glycosylated to give rise to the mature form that migrates slower on SDS-PAGE (referred to as the upper band; Figure 2) than the immature ER core-glycosylated form (the lower band). Western blots of lysates from cells expressing both fSUR1 and Kir6.2 showed that 17-AGG (100 nM for 12 h) significantly decreased the abundance of the mature complex-glycosylated SUR1 band (upper band), suggesting reduced processing efficiency of the channel protein (Figure 2A, left). In agreement, chemiluminescence assays showed that surface expression of KATP channels in COS cells treated with a range of 17-AGG concentrations (25 nM–2 μM) was reduced in a dose-dependent manner, with the effect being apparent at 25 nM and saturating at ~200 nM (Figure 2B). Similar results were obtained in INS-1 cells infected with KATP channel subunits viruses, with the effect

| Accession no. (NCBI) | Protein name | Sequence coverage (%) | Unique spectra | Total spectra |
|----------------------|--------------|-----------------------|----------------|--------------|
| fSUR1 only           | Q09427 SUR1  | 46                    | 143            | 588          |
|                      | Q61743 Kir6.2 | 11                    | 4              | 4            |
|                      | Q76G10 Hsp40  | 26                    | 7              | 9            |
|                      | P19378 Hsc70 | 57                    | 43             | 72           |
|                      | P46633 Hsp90α | 29                    | 20             | 26           |
|                      | P11499 Hsp90β | 39                    | 30             | 43           |
|                      | O54981 HOP    | 16                    | 6              | 6            |
|                      | Q8K3H8 Calnexin | 13                    | 7              | 10           |
| fSUR1 + Kir6.2       | Q09427 SUR1  | 48                    | 126            | 377          |
|                      | Q61743 Kir6.2 | 21                    | 10             | 18           |
|                      | Q76G10 Hsp40  | 37                    | 9              | 12           |
|                      | P19378 Hsc70 | 61                    | 33             | 57           |
|                      | P46633 Hsp90α | 26                    | 19             | 25           |
|                      | P11499 Hsp90β | 34                    | 24             | 35           |
|                      | O54981 HOP    | 7                     | 3              | 3            |
|                      | Q8K3H8 Calnexin | 13                    | 7              | 8            |
| HA-Kir6.2 only       | Q09427 SUR1  | 8                     | 7              | 9            |
|                      | Q61743 Kir6.2 | 30                    | 22             | 100          |
|                      | Q76G10 Hsp40  | 29                    | 6              | 10           |
|                      | P19378 Hsc70 | 57                    | 42             | 95           |
|                      | P46633 Hsp90α | 17                    | 9              | 13           |
|                      | P11499 Hsp90β | 23                    | 13             | 18           |
|                      | O54981 HOP    | 0                     | 0              | 0            |
|                      | Q8K3H8 Calnexin | 0                     | 0              | 0            |

* In total, 10 proteomics experiments were performed: three in INS-1 cells infected with fSUR1 virus only, five in INS-1 cells infected with fSUR1 and Kir6.2 viruses, and two in INS-1 cells infected with HA-Kir6.2 virus only. The fSUR1 or HA-Kir6.2 protein complexes in INS-1 cell lysates were affinity purified using FLAG- or HA-antibody–conjugated agarose beads and subjected to mass spectrometry analyses as described in Materials and Methods. Results from three proteomic experiments representing each condition are shown. Note Hsp40, Hsc70, and Hsp90α and -β are present in all experiments. The protein HOP is found in only a subset of experiments overexpressing fSUR1 alone or fSUR1 plus Kir6.2 (6 of 8 experiments), so is the protein calnexin (3 of 8 experiments); neither proteins showed up in cells overexpressing HA-Kir6.2 only. NCBI, National Center for Biotechnology Information.
Effects of 17-AGG on K<sub>ATP</sub> channel maturation and surface expression were further examined in INS-1 cells infected with fSUR1 and Kir6.2 adenoviruses. As in COS cells, Western blots show that 17-AGG causes a dose-dependent reduction in the amount of complex-glycosylated fSUR1. (B) Reduction in surface expression of K<sub>ATP</sub> channels was quantified by chemiluminescence assays. Each data point in the bar graphs represents the mean ± SEM of three experiments. All 17-AGG–treated cells showed statistically significant difference in surface expression compared with untreated cells (p = 0.02 for 25 nM and p < 0.02 for all other groups).

Discussion

Overexpression of Hsp90 Increases Surface Expression of K<sub>ATP</sub> Channels in COSm6 Cells

Given that the Hsp90 inhibitor reduces K<sub>ATP</sub> channel processing and surface expression, we next examined whether overexpression of Hsp90 might facilitate K<sub>ATP</sub> channel maturation and increase surface channel expression. There are two major cytosolic Hsp90 isoforms: the α isoform that is highly inducible and the β isoform that is thought to be constitutive (Sreedhar et al., 2004). For our experiments, Hsp90α or β CDNA or empty vector were transfected along with WT fSUR1 and Kir6.2 into COSm6 cells, and the resulting K<sub>ATP</sub> channel surface expression was analyzed by chemiluminescence assays. As shown in Figure 4A, overexpression of Hsp90β CDNA increased surface expression of K<sub>ATP</sub> channels by 29.64 ± 6.29%. Overexpression of Hsp90α also improved surface expression slightly (by 9.46 ± 4.82%), but this effect did not reach statistical significance. We therefore focused on Hsp90β in the experiments described below. To determine whether the increased surface expression of K<sub>ATP</sub> channels in cells overexpressing Hsp90β is a consequence of increased channel biogenesis, we performed metabolic pulse-chase labeling experiments to monitor the processing and maturation of newly synthesized SUR1 protein. Overexpression of Hsp90β was first confirmed by Western blots (Figure 4B). In metabolic pulse-chase experiments, the amount of SUR1 protein labeled at the end of the 1-h pulse period was higher in cells overexpressing Hsp90β than in control cells, indicating Hsp90β overexpression led to increased biogenesis of SUR1. Signals of both the lower and upper bands from seven experiments were quantified and are shown in Figure 4C. The signal of the mature complex-glycosylated SUR1 upper band in Hsp90β-cotransfected cells is consistently higher than that in control cells at all times during the chase period. These results provide evidence that Hsp90β function contributes to the biogenesis of endogenous SUR1 in INS-1 cells.
The folding/surface expression defects of mutant KATP channels, we examined the effect of Hsp90/H9252 and F1388 that show virtually no surface expression. These results suggest that although up-regulation of Hsp90 can rescue the processing and surface expression of some mutants, effects are dependent on the nature of the mutation and severity of processing defect.

**Hsp90 Exerts Its Effect on K<sub>ATP</sub> Channel Biogenesis via the SUR1 Subunit**

Unlike CFTR or hERG channels, the K<sub>ATP</sub> channel complex is formed by two membrane proteins of very different size and topological complexity. In coimmunoprecipitation experiments conducted in COS cells expressing fSUR1 or HA-Kir6.2 alone, we detected association of the Hsp90 chaperone complex proteins with SUR1 but not Kir6.2 (Figure 1), suggesting the chaperone complex might target the SUR1 subunit to exert its effect on channel biogenesis. To address the question of whether Hsp90 promotes channel maturation and surface expression by facilitating the folding and processing of SUR1, Kir6.2 or both, we examined how manipulation of Hsp90 function affects surface expression of SUR1 and Kir6.2 individually. For these studies, we used an fSUR1 construct in which the RKR ER retention/retrieval motif has been mutated to AAA (fSUR1<sup>RRK→AAA</sup> and a HA-Kir6.2 construct in which the RKR motif at the C terminus has been deleted (HA-Kir6.2ΔC36). Previous studies have shown that mutation of the RKR motif to AAA or deletion of this signal allows individual subunits to express at the cell surface independently of the other subunit (Zerangue et al., 1999). Also, the FLAG- and HA-epitope tags are inserted in the extracellular domain of SUR1 and Kir6.2 respectively, to permit quantification of surface expression of the individual protein by using the chemiluminescence assay described under Materials and Methods (Cartier et al., 2001; Lin et al., 2005). In COSm<sub>6</sub> cells expressing fSUR1<sup>RRK→AAA</sup> alone, inhibition of Hsp90 function by 17-AGG led to reduced steady state mature fSUR1<sup>RRK→AAA</sup> upper band level as well as the core-glycosylated immature lower band (Figure 6A), similar to that observed for fSUR1 coexpressed with Kir6.2 (Figure 2A). Chemiluminescence assays also revealed reduced surface expression of fSUR1<sup>RRK→AAA</sup> in a 17-AGG concentration-dependent manner (Figure 6B). In contrast, the steady state protein level of Kir6.2 or HA-Kir6.2ΔC36 expressed in COSm<sub>6</sub> cells in the absence of SUR1 was not affected by the 17-AGG treatment based on Western blots (Figure 6C), and surface expression of HA-Kir6.2 also unaffected as assessed by chemiluminescence assays (Figure 6D).

Next, either fSUR1<sup>RRK→AAA</sup> or HA-Kir6.2ΔC36 were co-expressed with Hsp90α or β in COSm<sub>6</sub> cells and surface expression of each channel subunit measured by chemiluminescence assays by using anti-FLAG or anti-HA antibody respectively. As shown in Figure 7A, coexpression of Hsp90β resulted in 29.52 ± 9.87% increase in surface expression of fSUR1<sup>RRK→AAA</sup> similar to that observed for WT SUR1/Kir6.2 channel complex (Figure 4A). By contrast, no significant difference in surface expression of HA-Kir6.2ΔC36 was observed between control cells (vector) and cells coexpressing either Hsp90α or β (Figure 7B). These results suggest that Hsp90 improves the surface expression of K<sub>ATP</sub> channels by facilitating folding and processing efficiency of SUR1 rather than Kir6.2.

**DISCUSSION**

Pancreatic β-cell K<sub>ATP</sub> channels have been extensively studied in the past two decades. However, as the key signal...
transduction protein complex linking glucose metabolism to insulin secretion remarkably little is known about the molecular mechanisms involved in the channel’s biogenesis process. In this work, we have begun to address these issues by identifying molecular chaperones that associate with the channel subunits in β-cells, by using the insulinoma cell line INS-1 as an experimental platform. We provide evidence that the Hsp90 chaperone complex participates in the biogenesis of K\(_{\text{ATP}}\) channels. First, K\(_{\text{ATP}}\) channels are associated with Hsp90, Hsc70, and Hsp40, components of the Hsp90 chaperone complex. Second, inhibition of Hsp90 function by 17-AGG markedly reduced surface expression of K\(_{\text{ATP}}\) channels, whereas overexpression of Hsp90β significantly increased surface expression. In addition, we show that overexpression of Hsp90β improved surface expression of select misprocessing/mistrafficking SUR1 mutants. Finally, we present evidence that Hsp90 facilitates channel biogenesis by increasing the folding/processing efficiency of SUR1.

**Comparison with Other Hsp90 Client Proteins**

Hsp90 is an abundant cytosolic chaperone (Pearl and Prodranou, 2006). Unlike Hsc/p70, which acts by holding newly synthesized proteins in a folding competent state, Hsp90 is required for the folding of a subset of client proteins that are thought to have difficulties in reaching a native conformation (Nathan et al., 1997; Young et al., 2001). Even with proteins that share structural similarity or complexity, there can be a high degree of specificity. We now identify the K\(_{\text{ATP}}\) channel as another Hsp90 client. K\(_{\text{ATP}}\) channels are unique in that they are heterooligomers of SUR1 and Kir6.2—two membrane proteins with very different topological complexity (Inagaki et al., 1995; Conti et al., 2001). In understanding the biogenesis of such heteromeric protein complex, one has to consider the folding and processing of both channel subunits. Our study using SUR1 and Kir6.2 mutants that are able to express at the cell surface in the absence of the other assembly partner subunit shows that the two subunits exhibit differential dependence on Hsp90 for surface expression. Although surface levels of SUR1 are reduced by 17-AGG and augmented by overexpression of

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**Figure 4.** Overexpression of Hsp90 increases surface expression of K\(_{\text{ATP}}\) channels by improving biogenesis efficiency of the channel. (A) COS cells were cotransfected with fSUR1 and Kir6.2 subunits and either 0.6 µg Hsp90 (α or β) or empty vector as control. Surface expression of K\(_{\text{ATP}}\) channels was analyzed by chemiluminescence assays in COS cells cotransfected with various K\(_{\text{ATP}}\) channel subunits (0.3 µg of WT or mutant SUR1, 0.2 µg of Kir6.2 per 35-mm dish of cells) and either 0.6 µg of Hsp90β or empty vector as control. Although Hsp90β improved surface expression of the N24K, D310N, and D1472N mutant (p = 0.01, 0.01, 0.05, and 0.03 for WT, N24K, D310N, and D1472N, respectively), it did not significantly increase surface expression of the A116F or ΔF1388 mutants.

**Figure 5.** Hsp90 improves surface expression of select misprocessed mutant K\(_{\text{ATP}}\) channels. The effect of Hsp90β abundance on surface expression of K\(_{\text{ATP}}\) channels harboring SUR1 mutations known to disrupt normal processing and trafficking of the channel was assessed by chemiluminescence assays in COS cells cotransfected with various K\(_{\text{ATP}}\) channel subunits (0.3 µg of WT or mutant SUR1, 0.2 µg of Kir6.2 per 35-mm dish of cells) and either 0.6 µg of Hsp90β or empty vector as control. Although Hsp90β improved surface expression of the N24K, D310N, and D1472N mutant (p = 0.01, 0.01, 0.05, and 0.03 for WT, N24K, D310N, and D1472N, respectively), it did not significantly increase surface expression of the A116F or ΔF1388 mutants.
Hsp90β, those of Kir6.2 are not affected. These findings are consistent with the coimmunoprecipitation results obtained in COS cells in which the Hsp90 complex proteins were only detectable in the SUR1 but not Kir6.2 immunoprecipitates. To our knowledge, this is the first example in which differential dependences of individual components of a membrane protein complex on Hsp90 has been documented. Identification of SUR1 as an Hsp90 client protein also underlines the question of how substrate specificity for this molecular chaperone is determined (Pearl and Prodromou, 2000). Although Hsp90 is important for the folding and maturation of CFTR and SUR1, it does not seem critical for the processing and maturation of two other ABC transporters, P-glycoprotein and MRP (Loo et al., 1998). In terms of sequence homology, P-glycoprotein belongs to the ABCB subfamily, whereas CFTR, SUR1, and MRP are in the ABCC subfamily (Vasiliou et al., 2009). With respect to membrane topology, CFTR and P-glycoprotein both contain a tandem repeat of six transmembrane helices, each set followed by an ATP-binding domain. SUR1 and MRP, in contrast, have an additional N-terminal transmembrane domain of five transmembrane helices that precedes the ABC core structure seen in the CFTR (Tusnady et al., 2006). Thus, prediction of Hsp90 substrates cannot be made simply based on similarity in sequence or topology. Future structure-function analyses of these closely related proteins may reveal features recognized by the Hsp90 chaperone complex.

Many cochaperone proteins interact with Hsp90 to regulate client protein folding and maturation. For example, Aha-1 is a cochaperone that stimulates the ATPase activity of Hsp90 and has been shown to play a role in CFTR folding. Knocking down Aha-1 by RNA interference rescued the trafficking defect caused by Δ508 (Wang et al., 2006). Another cochaperone, FK506-binding protein of 38-kDa (FKBP8; also called FKBP38), has been reported as a cochaperone of both CFTR and hERG potassium channels and knockdown of FKBP8 caused a reduction of processing and trafficking for both hERG and CFTR (Wang et al., 2006; Walker et al., 2007). Curiously, we did not observe Aha-1 or FKBP8 associated with wild-type K<sub>ATP</sub> channels in our proteomics experiments, and further studies are needed to determine whether these cochaperones are involved in the folding/processing of wild-type and mutant K<sub>ATP</sub> channels in INS-1 cells.
Role of Hsp90 in Modulating Surface Expression of Misprocessing/Mistrafficking K\textsubscript{ATP} Channel Mutants

Studies of mutations identified in patients with congenital insulin secretion disease have shown that many SUR1 and Kir6.2 mutations affect channel function by preventing normal processing and trafficking of the channel (Ashcroft, 2005; Lin et al., 2006; Yan et al., 2007). In the present study, we have examined five such SUR1 mutations identified in congenital hyperinsulinism. Interestingly, only mutants that are considered to have mild processing/trafficking defects showed improved surface expression upon overexpression of Hsp90. Because Hsp90 is thought to act on substrates at a late stage of folding, it is possible that the A116P and ΔF1388-SUR1 mutations render folding difficulties at an early stage that cannot be overcome by upregulation of Hsp90 function. Examination of more disease mutations that disrupt channel processing and trafficking to different degrees will further test this idea. Given our results, we speculate that the Hsp90 chaperone complex may play a role in modulating disease phenotype by modulating the expression of mutant channels at the cell surface. We have shown previously that many disease mutations disrupt both channel gating and biogenesis/surface expression (Lin et al., 2006; Yan et al., 2007; Pratt et al., 2009). In this case, the expression level of a mutant will modulate the severity of β-cell dysfunction by modulating the total channel functional output (Lin et al., 2006). It is conceivable that changes in Hsp90 expression under different physiological or pathological conditions could affect manifestation of certain disease mutations. This could potentially help explain why disease symptoms can vary among patients carrying the same mutations (Ashcroft, 2005).

Effects of the Hsp90β Inhibitor 17-AGG on K\textsubscript{ATP} Channels

In recent years, Hsp90 inhibitors such as 17-AGG have shown great promise in treating cancer by targeting oncogenic kinases for degradation (Solit and Rosen, 2006). Our study suggests that the use of these drugs may reduce the number of K\textsubscript{ATP} channels to alter glucose-stimulated insulin secretion response in pancreatic β-cells. It is also worth noting that although 17-AGG has little effect on K\textsubscript{ATP} channel gating at concentrations below 100 nM (those used in Figure 3), it does inhibit channel response to the stimulatory effect of Mg-nucleotides when applied to the cytoplasmic face of inside-out patches at concentrations above 200 nM (Pratt and Shyng, unpublished). Because K\textsubscript{ATP} channel stimulation by Mg-nucleotides is mediated by the NBDs of SUR1, 17-AGG may disrupt Mg-nucleotide binding or hydrolysis at NBDs to alter channel gating. Thus, 17-AGG at high concentrations probably targets ATPases other than Hsp90 (Chen, 2002). In this respect, functional data obtained with the use of 17-AGG need to be viewed with biochemical data and interpreted with caution. Recently, it has been reported that 17-AGG partially rescues the processing defect of a disease-causing aquaporin-2 mutation, although the precise mechanism of rescue is unknown (Yang et al., 2009). We, too, tested whether 17-AGG has a salutary effect on the five SUR1 trafficking mutants but saw no effects.

In summary, we have demonstrated in this study that Hsp90 plays a role in the biogenesis of SUR1/Kir6.2 K\textsubscript{ATP} channels, in particular the maturation of the SUR1 subunit. Alterations in Hsp90 expression or function affect the number of K\textsubscript{ATP} channels in the plasma membrane. Up-regulation of Hsp90 alleviates the processing/trafficking defects in some but not all disease-associated SUR1 mutations. Future studies examining the effect of overexpression or knock-down of other chaperones and cochaperones on the biogenesis of wild-type and mutant channel proteins will probably shed additional light on the molecular and structural mechanisms of channel folding and processing. Besides the SUR1/Kir6.2 K\textsubscript{ATP} channel subtype, several other K\textsubscript{ATP} channel subtypes containing the SUR2 isoforms are expressed in cardiac, vascular, and skeletal muscle tissues where they play important roles in linking metabolic changes to muscle activities (Minami et al., 2004). It would be important to determine whether Hsp90 similarly affects biogenesis and expression of these channels by interacting with the SUR2 protein.

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