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Expression, purification, and evidence for the interaction of the two nucleotide-binding folds of the sulphonylurea receptor

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Abstract

The ATP-sensitive potassium channel is made up of four pore forming Kir6.2 subunits, surrounded by four regulatory sulphonylurea receptor (SUR) subunits. The latter subunit contains two nucleotide-binding folds (NBFs) that confer the ability on the channel to sense changes in the metabolic status ([ATP]/[ADP]) of the cell and couple the changes to the membrane potential of the cell. In an attempt to better understand the mechanisms by which NBFs influence the activity of the channel, we have expressed the NBF domains with C-terminally added epitopes (FLAG to NBF1 and His_6 to NBF2) in *Escherichia coli* and the rabbit reticulocyte lysate system and examined the ability of these domains to interact with each other and with Kir6.2. Both NBFs could be expressed to high levels in *E. coli* and purified to homogeneity from inclusion bodies. Re-folding of the proteins proved to be unsuccessful. However, we were able to obtain small amounts of radio-labelled NBFs in a soluble state. Using co-immunoprecipitation, we demonstrate that the radio-labelled NBF1 and NBF2 interact with each other. Neither of the NBFs bound to Kir6.2 expressed in the presence of canine microsomes.

Keywords: ATP-sensitive potassium channel; Sulphonylurea receptor; Nucleotide-binding folds; Kir6.2; Interactions of NBFs

ATP sensitive potassium (K_{ATP}) channels are activated by ADP and inhibited by ATP; activation leads to hyperpolarisation and inhibition causes depolarisation of the cell membrane (for reviews see [1–3]). Cells expressing K_{ATP} channels therefore possess the unique ability to couple the metabolic status, i.e., [ATP]/[ADP], of the cell to its membrane electrical activity. This property is crucial for a range of physiological functions, including glucose stimulated insulin secretion by pancreatic β-cells.

The ability of K_{ATP} channels to respond to changes in [ATP]/[ADP] is associated with the nucleotide binding domains of the channel protein. The pancreatic channel is an octomer composed of four subunits each of Kir6.2 and the sulphonylurea receptor1 (SUR1) [4–6]. SUR1, a member of the ATP-binding cassette (ABC) protein superfamily, contains two intracellular nucleotide-binding folds (NBF), referred to as NBF1 and NBF2 (Fig. 1A) [4,7]. Kir6.2 is a member of the inwardly rectifying potassium (Kir) channel subfamily and forms the K^+ conducting pore of the channel. Although this subunit does not contain a classical consensus sequence capable of binding nucleotides, recent evidence suggests that residues of the intracellular N- and C-terminal domains can contribute to an inhibitory ATP binding site [8–10].

Mutations introduced into the NBFs of SUR1 alter the sensitivity of the channel to [ATP]/[ADP] ratio, indicating that nucleotides also control the gating of channel through these domains [11,12]. Furthermore, the majority of known mutations that cause persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI), a genetic disorder characterised by an excessive secretion of insulin leading to severe hypoglycaemia, are localised in these domains [13,14]. Heterologous expression studies showed that these mutations reduce the sensitivity of the channel to [ATP]/[ADP] ratio [15,16]. Thus there is compelling evidence that NBFs control the activity of K_{ATP} channels. Recent evidence strongly suggests that NBFs also play important roles in membrane trafficking, as certain PHHI mutations located in the NBFs prevent the trafficking of K_{ATP} channels to the cell surface [17,18].

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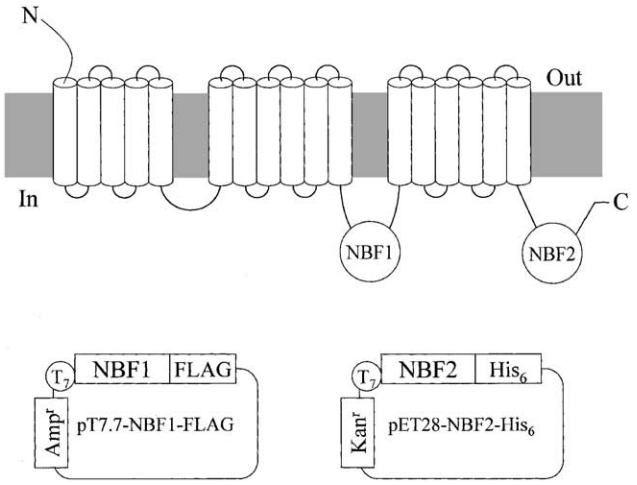

Fig. 1. Schematic representation of: (A) the SUR1 subunit of the K_{ATP} channel showing transmembrane helices (cylinders) and the intracellular nucleotide binding folds (NBF1 and NBF2); (B) the expression vector constructs used to express the NBF1–FLAG and NBF2–His_6 fusion proteins; T_7 represents the promoter.

Using 8-azido-[^{32}P]ATP as a photoaffinity label, Ueda and coworkers [19–22] demonstrated that SUR1 binds ATP at NBF1 and Mg-ADP at NBF2. Moreover, the authors [21] found that the binding of ADP at NBF2 stabilises the binding of ATP at NBF1, thereby suggesting a potential interaction between the two domains. This interaction may be critical for sensing changes in intracellular [ATP]/[ADP] and communicating the signal to the pore domain. But the question as to how the signal might be transmitted to the pore domain (Kir6.2) remains unanswered. It is possible that NBFs may transmit the signal to the pore domain via the transmembrane portion of SUR1 or by direct interaction with the cyptoplasmic portion of Kir6.2.

Here we have investigated if the two NBFs interact with each other directly and if any of these domains interact with Kir6.2. As mentioned above, K_{ATP} channels have a complex structure; this makes the interactions very difficult to investigate with the intact channel. To circumvent this difficulty, we have expressed the NBFs as independent domains in *Escherichia coli* and in the rabbit reticulocyte lysate system and used biochemical approaches to examine if they interact with each other and Kir6.2. Here we show that: (i) NBF1 and NBF2 could be overexpressed in *E. coli* and purified; however, the domains could not be re-folded (ii) NBF1 and NBF2 physically interact with each other, and (iii) NBFs expressed in vitro do not interact with the membrane bound Kir6.2.

Materials and methods

Materials. Plasmid vectors pET28a and $pcDNA_3$ were obtained from Novagen and Invitrogen, respectively. pQE70 was from Qiagen. pT7.7 was a gift from Dr. S. Tabor, Harvard University. BL21(DE3) *E. coli* cells were obtained from Novagen. Anti-FLAG M2 antibody and Anti-FLAG M2 affinity resin were purchased from Sigma Chemical. Ni–NTA agarose was from Qiagen. Protein G–Sepharose was from Upstate Biotechnology. The Quick TNT coupled transcription/translation kit was from Promega. [^{35}S]Methionine was from ICN. Biotinylated nitriloacetic acid was purchased from Molecular Probes. All other reagents were from Sigma Chemical. Mouse Kir6.2 (GenBank Accession No. D50581) Hamster SUR1 (GenBank Accession No. L40624) cDNA clones were gifts from Dr. S. Seino and Dr. J. Bryan, respectively.

Construction of expression vectors. The region of cDNA encoding NBF1 (amino acid residues 674–941) was amplified by PCR using a sense primer that contained an *Eco*RI site and an anti-sense primer that had a *Hin*dIII site at the 3′ end. The resultant PCR product was restricted with *Eco*RI and *Hin*dIII and subcloned into the pKS-FLAG vector [23] such that NBF1 is in-frame with the FLAG epitope. The resultant NBF1–FLAG fusion sequence was subcloned into the *E. coli* expression vector pT7.7. The resultant construct was referred to as pT7.7–NBF1–FLAG. To allow co-expression with the NBF2–His_6 fusion protein (see below), the NBF1–FLAG sequence was subcloned into pET28a vector (the resultant clone referred to as pET28a–NBF1–FLAG), which contained the kanamycin resistance gene. For NBF2 expression, the region of cDNA encoding NBF2 (residues 1342–1582) was amplified by PCR using primers that yielded a product with an *Eco*RI site at the 5′ end and a *Bam*HI site at the 3′ end. The product was subcloned into the *Eco*RI and *Bam*HI sites of pQE70. The resultant expression construct has the NBF2 sequence in-frame with a hexa-his (His_6) sequence at the 3′-end. The fusion sequence NBF2–His_6 was subsequently subcloned into pT7.7 (referred to as pT7.7–NBF2–His_6) to allow pulse labelling (see below).

Expression of NBFs in E. coli. To express the proteins, pT7.7–NBF1–FLAG and pT7.7–NBF2–His_6 were separately transformed into BL21(DE3) cells. The resultant transformants were cultured at 37 °C, with shaking at 200 rpm, in Luria–Bertani (LB) medium supplemented with 100 μg/ml ampicillin. Where co-expression of both proteins was desired, BL21(DE3) cells were transformed with pET28a–NBF1–FLAG and pT7.7–NBF2–His_6 and cultured in the presence of 100 μg/ml ampicillin and 30 μg/ml kanamycin. Expression was induced by the addition of 0.25 mM isopropyl-β-thio-D-galactoside (IPTG) when the A_{600} of the culture reached ~0.6; the cultures were incubated for a further 4 h at 30 °C before harvesting the cells by centrifugation at 5000*g* for 15 min.

Purification of NBFs from the inclusion bodies. To isolate the inclusion bodies the cell pellets were resuspended in 1/20th the original culture volume of 20 mM Tris–HCl/100 mM NaCl, pH 8.0, containing 50 μg/ml lysozyme, and incubated at 4 °C for 30 min. Cells were then lysed by sonication. After removing unbroken cells by a low speed centrifugation (1000*g* for 5 min), inclusion bodies were harvested by centrifugation at 13,000*g* for 10 min. Inclusion bodies containing the NBF1–FLAG fusion protein were solubilised in 8 M urea/20 mM Tris–HCl/50 mM NaCl, pH 7.5. After diluting the suspension 10-fold with 20 mM Tris–HCl/50 mM NaCl, NBF1–FLAG was purified using the anti-FLAG M2 affinity resin according to the instructions of the manufacturer. The resin bound protein was eluted with 0.1 M glycine–HCl, pH 3.5. Inclusion bodies containing the NBF2–His_6 fusion protein were dissolved in 6 M guanidium hydrochloride/0.1 M NaH_2PO_4/10 mM Tris–HCl, pH 8.0. NBF2–His_6 was purified using the Ni–NTA agarose according to the instructions of the manufacturer (Qiagen). NBF2–His_6 was eluted with 100 mM imidazole/100 mM EDTA/0.1 M NaH_2PO_4/10 mM Tris–HCl, pH 8.0.

SDS–polyacrylamide gel electrophoresis and Western blotting. Proteins were separated on a 12% polyacrylamide gel and stained with coomassie blue or, after transfer to nitrocellulose filters, subjected to Western blotting using anti-FLAG M2 antibodies and the HRP conjugate of anti-mouse antibodies to detect NBF1–FLAG. NBF2–His_6 was detected using Ni^{2+}-charged biotinylated nitriloacetic acid and the HRP-conjugate of streptavidin [24]. The proteins were detected using the enhanced chemiluminiscence (ECL) system (Amersham).

In vivo radio-labelling of NBFs with [^{35}S]methionine in E. coli. Exclusive radio-labelling of the NBF fusion proteins was performed using a method described previously [25]. Briefly, BL21(DE3) cells harbouring the expression vector constructs were grown in M9 medium supplemented with all amino acids except cysteine and methionine. Following induction with IPTG (30 min), cultures were treated with rifampicin (0.2 mg/ml) for 30 min. The cells were then pulsed with [^{35}S]methionine (10 μCi/ml) for 5 min. The cells were collected by centrifugation and resuspended in IP buffer (20 mM Tris–HCl/100 mM NaCl/1 mM EDTA 0.5% Triton X-100/0.2 mM phenylmethylsulphonylfluoride, pH 7.5) by thorough vortexing followed by incubation at 4 °C for 30 min with shaking. The suspension was centrifuged and the supernatant was collected for analysis.

In vitro radio-labelling of proteins with [^{35}S]methionine using rabbit reticulcyte lysate system. The pT-7.7 constructs of NBF1–FLAG and NBF2–His$_6$ were used as templates in the T7 promoter-based Quick TNT coupled transcription/translation system to label NBFs with [^{35}S]methionine (according to the instructions provided by Promega). The Kir6.2 sequence was translated in the presence of canine microsomal membranes using the pcDNA3–Kir6.2 construct. The membrane bound Kir6.2 was separated from the unbound by centrifugation at 105,000*g* for 60 min followed by washing with phosphate-buffered saline.

Assay of protein–protein interactions. For this, NBF1–FLAG and NBF2–His$_6$ were first labelled with [^{35}S]methionine either individually or together using methods described above. The labelled proteins were diluted with the IP buffer and incubated for 1 h (4 °C with shaking) with 10 μl of anti-FLAG M2 affinity resin in a final volume of 100 μl. The resin was washed with IP buffer three times by centrifugation. The resin was then washed quickly with 0.1 M glycine–HCl, pH 2.0. SDS–PAGE sample buffer was then added to the resin and after incubation at 95 °C for 5 min, the samples were subjected to SDS–PAGE/fluorography.

Assay of NBF binding to membrane bound Kir6.2. Kir6.2 was labelled with [^{35}S]methionine in the presence of microsomal membranes as described above. To aliquots of labelled Kir6.2, radio-labelled NBFs were added. Following incubation at 30 °C for 30 min, membrane fractions were isolated by centrifugation and, after washing with PBS, subjected to SDS–PAGE/fluorography.

Results

Expression of NBFs in E. coli

To express the nucleotide binding folds, sequences corresponding to NBF1 (residues 674–941) and NBF2 (residues 1342–1582) were amplified by PCR and subcloned into T7 promoter-based expression vectors (Fig. 1). FLAG and hexa-histidine (His$_6$) epitopes were fused to the C-terminal ends of NBF1 and NBF2, respectively to allow purification of the expressed proteins by affinity chromatography. The fusion constructs, NBF1–FLAG and NBF2–His$_6$, were expressed in BL21(DE3) *E. coli* cells. SDS–PAGE analysis (Fig. 2A) revealed that upon induction with IPTG, both domains could be expressed to high levels (lanes labelled I). On fractionation, almost all the expressed protein was found in inclusion bodies (lanes labelled In), with no detectable NBFs being found in the soluble fraction (data not shown). Attempts to increase the protein in the soluble fraction by a range of manipulations, including reducing the incubation temperature to 18–20 °C, were unsuccessful. The two NBFs were co-expressed in *E. coli* in the hope that, if they associate, the complex formation might prevent the inclusion body formation. Unfortunately, however, although both proteins could be co-expressed to high levels (Fig. 2A, lane I), inclusion body formation could not be prevented.

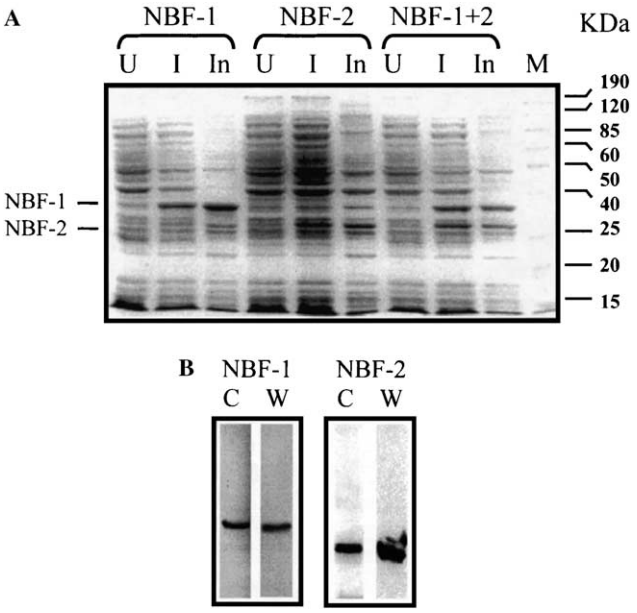

Fig. 2. Expression and purification of NBF1–FLAG and NBF2–His$_6$. (A) SDS–PAGE analysis of proteins isolated from BL21(DE3) cells harbouring expression vector constructs, before (lanes U) and after (lanes I) induction of protein expression with IPTG. Lanes In represent proteins present in the inclusion bodies. Lane M has the marker proteins with sizes indicated on the left. Positions of NBF1 and NBF2 are shown on the right. (B) Purified NBFs were separated on a 12% SDS–PAGE and stained with Coomassie blue (lanes C) or subjected to Western blotting (lanes W). For details see the Materials and methods.

Purification of NBFs

Inclusion bodies were dissolved in urea or guanidium hydrochloride and the NBFs were purified under denaturing conditions: anti-Flag M2 affinity resin was used to purify NBF1–FLAG and Ni^{2+}–NTA resin to purify NBF2–His$_6$. The purified proteins showed bands of expected size (32 kDa for NBF1–FLAG, 29 kDa for NBF2–His$_6$) on SDS–PAGE (Fig. 2B). Western blot analysis using anti-FLAG antibodies or nickel charged biotinylated NTA reagent (specific for His$_6$ epitope) confirmed the presence of epitopes in the purified proteins (Fig. 2B). Attempts were made to renature the purified proteins by dialysis against buffers containing decreasing concentrations of urea. However, as the urea concentration was lowered to below 4 M, the proteins began to aggregate (NBF1, as judged by gel filtration) or precipitate (NBF2). Inclusion of adenine nucleotides

and Mg^{2+} (ligands for NBFs) failed to improve the solubility. Similarly, use of nondetergent sulphobetaines (NSBD) compounds, which has been reported to improve re-folding of proteins [26], or DTT, which prevents disulphide bridges between protein molecules, also proved to be unsuccessful.

Expression of NBFs as [^{35}S]methionine-labelled proteins in a soluble form

The inability to obtain the NBFs in a soluble form prevented us from carrying out biochemical/structural studies on these domains. To circumvent this, we have labelled the NBFs with [^{35}S]methionine briefly (5 min) in the hope that it might be possible to isolate the labelled species in a soluble state, before they aggregate into inclusion bodies. We stopped the synthesis of the host cell proteins by treating the cells with rifampicin for 30 min prior to the addition of [^{35}S]methionine. Since rifampicin does not affect the synthesis of proteins from T7-based plasmid vectors, this has allowed us to label the NBFs exclusively. Fig. 3 shows radio-labelled NBF bands in the induced (I), but not the uninduced (U), lanes. Rifampicin treatment prevented the labelling of *E. coli* proteins without affecting the labelling of plasmid-encoded NBFs (lanes labelled R). A significant proportion (~20%) of the labelled proteins could be solubilised with denaturant-free buffers. These results demonstrate that even though NBFs have a high propensity to form inclusion bodies, it is possible to obtain small amounts of these proteins in a soluble form that could potentially be used to examine the biological function.

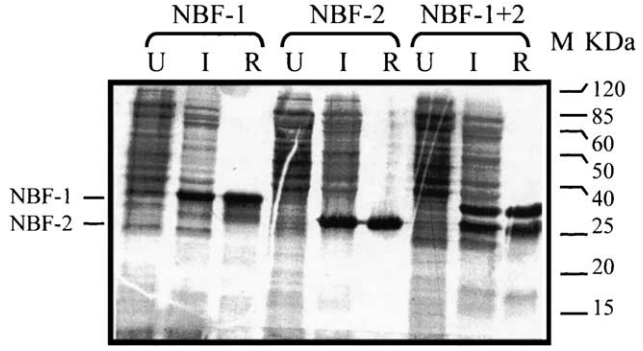

Fig. 3. Pulse-labelling of NBF1–FLAG and NBF2–His_6 with [^{35}S]methionine. BL21(DE3) cells harbouring expression vector constructs were treated with [^{35}S]methionine for 5 min to radio-label the proteins. The labelled proteins were separated on a 12% SDS–PAGE and detected by fluorography. Labelling was performed on cells before (lanes U) and after (lanes I) induction of protein expression with IPTG. Lanes R show proteins isolated from induced cells treated with rifampicin for 30 min prior to radio-labelling. Lane M has the marker proteins with sizes indicated on the left. Positions of NBF1 and NBF2 are shown on the left.

NBF1 co-immunoprecipitates NBF2

We have used the in vivo radio-labelled NBFs to test the proposition that NBF1 and NBF2 interact with each other. For this, we have extracted the labelled NBFs with the immunoprecipitation (IP) buffer and incubated the extracts with the anti-FLAG affinity resin. After washing the unbound proteins away, the resin bound proteins were analysed by SDS–PAGE/fluorography. Fig. 4A shows that, as expected, the anti-FLAG affinity resin binds NBF1–FLAG, but not NBF2–His_6 (lanes P). However, when both proteins were present, the resin retained NBF2–His_6 along with NBF1–FLAG. The corresponding supernatants (lanes SN) show depletion of labelled proteins from reactions containing NBF1–FLAG. These data support the proposition that NBF1 interacts with NBF2.

To confirm these findings, we have used an independent expression system. We have expressed the NBFs as [^{35}S]methionine-labelled proteins using the rabbit reticulocyte lysate in vitro translation system, and examined the ability of anti-FLAG antibody to co-immunoprecipitate NBF1–FLAG with NBF2–His_6. The data in Fig. 4B show that the anti-FLAG antibody indeed

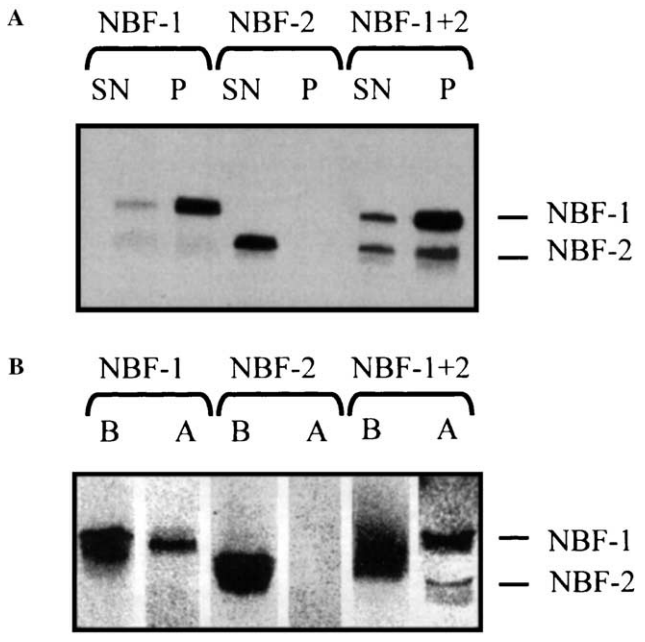

Fig. 4. Co-immunoprecitation of NBF1–FLAG and NBF2–His_6. (A) Co-immunoprecipitation of NBFs radio-labelled in *E. coli*. NBFs were uniquely radio-labelled with [^{35}S]methionine as described in Fig. 3. Proteins were extracted with IP buffer and adsorbed on the anti-FLAG M2 affinity resin. Proteins bound to the resin (lanes P) and left in the supernatants (lanes SN) were separated by SDS–PAGE and subjected to fluorography. (B) NBFs were radio-labelled with [^{35}S]methionine using the TNT transcription–translation system and subjected to immunoadsorption as described in (A). Samples before (lanes B) and after (lanes A) immunoadsorption were subjected to SDS–PAGE and fluorography. Positions of NBF1 and NBF2 are shown on the right.

precipitates NBF2–His_6 in the presence, but not in the absence, of NBF1–FLAG (see lanes labelled A).

In vitro NBFs do not appear to interact Kir6.2

Since K^+ conduction through Kir6.2 is regulated by the nucleotide binding folds of SUR1, we tested the possibility that NBFs might interact with Kir6.2. Since Kir6.2 is a membrane protein, we have labelled Kir6.2 with [^{35}S]methionine in the presence of canine microsomal membranes using the reticulocyte lysate (TNT) system. To confirm that Kir6.2 is incorporated into the membranes, we have isolated the microsomes by ultracentrifugation (105,000*g*) and, after extensive washing, subjected the supernatant and membrane fractions to SDS–PAGE/fluorography. We found that Kir6.2 is incorporated into the membranes (Fig. 5B, lane 1), although the majority of Kir6.2 was found in the supernatant (Fig. 5A, lane 1). To test if NBFs bind the membrane associated Kir6.2, we have incubated membranes containing radio-labelled Kir6.2 with radio-labelled NBFs. After centrifugation and washing, we subjected the supernatant (Fig. 5A) and pellet fractions (Fig. 5B) to SDS–PAGE/fluorography. The results (Fig. 5B) show that NBF2 (lanes 3,4), but not NBF1 (lanes 2,4), was present in the pellet fraction. Control experiments where Kir6.2 was omitted also showed NBF2 in the pellet fraction (lanes 6,7). Although these data initially suggested that NBF2 might interact with the membranes in the absence of Kir6.2, subsequent control experiments revealed that NBF2 forms a pellet even in the absence of membranes (Fig. 5C). Thus the initial observation that NBF2 might interact with membranes appears to be an artefact, resulting from protein aggregation.

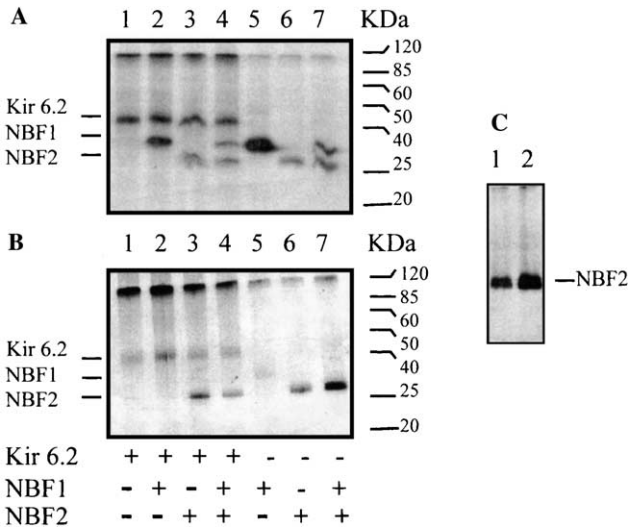

Fig. 5. Lack of binding of NBFs to Kir6.2. Kir6.2 and NBFs were labelled with [^{35}S]methionine using the TNT system. Kir6.2 was labelled in the presence canine microsomes. Labelled NBFs were incubated with membranes containing (lanes 1–4) or not containing (lanes 5–7) Kir6.2 as indicated with + or − signs at the bottom of panel B. (A, B). SDS–PAGE/autoradiography of aliquots of the supernatants (panel A) and membranes recovered by centrifugation and washing (panel B). (C) Labelled NBF2 (no membranes) was centrifuged and the pellet (lane 1) and the supernatant (lane 2) fractions were analysed as in (A) and (B).

Discussion

To examine the potential protein–protein interactions between the two NBF domains and the NBF domains and Kir6.2, we have expressed the NBF domains in *E. coli* and the rabbit reticulocyte lysate system and tested for interactions using biochemical approaches. We found that NBF1 and NBF2 physically interact with each other. However, we found no evidence for the interaction of NBFs with Kir6.2.

Expression and purification of NBFs

Both NBF1 and NBF2 could be expressed in large quantities in *E. coli* (Fig. 2); however, almost all of the expressed proteins appeared in the inclusion bodies. Attempts to improve the proteins in the soluble fraction of the cell were unsuccessful. Manipulations such as the use of other bacterial host strains, lowering the culture temperatures, decreasing the inducer concentration, inclusion of glycerol in the culture medium, all failed to shift the proteins from the inclusion bodies to soluble fraction of the cell (data not shown). Attempts to co-express the two NBFs also failed to prevent inclusion bodies. Thus the complex formation between the two NBFs within the cell does not appear to improve the solubility of the protein. For this reason, the NBFs were purified in a denatured state first. However, attempts to re-fold the protein by a variety of approaches proved to be unsuccessful. Thus we were unable to carry out several structural and functional studies that we have originally envisaged.

In choosing the boundaries for NBFs for expressing as independent domains, we were guided by the crystal structure of HisP, a bacterial homologue of NBFs and the sequence alignment of HisP with CFTR [27] and SUR1. Using a different section of NBF1 (residues 616–982 cf. 674–941), Mikhailov and Ashcroft [28] were able to obtain some protein in a soluble state and demonstrate that NBF1 forms a tetramer. There have been no reports on the expression and purification of NBF2.

Physical interaction of NBF1 with NBF2

The presence of NBFs is a hallmark of all ABC proteins [29]. In prokaryotes, they exist as independent protein subunits in the form of a dimer that exists in

association with the transmembrane portion of the transporter [27]. By contrast, in eukaryotes, they form part of a single polypeptide chain that includes both NBFs and the transmembrane portion of the protein. Although separated from one another by a six transmembrane domain, in most ABC proteins, including the P-glycoprotein (MDR) and CFTR, the two NBFs physically interact with each other [30–33]. These interactions and the hydrolysis of ATP by NBFs appear to be critical for the transport/channel activity of most ABC proteins.

Although SUR belongs to the ABC protein superfamily, it differs from the other members in several respects. Unlike CFTR and MDR, which possess both the catalytic and regulatory functions, SUR1 appears to lack catalytic activity. Its function appears to be to regulate the activity of Kir6.2, and help trafficking of Kir6.2 to the cell surface [23,34]. Another difference lies in its structural organisation: CFTR and MDR appear to function as monomers; by contrast, four molecules of SUR1 wrap round a central pore domain made up of four subunits of Kir6.2 to form a functional unit. Despite these structural and functional differences, the present data suggest that NBFs of SUR1 interact with each other physically. This is in agreement with the indirect evidence presented by Mikhailov and Ashcroft [28], where the authors showed that NBF1–GFP, when expressed singly in insect cells, is found in the cytoplasm; however, when co-expressed with the C-terminal half of SUR1, containing the six transmembrane segments and NBF2, more fluorescence was seen at the cell surface.

Implications of NBF1–NBF2 interactions

In MDR, and presumably in CFTR, hydrolysis of ATP by NBFs is thought to result in a conformational change that promotes the interaction between the two NBFs [33]. This in turn is believed to lead to the opening of the gates situated in the transmembrane portion of the transporter/channel, leading to the flux of substances across the membrane. It seems unlikely that a similar mechanism operates in K_{ATP} channels because Kir6.2, lacking the ER retention signal, can conduct K^+ ions even in the absence of SUR1 [35]. More importantly, the activity of Kir6.2 can be modulated by nucleotides even in the absence of SUR1. However, the affinity of Kir6.2 for ATP is ~10-fold lower in the absence of SUR1 [9]. This suggests that SUR1 somehow enhances the binding affinity of nucleotides to Kir6.2. One possible way by which this could be achieved is that in the native state of the channel, NBF1 might interact with Kir6.2 and transfer its ATP to the inhibitory site on Kir6.2. Alternatively, the interaction might increase the affinity of ATP to Kir6.2. It has been demonstrated that the affinity of ATP to NBF1 increases when the binding site on NBF2 is occupied by Mg-ADP [21,22]. This increase in binding affinity might prevent the transfer of ATP from NBF1 to Kir6.2, thereby favouring the open state of the channel. However, we were unable to demonstrate direct interactions between NBF1 and Kir6.2 to support this proposition. It should, however, be noted that these data were obtained with domains/proteins expressed in vitro using the rabbit reticulocyte translation system. Since we are not certain about the assembly of Kir6.2 in this system, we cannot rule out the possibility that NBF1 interacts with Kir6.2 in the native state of the channel.

Gel filtration and sucrose density gradient centrifugation analysis showed that the purified NBF1 forms a tetramer [28]. From our data, we cannot conclude whether the tetrameric NBF1 has one binding site for NBF2, or four, one for each NBF1 monomer. Since the K_{ATP} channel, like other K channels (both the core α- as well as the auxiliary β-subunits), appears to be a symmetric oligomer [5,34,36] it is reasonable to assume that the NBFs of SUR1 might be organised into a complex domain structure, comprised of four NBF1s bound to four NBF2s. Our attempts to test if NBF2 can assemble as an oligomer were unsuccessful. Thus we do not know whether the primary interface holding the domains together is contributed by NBF1 alone, or if NBF2 also contributes to it by forming a tetramer on its own. We speculate that the NBF1 tetramer binds to the intracellular phase of the Kir6.2 tetramer, thereby forming a functional interface via which the nucleotide effect is communicated to the pore. This organisation and regulation would be reminiscent of the way the regulatory β-subunits associate with, and influence the gating of, α-subunits of K_v channels [37].

Acknowledgments

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