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Novel nucleotide-binding sites in ATP-sensitive potassium channels formed at gating interfaces

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The coupling of cell metabolism to membrane electrical activity is a vital process that regulates insulin secretion, cardiac and neuronal excitability and the responses of cells to ischemia. ATP-sensitive potassium channels (K_{ATP}; Kir6.x) are a major part of this metabolic–electrical coupling system and translate metabolic signals such as the ATP:ADP ratio to changes in the open or closed state (gate) of the channel. The localization of the nucleotide-binding site (NBS) on Kir6.x channels and how nucleotide binding gates these K_{ATP} channels remain unclear. Here, we use fluorescent nucleotide binding to purified Kir6.x proteins to define the peptide segments forming the NBS on Kir6.x channels and show that unique N- and C-terminal interactions from adjacent subunits are required for high-affinity nucleotide binding. The short N- and C-terminal segments comprising the novel intermolecular NBS are next to helices that likely move with channel opening/closing, suggesting a lock-and-key model for ligand gating.

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Introduction

Potassium channels set the cell membrane potential, generate electrical signals in excitable cells, regulate cell volume and cell movement, and support net K^+ transport across epithelia. The movement of K^+ ions through these channels is governed by conformational changes resulting in channel opening or closing, that is, gating (Jiang *et al*, 2002b; Enkvetchakul and Nichols, 2003). Ion channels have evolved a number of gating sensors that respond to membrane voltage and/or a variety of extra- and intracellular molecules or ligands (Jiang *et al*, 2002a, 2003a). The mechanism for transducing ligand binding to gating remains an area of active investigation crucial to understanding channel function and how certain pharmacological agents work (Jiang *et al*, 2003a, b).

Inwardly rectifying K^+ channels (Kir1–7; Nichols and Lopatin, 1997; Nishida and MacKinnon, 2002; Kuo *et al*, 2003) are gated by the binding of intracellular ligands like $\beta\gamma$ G proteins, protons, phosphatidylinositol-4,5-bisphosphate (PIP_2) and ATP. ATP-gated Kir channels (K_{ATP}) are unique among the inward rectifiers in that they couple metabolism to cell electrical excitability and provide therapeutic targets for diseases including tissue ischemia, diabetes and hypertension. Plasma membrane K_{ATP} channels are formed by a 4:4 hetero-octameric assembly of Kir6.x pore-forming subunits and sulfonylurea receptors (SUR; Aguilar-Bryan *et al*, 1995) that belong to the ATP-binding cassette (ABC) family (Clement *et al*, 1997; Inagaki *et al*, 1997; Shyng and Nichols, 1997). On the other hand, mitochondrial K_{ATP} channels have been suggested to be composed of Kir6.x subunits in a multiprotein complex including succinate dehydrogenase that may participate in cellular protection against ischemic death (Lacza *et al*, 2003; Ardehali *et al*, 2004).

The binding of ATP to the Kir6.2 subunit is thought to inhibit channel activity by stabilizing the closed channel state (Enkvetchakul and Nichols, 2003), while interaction of Mg-nucleotides with the SUR subunit activates these channels. Negatively charged lipids like PIP_2 increase the open probability of Kir channels (Hilgemann and Ball, 1996; Fan and Makielski, 1997; Huang *et al*, 1998; Hilgemann *et al*, 2001) and antagonize the inhibition of K_{ATP} channels by ATP (Baukrowitz *et al*, 1998; Shyng and Nichols, 1998; Fan and Makielski, 1999; Shyng *et al*, 2000). PIP_2 competes with TNP-ATP (TNP: trinitrophenylcyclohexadienylidene) binding to recombinant Kir channel proteins such that ATP and PIP_2 bindings are mutually exclusive (MacGregor *et al*, 2002; Vanoye *et al*, 2002).

The absence of classical glycine-rich 'P-loop' motifs or sequence homology to any known nucleotide-binding protein (Saraste *et al*, 1990; Traut, 1994) has hampered defining the nucleotide-binding pocket on K_{ATP} channels and our understanding of nucleotide gating. Patch clamp studies of ATP inhibition of Kir6.2 channels (Tucker *et al*, 1997, 1998; Proks *et al*, 1999; Shyng *et al*, 2000; Cukras *et al*, 2002; John *et al*, 2003; Ribalet *et al*, 2003; Trapp *et al*, 2003) have identified N- and C-terminal residues affecting ATP sensitivity, however, interpretation is complicated by the difficulty in discerning between mutational effects on nucleotide binding versus gating (Enkvetchakul and Nichols, 2003) and is hampered by mutations that render the channel nonfunctional or that markedly alter open probability. Here, we use a direct approach to assess nucleotide binding that is based on fluorescence increases due to TNP-AXP (Hiratsuka, 2003) (2′,3′-*O*-(2,4,6-trinitrophenylcyclohexadienylidene) adenosine 5′-mono-, di- or triphosphate) interacting with maltose-binding (MBP) fusion proteins of the

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N- and C-termini of Kir channels (Vanoye *et al*, 2002; Hiratsuka, 2003).

Results

A Kir6.2 polypeptide linking the N-terminus directly to the C-terminus greatly enhances TNP-ATP, but not PIP_2, binding

Figure 1A shows that the fluorescent nucleotide, TNP-ATP, inhibits Kir6.2 channel activity in *Xenopus laevis* oocytes, and therefore, is an effective probe to study the structural basis of nucleotide binding in Kir6.x proteins. Although TNP-AXP analogs generally exhibit 10–10^3 times higher binding affinity to proteins compared to natural ATP (Vanoye *et al*, 2002; Hiratsuka, 2003), the IC_{50} values for inhibition of channel current by Na-ATP and TNP-ATP were similar (Figure 1A). This suggests that the TNP moiety on the ATP backbone may partially interfere with translating the higher nucleotide-binding affinity to a lower IC_{50} for channel gating.

Our previous studies had shown that TNP-ATP binding to a Kir6.2 C-terminal polypeptide comprising residues 170–390 exhibited a high K_d and a low fluorescence enhancement factor, γ ($\sim$11; Vanoye *et al*, 2002), suggesting that other regions of the channel likely contribute to formation of a high-affinity nucleotide-binding pocket in this K_{ATP} channel. We now show that the N-terminal polypeptide (6.2N, residues 1–72; Figure 1B) binds TNP-ATP and that directly linking the Kir6.2 channel N-terminus to the entire C-terminus significantly decreases K_d (1.2 ± 0.1 μM) and increases γ (24.1 ± 0.9) for TNP-ATP binding (Figure 1B and C). This TNP-ATP-binding affinity is about 30-fold higher than the IC_{50} for channel inhibition (Figure 1A) as is typical for this probe (Hiratsuka, 2003). Since the K_d was lower for the N–C-linked polypeptide compared to either of the N- or C-terminal proteins alone (Figure 1B and C), linking the Kir6.2 N- and C-termini had a synergistic effect on TNP-ATP-binding affinity. This synergistic effect on nucleotide binding of linking N- and C-termini was confirmed by Na-ATP competition of 8-azido-[γ-^{32}P]ATP binding (Figure 2). In contrast, the N-terminus of the H^+-gated Kir1.1 did not bind TNP-ATP (Vanoye *et al*, 2002) and had no significant effect on TNP-ATP binding to the Kir6.2 or Kir1.1 C-terminus (Figure 1C). However, the N-terminus of Kir6.2 significantly reduced the K_d for TNP-ATP binding to the Kir1.1 C-terminus (Figure 1C). This latter effect was also synergistic since the TNP-ATP affinity for the 6.2N–1.1C protein was higher than for either individual N- or C-terminal polypeptide. Although the isolated Kir6.1 N-terminal protein does not bind TNP-ATP (Figure 4C; Vanoye *et al*, 2002), it also increases nucleotide-binding affinity when

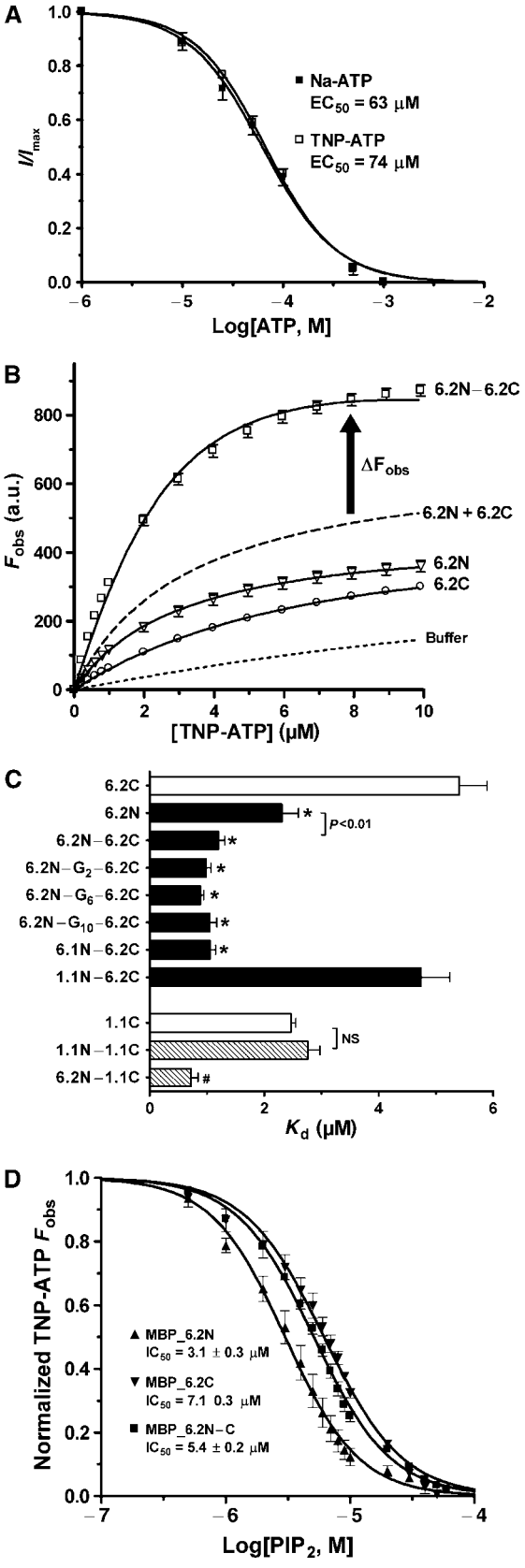

Figure 1 Kir6.2 N- and C-terminal interactions enhance TNP-ATP, but not PIP_2, binding. (**A**) TNP-ATP inhibits Kir6.2CΔ36 channel current expressed in *X. laevis* oocytes. TNP-ATP and Na-ATP inhibition curves of relative current (I/I_{max}; I_{max} is the current in the absence of nucleotide) are compared. (**B**) MBP fusion proteins of the cytoplasmic termini of Kir channels were produced in *Escherichia coli* and purified (Vanoye *et al*, 2002). TNP-ATP concentration-dependent fluorescence increases were determined for 5 μM proteins and fitted using equation (1) of Vanoye *et al* (2002). The arrow shows the increment in fluorescence, ΔF_{obs}, over that expected from the sum of 6.2N and 6.2C. The short dashed line is the intrinsic TNP-ATP fluorescence in buffer alone. (**C**) Comparison of the K_d (mean$\pm$s.e.m.) for TNP-ATP binding. $^*P<0.05$. (**D**) Competition of 10 μM TNP-ATP binding to Kir6.2 proteins by PIP_2. PIP_2 was prepared as described previously (MacGregor *et al*, 2002). Sequences for proteins were (amino-acid numbers in rat sequences) 6.2N (1–72), 6.2C (170–390), 6.1N (1–73), 6.1CT (221–424) and 1.1N (1–82).

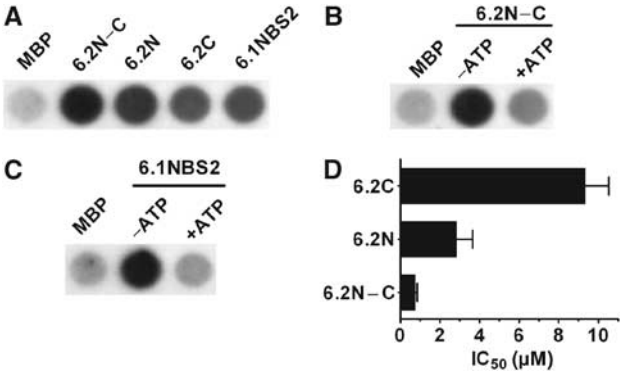

Figure 2 Enhancement of 8-azido-[γ-^{32}P]ATP by Kir6.2N–C. (**A, B**) Dot blot autoradiograms of 8-azido-[γ-^{32}P]ATP binding to Kir6.x proteins: MBP alone, 6.2N$^{1–72}$–C$^{170–390}$, 6.2N$^{1–72}$, 6.2C$^{170–390}$ and 6.1NBS2$^{319–404}$. The low ^{32}P signal with MBP (A–C) represents nonspecific binding, as it is not competed by 10 mM cold Na-ATP. The binding of 8-azido-[γ-^{32}P]ATP to Kir6.2N–C (B) or Kir6.1C$^{319–404}$ (**C**) is competed by 10 mM Na-ATP. (**D**) The IC_{50} values for Na-ATP competition of 8-azido-[γ-^{32}P]ATP binding to Kir6.2 proteins confirms the synergistic effect of N- and C-terminal interactions on nucleotide binding. IC_{50} values are as follows: N–C, 0.7±0.1 μM; N, 2.8±0.8 μM; C, 9.3±1.2 μM ($n=3$).

linked to the Kir6.2 C-terminus (6.1N–6.2C; Figure 1C). Thus, the N-termini of Kir6.x channels are unique because of their ability to enhance nucleotide binding in the context of the C-terminus. A role for the N-terminus in nucleotide binding is consistent with electrophysiological studies showing that N-terminal truncations or mutations alter ATP gating of Kir6.2 channels (Tucker *et al*, 1998; Koster *et al*, 1999; Proks *et al*, 1999; Cukras *et al*, 2002).

The magnitudes of the concentration-dependent increases in TNP-ATP fluorescence provide information about the nucleotide-binding environment. TNP-ATP fluorescence increases for the N–C-linked protein were significantly higher (ΔF_{obs} in Figure 1B) than expected from the sum of the F_{obs} values for the individual N- and C-termini. This observation, together with the increased enhancement factor for the N–C-linked protein, indicates N- and C-terminal interactions are involved in forming the high-affinity nucleotide-binding fold. Since the TNP-ATP-binding affinity and enhancement factor were not further increased by placing two, six or 10 glycine residues between the Kir6.2 termini (Figure 1C), flexibility between the directly linked N- and C-termini must be sufficient to form a stable N–C interaction for TNP-ATP binding. A linker polypeptide between the truncated cytoplasmic N- terminus and the C-terminus of Kir3.1 was also not required for crystal formation (Nishida and MacKinnon, 2002). In contrast to TNP-ATP binding, a synergistic effect of linking the Kir6.2 N- and C-termini was not seen for PIP_2 competition of TNP-ATP binding (Figure 1D), indicating that the Kir6.2 N- and C-terminal interactions are not required for PIP_2 binding.

Basic residues in the N- and C-termini of Kir6.2 interact with the phosphoryl groups of nucleotides

Mutational analyses, coupled with patch clamp methods, have identified three basic amino-acid residues in Kir6.2 (R^{50} in the N-terminus and K^{185} and R^{201} in the C-terminus) that modify ATP sensitivity (Tucker *et al*, 1998; Shyng *et al*, 2000; John *et al*, 2003; Ribalet *et al*, 2003; Trapp *et al*, 2003).

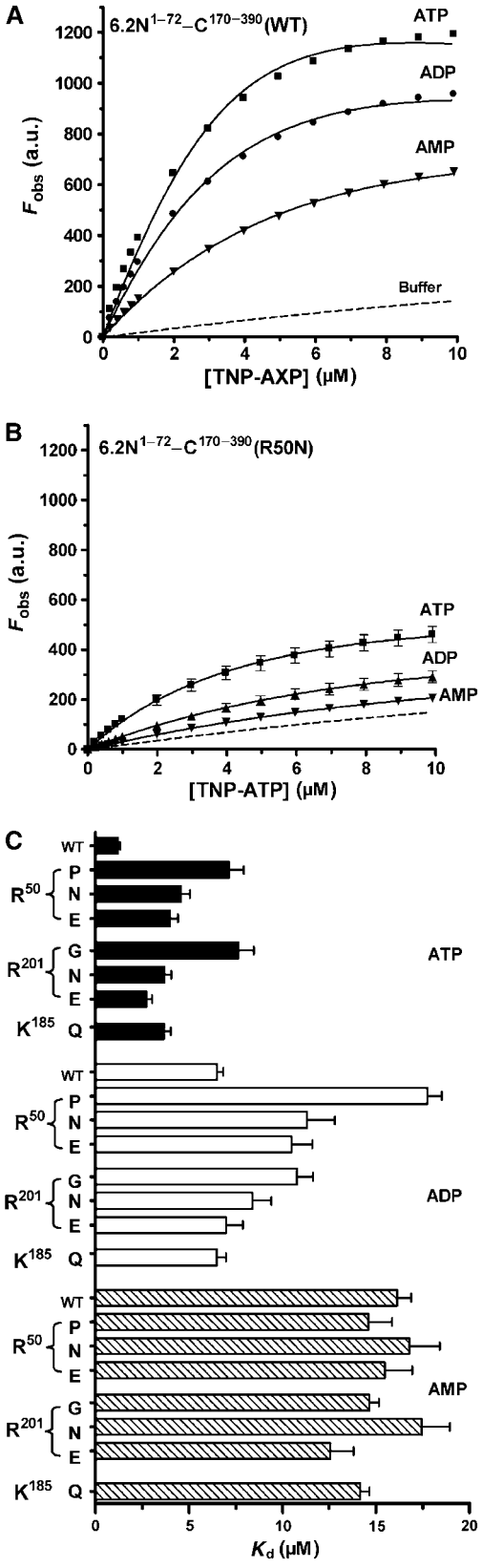

Figure 3 Mutation of basic residues in the N-terminus (R^{50}P) and C-terminus (K^{185}Q, R^{201}G) reduce TNP-AXP binding to the Kir6.2N–C-linked protein. TNP-ATP-, TNP-ADP- and TNP-AMP-binding curves for wild-type (WT, **A**), R^{50}P (**B**) and K^{185}Q (**C**) proteins. (**D**) Comparison of the K_d (mean±s.e.m.) values for TNP-AXP binding to wild-type and mutant Kir6.2N–C proteins. *$P<0.01$. Superscript numbers refer to rat sequence. The short dashed lines are the intrinsic TNP-ATP fluorescence in buffer alone.

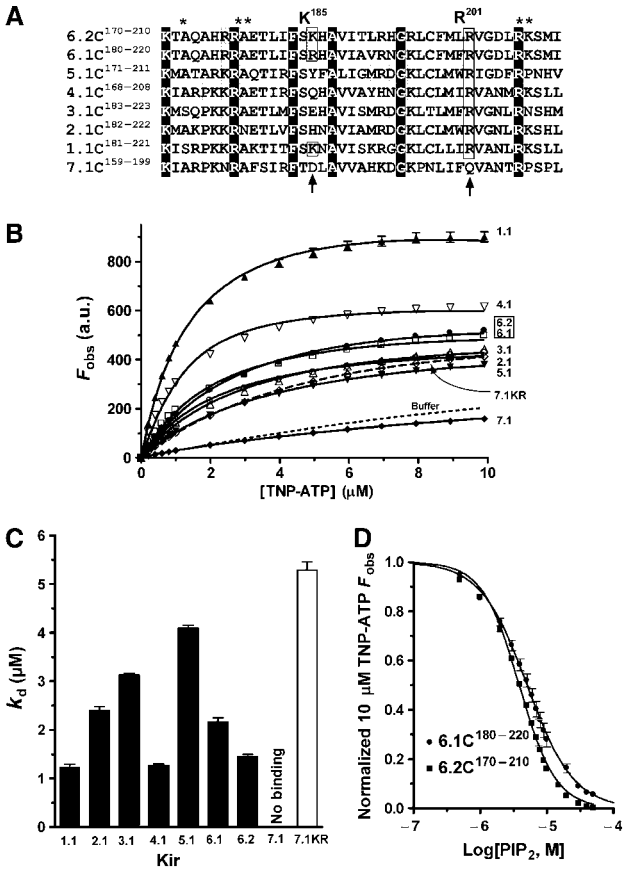

Figure 4 A conserved 39–42 amino-acid segment in the initial C-termini of Kir channels binds TNP-ATP. (**A**) Alignment of the initial 42 residues of the C-termini of Kir channels. Residues conserved throughout the Kir family are shaded black. Partially conserved similar residues are shaded gray. Boxed residues indicate conservation of the ATP phosphoryl group-binding positively charged residues at positions K^{185} and R^{201} in 6.2C. *Residues interacting with the phosphoryl groups of PIP_2. (**B**) Concentration dependence of TNP-ATP binding to the initial C-terminal segments of all Kir subfamily members. The short dashed line is the intrinsic TNP-ATP fluorescence in buffer alone. The long dashed line represents the K,R double mutant of Kir7.1. (**C**) Comparison of the K_d (mean ± s.e.m.) values for TNP-ATP binding. (**D**) PIP_2 competition of 10 μM TNP-ATP binding to the initial C-terminal segments of Kir6.x channels; IC_{50} ($6.2C^{170–210}$) = 3.84 ± 0.07 μM; IC_{50} ($6.1C^{180–220}$) = 5.13 ± 0.44 μM.

These positively charged residues have been proposed to interact with the phosphoryl groups on ATP by electrostatic contacts (Reimann *et al*, 1999; John *et al*, 2003; Ribalet *et al*, 2003; Trapp *et al*, 2003). We show in Figure 3 that individually mutating each of these basic residues increases the K_d for TNP-ATP binding, establishing their roles in nucleotide binding. In the wild-type Kir6.2N–C-linked protein, all three phosphoryl groups (α, β and γ) of ATP participate in nucleotide binding, with the γ phosphate being most critical (TNP-ATP K_d = 1.20 ± 0.11 μM < TNP-ADP K_d = 6.50 ± 0.34 μM < TNP-AMP K_d = 16.11 ± 0.77 μM; Figure 3C). Mutation of the N-terminal R^{50} to proline, R^{50}P, asparagine, R^{50}N, or glutamate, R^{50}E, increased the K_d for TNP-ATP (P > N = E; Figure 3C). All three mutants exhibited significant reductions in the maximal TNP-AXP F_{obs} (e.g., Figure 3B compared to Figure 3A), consistent with an altered nucleotide-binding environment. The R^{50}N and R^{50}E mutations had a modest effect on the binding of TNP-ADP but no effect on TNP-AMP (Figure 3B and C). These results indicate that R^{50} primarily interacts with the γ and β phosphates of ATP and a similar conclusion was reached in a recent electrophysiological study of nucleotide sensitivity of mouse Kir6.2 + rat SUR1 expressed in *Xenopus* oocytes (Trapp *et al*, 2003). The IC_{50} for nucleotide sensitivity of Kir6.2 channel activity being three- to 10-fold higher for ADP than for ATP (John *et al*, 2003; Ribalet *et al*, 2003) also supports a role for the γ phosphate in stabilizing binding of the α and β phosphates or in translation of nucleotide binding to channel gating. Mutation of the C-terminal R^{201} to G, N or E increased the K_d values for TNP-ATP (G > N = E), had a modest effect on TNP-ADP and no effect on TNP-AMP (R^{201}G, R^{201}N and R^{201}E; Figure 3C). Thus, like R^{50}, R^{201} primarily interacts with the γ and β phosphates of ATP rather than the α phosphate as suggested from studies of nucleotide gating sensitivities of Kir6.2 channels (John *et al*, 2003; Ribalet *et al*, 2003). In Kir6.1, these residues are conserved as R^{51} and R^{211}, the former likely contributing to the ability of the Kir6.1 N-terminus to enhance TNP-ATP binding to the Kir6.2 C-terminus (6.1N–6.2C; Figure 1C). Although mutation K^{185} to glutamine, K^{185}Q, greatly reduces ATP sensitivity of Kir6.2 channels (Tucker *et al*, 1998; Reimann *et al*, 1999; Shyng *et al*, 2000), this mutation only reduced TNP-ATP binding (Figure 2C and D). Moreover, the increase in TNP-ATP K_d for K^{185}Q was much less than that for R^{50}P or R^{201}G, suggesting that mechanisms other than altering the interaction of channel protein with nucleotide phosphoryl groups may mediate the large effect on the ATP sensitivity of Kir6.2 channels.

The highly conserved initial 39 amino-acid residues in the C-termini of Kir channels bind TNP-ATP

Although the H^+-gated Kir1.1 (ROMK) is not a classic K_{ATP} channel (Ashcroft and Ashcroft, 1990; Misler and Giebisch, 1992), it is inhibited by millimolar cytosolic ATP in native renal epithelial cells (Wang and Giebisch, 1991). Our earlier studies demonstrated nucleotide binding to the Kir1.1 C-terminus (Vanoye *et al*, 2002) and showed that a polypeptide comprising residues 183–221 in the initial C-terminus is a TNP-ATP- and PIP_2-binding region (Dong *et al*, 2002). Figure 4A shows that this C-terminal segment is highly similar among Kir channels and contains distinct basic residues that interact with the phosphoryl groups of ATP (Figure 3D; Dong *et al*, 2002; John *et al*, 2003; Ribalet *et al*, 2003; Trapp *et al*, 2003) or PIP_2 (Huang *et al*, 1998; Shyng *et al*, 2000; Lopes *et al*, 2002). Thus, we made individual polypeptides comprising the common segment from each of the Kir channels and examined TNP-ATP binding and PIP_2 competition. Each of the C-terminal segments, except for Kir7.1, binds TNP-ATP with relatively high affinity (Figure 4B and C) and with enhancement factors, γ, ⩾5. All of the Kir1–6 C-terminal segments that bind TNP-ATP conserve at least one of the two C-terminal basic residues (Figure 4A; K^{185} and/or R^{201}) that interact with the phosphoryl groups on ATP (Figure 3), while neither basic residue is conserved in Kir7.1. R^{201} is conserved in all TNP-ATP-binding segments and a basic residue at position of K^{185} is conserved only in Kir1.1, and Kir6.x channels. Mutation of both D^{174} to K and Q^{190} to R in Kir7.1 (Figure 4A) restored TNP-ATP binding to this C-terminal segment (Figure 4B and C), confirming the importance of the K^{185} and R^{201} residues in nucleotide binding in Kir6.x channels. PIP_2 competed with

TNP-ATP binding to this segment from Kir6.x channels (Figure 4D) as well as from Kir1.1 (Dong *et al*, 2002), consistent with the presence of conserved basic amino-acid residues (asterisk in Figure 4A) that are critical to PIP_2 binding in Kir6.2 (Shyng and Nichols, 1998; Shyng *et al*, 2000) and ATP-insensitive Kir channels (Huang *et al*, 1998; Dong *et al*, 2002; Lopes *et al*, 2002).

The N-termini and distal C-termini modulate nucleotide binding to the conserved initial C-terminal segment of Kir6.2

Why is nucleotide gating restricted to the Kir6.x subgroup of channels, if each of the first 42 amino acids of the C-termini of Kir channels can bind nucleotides? The results shown in Figures 1–3 indicated that the N-terminus is crucial for high-affinity nucleotide binding in Kir6.2. To explore this issue further, we examined the influence of the N-terminus (segment ① in Figure 5A) on TNP-ATP binding to the initial C-terminus in Kir6.x proteins (segment ② in Figure 5A). Linking either of the Kir6.x N-terminal segments ① to the Kir6.2 C-terminal segment ② lowered the K_d for TNP-ATP binding (6.2①–6.2② = 0.50 ± 0.04 μM; 6.1①–6.2② = 0.52 ± 0.02 μM; Figure 5D) compared to either segment alone (K_d for 6.2① = 2.31 ± 0.29 (Figure 1C) or for 6.2② = 1.45 ± 0.05 μM (Figure 5D)) or the entire Kir6.2N–C construct (①–②–③; K_d = 1.20 ± 0.11 μM; Figures 5B, D and 1C). In contrast,

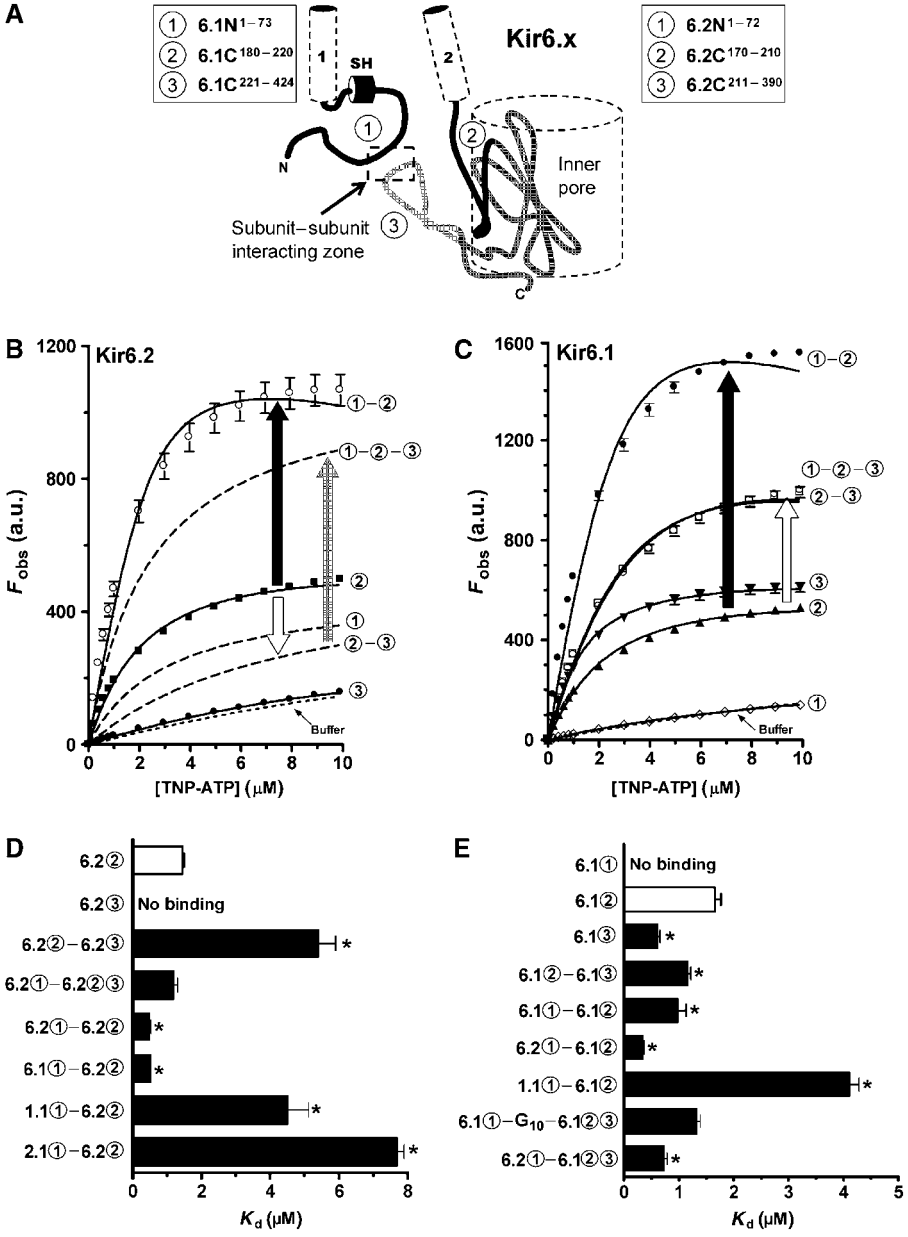

Figure 5 Modulation of TNP-ATP binding to the conserved initial C-terminal segment by N- and distal C-terminal segments. (**A**) Cartoon showing the location of the three cytoplasmic segments that determine TNP-ATP binding in Kir6.2. The N-terminus ①, and initial C-terminus ② and more distal C-terminal ③ segments are indicated. SH, slide helix. (**B, C**) TNP-ATP fluorescence increases in Kir6.2 and Kir6.1, respectively, showing the effects of linking N-terminal ① and distal C-terminal ③ segments to the initial C-terminal nucleotide-binding segment ② of Kir6.2. The long dashed lines in (B) show data from Figure 1B. The short dashed lines are the intrinsic TNP-ATP fluorescence in buffer alone. (**D, E**) Comparison of TNP-ATP K_d (mean ± s.e.m.) values for Kir6.2 and Kir6.1 segments, respectively. $^*P < 0.05$ compared to 6.2C or 6.1C.

neither segment ① from Kir1.1 or Kir2.1 enhanced TNP-ATP binding to 6.2② (1.1①–6.2②, $K_d = 4.52 \pm 0.60\,\mu M$; 2.1①–6.2②, $K_d = 7.70 \pm 0.20\,\mu M$; Figure 5D). These results provide further support for the unique role of the Kir6.x N-termini in enhancing nucleotide binding.

The N-terminus and distal C-terminal segment ③ from adjacent subunits in Kir channels interact (Figures 5A and 6B) and the contact between these flexible segments has been hypothesized to be an important component of the gating mechanism (Kuo *et al*, 2003). The X-ray crystal structure of the bacterial Kir, KirBac1.1, shows that the N-terminal residues A^{48}YG interact with the distal C-terminal residues I^{298}DY, corresponding to V^{328}DY in Kir6.x channels (Figure 6A and B). The sequence corresponding to residues AYG in Kir6.x channels is H^{46}KN just two residues before the critical R^{50}. Therefore, the distal C-terminus could influence TNP-ATP binding either by contributing charged amino-acid residues to the nucleotide-binding pocket formed by the linked segments ①–② or by N–C interactions that stabilize nucleotide binding to these segments. Figure 5B and D shows that TNP-ATP does not bind to segment ③. However, the binding affinity is lower in the whole C-terminus when segments ② and ③ are linked compared to segment ② alone (Figure 5D; white arrow in Figure 5B). The importance of N- and C-terminal interactions on binding is illustrated by the reversal of the inhibitory effect of segment ③ on segment ② by addition of the N-terminal segment ① (②–③ versus ①–②–③; gray arrow in Figure 5B and D).

The sequence F^{333}GNT(I/V)K in the distal C-terminal segment ③ of Kir6.2 (Figure 6A) may be involved in nucleotide binding since mutation of the four residues, G^{334}NTI(V in rat), or the single mutation of $G^{334} \rightarrow$D significantly lowered the ATP sensitivity of Kir6.2 channels (Drain *et al*, 1998). This C-terminal sequence is two residues distal to the conserved (V^{328}/I)DY that interacts with the N-terminus and includes the FX4K motif that is conserved in ion-motive ATPases (McIntosh *et al*, 1996, 2003). In the crystal structure of the sarcoplasmic reticulum Ca^{2+}-ATPase, the FX4K sequence lies within the TNP-AMP-binding pocket together with a lysine residue that interacts with the α phosphate of the nucleotide (Toyoshima *et al*, 2000). Mutation studies have shown that residues within the FX4K motif are critical for ATP binding in the absence of Mg (Toyoshima *et al*, 2000; McIntosh *et al*, 2003). Thus, we examined the role of this sequence in nucleotide binding. Mutation of F^{333}GNT$\rightarrow$4A did not alter TNP-ATP, -ADP or -AMP binding to the 6.2N–C protein (Figure 6C and D). In addition, TNP-AXP-binding affinities were not affected in the $K^{338} \rightarrow$D/A mutant proteins (Figure 6D).

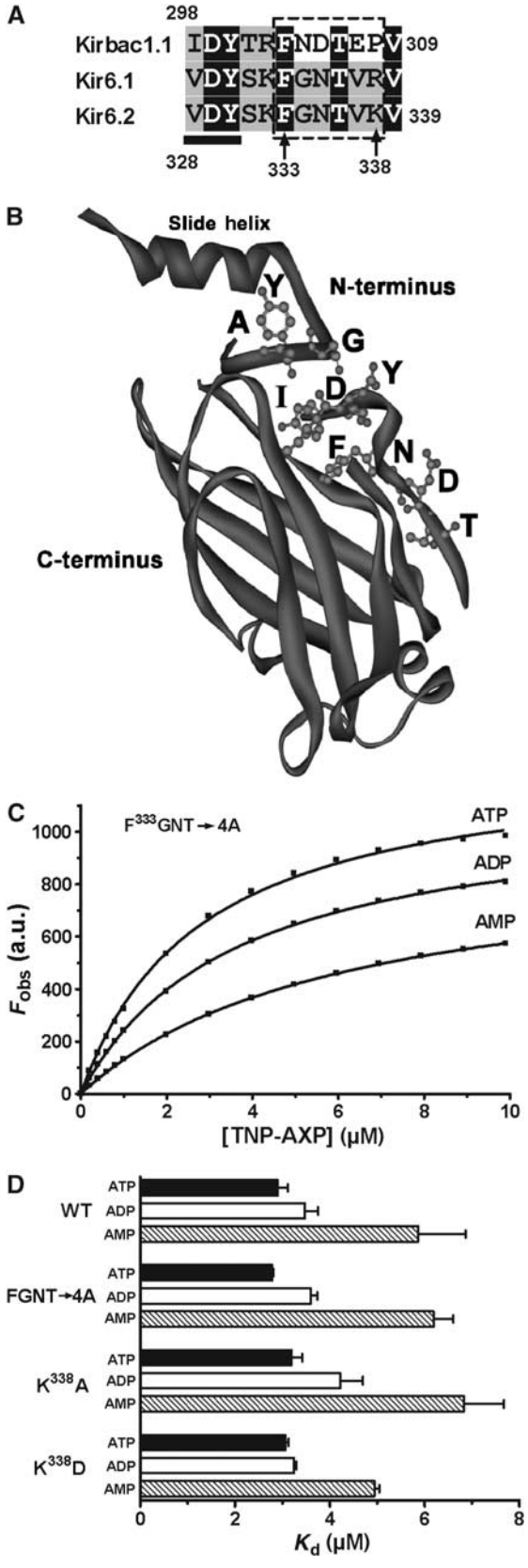

Figure 6 Role of the distal C-terminal 'FGNTVK' sequence in nucleotide binding in Kir6.2. (**A**) Amino-acid comparison for the 'FGNTVK' (boxed) and the nearby '(V/I)DY' (underlined) sequences in rat Kir6.x and bacterial KirBac1.1 channels. The '(V/I)DY' sequence (298–300) in the KirBac1.1 X-ray structure interacts with residues 40–42 in the N-terminus of the adjacent subunit and has been proposed to be important in channel gating (Kuo *et al*, 2003). (**B**) X-ray structural view of the interaction between the N- (red; residues 36–60) and C- (green; residues 151–309) terminal regions of adjacent subunits in KirBac1.1 (Kuo *et al*, 2003). Critical residues are indicated. (**C**) Concentration dependence of TNP-AXP binding in the 6.2N–C-linked protein where F^{333}GNT has been mutated to four alanine residues. (**D**) Comparison of TNP-ATP K_d (mean$\pm$s.e.m.) values for Kir6.2 proteins with the indicated mutations in, or truncations of, the 'FGNTVK' region.

Computer-generated models of Kir6.2 (Enkvetchakul and Nichols, 2003; John *et al*, 2003; Trapp *et al*, 2003) that are based on the Kir3.1 crystal structure have suggested that G^{334} is close to K^{185} (Figure 7A). Thus, we also examined the influence of the distal C-terminus on nucleotide binding to the 6.2N–C construct by assessing the effect of segment ③ truncations and G^{334} mutations on the ability of $K^{185}Q$ to reduce TNP-ATP binding (Figure 7B–D). Although mutation of G^{334} to an acidic residue (D or E) did not alter TNP-AXP binding compared to wild-type 6.2N–C (Figure 7B), the modulatory effect of the $K^{185}Q$ mutation was abrogated (Figure 7C and D). Furthermore, truncation at W^{311} or F^{333}, which removes G^{334} (Figure 7A), or the $G^{334}A$ mutation enhanced TNP-ATP binding while also abolishing the effect of the $K^{185}Q$ mutation (Figure 7C and D). The influence of G^{334} mutation or deletion on ATP binding in the context of the $K^{185}Q$ mutation is consistent with their proximity proposed from computer-generated structural models (Enkvetchakul and Nichols, 2003; John *et al*, 2003; Trapp *et al*, 2003). Thus, F^{333}GNT(I/V)K in segment ③ likely alters ATP sensitivity in Kir6.2 channels by modulating the influence of K^{185} and/or by altering the translation of nucleotide binding to channel gating via N- and C-terminal interactions. Such a mechanism would also explain the rather weak effect of $K^{185}Q$ on TNP-ATP binding (Figure 3) compared to the dramatic reduction in ATP sensitivity of channel gating (Tucker *et al*, 1998; Reimann *et al*, 1999; Shyng *et al*, 2000). Interactions between K^{185} and G^{334} appear to be important in this context.

The minimal nucleotide-binding site in Kir6.2

We further refined the segments interacting with ATP phosphoryl groups in the Kir6.2①–②-linked protein to a 24-residue segment (R^{31}–R^{54}) of the N-terminus and a 32-residue region of the C-terminus (E^{179}–I^{210}; Figure 8A). Truncation of the N-terminal segment ① by ⩽30 residues did not affect ATP sensitivity of Kir6.2 channels (Koster *et al*, 1999; Tucker and Ashcroft, 1999) and similarly did not alter TNP-ATP binding to the 6.2N–$C^{170–210}$ protein (Figure 8B and D). In contrast, truncations of greater than 30 residues that result in non-functional channels (Babenko *et al*, 1999; Koster *et al*, 1999; Tucker *et al*, 1999) increased the K_d for TNP-ATP binding (Figure 8B and D). Truncation of the carboxyl end of the N-terminus that deletes the slide helix and linker to the membrane helix #1 (Figure 8E) did not alter TNP-ATP binding to the N-terminal protein or the N–C-linked protein, 6.2$N^{31–42}$–$C^{170–210}$. Further truncations of the amino-terminal end of the C-terminal segment ② identified the minimal nucleotide-binding segment as 6.2$N^{31–54}$–$C^{179–210}$ (Figure 8C–E). This NBS contains residues involved in the maintenance of channel function, N- and C-terminal communication, interaction with PIP_2 and sensitivity to ATP (Koster *et al*, 1999; Tucker *et al*, 1999; Shyng *et al*, 2000; Enkvetchakul and Nichols, 2003; John *et al*, 2003; Ribalet *et al*, 2003; Trapp *et al*, 2003).

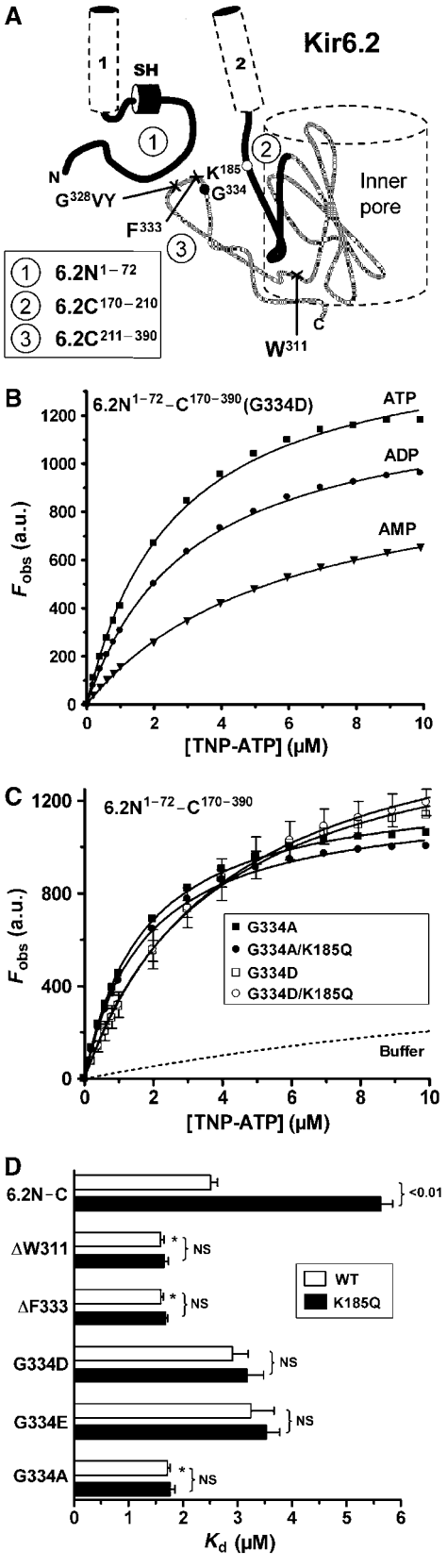

Figure 7 The distal C-terminal segment ③ modulates nucleotide binding in Kir6.2. (**A**) Cartoon of the cytoplasmic segments in Kir6.2 showing the location of mutated residues or truncations. SH, slide helix. (**B**) Concentration dependence of TNP-AXP in the Kir6.2N–C protein with mutation of G^{334} to aspartate. TNP-ATP concentration dependence curves (**C**) and K_d (mean±s.e.m.) values (**D**) showing that G^{334}A/D mutations do not alter TNP-ATP binding but abrogate the effect of $K^{185}Q$ on binding. While truncations that eliminate the 'FNGTVK' region (ΔW311 and ΔF333) enhanced TNP-ATP binding, they also abrogated the effect of $K^{185}Q$ on binding. $^*P<0.05$ compared to 6.2N–C WT.

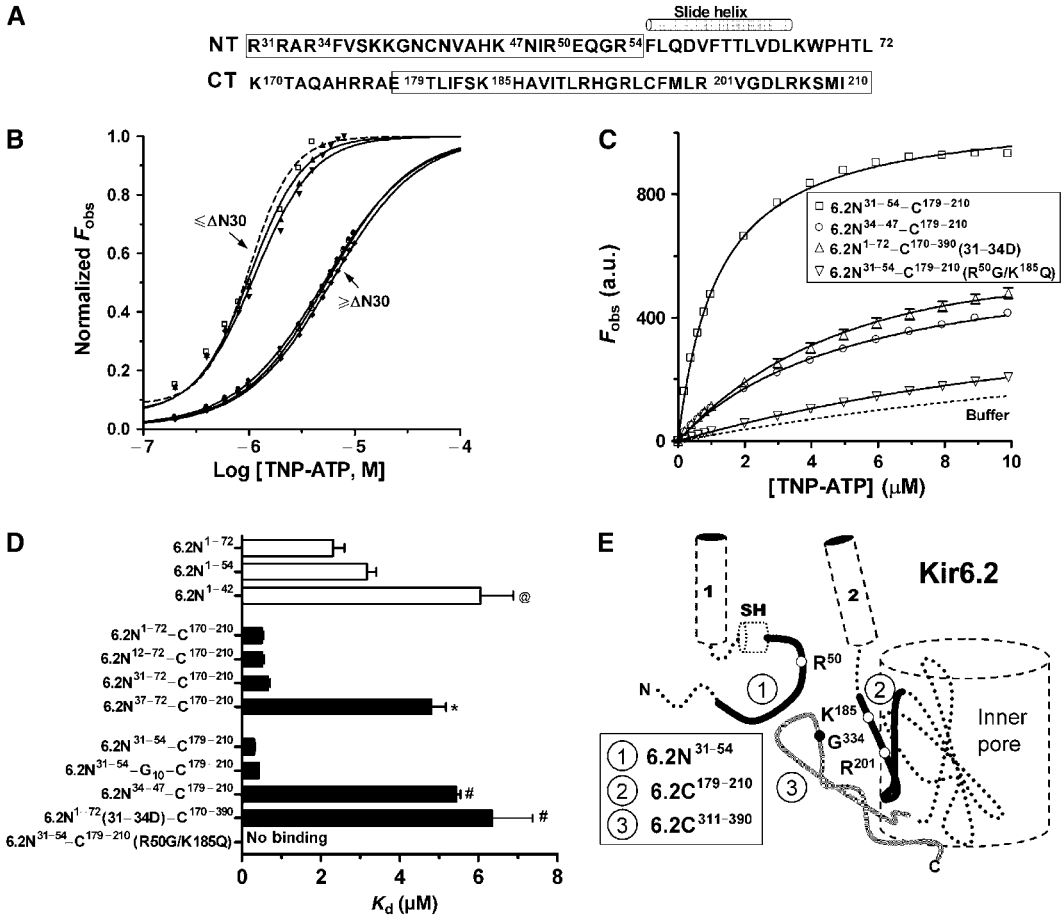

Figure 8 The minimal N- and C-terminal sequences that form the NBS (NBS1) in Kir6.2. (**A**) The minimal N- (NT) and C-terminal (CT) segments comprising the NBS in rat Kir6.2 are boxed. Sequence locations are indicated by superscript numbers. (**B**) N-terminal truncations up to 30 residues of the N-terminus do not alter TNP-ATP binding in the Kir6.2N–C-linked protein. (**C, D**) The Kir6.2N^{31-54}–$C^{179-210}$-linked protein contains the minimal N- and C-terminal segments critical for high-affinity TNP-ATP binding. TNP-ATP binding was abolished in the double R^{50}G-K^{185}Q mutant. (**D**) K_d (mean ± s.e.m.) values. Clear bars show effect of truncation of the C-terminal end of 6.2N. Solid bars show truncations defining the minimal 6.2NBS. (**E**) Cartoon showing the minimal nucleotide-binding segments in Kir6.2 and the location of residues that modulate binding. SH, slide helix. $^{*}P<0.01$ compared to 6.2N^{1-72}–$C^{170-210}$; $^{\#}P<0.01$ compared to 6.2N^{31-54}–$C^{179-210}$; $^{@}P<0.01$ compared to 6.2N.

The R^{50}G-K^{185}Q double mutant abolished TNP-ATP binding to this nucleotide-binding segment (Figure 8C and D). The highly basic sequence R^{31}RAR^{34} (Figure 8A) is also involved in nucleotide binding, as either removal of this sequence, 6.2N^{34-47}–$C^{179-210}$, or mutation of the four residues to aspartic acid in the whole N–C-linked protein, 6.2N^{1-72}–$C^{170-390}$(31–34D), significantly reduced TNP-ATP binding (Figure 8B and C). The role of this latter region in the ATP sensitivity of Kir6.2 + SUR1 channels could not be fully explored by another group due to effects on channel trafficking (Ribalet *et al*, 2003).

Kir6.1 contains two TNP-ATP-binding sites—one conserved with Kir6.2 and a second site in the distal C-terminus

We previously showed that the whole Kir6.1 C-terminus bound TNP-ATP with high affinity and that the isolated Kir6.1 N-terminus exhibited no nucleotide binding (Vanoye *et al*, 2002; confirmed here in Figure 5C and E). The Kir6.1 C-terminus contains the TNP-ATP-binding segment ② homologous to that in Kir6.2 but diverges from Kir6.2 after V^{348} in segment ③ (V^{339} in Kir6.2). While binding to the whole Kir6.1 C-terminus (segments ②–③) was not enhanced by segment ① of Kir6.1 (6.1①–G_{10}–6.1②–③; Figure 5C and E), this segment ① significantly increased nucleotide binding to Kir6.1 segment ② (linked segments ①–②; Figure 5C and E). The N-terminal segment ① from Kir6.2, but not Kir1.1, also increased TNP-ATP binding to Kir6.1 segment ② (Figure 5E) and the whole Kir6.1 C-terminus (Figure 5C and E). When taken together with the observations that the Kir6.1 N-terminal segment ① also decreased the K_d of the whole 6.2 C-terminus equivalently to segment ① from Kir6.2 (Figure 1C) and that the Kir6.1 N-terminus conserves the critical R^{51} (R^{50} in Kir6.2;Figure 3B and D) residue, Kir6.1 segments ① and ② appear to form a nucleotide-binding fold homologous to Kir6.2.

The inability of the Kir6.1 N-terminus to enhance TNP-ATP binding to the whole C-terminus may relate to the unique properties of the distal C-terminal segment ③. In contrast to Kir6.2, the Kir6.1 C-terminal segment ③ binds TNP-ATP with high affinity and γ ($K_d = 0.62 \pm 0.04\,\mu M$ and $\gamma = 22.6$; Figure 5C and E). This appears to be a second nucleotide-binding fold since the K_d for the entire C-terminus (segments ②–③; $1.17 \pm 0.05\,\mu M$; Figure 4E) is the average of the K_d values for the two contributing segments (average $K_d = (② \ 1.65 + ③$

0.62)/2 = 1.14 μM) and the binding stoichiometry, N_0 (molar ratio of TNP-ATP/protein; equation (1) in Vanoye *et al*, 2002), of the C-terminus was the sum of those for segments ② and ③ (②–③ = 0.52, ② = 0.22 and ③ = 0.20, respectively). Progressive truncations of the Kir6.1 C-terminal segment ③ identified $6.1C^{319–404}$ (Figure 9A–C) as the second high-affinity TNP-ATP-binding site (NBS2) in Kir6.1. This NBS2 ($6.1C^{319–404}$) also binds 8-azido-[γ-^{32}P]ATP (Figure 2A) and this interaction is competed by 10 mM cold Na-ATP (Figure 2C), confirming that this segment binds nucleotides. The $Kir6.1C^{319–404}$ segment contains the $V^{337}DY$ triad that interacts with the N-terminus (Kuo *et al*, 2003) and the $FG^{343}NTVR$ sequence that in Kir6.2 modulates TNP-ATP binding in the $K^{185}Q$ mutant (Figures 6 and 7). PIP_2 competed with TNP-ATP binding to each of these NBS (NBS1, $6.1C^{1–73}$–$C^{180–220}$; NBS2, $6.1C^{319–404}$; Figure 9D).

Discussion

The X-ray crystal structures of the channel tetramers for the two inward rectifiers, Girk1 (Nishida and MacKinnon, 2002) and KirBac (Kuo *et al*, 2003), provide models for understanding the interaction between the N- and C-terminal segments forming the common NBS in Kir6.x channels. In Girk1, the N- and C-termini were directly linked and the N–C interaction is formed within each subunit (Nishida and MacKinnon, 2002). In contrast, in KirBac, each subunit contains virtually the entire protein, including the selectivity filter and transmembrane helices, and the N-terminus of one subunit interacts with the C-terminus of the adjacent subunit (Kuo *et al*, 2003). Nevertheless, the N- and C-terminal segments of single subunits from both structures superimpose closely (Gulbis and Doyle, 2004), supporting the validity of binding information obtained from our MBP fusion proteins in which N- and C-termini are linked. However, the assemblies of these cytosolic segments in Girk1 and KirBac tetramers are different. In KirBac, these segments are tilted with respect to Girk1, thereby forming a constriction at the interface with the membrane (Gulbis and Doyle, 2004) consistent with the ion conduction pathway being closed in KirBac (Kuo *et al*, 2003). These two types of tetrameric configurations for the cytosolic segments have been suggested to represent the closed (KirBac) and open (Girk1) conformations of Kir channels (Gulbis and Doyle, 2004). Since ATP stabilizes the closed state of Kir6.x channels, the binding of nucleotides to these channels would be expected to shift the tetrameric assembly of cytosolic domains to the KirBac conformation where N–C interactions are between subunits (i.e., are intermolecular).

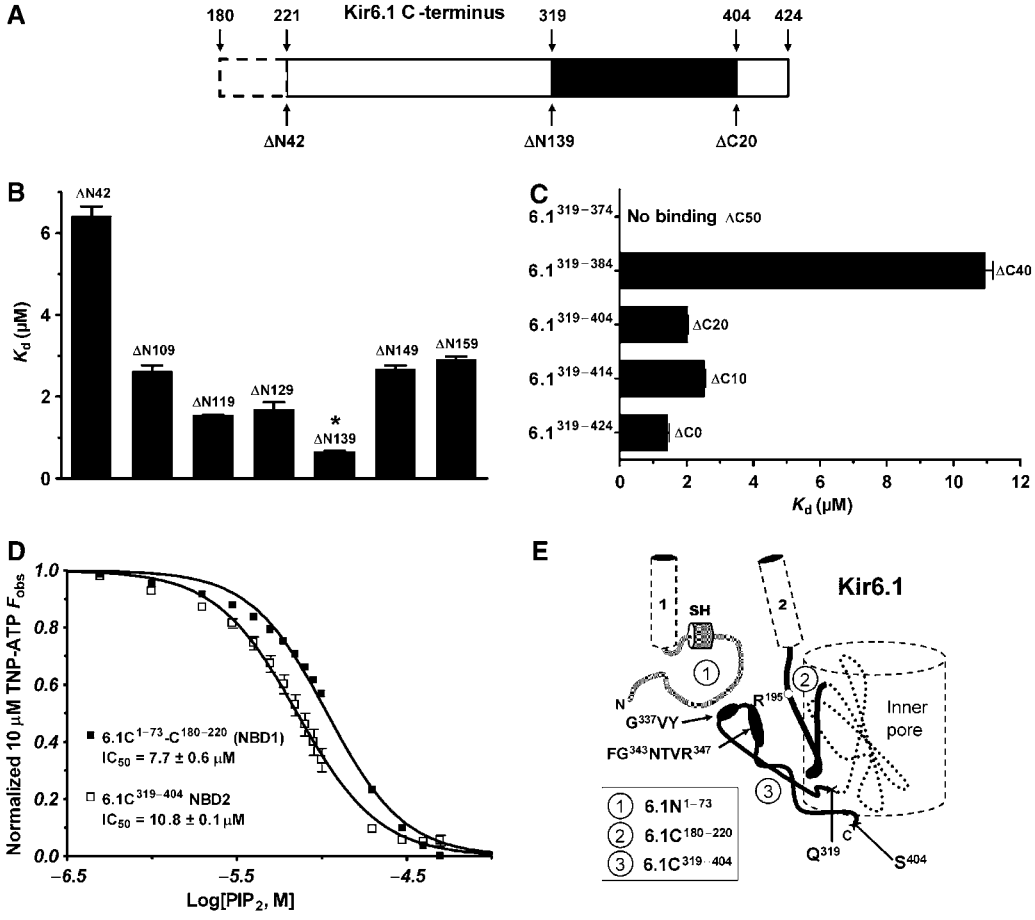

Figure 9 The minimal C-terminal sequence that forms the second unique NBS (NBS2) in Kir6.1. (**A**) Cartoon of the C-terminus of kir6.1 showing the location of truncations that define the minimal sequence of the TNP-ATP-binding site unique to this K_{ATP} channel. Effect of graded truncations from the amino- (**B**) or carboxyl-terminal (**C**) ends of the Kir6.1 C-terminus on the K_d for TNP-ATP binding. (**D**) PIP_2 competition of 10 μM TNP-ATP binding to the two NBS (NBS1 and NBS2) in Kir6.1. NBS1 is composed of the N-terminus linked to the conserved initial C-terminal segment. NBS2 is formed by residues 319–404 in the distal C-terminal segment ③. (**E**) Cartoon showing the nucleotide-binding segments in Kir6.1 and the location of important residues. SH, slide helix.

Figure 10 shows the locations of the segments forming the shared NBS (NBS1) in Kir6.x channels mapped onto the homologous KirBac tetrameric channel, which is in the closed state for ion conduction (Gulbis and Doyle, 2004). While the segments that comprise NBS1 (colored in Figure 10A and B) are separated by long stretches of residues that form the transmembrane ion pore and gate of the channel, they come together on the cytoplasmic surface of the channel tetramer to form four identical nucleotide-binding folds. While the NBS does not contain recognized nucleotide triphosphate (NTP)-binding consensus sites, a general feature of nucleotide-binding proteins shared with this Kir6.x NBS1 is the contribution of at least two separate peptide segments to the NTP-binding site (Traut, 1994). However, a novel feature of this NBS is that the two segments forming the NTP-binding site are not contained in the same protein but are from two adjacent Kir subunits, with each subunit contributing its N-terminal segment ① to one NBS and the C-terminal segment ② to a second NBS (Figure 10B). In other words, the nucleotide-binding pocket is formed at an intermolecular interface. A four-site ATP-binding model has also been proposed to account for the complex gating behavior of K_{ATP} channels (Enkvetchakul *et al*, 2000; Enkvetchakul and Nichols, 2003). In this model, ATP binding to any of the four sites (Figure 10B) will close the channel, while at saturating ATP concentrations, all four sites must be unoccupied for the channel to open.

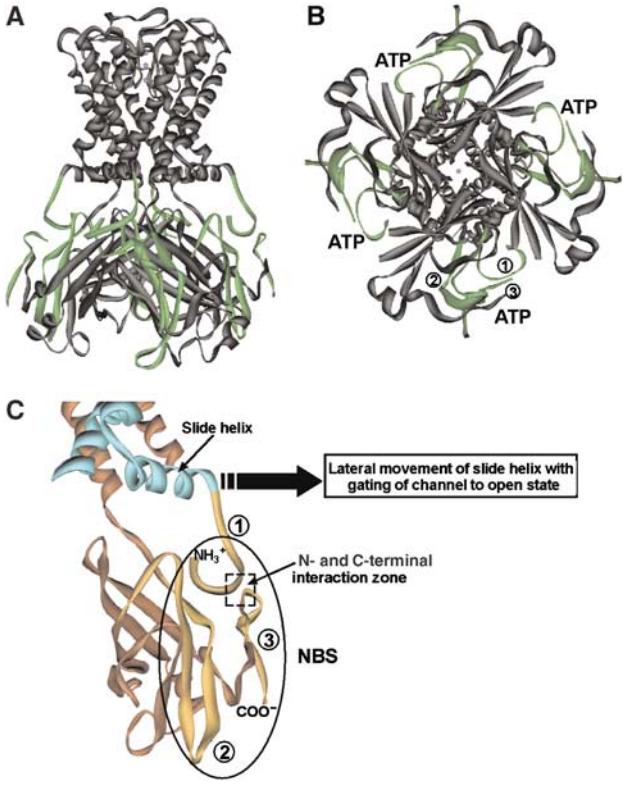

Figure 10 Structural models of the ligand-binding segments mapped onto the KirBac1.1 X-ray structure. Side (**A**) and bottom (**B**) views of KirBac1.1 showing the location of the homologous Kir6.x nucleotide-binding segments ①–③ colored green. (**C**) Solid ribbon model of KirBac1.1 showing how nucleotide binding might alter gating in a lock-and-key fashion. Binding of the phosphate groups on ATP (key) to basic residues in segments ① and ② (lock) could limit the lateral movement of the slide helix. The latter is required for bending of the second membrane spans that open the inner gate at the cytoplasmic face of the transmembrane spans allowing potassium ions to flow through the channel.

What distinguishes ATP-sensitive, from insensitive, Kir channels is the presence of a unique N-terminal segment ① that interacts with the conserved C-terminal segment ② to form the NBS1 (Figure 10A and B). Conserved basic residues in segment ② of Kir channels (see Figure 4A) also electrostatically interact with the phosphates of PIP_2 (Shyng *et al*, 2000; Hilgemann *et al*, 2001; Dong *et al*, 2002; Lopes *et al*, 2002; MacGregor *et al*, 2002). The evolutionary conservation of this PIP_2-binding C-terminal segment ② in Kir subfamilies provides both for the gating of ATP-insensitive channels and for the modulation of ATP sensitivity in K_{ATP} channels by phospholipids (Shyng *et al*, 2000; Hilgemann *et al*, 2001; Lopes *et al*, 2002; Enkvetchakul and Nichols, 2003). In addition, Kir6.1 has a unique region in the distal C-terminus (segment ③) that binds TNP-ATP and appears to form a distinct second NTP- and PIP_2-binding site. The role of the NBS2 in Kir6.1 channel gating is unknown. However, given the heteromeric composition of Kir6.x channels with SUR that contain two classic nucleotide-binding domains, regulation of Kir6.1 channel gating by nucleotide may be complex and involve interactions of these different NBS.

How might ATP binding affect gating in Kir6.x channels? The gating behavior of single Kir6.2 channels is complex with periods of rapid opening and closings (bursting) interspersed between periods where the channel is predominantly closed (interburst period; Enkvetchakul and Nichols, 2003). Two 'gates' have been suggested for K_{ATP} channels—one at the selectivity filter (outer gate; Proks *et al*, 2001, 2003) and one at the cytoplasmic ends of the second transmembrane (M2) segments (inner gate; Kuo *et al*, 2003). Which of these potential 'gates' is the ligand-sensitive gate is controversial (Enkvetchakul and Nichols, 2003; Kuo *et al*, 2003; Phillips and Nichols, 2003; Phillips *et al*, 2003; Proks *et al*, 2003). The location of the homologous nucleotide-binding segments identified here is most compatible with gating by the 'inner gate' (Figure 10). Based on the KirBac1.1 X-ray crystal structure, Doyle and co-workers (Kuo *et al*, 2003) suggested that the bending of the inner helix (second membrane span, M2) at a conserved glycine residue opens the inner gate to allow potassium ions to pass through the Kir pore. According to their gating model, the bending of M2 is sterically restricted by the outer M1 helices, and the N-terminal slide helix must move laterally along the plane of the plasma membrane to allow room for the M2 to bend (Figure 10C). The binding of the phosphoryl groups of ATP to the initial part of the C-terminus following M2 and the N-terminus proximal to the slide helix of the adjacent subunit (the lock mechanism) could limit the lateral movement of the slide helix, and thus, ATP could act as a key that stabilizes (locks) the channel in the closed state (Figure 10C). The interaction between N- and C-terminal segments is likely a conserved feature of Kir channels and the location of the ATP-binding region at this site suggests a common gating strategy for other ligands (e.g., H^+, G protein $\beta\gamma$, etc.) in ATP-insensitive inward rectifier channels.

Materials and methods

TNP-ATP inhibits Kir6.2ΔC36 channels expressed in X. laevis oocytes

Oocytes were injected with 10 ng of Kir6.2ΔC36 cRNA. After 48 h of expression, macroscopic currents were recorded in the inside-out

macropatch configuration at −50 mV holding potential using a Warner patch clamp (model L/M-EPC 7, Heka Elektronik, Lambrecht/Pfalz, Germany) and passed through an eight-pole Bessel filter at 1000 Hz (Warner Instruments, Hamden, CT). Bath solution contained (in mM) 150 KCl, 2 EDTA and 10 MES/TRIS, pH 7.4. Pipette solution contained (in mM) 150 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 MES/TRIS, pH 7.4. Different concentrations of either Na-ATP or TNP-ATP were delivered by a multibarrel quick exchange perfusion system (SF-77B Perfusion Fast Step, Warner, CT).

Construction of vectors and mutagenesis

cDNA encoding the N- and C-termini of Kir1.1a (ROMK2), Kir6.1 and Kir6.2 were derived from rat kidney or brain. Deletions, single amino-acid mutations and chimeric constructs were generated using polymerase chain reaction (PCR) amplification. All targeted cDNA fragments were inserted into the modified pMAL™-c2 vector (New England Biolabs).

Protein purification

The proteins were produced in BL21-CodonPlus (DE3)-RIL cells (Stratagene) induced with 0.3 mM isopropyl β-D-thiogalactoside for 1–3 h as described (Vanoye *et al*, 2002). Samples were lysed with a probe sonicator in an ice-water bath. The supernatant was kept in 20 mM Tris–HCl, 200 mM NaCl and 1 mM EDTA, pH 7.4, and fusion protein purified by amylose resin affinity chromatography. Fusion proteins were eluted with 10 mM maltose and dialyzed against 50 mM Tris–HCl, pH 7.5.

TNP-ATP binding

We used fluorescent TNP-AXP (Molecular Probes Inc.) to assess the binding of AXP to the fusion proteins as described (Vanoye *et al*, 2002). A 5 μM portion of fusion protein was dissolved in 50 mM Tris–HCl at pH 7.5, and TNP-AXP fluorescence increases were detected by the increase in fluorescence upon binding to the protein using a SPEX Fluromax-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ; Ex 403 nm; Em 546 nm). The temperature was maintained at 22 ± 0.1°C.

Analysis of TNP-ATP binding

The observed TNP-AXP fluorescence intensity (F_{obs}) in arbitrary units is given by the following equation (Vanoye *et al*, 2002):

$$\begin{aligned} F_{obs} =& (Q[\mathrm{TNP-AXP}] + Q_2[\mathrm{TNP-AXP}]^2) \\ &+ \frac{Q_c}{2}(\gamma - 1)\{([\mathrm{TNP-AXP}] + N_oP + K_d) \\ &- [([\mathrm{TNP-AXP}] + N_oP + K_d)^2 - 4N_oP[\mathrm{TNP-AXP}]^{1/2}]\} \end{aligned}$$

where P is the protein concentration (μM), Q and Q_2 are constants (fluorescence intensity/μM or μM^2 of free TNP-ATP, respectively) derived independently from the concentration dependence of TNP-ATP fluorescence intensity in buffer alone (F_{Buffer}) and account for the 'inner filter' effect ($F_{Buffer} = Q[\mathrm{TNP\text{-}ATP}] + Q_2[\mathrm{TNP\text{-}ATP}]^2$) and Q_c is the slope of the F_{Buffer} versus [TNP-ATP] curve in buffer alone, where

$$\frac{dF_{Buffer}}{d[\mathrm{TNP-AXP}]} = Q + Q_2[\mathrm{TNP-AXP}]$$

Kinetic parameters were calculated using GraphPad Prism version 4 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com.

8-Azido-[γ-^{32}P]ATP binding

Aliquots of 5 μM protein were incubated on ice without or with Na-ATP (0–10 mM) for 15 min in reaction buffer (mM): 50 HEPES, 10 Tris, 10 $CaCl_2$ and 0.5 $MgCl_2$ at pH 7.4. 8-Azido-[γ-^{32}P]ATP (1 μCi; MP Biomedicals Inc.) was added to the reaction, the mixture incubated on ice for an additional 10 min and then exposed to UV (λ = 254 nm; 10 cm distance) for 5 min using a Stratalinker UV crosslinker. Equal amounts of the 8-azido-ATP-crosslinked proteins were dot blotted onto a nitrocellulose membrane and the ^{32}P activity of each dot quantified using a phosphorimager-SI (Molecular Dynamics) with a wide dynamic range.

PIP_2 competition

L-α-Phosphatidyl-D-*myo*-inositol-4,5-bisphosphate ($PI(4,5)P_2$; Avanti Polar-Lipids Inc.) was dissolved in chloroform, dried under a stream of N_2, hydrated (50 mM Tris–HCl, pH 7.5) and sonicated for 30 min until an optically clear suspension was formed. The competition of TNP-ATP binding was assessed by incremental additions of PIP_2 as described (MacGregor *et al*, 2002). Competition curves were analyzed as

$$\frac{F_{obs}}{F_{obs}^{max}} = \frac{1}{1 + 10^{(\log EC_{50} - [\mathrm{TNP-ATP}]:\mathrm{Hill})}}$$

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