HHS Public Access

Author manuscript

J Proteome Res. Author manuscript; available in PMC 2021 July 16.

Published in final edited form as:

J Proteome Res. 2012 February 03; 11(2): 1364–1372. doi:10.1021/pr201003g.

Human ABCC1 Interacts and Colocalizes with ATP Synthase α, Revealed by Interactive Proteomics Analysis

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Abstract

Human ABCC1 is a member of the ATP-binding cassette (ABC) transporter superfamily, and its overexpression has been shown to cause multidrug resistance by active efflux of a wide variety of anticancer drugs. ABCC1 has been shown to exist and possibly function as a homodimer. However, a possible heterocomplex involving ABCC1 has been indicated. In this study, we performed an interactive proteomics study to examine proteins that bind to and form heterocomplexes with ABCC1 using coimmunoprecipitation and tandem mass spectrometry (MS/MS) analyses. We found that ATP synthase α binds to ABCC1 in plasma membranes with a ratio of 2:1. The ATP synthase α binding site in ABCC1 is located in the linker domain at the carboxyl core of ABCC1, and phosphorylation of the linker domain at the protein kinase A site enhances ATP synthase α binding. The interaction between ABCC1 and ATP synthase α in a heterocomplex may indicate a novel function of ABCC1 in regulating extracellular ATP level and purinergic signaling cascade.

Keywords

ABC transporter; MRP1; ABCC1; drug resistance; ATP synthase; oligomers

INTRODUCTION

Human ABCC1 is a member of the ATP-binding cassette (ABC) transporter superfamily that consists of more than one thousand members ranging from bacteria to human.[1] Overexpression of ABCC1 has been demonstrated to be one of the major causes of multidrug resistance (MDR) in both laboratory cell models and clinical settings.[2-4] It functions as an ATP-dependent efflux pump of a wide variety of anticancer drugs, xenobiotics, and a broad spectrum of organic anions including oxidized and reduced

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glutathione (GSSG and GSH) as well as anionic conjugates of GSH, glucuronide, and sulfate.[5] Unlike most other human ABC family members, such as ABCB1, which contain two membrane-spanning domains (MSD) and two nucleotide-binding domains (NBD) with a domain structure of MSD1-NBD1-MSD2-NBD2, ABCC1 has an additional membrane-spanning domain (MSD0) that consists of five predicted transmembrane segments with a putative extracellular amino terminus, which has recently been shown to be functionally important[6] and may form a U-shaped structure with a gating function for the transport activity of ABCC1.[7,8]

Human ABCC1 has recently been shown to exist and may function as a homodimer[9] with the dimerization site mapped to the domain including transmembrane segment 5 (TM5) and extracellular loop 3 (ECL3) in MSD0.[10] However, it is not known if human ABCC1 also possibly binds to other proteins to regulate or to be regulated by these binding partners. Previously, a minor population of chemically cross-linked ABCC1 with an apparent molecular weight between monomeric and dimeric ABCC1 was observed,[9] and ABCB1 has been shown to bind to tubulin.[11] We, thus, hypothesize that human ABCC1 may also bind to other proteins to form heterocomplexes. In this study, we tested this hypothesis using coimmunoprecipitation and tandem mass spectrometry (MS/MS) and found that ABCC1 binds to ATP synthase α. The binding of ABCC1 to ATP synthase α was verified by Western blot analysis and by colocalization study using indirect immunofluorescence. The binding site of ATP synthase α appears to localize to the linker domain at the carboxyl core of ABCC1 and the binding can be enhanced by phosphorylation of the linker domain that has several potential phosphorylation sites including sites for PKA and PKC.

MATERIALS AND METHODS

Materials

Monoclonal anti-ABCC1 antibody MRPr1 and anti-HA antibody were from Kamiya and Covance, respectively. Anti-FLAG antibody M2, HRP-conjugated goat anti-mouse IgG and rabbit anti-rat IgG were from Sigma. Polyvinylidene difluoride (PVDF) membranes, concentrated protein assay dye reagents were from Bio-Rad. LipofectAMINE, G418, and cell culture media and reagents were obtained from Invitrogen. Calyculin A and enhanced chemiluminescence (ECL) system were purchased from Cell Signaling and Amersham/Pharmacia, respectively. Anti-ATP synthase α antibody and FITC-conjugated donkey anti-rat IgG were from BD Transduction Laboratories and Jackson Immunoresearch Laboratories, respectively. PE-conjugated goat anti-mouse IgG and protein G-agarose were from Santa Cruz Biotechnology. All other reagents of molecular biology grade were purchased from Sigma or Fisher Scientific.

Cell Lines, Transfection, and Treatments

HEK293 cells with or without stable expression of $ABCC1^{F\text{-}WT}$, $ABCC1^{F\text{-}FLAG}$ or $ABCC1^{F\text{-}HA}$ were established previously[9] and maintained in DMEM supplemented with 10% fetal bovine serum in the presence of 100 units/mL penicillin and 100 μg/mL of streptomycin. For transient transfection, 2.5 to 15 μg of plasmid DNAs were transfected into HEK293 cells in 150 mm dishes using LipofectAMINE according to the manufacturer's

instructions. Forty-eight hours after transfection, cells were harvested for preparation of cell lysates as described previously for coimmunoprecipitation.[6] For the phosphorylation enhancement study, HEK293 cells expressing FLAG-tagged ABCC1 were treated with 100 nM calyculin A for 45 min at 37 °C followed by lysis of cells for immunoprecipitation.

Metabolic Labeling

Metabolic labeling was performed as previously described.[12] HEK293 cells (3×10^6) with or without stable expression of $ABCC1^{F\text{-}WT}$, $ABCC1^{F\text{-}FLAG}$ or $ABCC1^{F\text{-}HA}$ were seeded in 100-mm dish and cultured for 24 h followed by washing 3 times with DMEM containing no methionine and cysteine. The cells were then labeled with 300 μCi of ^{35}S-methionine in DMEM containing cysteine, glutamine, 2% dialyzed FBS for 16 h. The cells were then washed 3 times with ice-cold PBS and harvested for cell lysate preparation as described previously.[9]

Immunoprecipitation

Immunoprecipitation was performed as previously described.[9,13] Briefly, cell lysates (1.5 mg) were first precleared by incubation with 1 μg of normal mouse IgG at 4 °C for 1 h, mixed with 50 μL of protein G agarose beads (50% slurry) and incubated at 4 °C for another hour followed by centrifugation at 500× g for 5 min. The cleared supernatants were incubated with 10 μg of anti-FLAG or anti-HA antibodies at 4 °C for 4 h, mixed with 50 μL of 50% protein G-agarose slurry. After overnight incubation at 4 °C, the reaction was centrifuged to collect precipitates which were then washed five times with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) before being subjected to SDS-PAGE analysis for autoradiography or Western blot analysis.

2-Dimensional Gel Electrophoresis

The 2-dimensional gel electrophoresis was performed as previously described.[14-16] Briefly, immunoprecipitates were diluted to 0.9 μg/μL in a total volume of 185 μL with BioRad premade sample loading buffer (8 M urea, 50 mM DTT, 4% CHAPS, 0.2% carrier ampholytes, 0.0001% bromophenol blue) and loaded onto the IPG strips (pH 3–10 and 5–8) by overnight passive absorption. The first dimensional separation was done in three steps of isoelectric focusing (linear 250 V for 20 min, linear 8000 V for 2.5 h, rapid 8000 V for 20000 V-hours) and the second dimensional separation was done by SDS-PAGE using BioRad precast 10–20% gradient gel at 130 V.

Indirect Immunofluorescence Imaging

Indirect immunofluorescence imaging was performed as previously described.[6,7] Briefly, HEK293 cells with stable expression of $ABCC1^{F\text{-}WT}$ cultured on coverslips were washed with phosphate-buffered saline, fixed with acetone/methanol, blocked with bovine serum albumin, and probed with anti-ABCC1 antibody MRPr1, anti-ATP synthase α, or anti-ATP synthase β antibody at room temperature for 1 h followed by incubation with FITC-conjugated donkey anti-rat IgG or PE-conjugated goat anti-mouse IgG at room temperature for 30 min. The coverslips were then mounted on slides before viewing with a confocal microscope.

Tryptic Digestion

Coomassie blue-stained protein bands of 180, 54 and 50 kDa were excised manually from SDS-PAGE, cut into smaller (less than 1 mm in each dimension) pieces, covered with 200 μL of 200 mM ammonium bicarbonate in 40% acetonitrile in an Eppendorf tube and incubated at 37 °C for 30 min to remove dye, salts and SDS. The gel pieces were then completely dehydrated followed by rehydration in 20 μL of 20 μg/mL trypsin solution (36 mM ammonium bicarbonate, 8% acetonitrile) with addition of 50 μL of 40 mM ammonium bicarbonate in 9% acetonitrile and proteins were digested for 18 h at 37 °C. The liquid containing digested proteins was removed from gel pieces, dried and reconstituted in 10 μL of 1% formic acid for further analyses.

Nano-LC–MS/MS

The nano-LC–MS/MS was performed as previously described.[15] Briefly, 3 or 6 μL of the digested proteins were loaded onto a trapping column (15 mm × 100 μm i.d.) packed in-house with 5 μm 200 Å Magic C18AQ packing media (Michrom Bioresources, Auburn, CA) followed by washing to remove any salts and unretained materials prior to elution and separation of the retained peptides on a pulled-tip capillary column (150 mm × 75 μm i.d.) packed with the same materials for the trapping column. At the end of the column, ions were electrosprayed directly into a ThermoFinnigan (San Jose, CA) LCQ Deca XP ion-trap mass spectrometer, which recorded alternating mass spectra from m/z 300 to 1600 and data-dependent tandem mass spectra of the separated peptide ions. Precursor ions exceeding the intensity threshold of 5×10^5 were selected with a 2.0-Da isolation window for collision-induced dissociation at 35% normalized collision energy. Precursors were dynamically excluded from selection for 60 s if they were selected twice within a 60-s window. The acquired MS/MS spectra were searched against human protein sequences in the Swiss-Prot database (downloaded June 15, 2010) using Mascot version 1.9[17] for peptide scoring and consequently protein identification. MS/MS spectra were extracted as Mascot Generic Format files using TurboRaw2 mgf v.1.0.9 converter written in-house and requiring minimum spectral intensity of 1×10^6 and a minimum of 15 ions in the spectrum. Database searches of the spectra as doubly- or triply charged precursors were restricted to trypsin specificity allowing for one missed cleavage per peptide. Monoisotopic precursor mass tolerance was set to ±2.0 Da while monoisotopic fragment ion tolerance was ±0.8 Da. Variable modifications of carbamidomethylation or propionamidination of cysteine and oxidation of methionine, histidine or tryptophan were included in the search. Spectral matches with scores equal to or greater than 39, the score threshold determined by Mascot to represent $p < 0.05$, were reported as identified peptides. These peptide scores were then summed for each assigned protein and reported in Table 1 along with the sequence coverage for each protein in replicate experiments.

GST-linker Domain Fusion Protein, Synthetic Peptides and Pull-down Assay

The construct encoding GST-linker domain fusion protein was engineered by first amplifying the linker domain-encoding sequence using ABCC1 cDNA as a template with specific primers (forward: 5′-ggatcccccgggatctctgagatgggctcct-3′ and reverse: 5′-gtcgacgcggccgcctattagagtccgatggccttca-3′) and cloning the PCR products into TOPO

BLUNT II (Invitrogen), and subsequently into pGEX 6.1 (Amersham-Pharmacia) between Bam HI and *Sal* I restriction sites. The final construct was verified by double strand DNA sequencing.

Competent *E. coli* strain BL21 was transformed with the above construct or vector control and grown in LB broth containing 100 μ/mL ampicillin to an A600 of 0.6 at 37 °C followed by induction of expression with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Invitrogen) at 20 °C for 16 h. Bacteria were harvested by centrifugation, subsequently lysed by sonication in PBS supplied with 2 mM phenylmethylsulfonyl fluoride and then removal of insoluble material by centrifugation. The GST and GST-linker domain fusion protein were purified using glutathione-Sepharose 4B beads according to the manufacturer's instructions (Amersham-Pharmacia). GST and GST-linker domain fusion proteins were quantified and the purity of the preparations were verified by SDS-PAGE followed by Coomassie blue staining and Western blot.

For pull down assay, the purified GST and GST-linker domain fusion proteins bound with glutathione-Sepharose 4B beads were mixed with lysate of HEK293 cells followed by incubation on a rotator for 3 h at 4 °C. The bound complexes were separated from unbound materials by centrifugation followed by washing for 4 times. The beads were incubated with 1× SDS sample buffer for 30 min at room temperature followed by SDS-PAGE and Western blot analyses.

Heptapeptides representing different potential phosphorylation sites in the linker domain of human ABCC1 were synthesized using Fmoc-protected amino acids as previously described.[11] Polypropylene pins with synthetic peptides were then incubated with HEK293 cell lysates overnight at 4 °C followed by washing with phosphate-buffered saline including 2 mM phenylmethylsulfonyl fluoride to remove unbound materials. The bound proteins were then eluded by incubating the pins in 1× SDS sample buffer (without bromo-phenol blue) for 30 min at room temperature followed by SDS-PAGE and Western blot analyses as previously described.

RESULTS

Observation of Chemically Cross-linked Hetero-ABCC1 Complex

Previously, we observed that chemically cross-linking ABCC1 resulted in a possible heterocomplex of ABCC1 with an apparent molecular weight of ~280 kDa in addition to the cross-linked homodimeric ABCC1.[9] To further examine this possible heterocomplex, we first performed the same experiment to reversibly cross-link ectopic ABCC1 in HEK293 cells using dithiobis succinimidylpropionate (DSP). As shown in Figure 1, human ABCC1 has an apparent molecular weight of 180 kDa on SDS-PAGE without chemical cross-linking. A dimeric ABCC1 of ~360 kDa appeared following chemical cross-linking, which can be reversed in the presence of reducing agent dithiothreitol (DTT). Interestingly, another minor cross-linked complex is also detected that has an apparent molecular weight of ~280 kDa between the dimeric and monomeric ABCC1 (indicated by an arrow in Figure 1). This additional cross-linked product may represent a possible heterocomplex between human

ABCC1 and another protein with a size of ~100 kDa considering that monomeric ABCC1 has an apparent molecular weight of ~180 kDa on SDS-PAGE.

Identification of ABCC1-associated Proteins

To determine if ABCC1 possibly binds to other proteins and forms a heterocomplex, we performed a coimmunoprecipitation of HA- or FLAG-tagged ABCC1 in HEK293 cells following metabolic labeling with [^{35}S]methionine and analyzed the precipitate by SDS-PAGE and autoradiography. As shown in Figure 2A, one major protein with an estimated molecular weight of ~54 kDa and another minor protein around 45 kDa are observed which are not found in vector-transfected control cells that do not express ABCC1. Coomassie blue staining of the coprecipitate of HA- or FLAG-tagged ABCC1 on SDS-PAGE also revealed these proteins (Figure 2B).

We next analyzed the coimmunoprecipitate using 2-dimentional gel electrophoresis followed by Coomassie blue staining. As shown in Figure 2C, the only distinctive difference between vector-transfected and ABCC1-overexpressing cells is a protein spot with an estimated molecular weight of ~54 kDa and an estimated pI of 9.4, consistent with the observation using SDS-PAGE. Consequently, the 54-kDa protein was excised from Coomassie blue-stained SDS-PAGE and subjected to MS/MS analysis. We also analyzed the 180-kDa protein to determine if it is ABCC1 as well as the 45-kDa proteins from Coomassie blue-stained SDS-PAGE gels using MS/MS. As shown in Table 1, the 180-kDa protein was identified to be ABCC1 as expected and the 54- and 45-kDa proteins were identified as ATP synthase α and elongation factor 1-α1, respectively, in two separate experiments, indicating that these experiments are reproducible. The calculated pI of ATP synthase α is 9.5, consistent with the estimated pI (9.4) of the 54-kDa protein on 2-dimensional gel (Figure 2C). β-Tubulin was also identified as a possible 54-kDa interactive partner. However, its pI (4.8) does not match the pI of the protein on the 2-dimensional gel; we thus elected not to further study tubulin.

To verify the findings from MS/MS analysis, we chose to focus on ATP synthase α because the peptide coverage in both experiments is consistently high (Table 1) and it is a major ABCC1-associated protein that is consistently observed in both SDS-PAGE and 2-dimensional gel profiles. We first performed coimmunoprecipitation using HA antibody for HA-tagged ABCC1 followed by Western blot analysis of the precipitate using ATP synthase α and HA antibodies. As shown in Figure 2D, ATP synthase α was identified in the coprecipitate of HA-tagged ABCC1 but not in the precipitate of vector-transfected control cells without ABCC1. We also tested ATP synthase β but did not find it in the coprecipitate of ABCC1. Thus, likely ABCC1 interacts with ATP synthase α but not ATP synthase β. To determine the binding of ABCC1 to ATP synthase is not cell line specific, we next transfected Flag-tagged ABCC1 into HeLa cells and performed coimmunoprecipitation analysis. We found that ATP synthase α in HeLa cells could also be efficiently precipitated by ABCC1 (data not shown), suggesting that the interaction between ABCC1 and ATP synthase α is not cell line specific.

ABCC1 Colocalizes with ATP Synthase α on Plasma Membranes

Since ATP synthase α is a subunit of F_1/F_0 ATP synthase that is known to be located in mitochondria while ABCC1 is known to localize on plasma membranes, it is peculiar to find that ABCC1 interacts with ATP synthase α. However, it has recently been demonstrated that ATP synthase α also localizes on plasma membranes where it resides in lipid rafts[18] possibly participating in synthesis of extracellular ATP.[19] Furthermore, ABCC1 has also been suggested to be enriched in lipid rafts.[20] Thus, it is possible that ABCC1 and ATP synthase α interact on plasma membranes in lipid rafts. To determine if ABCC1 possibly colocalizes with ATP synthase α on cell surface, we performed indirect double immunofluorescence staining of HA-tagged ABCC1 and ATP synthase α. Figure 3 shows that some ATP synthase α colocalizes with ABCC1 on plasma membranes although the majority of ATP synthase α is located intracellularly presumably in mitochondria, (Figure 3A-C). Some ATP synthase β also colocalizes with ABCC1 on plasma membranes (Figure 3D-F). The latter is not unexpected since ATP synthase β has also been suggested to coexist with ATP synthase α on plasma membranes[21] although it does not appear to interact with ABCC1 in coimmunoprecipitation assay. Thus, the interaction between ABCC1 and ATP synthase α likely occurs on plasma membranes and that cell surface ATP synthase α and β may not be obligatory interacting subunits (see also Discussion below).

Binding Ratio between ABCC1 and ATP Synthase α

We next determined how many ABCC1 are possibly involved in interaction with ATP synthase α by examining the ratio of ABCC1/ATP synthase α in the coprecipitated materials by taking advantage of metabolic labeling and the known content of methionine in ABCC1 and ATP synthase α. For this purpose, the [^{35}S]methionine-labeled ABCC1 and ATP synthase α following coimmunoprecipitation and separation by SDS-PAGE were excised and used for scintillation counting. As shown in Table 2, the ratio of ABCC1:ATP synthase α as revealed by their respective [^{35}S]methionine content on SDS-PAGE is 2:1. Considering that only a small fraction of ABCC1 exists as heterocomplex as determined by chemical cross-linking and that the non-ABCC1 component contributes ~100 kDa of the 280-kDa cross-linked heterocomplex (Figure 1), we estimate that at maximum ~25% of ABCC1 is involved in binding ATP synthase α and each ABCC1 may bind to two ATP synthase α molecule with each ATP synthase α contributes ~54 kDa to the heterocomplex.

ATP Synthase α Binds to the Linker Domain in the Carboxyl Core Domain of Human ABCC1

To determine the ATP synthase α-binding site in ABCC1, we first examined two ABCC1 constructs containing MSD0 alone or the core structure with MSD1-NBD1-MSD2-NBD2 by coimmunoprecipitation. For this purpose, HEK293 cells were transiently transfected with previously engineered FLAG-tagged constructs ABCC1$^{\text{F-FLAG}}$, ABCC1$^{\text{281N-FLAG}}$, and ABCC1$^{\text{CORE-FLAG}}$[6,9] (Figure 4A) followed by immunoprecipitation with FLAG antibody and Western blot analysis of the coprecipitates for ABCC1 and ATP synthase α. Figure 4B and 4C show that ABCC1$^{\text{281N-FLAG}}$ coprecipitates much less ATP synthase α than ABCC1$^{\text{F-FLAG}}$ and ABCC1$^{\text{CORE-FLAG}}$. Thus, we conclude that ATP synthase α likely binds mainly to a site within the carboxyl core domain of ABCC1.

Previously, it was found that the binding site of tubulin in ABCB1 is located in the linker domain that connects NBD1 and MSD2.[11] Thus, it is possible that the analogous linker domain in the carboxyl core of ABCC1 may also be responsible for binding of ATP synthase α. To test this possibility, we first generated a GST-linker domain fusion protein containing amino acid residues 846–972 of ABCC1 and performed a pull-down assay of HEK293 cell lysate using GST-linker domain-bound to glutathione-conjugated beads as a bait. As shown in Figure 5A, the GST-linker domain fusion protein could efficiently pull down ATP synthase α whereas the control GST alone did not. As a control, we also tested ATP synthase β but did not find it in the pull-down materials. Thus, the ATP synthase α-binding site in ABCC1 is likely located in the linker domain of ABCC1.

Role of ABCC1 Phosphorylation in Binding to ATP Synthase α

The linker domain of ABCC1 contains several potential phosphorylation sites including a protein kinase A (PKA) site (Figure 6A). To further determine if the phosphorylation status of the linker domain potentially affects the binding of ATP synthase α to ABCC1, we next phosphorylated the purified GST-linker domain fusion protein in vitro using PKA (Figure 5B). The phosphorylated GST-linker domain and the nonphosphorylated control protein were then used to pull down ATP synthase α from HEK293 cell lysate followed by Western blot analysis. Figure 5B shows that the amount of ATP synthase α pulled down by the phosphorylated GST-linker domain is 2-fold of that by the nonphosphorylated control GST-linker domain protein. Thus, phosphorylation of the linker domain likely enhances ABCC1 binding to ATP synthase α.

To further examine the contribution of phosphorylation of all four sites in the linker domain (Figure 6A) to ATP synthase α binding, we synthesized peptides representing these potential phosphorylation sites and these peptides on pins were used to pull down ATP synthase α from HEK293 cell lysate followed by Western blot analysis. As shown in Figure 6B, compared to the first peptide 866–872 (LRTYAST), all others had significantly less activity in binding to ATP synthase α. Next, peptides with mutation of the Ser to Asp to mimic phosphorylation were synthesized and were used to pull down ATP synthase α in comparison with the wild type sequence. As shown in Figure 6C, mutating Ser to Asp of peptides 910–916 (LQRQLSS) and 926–931 (RHHNST) dramatically increased the binding of these peptides to ATP synthase α. The control mutation of Ser to Ala in these two peptide sequences had no effect on ATP synthase α binding. Mutation of the Ser in peptide 956–962 (GQVKLSV) to either Asp or Ala did not increase its binding to ATP synthase α. While mutation of Ser in peptide 866–872 (LRTYAST) to Asp had no effect on ATP synthase α binding, its mutation to Ala significantly reduced the binding. These findings suggest that while the wild type sequence LRTYAST may be involved in ATP synthase α binding, the contribution of the sequences LQRQLSS and RHHNST to this binding is likely dependent on their phosphorylation. The last sequence GQVKLSV may not contribute to ATP synthase α binding with or without phosphorylation.

Effect of Phosphorylation on the Binding of Endogenous ABCC1 to ATP Synthase α

To determine if the interaction between endogenous ABCC1 and ATP synthase α could be elevated by increasing ABCC1 phosphorylation, we treated HEK293 cells with calyculin A,

an inhibitor of protein phosphatase PP1 and PP2A (serine/threonine phosphatase), followed by coimmunoprecipitation and Western blot analysis. As shown in Figure 7, ABCC1 serine phosphorylation is indeed increased by calyculin A treatment. Significantly more ATP synthase α was also coimmunoprecipitated with ABCC1 following calyculin A treatment than the control treatment group. Taken together with findings shown above, we conclude that the binding site of ATP synthase α in ABCC1 is located in the linker domain and that phosphorylation of the PKA and possibly also RSK site in the linker domain increases the binding activity of ABCC1 to ATP synthase α.

DISCUSSION

In this study, we showed that human ABCC1 participates in forming hetero-oligomeric complexes with ATP synthase α using interactive proteomics approach. The interaction between ABCC1 and ATP synthase α has also been confirmed using Western blot analysis in combination with coimmunoprecipitation and by colocalization analysis using indirect immunofluorescence. It appears that ATP synthase α binds to the carboxyl core of ABCC1 at the linker domain and its binding could be enhanced by phosphorylation at the PKA and RSK phosphorylation site in the linker domain.

While ATP synthase α has a molecular mass of 54 kDa, the protein that chemically cross-linked to ABCC1 appears to have a size of ~100 kDa. Based on these estimated sizes, it is tempting to speculate that ABCC1 may bind to two ATP synthase α. However, Western blot analysis of ATP synthase α following chemical cross-linking to determine if the cross-linked products possibly contain ATP synthase α was unsuccessful (unpublished observation). This negative observation may be due to the possibility that the antibody epitope in ATP synthase α is damaged by the cross-linking agent, which renders the molecule undetectable in the cross-linked complex. Another minor possible interactive protein was identified as elongation factor 1α1. However, since it is not observed in the 2-dimensional gel, it is not clear if it is truly interactive with ABCC1. We also found tubulin as a possible ABCC1-binding partner from the MS analysis. However, we were unable to verify this possible interaction by 2-dimensional gel electrophoresis.

The binding site of ATP synthase α appears to reside in the carboxyl core domain of ABCC1 and likely in the linker domain preceding TM12. Previously, it has also been shown that the linker domain of ABCB1 contains a binding sequence to α- and β-tubulins.[11] Thus, the linker domain of ABC transporters may play important roles in organization of hetero-oligomeric ABC transporter complexes with other proteins. At least three phosphorylation sites (amino acid residues 866–872, 910–916, and 926–931) in the linker domain of ABCC1 were shown to contribute to ATP synthase α binding. While the wild type sequence of the first site (LRTYAST) may be involved in ATP synthase α binding, the other two sites (910–916, and 926–931) do not efficiently bind to ATP synthase α unless the potential phosphorylation site (Ser) is mutated to Asp, mimicking phosphorylation, suggesting that phosphorylation might be required.

The finding that ABCC1 binds to ATP synthase α is peculiar since ATP synthase α is a subunit of the mitochondrial F_1/F_0 ATP synthase and ABCC1, a plasma membrane

transporter, has never been reported to localize in mitochondrial membranes. However, as a known subunit of mitochondrial ATP synthase, the α subunit has been demonstrated to exist also on cell surface[22] and possibly in lipid rafts.[18] The cell-surface ATP synthase has been suggested to play several important functions such as regulating intracellular pH and cholesterol homeostasis (for review see ref 21). Cell surface ATP synthase may also participate in synthesis of extracellular ATP which activates P2X class purinergic receptors.[19] Thus, the interaction between ABCC1 and ATP synthase α likely occurs on plasma membranes. It is noteworthy that ABCC1 has also been found in lipid rafts although it does not interact with caveolin[20,23] whereas ATP synthase α has been shown to interact with caveolin-1.[21]

Currently, the physiological significance of ABCC1 binding to ATP synthase α is unknown. However, since both proteins have a common relationship with ATP as one synthesizes ATP while the other uses and hydrolyzes ATP, it is tempting to speculate that the interaction between these two proteins may help regulate the levels of both intracellular and extracellular ATP. Although other subunits of the F_1/F_0 ATP synthase which are essential in ATP synthesis were not observed as interactive with ABCC1, it cannot be ruled out that they are in fact part of the large complex but were dissociated under the condition used in this study. The finding that ATP synthase β is also colocalized with ABCC1 on plasma membranes supports this argument.

The interaction between ATP synthase α and ABCC1 is apparently dependent on phosphorylation status of the linker domain of ABCC1, further suggesting that their interactions may be regulated. Thus, it is tempting to speculate that high levels of extracellular ATP activates P2X class purinergic receptors which in turn activates PKA/RSK and phosphorylates ABCC1 to enhance the binding of ABCC1 to surface ATP synthase α, serving a feed back regulation of ATP synthase α. This possibility awaits further investigation.

ACKNOWLEDGMENTS

This work was supported in part by a National Institute Health grant CA113384 (J.T.Z.) and by Natural Sciences and Engineering Research Council of Canada (E.G.). Y.Y.Y. was supported in part by the NRSA T32 HL07910 from the National Institutes of Health. The mass spectrometry studies were facilitated by the instrumentation provided through Indiana Genomics Initiative (INGEN), a grant from the Lilly Endowment, Inc.

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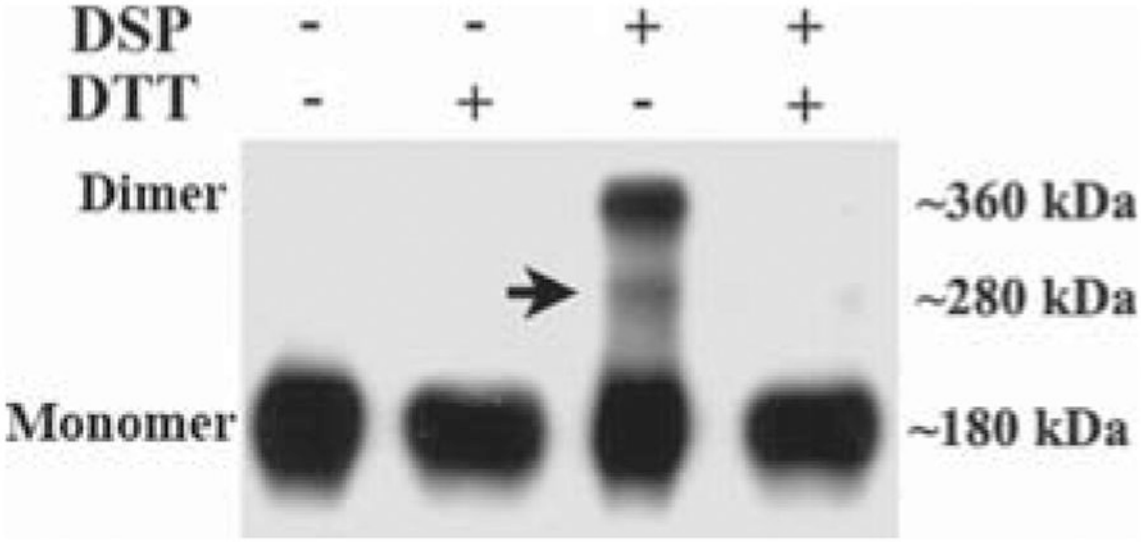

Figure 1.

Chemical cross-linking of human ABCC1. Live HEK293 cells expressing human ABCC1 were treated without or with DSP as indicated. Crude membranes were then prepared for separation by SDS-PAGE in the absence or presence of 100 mM DTT followed by Western blot analysis using monoclonal antibody MRPr1 as a probe. The molecular weight of each band was estimated by linear regression of molecular weight marker standard as previously described.[9]

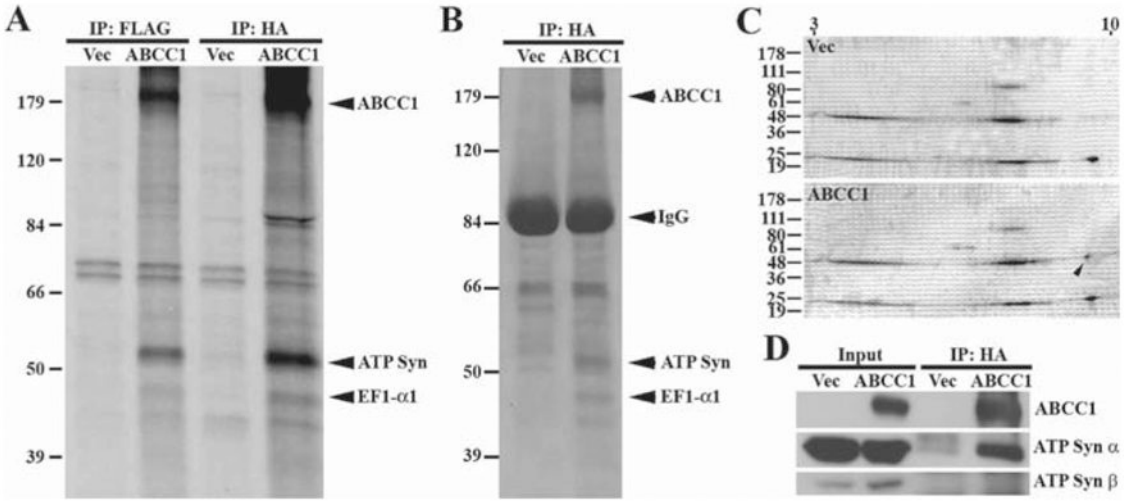

Figure 2.

Identification and validation of ABCC1-bound ATP synthase α. (A–C) Profile of ABCC1-bound proteins. Stable HEK293 cells expressing HA- or FLAG-tagged ABCC1 were labeled with [^{35}S]methionine followed by immunoprecipitation with HA or FLAG antibodies. The immunoprecipitates were then separated by SDS-PAGE (A and B) or 2-dimension PAGE (C). The gel was either dried for autoradiography (A) or stained by Coomassie blue (B and C). (D) Validation of ABCC1 interaction with ATP synthase α. Stable HEK293 cells expressing HA-tagged ABCC1 were subjected to lysis and immunoprecipitation with HA antibody followed by Western blot analysis of ATP synthase α, ATP synthase β, and ABCC1.

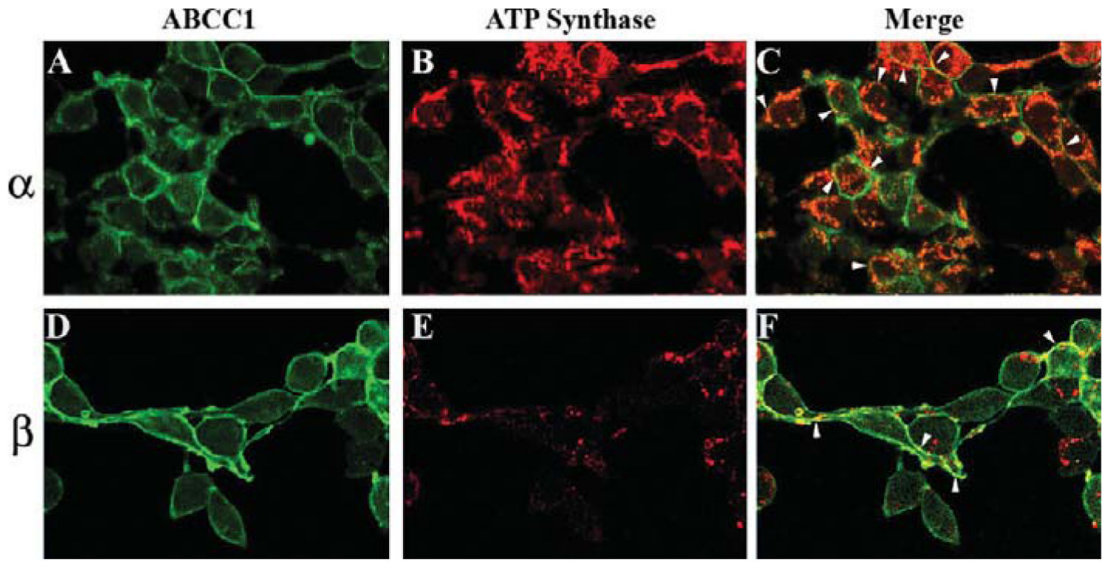

Figure 3.

Colocalization of ABCC1 with ATP synthase on plasma membranes. Stable HEK293 cells expressing FLAG-tagged ABCC1 were stained with anti-ABCC1 antibody MRPr1 (A and D), anti-ATP synthase α (B) and anti-ATP synthase β (E) antibodies followed by incubation with FITC-conjugated donkey anti-rat IgG (for ABCC1) or PE-conjugated goat anti-mouse IgG (for ATP synthase) and viewing on a confocal microscope.

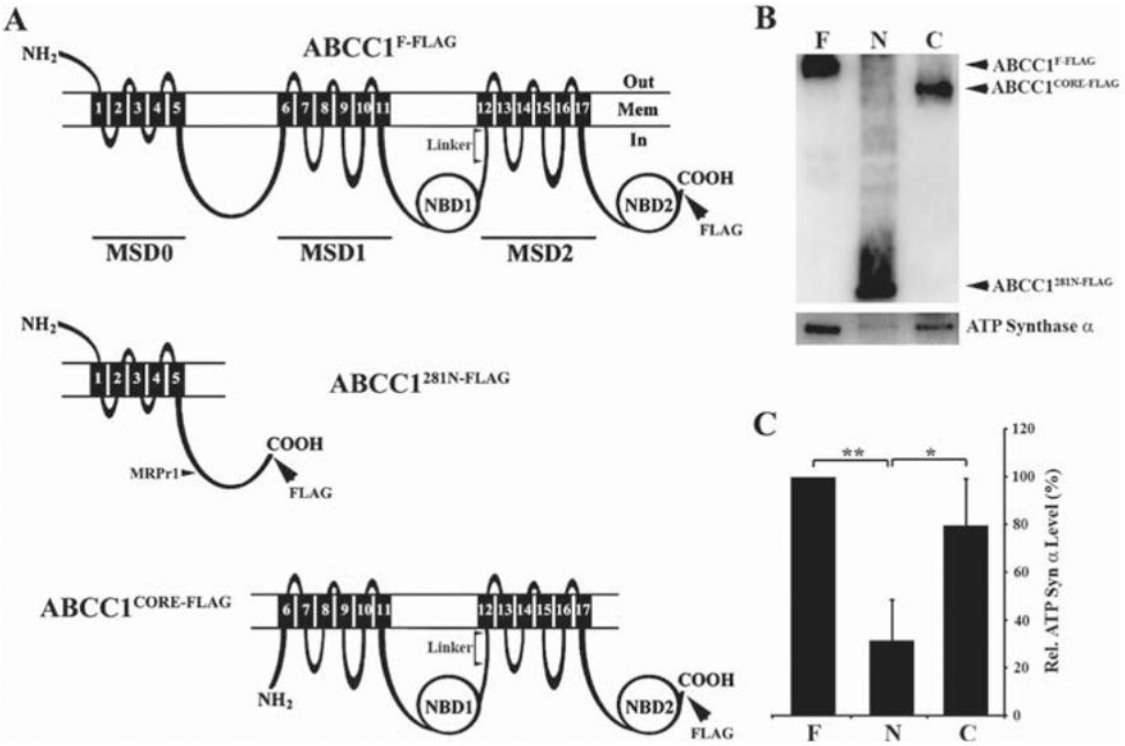

Figure 4.

Localization of ATP synthase α-binding site in ABCC1. (A) Schematic diagram of domain structure and constructs of full-length human ABCC1 ($ABCC1^{F\text{-}FLAG}$), amino terminal $ABCC1^{281N\text{-}FLAG}$, and carboxyl core $ABCC1^{CORE\text{-}FLAG}$. The transmembrane segments in membrane-spanning domains (MSD) are shown as numbered boxes and nucleotide-binding domains (NBD) are shown as circles. The position for FLAG tags, MRPr1 epitope, and the linker domain are indicated. (B) Co-immunoprecipitation of different ABCC1 constructs with ATP synthase α. $ABCC1^{F\text{-}FLAG}$, (F) $ABCC1^{281N\text{-}FLAG}$ (N) and $ABCC1^{CORE\text{-}FLAG}$ (C) were transiently transfected into HEK293 cells followed by coimmunoprecipitation with FLAG antibody, elution with FLAG peptide, and Western blot analysis of the eluted materials for ABCC1 protein and ATP synthase α. (C) Relative level of coimmunoprecipitated ATP synthase α. The amount of ATP synthase α coimmunoprecipitated by different ABCC1 constructs in panel A were measured using AlphaEaseFC (Alpha Innotech corporation) ($n = 3$ **$p < 0.01$; *$p < 0.05$).

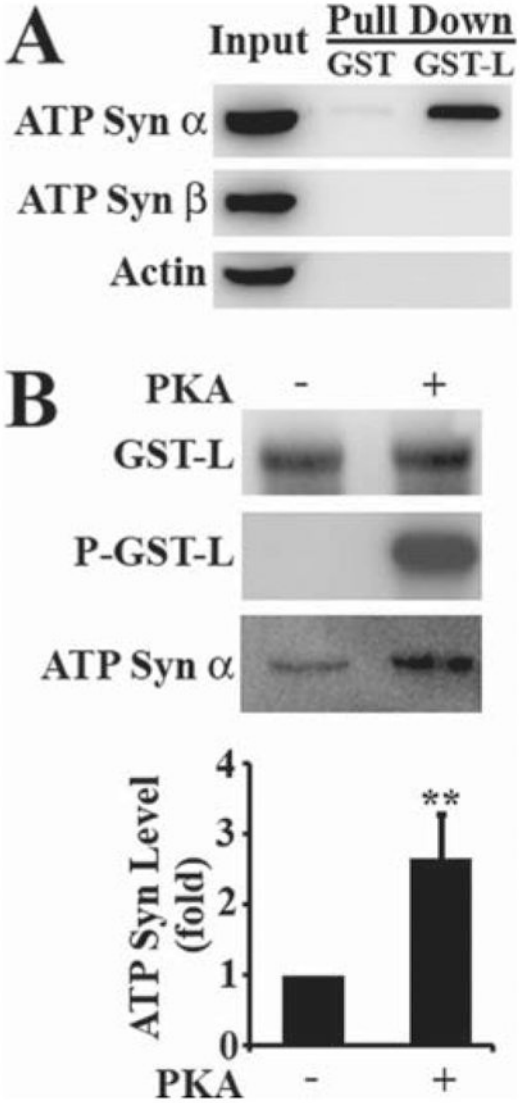

Figure 5.

Binding of GST-linker domain to ATP synthase α. (A) Binding between ATP synthase α and the linker domain of ABCC1. Purified GST-linker domain fusion protein and GST control were used to pull down ATP synthase from HEK293 cell lysate followed by Western blot analysis probed with antibodies against ATP synthase α, β, and negative control actin. (B) Effect of phosphorylation of the linker domain on ATP synthase α binding. Purified GST-linker domain was treated with or without protein kinase A (PKA) in the presence of $\gamma[^{32}P]$ATP followed by SDS-PAGE and autoradiographic analysis of phosphorylation of the protein. The phosphorylated GST-linker domain was then used for pull-down assay of HEK293 cell lysate followed by Western blot analysis probed with antibodies against ATP synthase α. The histogram shows the quantitative analysis of phosphorylation effect on the amount of ATP synthase α pulled down by GST-linker domain fusion protein ($n = 3$, $^{**}p \leq 0.01$). GST-*L* = GST-linker domain.

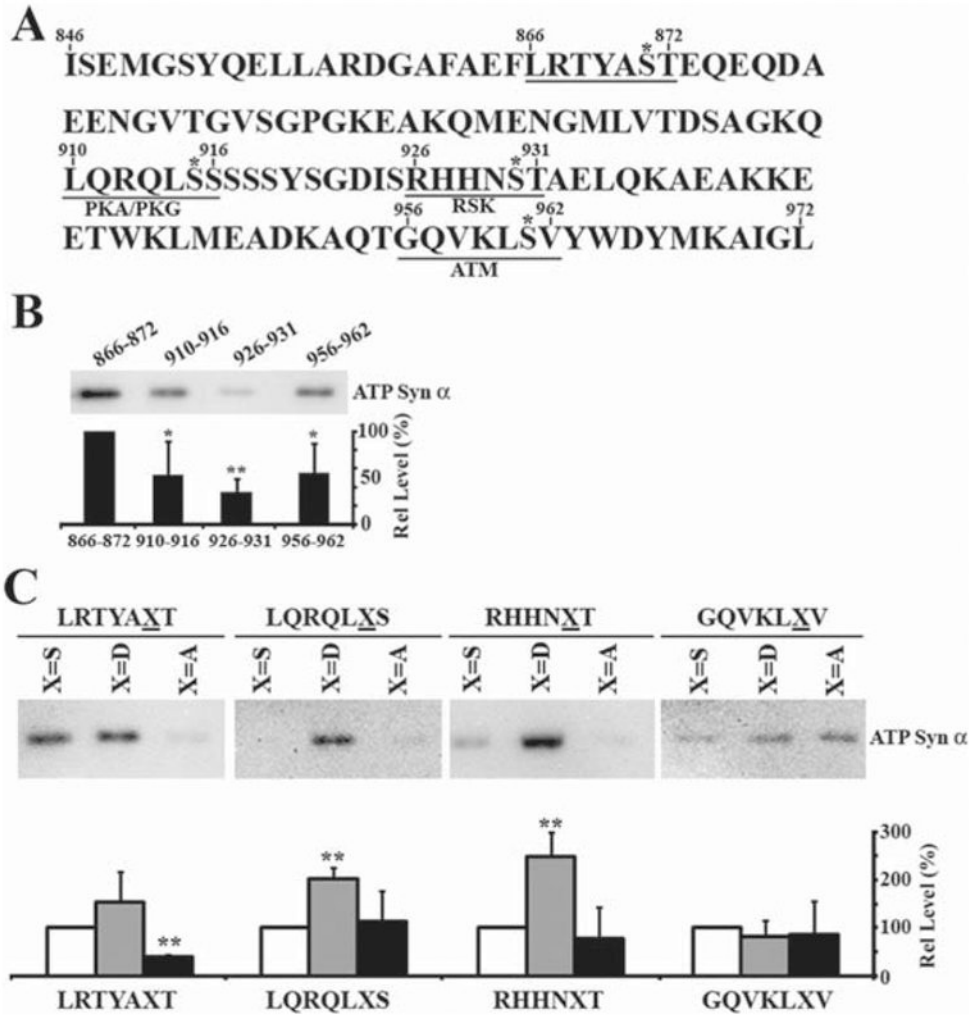

Figure 6.

Contribution of phosphorylation of the linker domain to ATP synthase α binding. (A) Sequence of the linker domain of ABCC1. Potential phosphorylation sites are indicated by * with the sequence of synthetic peptides underlined. The predicted kinases involved are also indicated. (B and C) Binding between ATP synthase α and synthetic peptides on pins. HEK293 cell lysates were used to incubate with synthetic peptides on pins followed by washing and elution of bound materials for Western blot analysis of ATP synthase α. The histogram shows relative level of peptide-bound ATP synthase α ($n = 4$–5, $^{**}p < 0.01$; $^{*}p < 0.05$).

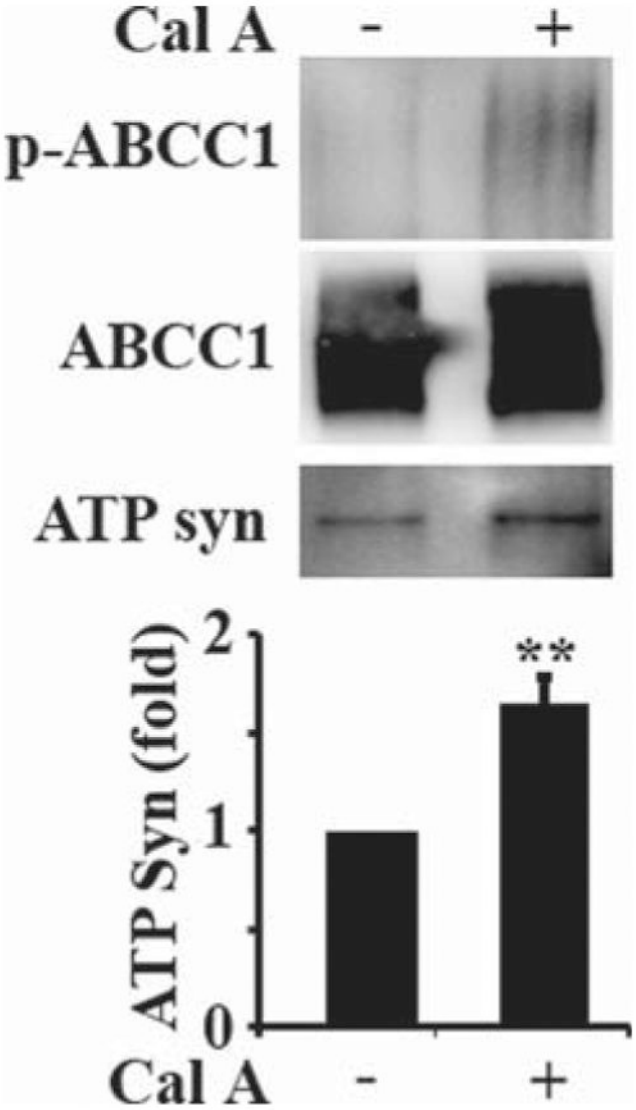

Figure 7.

Effect of phosphorylation on binding of endogenous ABCC1 to ATP synthase α. HEK293 cells expressing stable FLAG-tagged ABCC1 were treated with calyculin A (Cal A) followed by immunoprecipitation with FLAG antibody and Western blot analysis probed with phospho-serine, FLAG, and ATP synthase α antibodies. The histogram shows quantitative analysis of calyculin A effect on ABCC1 binding to ATP synthase α normalized to ABCC1 ($n = 3$, $^{**}p < 0.01$).

Table 1.

Mass Spectrometry Analysis of Coprecipitated Materials

sample (kDa)	protein	access #	coverage (%)	amino acids	peptides	score
180[a]	ABCC1	P33527	37.43	573/1531	43	2589
180[b]	ABCC1	P33537	28.22	432/1531	38	2175
54[a]	ATP Syn α	P25705	36.67	187/510	15	946
54[b]	ATP Syn α	P25705	17.25	88/510	7	522
50[a]	EF-1-α-1	P68104	18.40	85/462	8	431
50[b]	EF-1-α-1	P68104	4.11	19/462	2	120

[a]First experiment.

[b]Second experiment.

Table 2.

ABCC1/ATP Synthase α Ratio in Coimmunoprecipitate

	radioactive coprecipitates			number of methionines			
exp.	ABCC1 (cpm)	ATP syn α (cpm)	ratio[a]	ABCC1	ATP syn α	ratio	adjusted molar ratio[b]
1	5050	746	6.8	38	12	3.2	2.1
2	11322	2092	5.4	38	12	3.2	1.7
3	10541	1427	7.4	38	12	3.2	2.3
4	7143	1394	5.1	38	12	3.2	1.6
Mean ± SD							1.9 ± 0.3

[a] Ratio of ABCC1/ATP synthase α.

[b] Adjusted molar ratio equals the ratio of radioactive coprecipitates divided by that of methionine residues.