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Is Kir6.1 a subunit of mitoK_{ATP}?

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Abstract

The subunit composition of the mitochondrial ATP-sensitive K⁺-channel (mitoK_{ATP}) is unknown, though some suspect a role for the inward rectifier, Kir6.1, based largely antibody studies of heart mitochondria. To ascertain the molecular identity of mitoK_{ATP} we therefore sought to purify this putative mitochondrial Kir6.1, and conclusively identify mitoK_{ATP} subunits by mass spectrometry. Immunoblots conducted with two commercially-available antibodies, revealed two distinct signals in isolated heart mitochondria, of 51 kDa and 48kDa respectively. Localization was confirmed by either immuno-gold electron microscopy or by immunofluorescence. Each putative Kir6.1 species was extracted, purified and identified by LC-MS/MS. The 51 kDa band, was identified as NADH dehydrogenase flavoprotein 1, while the preponderant protein the 48kDa band was mitochondrial isocitrate dehydrogenase (NADP form). 1D-, 2D- and native gel analyses were consistent with these assignments. The data suggest it is premature to assign Kir6.1 a role in mitoK_{ATP} on the basis of immunoreactivity alone.

Keywords

Potassium channel; antibodies; protein purification; mass spectrometry; mitochondria

Introduction

ATP-sensitive potassium channels (K_{ATP}) act as metabolic sensors that couple electrical excitability of biological membranes to the cellular energy pool[1], which in turn regulates processes ranging from insulin secretion from the pancreas[2] to beating of the heart[3]. The channels are found at both the cell surface and intracellularly, within the mitochondria. Surface K_{ATP} channels consist tetramers of pore-forming inward-rectifying subunits (either Kir6.1 or Kir6.2), surrounded by four regulatory subunits called sulfonylurea receptors (SURs), of which there are three characterized forms SUR1, SUR2A and SUR2B. The precise combination of SUR and Kir6.x subunits that comprise the plasmalemmal K_{ATP} channels varies from tissue to tissue. By contrast, the molecular composition of mitoK_{ATP} has yet to be determined conclusively[4].

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K_{ATP} channels have garnered attention in the field of cardioprotection, as pharmacological studies have shown that activation of K_{ATP} channels mitigates the effects of myocardial ischemia/reperfusion injury. Compounds called potassium channel openers (KCOs) mimic the cardioprotective effects of ischemic and pharmacological preconditioning (PC) [5;6], whereas protection is abolished by K_{ATP} channel inhibitors[7]. Subsequent work has shown that salutary effects of KCOs are mediated by mitochondrial rather than sarcolemmal K_{ATP} (sarcK_{ATP}) channels. Garlid *et al.*[8] showed that diazoxide activates mitoK_{ATP} channels with 2000-fold higher potency than sarcolemmal channels. Capitalizing on the relative potencies of these compounds, Garlid *et al.*[9] showed that mitoK_{ATP}, not sK_{ATP}, is responsible for the PC induced by the KCO, diazoxide, a conclusion we reached independently[10].

The overlapping pharmacology of the sarcK_{ATP} and mitoK_{ATP} channels suggests a certain structural homology[4], which has prompted several groups to use immunological approaches toward identifying the mitoK_{ATP} subunits. Lacza *et al.*[11] used immunoblotting and immunogold electron microscopy to show that both Kir6.1 and Kir6.2 are present in isolated heart mitochondria. Singh *et al.*, also noted colocalization of Kir6.1 and Kir6.2 with Mitofluor Red in intact rat ventricular myocytes[12]. By contrast, Jiang *et al.* reported only Kir6.2 in their mitochondrial membrane preparations, from which they made single channel measurements [13]. Further yet, Kuniyasu *et al.*[14] detected neither Kir6.1 nor Kir6.2 in rat heart mitochondria, despite ample signal in a microsomal fraction. Clearly, there is little consensus; This, in turn has hampered progress in the field of K⁺ channel-mediated cardioprotection.

In this study, we sought to clarify whether Kir6.1 could be a subunit of bovine heart mitoK_{ATP}. We report identification of two distinct mitochondrial proteins that were tracked and purified on the basis of Kir6.1 immunoreactivity, using antibodies available commercially. Mass spectrometry of the isolated proteins revealed that neither antibody bound *bona fide* Kir6.1 from bovine heart mitochondria. Nor was Kir6.1 present in a thorough, scaled-up proteomic analysis of the purified fractions. The data are discussed in light of published literature and future prospects for the identification of mitoK_{ATP} subunits.

Methods

Isolation of Mitochondria and mitochondrial inner membranes

Hearts were minced and homogenized in 10 mM HEPES, 220 mM mannitol, 70 mM Sucrose, 1mM EGTA, pH 7.4 (MSE buffer). pH was adjusted to 7.8 with NaOH prior to centrifuging at 1100 × g. The supernatant was collected and re-adjusted to pH 7.8 prior to centrifugation at 8000 × g to obtain a crude mitochondrial pellet which was then purified by discontinuous gradient centrifugation according to Rehncrona *et al.*[15] (Figure 1A) or Storrie & Madden [16] (all other figures). Mitoplasts and mitochondrial inner membranes were subsequently isolated as per Maisterrena *et al.* [17].

Immunoblot analysis

Proteins samples were subjected to SDS-PAGE, blotted, and probed with the following antibodies: Na⁺/K⁺-ATPase α1 (Santa Cruz, SC-21712) , SERCA (Affinity BioReagents, MA3-910) , COXIV (Invitrogen, A21349), Kir6.1 “R-14” (Santa Cruz, , SC-11224), Kir6.1 (Alomone Labs, APC105), VDAC “D-16” (Santa Cruz, SC-3203) HRP-conjugated anti-mouse (NXA931) and anti-rabbit (NA9340V) secondary antibodies were obtained from GE-Biosciences. Blots were finally treated with SuperSignal West Pico Reagent (Pierce).

Immunoelectron microscopy

Small minced pieces of rat heart (approx 2mm × 2 mm) were fixed in 3% paraformaldehyde and prepared for immunoelectron microscopy as described by Tokuyasu [18]. Sections were

finally stained in 2% methylcellulose, 0.3% uranyl acetate for 10 min at 4°C. Sections were viewed on a Hitachi H-7600 Transmission Electron Microscope equipped with an AMT CCD-based camera system.

Confocal Microscopy

Bovine ventricles were cut into small pieces, fixed (3% paraformaldehyde), frozen, sectioned (8 µm), permeabilized (2 × 15 min; 0.1% (w/v) Triton X-100 in Tris-buffered saline), and blocked (5% w/v milk powder in TBS) prior to incubation with primary antibody (~4µg/mL; Kir6.1, Alomone; ATP synthase, Invitrogen). After 1hr at room temperature (or overnight at 4°C), sections were washed with TBS prior to application of secondary antibody (~4µg/mL; anti-rabbit-FITC; anti-mouse Alexa 568) for 1hour at room temperature. Secondary antibody was washed away with TBS prior to applying the mounting medium, VectaShield (containing DAPI), and sealing the coverslips. Sections were analyzed on a Zeiss LSM510 Meta, confocal microscope, using Plan-Apochromat 63x/1.4 Oil DIC lenses.

Purification of Kir6.1-Immunoreactivity from Bovine mitochondrial inner membranes Density gradient centrifugation

Mitochondrial membranes (5 mg protein/mL) were solubilized, clarified by centrifugation and subjected to discontinuous sucrose gradient centrifugation essentially as described by Hanson *et al.*[19].

FPLC Chromatography

Sucrose gradient fractions that harbored immunoreactivity to Kir6.1 were pooled and subjected to FPLC chromatography (ÄKTA system; GE Biosciences) using either a Resource Q column (to resolve the 51kDa band detected by anti-Kir6.1 (SC)) or a Mono S column (to resolve the 48kDa band detected by anti-Kir6.1 (Alo)). Proteins were eluted with linear gradients of NaCl as depicted in the figures 2 & 4. Detailed descriptions of the buffer conditions and chromatographic parameters are found in the online supplement.

Separation of Proteins & Preparation of Gel Bands for Mass Spectrometry

Proteins were separated using SDS-PAGE, Blue-native PAGE, and 2D gels (Invitrogen). Gels were stained with colloidal Coomassie (Simply Blue, Invitrogen; Imperial Blue, Pierce). Gel bands were excised from the gel, minced into 1 mm × 1mm pieces, and processed for in-gel trypsinization as described by Schevchenko *et al.* [20]

LC-MS/MS analysis

Tryptic digests (from gel bands or bulk digestion) were subjected to Nano-flow HPLC on a C18 column. Peptides were eluted, at 300 nanoliters per min, into an LTQ ion trap mass spectrometer (ThermoFinnigan) for MS/MS. Detailed HPLC gradient and LTQ instrument parameters can be found in the online supplement. Database searches (NCBI nr GB_158 20070316) were conducted with Mascot (Matrix Science, London, UK; version 2.2.0), and X! Tandem (www.thegpm.org; version 2007.01.01.1) restricted to mammals (429574 sequences). Carbamidomethylation of cysteine and oxidation of methionine were allowed as variable modifications. The peptide mass tolerance was set at ± 1.5 Da and the fragment mass tolerance was ± 0.8 Da. Protein identification was validated using Scaffold (version Scaffold-01_07_00, Proteome Software Inc., Portland, OR). Tabulated proteins contain at least two peptides identified with >80% confidence, yielding protein identification at >95% confidence.

Results

1. Localization, Purification and Identification of a 51-kDa Kir6.1-immunoreactive band

Initial blotting experiments revealed a 51-kDa Kir6.1-immunoreactive protein in bovine heart homogenates, using anti-Kir6.1 (R-14) from Santa Cruz Biotechnologies. Figure 1 shows the specific immunoreactivity (i.e. immunoreactivity per μg protein loaded in a given lane) of the 51-kDa band increased relative that found in to whole tissue homogenates (fig. 1 *cf.* lanes 6 or 7 vs. lane 1) and was present in mitochondrial inner membranes across species (cow, fig. 1A; rat, fig. 1B) and tissues (heart, fig. 1A; liver and brain, fig. 1B).

The results of mitochondrial membrane fractionation were corroborated by immunogold electron microscopy (Figure 1C, D). Ultrathin sections of rat heart probed with the Kir6.1 (R-14) and gold-conjugated secondary antibody clearly showed that Kir6.1-immunoreactivity was present in mitochondria in an otherwise clear field of myofilaments. At higher magnification (fig. 1D), localization to the inner membrane can be discerned.

Mindful that immunoreactivity implies but does not prove identity, we sought to purify the “mitoKir6.1” to the point where it might be identified by MS/MS analysis. As shown in figure 2A, the 51-kDa band copurified with a defined set of proteins of fixed stoichiometry, after sorting mitochondrial membrane extracts by molecular weight (sucrose gradient centrifugation; fig. 2A) and by charge (Resource Q FPLC; fig. 2B). Native-gel electrophoresis (fig. 2C) indicated that the Kir6.1-immunoreactive protein did not exist on its own, but co-migrated with the major Coomassie Blue-stained protein complex running at approx. 800 kDa, whose composition was determined by LC-MS/MS to be OXPHOS complex I (Online Table 1).

Given that Complex I has its own 51-kDa subunit, we reasoned that “mitoKir” could be distinguished by its characteristic isoelectric point under denaturing conditions. Immunoblot analysis of 2D gels revealed that the immunoreactive bands co-migrated with the main spots stained with Coomassie Blue (fig. 2D). From the 2D gels, the experimentally-determined molecular weights of 51 kDa and pI of 8.4 were consistent with the theoretical mass (50651.8 Da) and pI (8.37) of the 51 kDa subunit of complex I. (i.e. NADH dehydrogenase flavoprotein I gi:109939909). By contrast, the theoretical molecular weight and isoelectric point of bovine Kir6.1 (gi:95147668) are 47967 Da and 9.38 respectively.

The identity of the 51-kDa band was subsequently determined by excising it from a denaturing SDS gel and conducting MS/MS analysis (Figures 2E & 2F). As foreshadowed by the 2D analysis, the preponderant protein within the gel band was indeed 51-kDa subunit of Complex I. Moreover, though we also identified four other contaminating proteins (Online Table 2), no Kir6.1 was found. Finally, given that Kir6.1, if present, might be less abundant than Complex I, we performed MS/MS analysis on a bulk digest of 100 μg of purified Complex I. Again this allowed us to identify up to 39 subunits of Complex I and several trace contaminants, but no Kir6.1 (Online Table 3).

2. Localization, Purification and Identification of 48-kDa Kir6.1-immunoreactive band

For subsequent work we used a Kir6.1 antibody from Alomone labs. As shown in fig. 3A, the antibody recognized a 48-kDa protein by immunoblot analysis that was enriched substantially in bovine heart mitochondria. Localization to the mitochondria was confirmed by immunofluorescence (fig. 3B). A putative Kir6.1 signal (FITC) co-registered with the location of ATP synthase (Alex-568).

To isolate the immunoreactive band, mitoplasts were solubilized as before and subjected to sucrose density gradient centrifugation. Immunoreactivity was found in fractions 8 and 9 (fig

4A). These fractions were pooled and refractionated by FPLC chromatography on a Mono S column as depicted in fig. 4B. Unexpectedly, the immunoreactivity eluted broadly across the applied gradient, though other proteins eluted as distinct peaks. The cause of the broad elution profile is unclear. The fractions containing the 48-kDa band were pooled and concentrated in a Centriprep YM-30 prior to gel and MS analysis.

Coomassie blue-stained SDS-gel analysis of the Pooled Mono S fraction reveals 5 or 6 prominently stained protein bands. The 48-kDa band (Figure 4C) was excised for mass spectral analysis. LC-MS/MS identified the prominent 48-kDa band as mitochondrial isocitrate dehydrogenase (NADP-binding form; IDH2; Figure 4D), though 9 other proteins at or near 48-kDa found in the gel band are listed in Online Table 4. Three of the nine proteins (IDH2, acetyl-CoA acetyltransferase 1 and alpha-methylacetyl-CoA racemase) have theoretical isoelectric points consistent with that observed for the 48-kDa immunoreactive band ($pI \approx 9$; fig. 4E).

As before, we also performed larger bulk tryptic digests on fractions spanning the broad peak Mono S peak containing Kir6.1(Alo) immunoreactivity. Owing to the sensitivity of the analysis over 135 proteins were identified with greater than 95% confidence. (Online Table 5). Neither members of the Kir6.x family, nor the SUR family were found in this fraction.

Discussion

The existence of the mitoK_{ATP} channel has been amply validated by measuring single channel conductances in mitoplasts or mitochondrial membranes reconstituted into planar lipid bilayers (reviewed in ref. [4]). Nevertheless, sixteen years after its discovery[21], there is no consensus regarding the subunit composition of mitoK_{ATP}. Yet, establishing the identity of the subunits is critical to the development of genetic tools with which to manipulate channel function. Such tools would, no doubt, inform the emerging debate as to whether the salutary effects of KCOs stem from their specific K⁺-channel-dependent actions or their non-specific, K-channel-independent, effects on mitochondrial respiration. [22].

The overlapping pharmacology of sK_{ATP} and mitoK_{ATP} channels suggests a certain structural homology between the channels. For instance, by co-expressing the various combinations of SUR and Kir6.x subunits, we showed that the pharmacology of mitoK_{ATP} activation and inhibition can be approximated by SUR1-Kir6.1 channels and, to a lesser degree, by SUR2B-Kir6.1 channels[23]. Yet we found little evidence for Kir6.1 (or Kir6.2) immunolocalization in mitochondria by immunofluorescence using antibodies available at the time[24], an observation corroborated Kuniyasu *et al.*[14]. Others have however[11;12;25;26;27]. Resolving this discrepancy may prove problematic, given the confusion over something as simple as the molecular weight of the Kir6.x subunits *in vivo*. Estimates for Kir6.1 ($MW_{theo}=48$ kDa) vary between 42, 48, and 51 kDa [11;12;25;26]. Equally perplexing, reports peg the observed molecular weight of Kir6.2 anywhere between 40 and 56 kDa ($MW_{theo}=42$ kDa)[13;27]

To resolve some of the confusion, we endeavoured purify and identify any Kir6.x immunoreactive protein that might be detected in mitochondria, using commercially available antibodies. Anti-Kir6.1 “R-14”, from Santa Cruz Biotechnologies, revealed a strong immunoreactive band in heart homogenates at 51 kDa, consistent, within reason, not only with the predicted molecular weight of 48 kDa, but also with published studies[11;26]. We then purified the 51-kDa band, and ultimately identified it by MS/MS as the matrix-facing NADH dehydrogenase flavoprotein (fp) 1 subunit of Complex I. This assignment is corroborated by the fact that the isoelectric point of the 51-kDa band is consistent that of NADH dehydrogenase

fp 1, but not Kir6.1. Moreover, the immunoreactivity co-migrated with intact OXPHOS Complex 1 by native gel electrophoresis.

Using a similar purification strategy and a Kir6.1 antibody from Alomone Labs we enriched a Kir6.1 immunoreactive species to point where its physical properties (MW and pI) were consistent with 3 proteins found in 48 kDa gel band, the most abundant of which was isocitrate dehydrogenase 2 (IDH2). Though these enzymes share a similar molecular weight and isoelectric point with Kir6.1, the latter is, of course, an integral membrane protein. We note that the 48-kDa band co-segregated with the matrix fraction following sonication of the mitoplasts and centrifugation to remove the membranes (Figure 4F). This experiment was particularly revealing since this Kir6.1 antibody did recognize Kir6.1 overexpressed transiently in HEK293 cells (Online Data Supplement). Therefore, if Kir6.1 were, in fact, a subunit of mitoK_{ATP}, one would expect residual Kir6.1 signal in the mitochondrial membranes in Figure E, notwithstanding the cross-reactivity in to IDH2 in the matrix fraction. Thus, it seems there is little Kir6.1 to be found in bovine heart mitochondria. Our data is consistent with Kuniyasu *et al.* [14], who observed Kir6.1 in rat heart, but not their mitochondria.

Our data may bear on the interpretation of select observations from published reports. Lacza *et al.* detected a 51-kDa Kir6.1 band in isolated rat heart mitochondria [11], using a Kir6.1 antibody from Santa Cruz. A 51-kDa band was also observed by Suzuki *et al.* [25] in rat skeletal muscle and rat liver mitochondria, using a 'home-grown' antibody whose antigen corresponds to amino acid residues 375-386 of Kir6.1. In their study, the mitochondrial band (51 kDa) ran higher on SDS-gels than that of transiently expressed Kir6.1 (49 kDa) and major immunoreactive bands from skeletal muscle homogenates (49 kDa), and hepatocyte plasma membrane vesicles (47 kDa). Barring extensive post-translational modification, it is conceivable that the mitochondrial 51-kDa band observed in their studies may correspond to the NADH dehydrogenase identified by the Santa Cruz Kir6.1 R-14 antibody.

The data underscore one of the potential sources of discrepancy between published reports on mitoK_{ATP}. Namely, though many groups have noted Kir6.x or SUR subunit immunoreactivity in mitochondria, few have endeavoured to purify the channel to homogeneity and confirm the identity of these immunoreactive proteins unequivocally by tandem mass spectrometry. We submit that though researchers may tacitly acknowledge that antibody-based studies can yield misleading results, the probability may well have been underestimated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Detection of Kir6.1 Immunoreactivity in Mitochondrial Membranes

Mitochondrial membrane isolation. Kir6.1 immunoreactivity (anti-Kir6.1 (SC)), was detected in bovine (panel A), rat (panel B) mitochondrial membranes. This immunoreactivity paralleled that of CoxIV, an inner membrane marker, even as markers of the plasma membrane (Na⁺/K⁺ ATPase) and sarcoplasmic reticulum (SERCA) declined. This trend was also observed in rat tissues, including liver and brain (Panel B). *Immuno-electron microscopy.* The Kir6.1 (SC) antibody labels mitochondria specifically. Magnification: 40,000 × (Panel C); 100,000 × (Panel D).

Figure 2. Purification, Characterization and Identification of the 51-kDa Immunoreactive Band
Mitochondrial membranes were solubilized separated by density gradient centrifugation. Kir6.1(SC) antibody recognized a 51kDa protein in fractions containing 30% and 27.5% (w/v) sucrose (Panel A). The fractions were pooled and subjected to FPLC on a Resource Q column eluted with a linear gradient of KCl (Panel B). Fractions containing the 51kDa band were pooled for further analysis. Native gel electrophoresis (Panel C) revealed that the proteins within the fraction migrate as a macromolecular complex with a MW of approx 800 kDa. 2-D gels (IEF/SDS-PAGE; Panel D) show that Kir6.1 immunoreactivity migrates to isoelectric points between 7.5 and 8.4. The 51-kDa Coomassie-stained band that co-migrated with the Kir6.1-immunoreactive band was resolved by SDS-PAGE, excised and processed for MS/MS analysis (Panel E), which identified the predominant protein as NADH-dehydrogenase flavoprotein I. Panel F depicts the MS/MS spectrum of a unique peptide, NACGSYDFDVFVVR (Mascot ion score: 113, Peptide confidence: 95%, Protein confidence 100%).

Figure 3. Mitochondrial Kir6.1-Immunoreactivity Revealed by a Second Antibody

Kir6.1 immunoreactivity, assessed with an antibody from Alomone Labs, was detected in bovine mitoplasts (Panel A). Immunoreactivity to Kir6.1 paralleled that of ATP synthase, (beta subunit) through mitochondrial isolation, even as myofilament (cTnI) plasma membrane (Na⁺/K⁺ ATPase) and sarcoplasmic reticulum (SERCA) markers declined. Mitochondrial localization of the Kir6.1 signal was confirmed by immunofluorescence microscopy (Panel B). Sections from rat heart ventricles were fixed, prepared and probed as described in the methods section. Kir6.1-immunoreactivity coregisters with that of ATP synthase in sections from bovine ventricle.

Figure 4. Purification, Characterization and Identification the 48-kDa immunoreactive band
Mitochondrial membranes were solubilized and separated by density gradient centrifugation (Panel A). Kir6.1 antibody from Alomone Labs recognized a 48-kDa protein predominantly in fractions containing 17.5% and 15% (w/v) sucrose. The fractions were pooled and subjected to FPLC on a Mono S column eluted with a linear gradient of KCl. (Panel B). Fractions containing the 48-kDa band were pooled for further characterization. The Coomassie-stained band at 48 kDa was resolved, excised and prepared for MS/MS analysis (Panel C). The MS/MS spectrum (Panel D) corresponding to the sequence, DQTNDQVTIDSALATQK (Mascot Score: 135, Peptide confidence: 95%, Protein confidence: 100%) is one of the unique peptides that identifies mitochondrial isocitrate dehydrogenase (NADP-binding form) as the preponderant 48kDa band. The isoelectric point of the Kir6.1 immunoreactivity (pI≈9; Panel E) was consistent with IDH2. Finally, sonication of mitochondria and subsequent centrifugation to separate membrane proteins from those of the matrix (Panel E) revealed that Kir6.1 immunoreactivity was found in the supernatant (matrix) fraction, inconsistent with a *bona fide* Kir6.1 channel.