MECHANISM OF

K_{ATP} CHANNEL REGULATION BY SUR

MICHINORI MATSUO

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ABBREVIATIONS

ABC	ATP-binding cassette
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
CFTR	cystic fibrosis transmembrane conductance regulator
EDTA	ethylenediamine tetraacetic acid
K_{ATP}	ATP-sensitive potassium
kDa	kilodalton
MDR	multidrug resistance
MRP	multidrug resistance-associated protein
NBF	nucleotide-binding fold
NEM	*N*-ethylmaleimide
pCMPS	*p*-chloromercuriphenylsulphonate
PHHI	persistent hyperinsulinemic hypoglycemia of infancy
SDS	sodium dodecyl sulfate
SUR	sulfonylurea receptor
Tris	tris (hydroxymethyl) aminomethane

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INTRODUCTION

ATP sensitive potassium (K_{ATP}) channels are inwardly rectifying potassium channels, which are inhibited by ATP and activated by MgADP (1-4). They link cellular metabolic level to membrane potential by sensing intracellular ATP and ADP level in various tissues, such as pancreatic β-cell, heart, brain, and skeletal muscle. The K_{ATP} channel is a hetero-octamer composed of sulfonylurea receptor (SUR) and Kir6.x subunits in a 4:4 stoichiometry (5-8) (Fig. 1). SUR is a member of the ATP binding cassette (ABC) superfamily including P-glycoprotein (MDR1), multidrug resistance-associated protein (MRP1), and the cystic fibrosis transmembrane conductance regulator (CFTR) (9,10), all of which have two nucleotide-binding folds (NBFs) in the molecule; Kir6.x is a member of the inwardly rectifying potassium channel family (11-13).

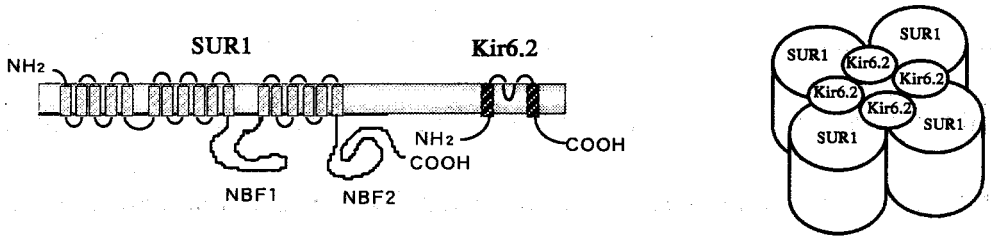

Fig. 1. **Predicted secondary structures of SUR1 and Kir6.2 and heterooctemeric structure of K_{ATP} channel.**

Both SUR and Kir6.x have some subtypes; SUR1, SUR2A, SUR2B, Kir6.1, and Kir6.2. SUR1 has been cloned as high-affinity binding protein to sulfonylurea, which is a mostly

common used drug for type-2 diabetic patients (10). SUR2A shares 68% amino acid identity with SUR1, and SUR2B is a splicing variant of SUR2A and have same sequences with SUR2A except its C-terminal 42 amino acids which are similar to SUR1 (14,15). Kir6.1 and Kir6.2 share 71% amino acids identity with each other, both of which have two putative transmembrane domains and ion pore-forming (H5) region (11,13).

Pancreatic β-cell K_{ATP} channels, composed of SUR1 and Kir6.2, regulate the insulin secretion by altering the β-cell membrane potential (1-4). Coexpression of SUR2A/Kir6.2, SUR2B/Kir6.2, and SUR2B/Kir6.1 reconstitutes cardiac, smooth muscle, and vascular smooth muscle K_{ATP} channels, respectively. Their physiological role is best understood in the pancreatic β-cell, where they link changes in blood glucose concentration to insulin secretion (Fig. 2). Under normal conditions, the K_{ATP} channels are open and set the β-cell resting membrane potential. Elevation of blood glucose concentration results in increased glucose uptake and metabolism by the β-cell. This closes the K_{ATP} channels, producing a membrane depolarization that activates voltage-gated calcium channels and thereby induces a rise in intracellular calcium concentration which stimulates insulin release. The physiological importance of the regulatory role of SUR1 subunit is demonstrated by the fact that mutations in this subunit have been found in patients with persistent hyperinsulinemic hypoglycemia of infancy (PHHI), a serious disorder characterized by excessive and unregulated insulin secretion (16-18). Therefore, the analysis of the mechanism of K_{ATP} channel regulation by SUR1 is important and interesting not only for scientific aspects but also for medical aspects.

It has been reported that a COOH-terminal truncated Kir6.2 can reconstitute potassium channel in the absence of SUR1 and the channel current is inhibited by ATP, suggesting Kir6.2

is the primary site for the channel inhibition by ATP (19,20). SUR1 is the primary site for the channel activation by MgADP and sensitivity to drugs such as sulfonylurea drugs and potassium channel openers. It is thought that SUR1 senses intracellular ATP and ADP concentrations with its NBFs, and activates K_{ATP} channels. However, the molecular mechanism is not clear. To elucidate it, the author analyzed the nucleotide binding properties of NBFs of SUR1. As a result, the author proposed a model for the mechanism of activation of K_{ATP} channel by SUR1.

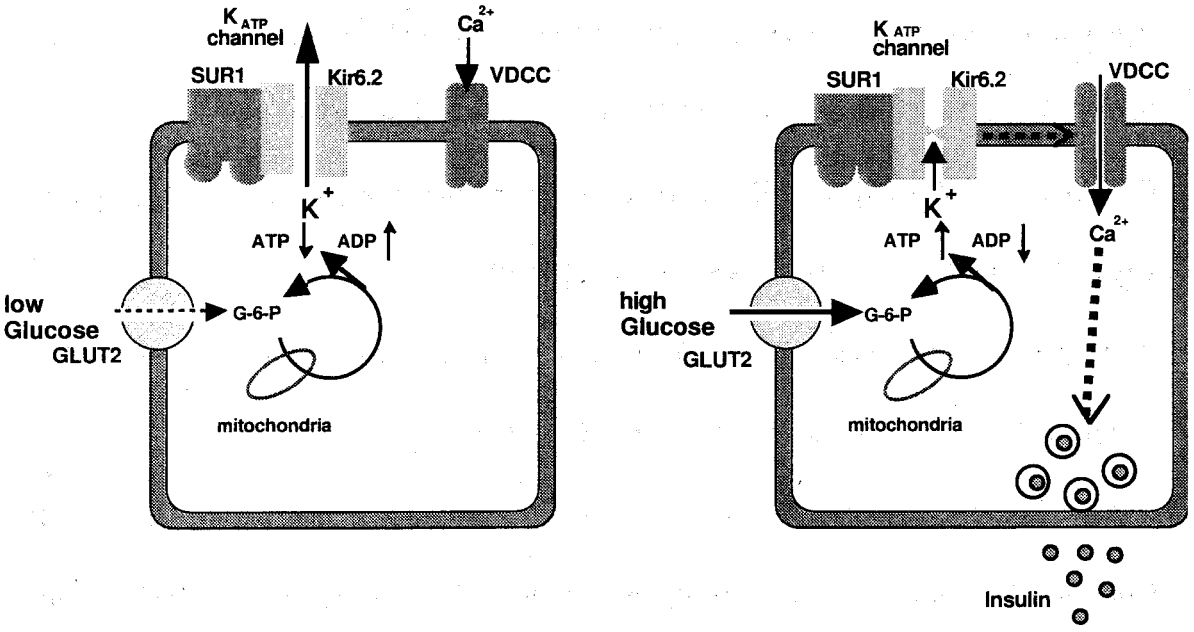

Fig. 2. **Mechanism of glucose-induced insulin secretion.** The K_{ATP} channels are thought to be ATP and ADP sensors that couple glucose metabolism to electrical activity, to stimulate insulin secretion. GLUT2: glucose transporter 2, VDCC: voltage-dependent calcium channel.

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Chapter 1

Inhibition of the high-affinity ATP biniding to the first nucleotide-binding fold of SUR1

ATP sensitive potassium (K_{ATP}) channels of pancreatic β-cells, composed of SUR1 and Kir6.2, regulate insulin release by modulating the β-cell membrane potential (1-4). Because these channels are inhibited by ATP, and activated by MgADP, they are thought to act as sensors of intracellular nucleotides. However, it is not yet clear how they monitor changes in the concentrations of intracellular ATP and ADP. Like MDR1, MRP1, and CFTR, SUR1 is a member of the ATP binding cassette (ABC) superfamily (5,6). These proteins have two nucleotide-binding folds (NBFs).

Recently, it was reported that SUR1 is efficiently photoaffinity-labeled with 8-azido-[^{32}P]ATP even in the absence of Mg^{2+} and that unlike MDR1, high-affinity labelling occurs preferentially at a single NBF (7). These features of ATP binding by SUR1 are different from that of MDR1, in which both NBFs can be labeled in an orthovanadate- and Mg^{2+}-dependent manner (8,9).

Chemical modification of specific amino acids provides a means of analyzing structure-function relationships. In particular, cysteine residues are useful targets for chemical modification because of their specific and covalent modification by many different thiol-modifying agents. Effects of the thiol-modifying agent *N*-ethylmaleimide (NEM) have been well studied on the ATP binding and hydrolysis properties of MDR1. Although human MDR1 contains seven cysteines, only two of them, located in the Walker A motifs of the

NBFs, can be readily modified by NEM (9,10). Covalent modification of a single cysteine in the Walker A motif of either NBF is sufficient to inactivate ATPase activity (10) or vanadate-induced nucleotide trapping of MDR1 (9). However, the effects of NEM modification of the cysteine in NBF1 and NBF2 on 8-azido-ATP binding to MDR1 are not equivalent. NEM modification of the cysteine in NBF2 did not appear to affect 8-azido-ATP binding to MDR1, whereas NEM modification of the cysteine in NBF1 significantly reduced it (9). Like MDR1, the Walker A motif of NBF1 of SUR1 contains a cysteine residue. The author therefore examined the effect of NEM on 8-azido-ATP binding to SUR1. In this chapter, the author demonstrated that NEM inhibits the high-affinity ATP-binding to NBF1 of SUR1, as it does for MDR1 (9), despite the fact that several other properties of ATP binding differ for these two ABC proteins.

MATERIALS AND METHODS

Materials. 8-azido-[α-^{32}P]ATP and 8-azido-[γ-^{32}P]ATP were purchased from ICN Biomedicals. Hamster SUR1 (K719M) cDNA was generously provided by Dr. Susumu Seino (Chiba University, Japan). Wild-type rat SUR1 cDNA was the kind gift of Dr G. Bell (University of Chicago, USA). The C717S and K1385M mutations used in this study were made in rat SUR1 and generously provided by Dr. Frances M. Aschcroft (Oxford University, UK). Hamster SUR1 and rat SUR1 exhibit identical ATP binding properties (data not shown).

Covalent modification of SUR1 with NEM. Membranes from COS-7 cells expressing SUR1, prepared as described (7), were incubated with NEM in TE buffer (40 mM Tris-Cl (pH 7.5), 0.1 mM EGTA) for 10 min at 25°C and washed twice with TE buffer containing 50 mM dithiothreitol.

Photoaffinity labeling with 8-azido-[^{32}P]ATP. Membranes treated with NEM were incubated with 5 μM 8-azido-[α-^{32}P]ATP or 8-azido-[γ-^{32}P]ATP in 10 μl TEM buffer (40 mM Tris-Cl (pH 7.5), 0.1 mM EGTA, 1 mM $MgSO_4$) containing 2 mM ouabain for 10 min at 37°C. The reactions were stopped by the addition of 500 μl of ice-cold TEM buffer, and free 8-azido-[^{32}P]ATP was removed after centrifugation (15,000 x g, 5 min, 2°C). Pellets were resuspended in 8 μl of TEM buffer and irradiated for 5 min (at 254 nm, 5.5 mW/cm^2) on ice. Samples were electrophoresed on a 7% SDS-polyacrylamide gel and autoradiographed. The 8-azido-[^{32}P]ATP labeled SUR1 was measured by scanning with a radioimaging analyzer (BAS2000, Fuji Photo Film Co.) Experiments were done in triplicate.

RESULTS

Covalent modification with thiol-modifying agents prevents ATP hydrolysis and ATP binding by the ABC superfamily proteins MDR1 (9-12) and CFTR (13). To learn more about the interaction of SUR1 with nucleotides, the author examined the effect of covalent modification by NEM on ATP binding to SUR1.

Membranes from COS-7 cells transiently expressing SUR1 were incubated with 5 μM

8-azido-[α-^{32}P]ATP or 8-azido-[γ-^{32}P]ATP. After removal of the unbound ligands, the proteins were irradiated with UV light. SUR1 was efficiently photoaffinity-labeled by both ligands (Fig. 1). When membranes were pretreated with NEM, photoaffinity labeling of SUR1 by either ligand was inhibited by NEM in a concentration-dependent manner (Fig. 1A). The data were fit by the Hill equation with K_i values of 1.8 μM and 2.4 μM, and Hill coefficients of 0.94 and 1.1, for binding of 8-azido-[α-^{32}P]ATP and 8-azido-[γ-^{32}P]ATP, respectively (Fig. 1B). Similar results were obtained when the reaction was carried out in the absence of Mg^{2+} (data not shown). These results confirm that the labeling observed is due to binding of ATP rather than phosphorylation. The Hill coefficient of unity further suggests that modification of a single site by NEM is sufficient to prevent ATP binding.

Fig. 2 compares the amino acid sequences of the Walker A motifs of NBF1 and NBF2 of SUR1, MDR1 and CFTR. A cysteine residue is present in both NBFs of MDR1 but neither NBF of CFTR. NBF1 of SUR1 contains a cysteine residue at the analogous site to that in NBF1 of MDR1. It has been reported previously that modification of the Walker A cysteine residue in NBF1 is responsible for the inhibition of ATP binding to MDR1 by NEM (9), and that modification of a cysteine engineered into the Walker A motif of either NBF of CFTR partially inhibited Cl^- channel activity, possibly via decreased ATP binding (14). This suggests that the cysteine residue within the Walker A motif of NBF1 is the site of NEM modification of ATP binding to SUR1. The author therefore examined the effects of NEM on 8-azido-ATP binding to a mutant form of SUR1, in which the cysteine residue within the Walker A motif of NBF1 was replaced with serine (C717S).

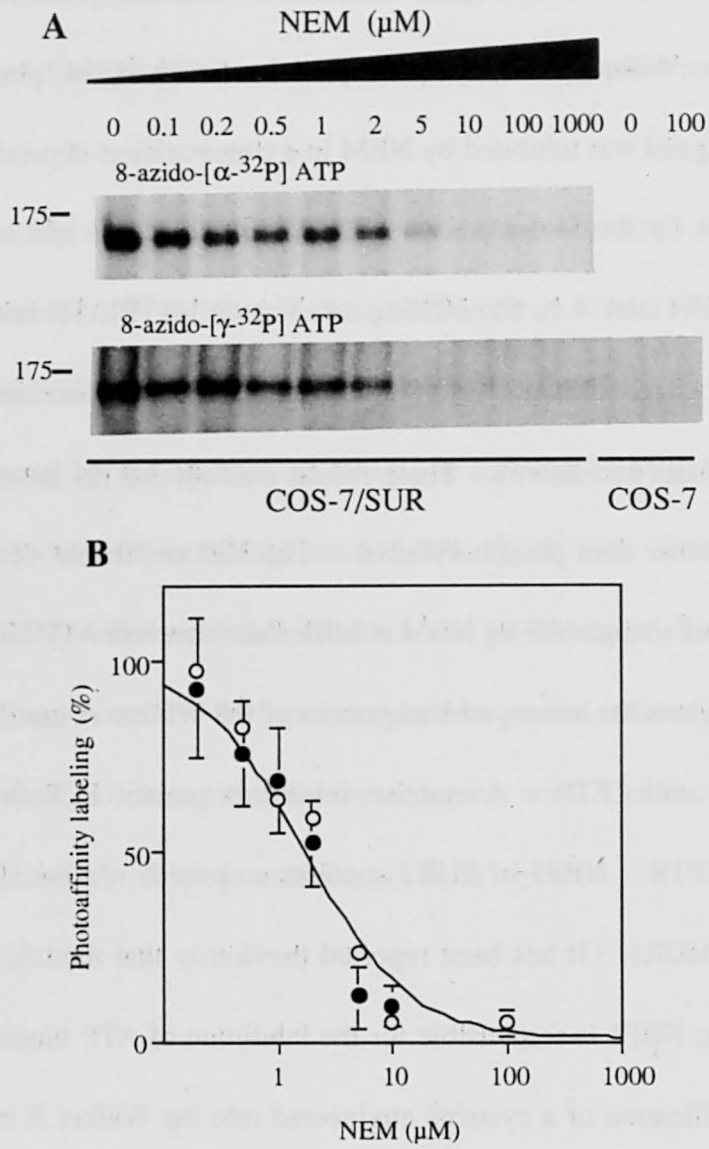

Fig. 1. **Inhibition of high-affinity 8-azido-ATP labeling of SUR1 by NEM.** A, Membrane proteins (20 μg) from COS-7 cells expressing SUR1 were treated with the indicated concentrations of NEM and incubated with 5 μM 8-azido-[α-^{32}P] or 8-azido-[γ-^{32}P] ATP at 37°C for 10 min. Proteins were photoaffinity-labeled as described under "Materials and Methods." B, Relative photoaffinity labeling of SUR1 after NEM treatment with 8-azido-[α-^{32}P] ATP (○) or 8-azido-[γ-^{32}P]ATP (●), expressed as a percentage of that obtained for NEM-untreated SUR1. The data were fit using the Hill equation. Experiments were done in triplicate.

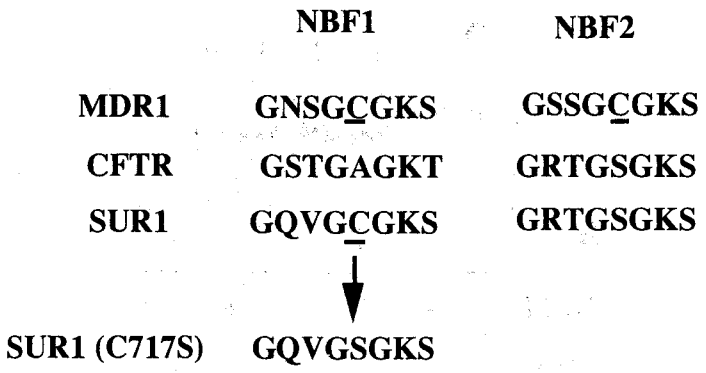

Fig. 2. **Comparison of the Walker A sequences of MDR1, CFTR, and SUR1.** The cysteine residue within the Walker A motif of NBF1 of SUR1 was replaced with serine in the C717S mutant form.

Membranes containing equal amounts of the wild-type and the C717S mutant form of SUR1 were treated with 100 μM NEM, and photoaffinity labeling with 5 μM 8-azido-[γ–^{32}P]ATP was then examined (Fig. 3). Both wild-type and C717S SUR1 were photoaffinity-labeled to the same extent in the absence of NEM. However, by contrast to wild-type SUR1, photoaffinity labeling of C717S SUR1 was unaffected by pretreatment with NEM. Like wild-type SUR1, the K1385M mutant form of SUR1 (in which the lysine residue in the Walker A motif of NBF2 was replaced with methionine) was photoaffinity-labeled by 8-azido-ATP and this labeling was inhibited by NEM treatment. However, the K719M mutant form of SUR1 (in which the lysine residue within the Walker A motif of NBF1 was replaced with methionine) was not photoaffinity-labeled either in the absence or presence of NEM. These results indicate that cysteine-717 within NBF1 of SUR1 is responsible for inhibition of high-affinity 8-azido-ATP binding by NEM, and suggest that NBF1 of SUR1 has a NEM-sensitive structure similar to that of NBF1 of MDR1.

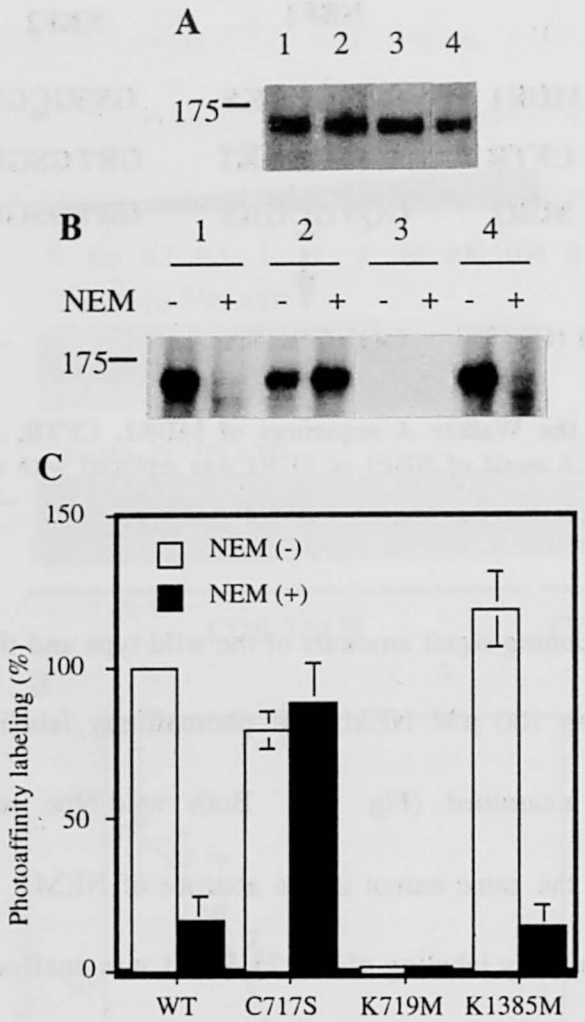

Fig. 3. **Effect of NEM on photoaffinity labeling of SUR1 mutants.** A, Immunoblotting of membrane proteins (7-20 µg) from COS-7 cells expressing wild-type (lane 1), C717S (lane 2), K719M (lane 3), or K1385M (lane 4) mutant forms of SUR1 using an antibody against the C-terminal 21 amino acids of rat SUR1. B, Membranes containing equivalent amounts of wild-type and mutant forms of SUR1 were treated with or without 100 µM NEM, and photoaffinity-labeled. C, Relative photoaffinity labeling of SUR1 expressed as a percentage of that of NEM-untreated wild-type SUR1. White bars, NEM untreated; black bars, NEM treated. Experiments were done in triplicate.

DISCUSSION

It has been reported previously that mutations in either the Walker A or B motifs of NBF1, K719M and D854N, abolish high-affinity 8-azido-ATP binding to SUR1, whereas

equivalent mutations in NBF2 do not affect ATP binding (7). The inhibition of this high-affinity 8-azido-ATP binding to SUR1 by NEM and the lack of inhibition found with the C717S mutation now confirms that NBF1 is the high-affinity ATP binding site identified on SUR1.

It has been reported that thiol-modifying agents, including NEM and *p*-chloromercuriphenylsulphonate (pCMPS), inhibit both β-cell and cardiac muscle K_{ATP} channels irreversibly and that the presence of ATP protects against this effect (15-17). However, the inhibitory effect of pCMPS on the β-cell K_{ATP} channel is due to interaction of this thiol-modifying agent with the cysteine-42 of Kir6.2 (18) and not cysteine-717 of SUR1. Unlike NEM, pCMPS does not inhibit high-affinity ATP binding to SUR1 (data not shown). This implies that, in comparison to NEM, the bulkier and more hydrophobic pCMPS reagent probably cannot access cysteine-717 of SUR1 in the Kir6.2/SUR1 channel complex. Therefore, it might be worth examining whether the ability of MgATP to activate Kir6.2/SUR1 currents (19), which is mediated by the NBFs of SUR1, can be influenced by NEM modification.

In summary, NBF1 of SUR1 has a NEM-sensitive structure similar to NBF1 of MDR1, although their ATP binding properties appear quite different. This study adds further support for the idea that the NBFs of SUR1 and MDR1 differ in their interaction with ATP and provides strong evidence that the high-affinity ATP binding site on SUR1 resides in the first nucleotide-binding fold.

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Chapter 2

Cooperative nucleotide binding of nucleotide-binding folds of SUR1

It has been reported that the Walker A lysine residue of both NBFs of SUR1 is essential for the activation of K_{ATP} channel, because K_{ATP} channel composed of Kir6.2 and Walker A lysine mutant (K719A or K1384M) SUR1 was not activated by MgADP (1). This suggests two possibilities. One is that both NBFs are necessary for the K_{ATP} channel activation. The other is that mutation at one NBF affects the nucleotide binding or hydrolysis at the other NBF because of the presence of cooperativity between two NBFs. It was reported that the Walker A lysine mutation at either NBF abolishes the ATP binding and hydrolysis at the other NBF in MDR1 (2,3). Then, the author intended to investigate the cooperative nucleotide binding of both NBFs of SUR1 in this chapter.

In chapter 1, the author demonstrated that SUR1 binds 8-azido-ATP strongly at NBF1. This property is quite different from MDR1, which binds 8-azido-ATP strongly only in the presence of both Mg^{2+} and vanadate by vanadate-induced nucleotide trapping (4-6). The high-affinity ATP binding of SUR1 makes it possible to investigate the biochemical basis of cooperative interaction between two NBFs. In this chapter, the author provides direct biochemical evidence of the cooperative interaction in nucleotide binding of the two NBFs of SUR1.

Persistent hyperinsulinemic hypoglycemia of infancy (PHHI) is characterized by inappropriately elevated insulin secretion in the presence of hypoglycemia. To date, many

mutations have been identified in the *Sur1* gene in PHHI patients (7-15). Some are nonsense or frameshift mutations, and they apparently result in the expression of a truncated SUR1 protein. Others are missense mutations in which a single amino acid has been substituted. Many of these missense mutations were identified in two NBFs of SUR1. Since these domains are the primary sites of nucleotide interactions with SUR1, the author speculates that these mutations impair K_{ATP} channel activity, at least in part, by disrupting nucleotide interactions with SUR1. Electrophysiological studies have showed that some PHHI mutant SUR1s do not reconstitute K_{ATP} channels (11,12), and other PHHI mutant SUR1s reconstitute K_{ATP} channels that are not acitivated by MgADP (8,14). However, direct molecular mechanisms causing PHHI is not clear.

Recently, two missense mutations (R1420C and R1436Q) of SUR1 were identified in Japanese PHHI patients. Both arginine-1420 and arginine-1436 locate between Walker A motif and SGGQ signature of NBF2 of SUR1. Electrophysiological study revealed that K_{ATP} channel composed of R1420C mutant form of SUR1 and Kir6.2 was not fully activated by metabolic inhibition and that R1436Q mutant form of SUR1 and Kir6.2 did not reconstitute K_{ATP} channel activity. In this chapter, the author shows that R1420C mutation impairs cooperative nucleotide binding to SUR1 and that R1436Q mutation affects SUR1 expression.

MATERIALS AND METHODS

Materials. 8-azido-[α-^{32}P]ATP and 8-azido-[γ-^{32}P]ATP were purchased from ICN

Biomedicals. Hamster SUR1 cDNA was gifted from Dr. Joseph Bryan (Baylor College of Medicine, USA). Hamster SUR1 (K1385M, D1506N) cDNA was generously provided by Dr. Susumu Seino (Chiba University, Japan). Mouse SUR1 (wild-type, R1420C, R1436Q) cDNA was generously provided by Dr. Yukio Tanizawa (Yamaguchi University, Japan).

Reaction of SUR1 with nucleotides (post-incubation procedure). Membrane proteins from COS-7 cells expressing SUR1, prepared as described (5), were incubated with 10 μM 8-azido-[α-^{32}P]ATP or 8-azido-[γ-^{32}P]ATP in 2.5 μl TE (+Mg) buffer (40 mM Tris-Cl (pH 7.5), 3 mM $MgSO_4$, 0.1 mM EGTA) containing 2 mM ouabain for 3 min at 37°C. The reactions were stopped by the addition of 500 μl of ice-cold TE (+Mg) buffer, and free 8-azido-[^{32}P]ATP was removed after centrifugation (15,000 x g, 5 min, 2°C). Pellets were resuspended in 5 μl of TE (+Mg) or TE (-Mg: without $MgSO_4$) buffer containing 2 mM ouabain. Membrane proteins were then mixed with 5 μl of TE (+Mg) or TE (-Mg) buffer containing 2 mM ADP or ATP. The mixture was incubated for 15 min at 0 or 37°C, and irradiated for 5 min (at 254 nm, 5.5 mW/ cm^2) on ice. Samples were electrophoresed on a 7% SDS-polyacrylamide gel and autoradiographed. The trapped 8-azido-[^{32}P]ATP in SUR1 was measured by scanning with a radioimaging analyzer (BAS2000, Fuji Photo Film Co.). Experiments were done at least in triplicate.

Statistical analysis. The statistical significance of differences in quantitative variables between groups was analyzed by unpaired (two-tailed) t-tests. P values less than 0.05 were considered significant.

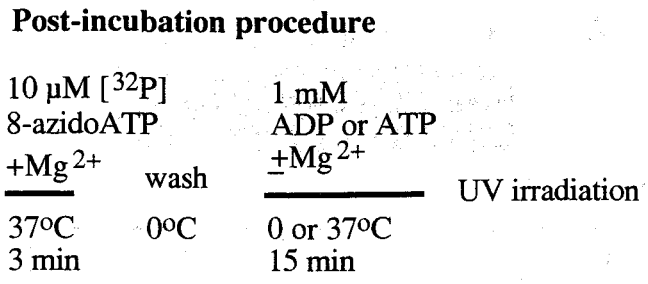

Fig. 1. **Schematic diagram of the reaction of SUR1 with nucleotides on post-incubation procedure.** Membrane proteins from COS-7 cells expressing SUR1 were incubated with 10 μM 8-azido-[α-^{32}P]ATP or 8-azido-[γ-^{32}P]ATP in 2.5 μl TE (+Mg) buffer containing 2 mM ouabain for 3 min at 37°C. The reactions were stopped, and free 8-azido-[^{32}P]ATP was removed as described under "Materials and Methods." Pellets were washed in the same buffer and resuspended in 5 μl of TE (+Mg) or TE (-Mg) buffer containing 2 mM ouabain. Membrane proteins were then mixed with 5 μl of TE (+Mg) or TE (-Mg) buffer containing 2 mM ADP or ATP. The mixture was incubated for 15 min at 0 or 37°C and irradiated on ice. Samples were electrophoresed on a 7% SDS-polyacrylamide gel, and autoradiographed. The trapped 8-azido-[^{32}P]ATP in SUR1 was measured by scanning with a radioimaging analyzer.

RESULTS

Effects of MgADP and MgATP on the prebound 8-azido-[^{32}P]ATP

When SUR1 was incubated with 8-azido-[α-^{32}P]ATP in the presence of Mg^{2+} (Fig. 1, post-incubation procedure), 8-azido-[α-^{32}P]ATP continued to bind to SUR1 for 15 min at 0°C, but dissociated gradually at 37°C (Fig. 2). In the presence of MgADP, 8-azido-[α-^{32}P]ATP remained tightly bound to SUR1 for 15 min at 37°C. When SUR1 was incubated with ADP or ATP in the absence of Mg^{2+} after preincubation with 8-azido-[α-^{32}P]ATP, 8-azido-[α-^{32}P]ATP dissociated from SUR1 in 15 min. About 80% of prebound 8-azido-[α-^{32}P]ATP dissociated from SUR1 in 5 min in the absence of Mg^{2+} at 37°C (data not shown), showing that 8-azido-[α-^{32}P]ATP readily dissociates from SUR1 in the absence of Mg^{2+} at 37°C. Accordingly, MgADP, by binding to one NBF, stabilizes prebound 8-azido-[α-^{32}P] ATP

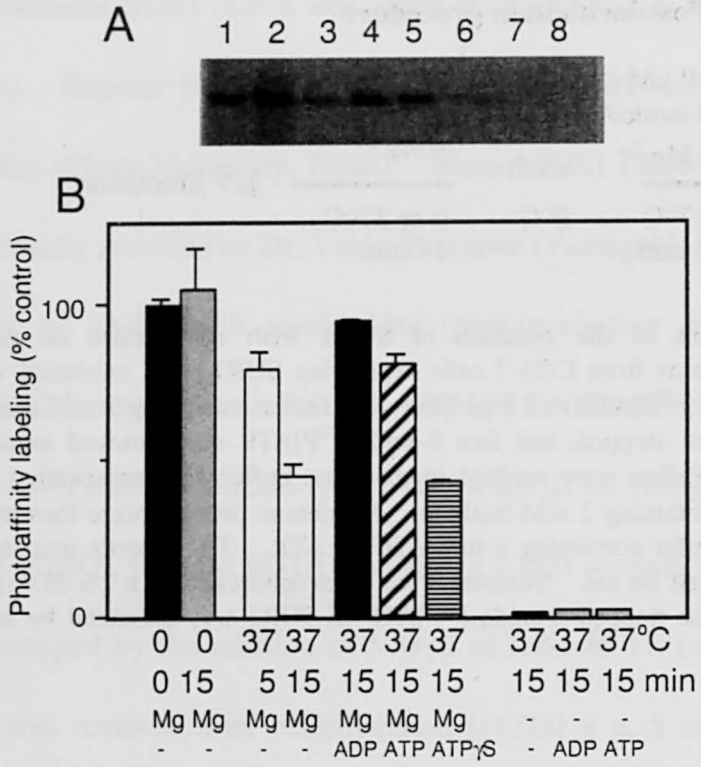

Fig. 2. **Cooperative binding of MgADP and 8-azido-[α-^{32}P]ATP.** Membrane proteins from COS-7 cells expressing SUR1 were preincubated with 10 μM 8-azido-[α-^{32}P]ATP. A, After free 8-azido-[α-^{32}P]ATP was removed, proteins were photoaffinity-labeled immediately (lane 1), or after incubation with 3 mM $MgSO_4$ for 15 min at 0°C (lane 2), with $MgSO_4$ for 15 min at 37°C (lane 3), with $MgSO_4$ and 1 mM ADP (lane 4), with $MgSO_4$ and 1 mM ATP (lane 5), with $MgSO_4$ and 1 mM ATP-γS (lane 6), without $MgSO_4$ or nucleotide (lane 7), and with 1 mM ADP (lane 8) for 15 min at 37°C as described for Fig. 1. B, Photoaffinity labeling is expressed as percentage of control (photoaffinity-labeled immediately) of those shown in A, lane 1.

binding to SUR1 with slightly less efficiency, compared with MgADP. In contrast, the slowly-hydrolysable ATP analogue ATP-γS, had no greater stabilizing effect than Mg^{2+} alone. The effects of MgADP and MgATP on the stabilization of prebound 8-azido-[α-^{32}P]ATP binding to SUR1 were concentration-dependent (maximal effects at 0.5 mM for both) (Fig. 3), suggesting that MgADP, either by direct binding or by hydrolysis of bound MgATP, stabilizes 8-azido-ATP binding. When SUR1 was incubated with MgADP or MgATP after preincubation with 8-azido-[γ-^{32}P]ATP, SUR1 was photoaffinity-labeled as efficiently as it

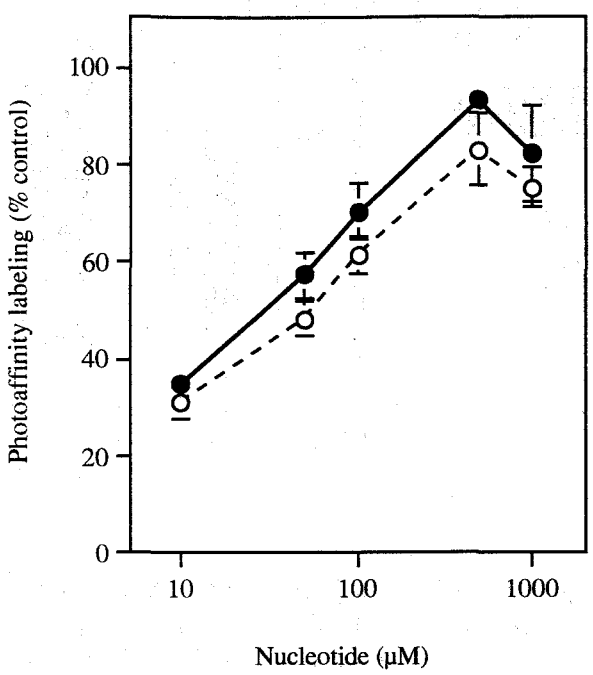

Fig. 3. **Effects of ADP and ATP on photoaffinity labeling.** Membrane proteins from COS-7 cells expressing SUR1 were preincubated with 10 μM 8-azido-[α-^{32}P]ATP. After free 8-azido-[α-^{32}P]ATP was removed, proteins were photoaffinity-labeled after incubation with 3 mM $MgSO_4$ and ADP (●) or with $MgSO_4$ and ATP (O) for 15 min at 37°C. Photoaffinity labeling is expressed as percentage of control (photoaffinity-labeled immediately).

was with 8-azido-[α-^{32}P]ATP (Fig. 4), indicating that prebound 8-azido-[γ-^{32}P]ATP is not hydrolyzed during incubation with MgADP or MgATP.

Effects of mutations in NBF2 on photoaffinity labeling

K_{ATP} channel activation requires both NBFs of SUR1 to be functional (1,8). To clarify the role of NBF2 in stabilization of nucleotide binding, the effects of mutations in NBF2 on photoaffinity labeling were examined by post-incubation procedure. Mutations in

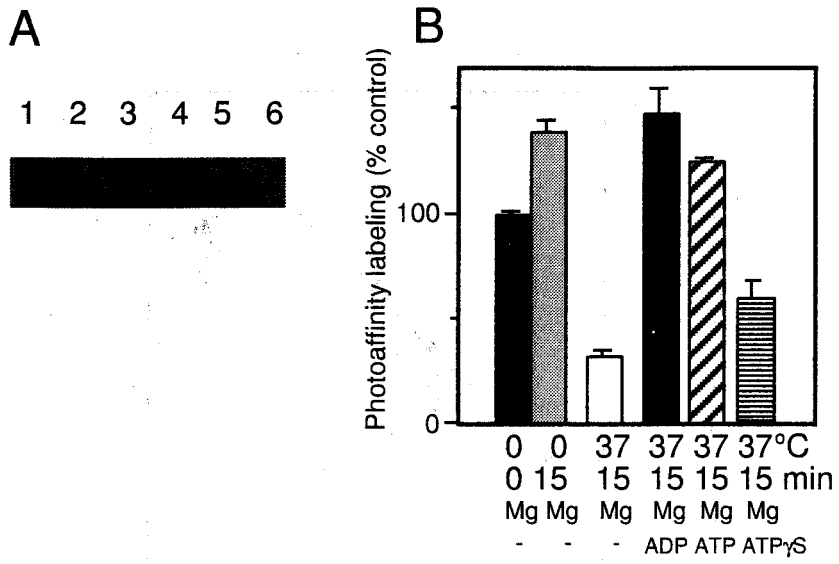

Fig. 4. **Cooperative binding of MgADP and 8-azido-[γ-^{32}P]ATP.** Membrane proteins from COS-7 cells expressing SUR1 were preincubated with 10 μM 8-azido-[γ-^{32}P]ATP. A, After free 8-azido-[γ-^{32}P]ATP was removed, proteins were photoaffinity-labeled immediately (lane 1), or after incubation with 3 mM $MgSO_4$ for 15 min at 0°C (lane 2), with $MgSO_4$ for 15 min at 37°C (lane 3), with $MgSO_4$ and 1 mM ADP (lane 4), with $MgSO_4$ and 1 mM ATP (lane 5), and with $MgSO_4$ and 1 mM ATP-γS (lane 6) for 15 min at 37°C as described for Fig. 1. B, Photoaffinity labeling is expressed as percentage of control (photoaffinity-labeled immediately) as those shown in A, lane 1.

the Walker A and B motifs, K1385M and D1506N, abolished the stabilizing effects of MgADP on 8-azido-ATP binding, although they had almost no effect on 8-azido-ATP (10 μM) binding overall (Fig. 5). Mutations at the corresponding sites in other ABC proteins reduce MgATP binding at low concentrations (< 10 μM) but not at high concentrations (~1 mM), and abolish ATP hydrolysis (3,6). The results suggest that SUR1 binds 8-azido-ATP strongly at NBF1, and that MgADP, by binding at NBF2, stabilizes prebound 8-azido-ATP binding at NBF1. Because MgADP had maximal effect on wild-type SUR1, it is unlikely that ATP hydrolysis is required to stabilize 8-azido-ATP binding. MgADP binding to NBF2 more likely induces a conformational change at NBF2 that transduces another conformational

change in NBF1 to stabilize ATP binding at NBF1. Mutations in NBF2 could well impede these changes.

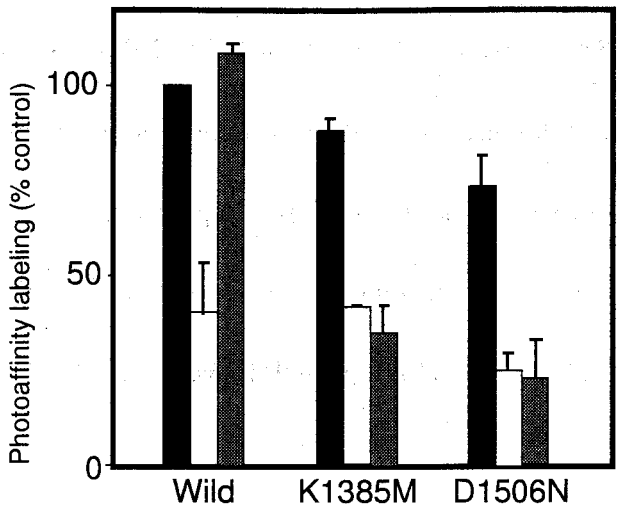

Fig. 5. **Photoaffinity labeling of the wild-type SUR1 and the K1385M and D1506N mutants of SUR1.** Membrane proteins from COS-7 cells expressing SUR1 were preincubated with 10 μM 8-azido-[α-^{32}P]ATP. After free 8-azido-[α-^{32}P]ATP was removed, proteins were photoaffinity-labeled immediately (black bars), after incubation with 3 mM $MgSO_4$ (white bars), or with $MgSO_4$ and 0.5 mM ADP (shaded bars) for 15 min at 37°C. Photoaffinity labeling is expressed as percentage of control (photoaffintiy labeled immediately) of the wild-type SUR1.

Expression of mutant SURs

Two mutations of SUR1, that are missense (R1420C, R1436Q) mutations, were identified in Japanese PHHI patients. None of these mutations were found in control Japanese subjects. Siblings homozygous for the R1420C mutation had a mild form, whereas patient heterozygous for the R1436Q mutations exhibited a severe form of PHHI. Both arginine-1420 and arginine-1436 locate between Walker A motif and SGGQ signature in NBF2 of SUR1 and are conserved between human and mouse SUR1. When mouse Kir6.2 and SUR1, in which mutations were introduced to the corresponding position of arginine-1420

and arginine-1436, were transiently coexpressed in COS-7 cells, R1420C mutant SUR1 formed K_{ATP} channels which activity was reduced 50% when examined under the metabolic inhibition compared to wild-type K_{ATP} channels, and R1436Q mutant form of SUR1 did not show functional K_{ATP} channel activity. Since, R1420C and R1436Q mutations are missense mutations, the protein expression level of mutant proteins were examined by western blotting analysis. Membranes from COS-7 cells transiently expressing wild-type SUR1 and mutant SUR1 were detected by the anti-C terminus antibody of SUR1 in Western blotting. The expression levels of R1420C and R1436Q SUR1 were about 1/2 and less than 1/10, respectively (Fig. 6).

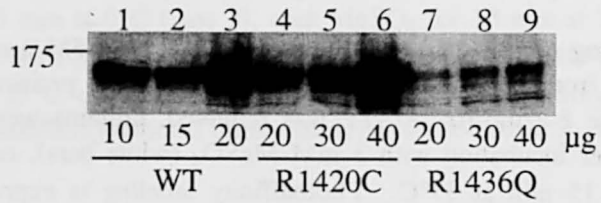

Fig. 6. **Western blotting analysis of wild-type and mutant SUR1.** Membrane fractions from COS-7 cells expressing wild-type SUR1 (lanes 1-3; 10, 15, 20 μg membrane proteins, respectively), R1420C SUR1 (lanes 4-6; 20, 30, 40 μg membrane proteins, respectively), and R1436Q SUR1 (lanes 7-9; 20, 30, 40 μg membrane proteins, respectively) were electrophoresed, blotted and detected with the anti-C terminus of SUR1 antibody.

Effect of R1420C mutation on cooperative binding of adenine nucleotides in SUR1

There is a cooperative regulation of ATP and ADP binding to SUR1 and this cooperativity may be involved in MgATP and MgADP regulation of the K_{ATP} channels. Prebound ATP at NBF1 is stabilized by MgADP or MgATP. Effect of the only R1420C

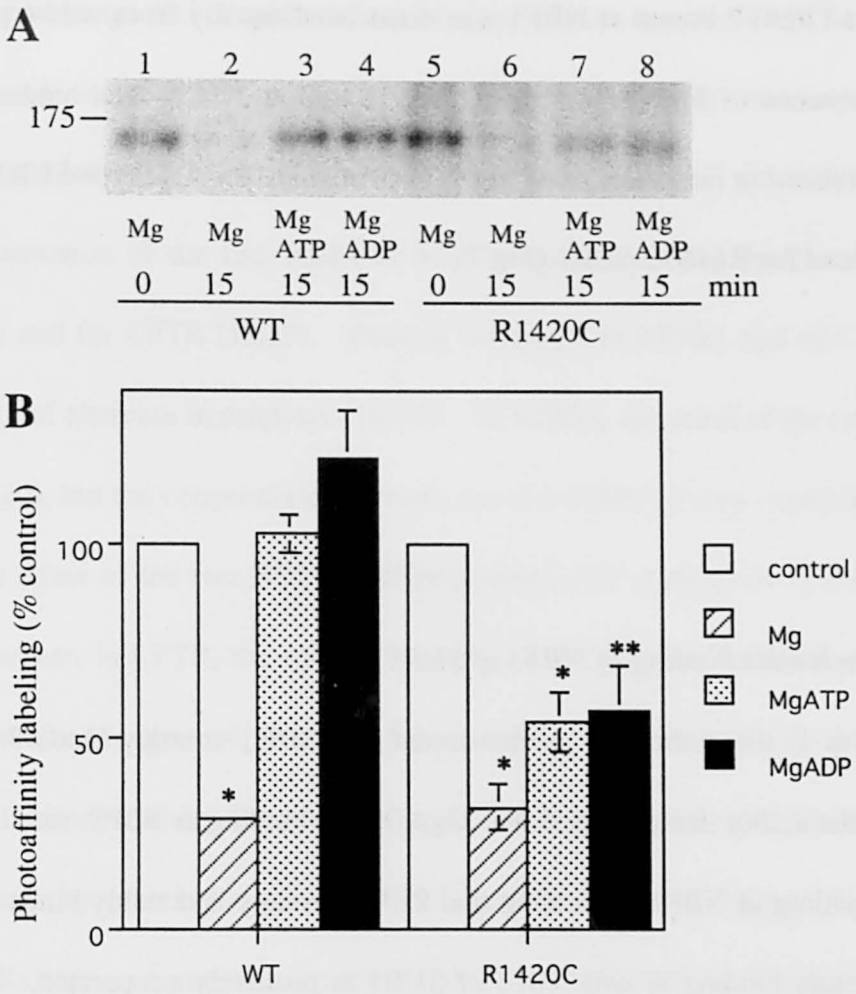

Fig. 7. **Cooperative binding of MgADP and 8-azido-[α-^{32}P]ATP of wild-type and mutant SUR1.** A, Membrane proteins from COS-7 cells expressing wild-type (lanes 1-4) or R1420C (lanes 5-8) SUR1 were preincubated with 10 μM 8-azido-[α-^{32}P]ATP. After free 8-azido-[α-^{32}P]ATP was removed, proteins were photoaffinity-labeled immediately (lanes 1 and 5), or after incubation with 3 mM $MgSO_4$ (lanes 2 and 6), with $MgSO_4$ and 0.5 mM ATP (lanes 3 and 7), with $MgSO_4$ and 0.5 mM ADP (lanes 4 and 8) for 15 min at 37°C as Fig. 1 (post-incubation procedure). B, Photoaffinity labeling is expressed as percentage of control (photoaffinity-labeled immediately) as those shown in A, lane 1. * $p<0.01$, ** $p<0.05$.

mutation in NBF2 of SUR1 on cooperative nucleotide binding was analyzed by post-incubation procedure, because R1436Q SUR1 was expressed only little. When membranes expressing equal amounts of wild-type and R1420C SUR1 were photoaffinity-labeled with 10 μM 8-azido-[α-^{32}P]ATP, R1420C SUR1 bound the ligand as effectively as wild-type SUR1.

The 8-azido-[α-^{32}P]ATP bound at NBF1 was dissociated equally from wild-type and mutant SUR1 in the absence of MgATP or MgADP in 15 min at 37°C. The binding was almost completely stabilized in the presence of MgATP or MgADP for wild-type SUR1, whereas the effect was reduced for R1420C SUR1 (Fig 7).

DISCUSSION

Cooperative nucleotide binding of NBFs of SUR1

In Chapter 1, the author has demonstrated that SUR1 strongly binds 8-azido-ATP at NBF1. Here the author demonstrated that MgADP, by binding at NBF2, stabilizes prebound 8-azido-ATP binding at NBF1, indicating that SUR1 strongly and stably binds ATP at NBF1 and that nucleotide binding at two NBFs of SUR1 is positively cooperated. In this study, SUR1 was first incubated with 8-azido-[^{32}P]ATP and then post-incubated with MgADP after removing free 8-azido-[^{32}P]ATP as diagrammed in Fig. 1. In this procedure, SUR1 was photoaffinity-labeled only with the 8-azido-[^{32}P]ATP prebound at NBF1 and continuously bound during post-incubation, otherwise free cold nucleotides would compete with dissociated and diluted 8-azido-[^{32}P]ATP. This indicates that prebound 8-azido-ATP is very stable and dissociates very slowly, if SUR1 binds MgADP at NBF2.

MgATP, like MgADP, is able to stimulate the activity of K_{ATP} channels containing a mutation (R50G) in Kir6.2 that impairs ATP inhibition (16). The degree of MgADP activation of K_{ATP} channels is greater than that of MgATP activation, which occurs over the

same concentration range (0.1-1 mM). Both of these effects are abolished, however, when mutations are made in the NBFs of SUR1 (16). The present study thus demonstrates that positive interaction between the two NBFs of SUR1 is important for K_{ATP} channel regulation. Cooperative interaction of the two NBFs of ABC proteins has been previously shown for MDR1 (3,6,17) and for CFTR (18,19). Both of the NBFs of MDR1 and of CFTR seem to hydrolyze ATP and alternate in catalysis (18-20). In MDR1, the roles of the two NBFs may be equivalent (20), but the cooperativity between the two NBFs is very strong; a mutation or modification in either of the two NBFs in MDR1 blocks ATP hydrolysis by the other intact NBF (6). However, in CFTR, the roles of the two NBFs may not be equivalent; NBF1 is thought to be involved in channel opening, whereas NBF2 is thought to be involved in channel closing (18,19). Cooperativity between the two NBFs is induced by phosphorylation of the regulatory domain by protein kinase A, which is also necessary for channel opening (19). However, CFTRs bearing a mutation in either of the two NBFs still function as channels even though the gating kinetics are altered (18,19).

When membrane was incubated for 15 min in the presence of Mg^{2+} at 0°C or in the presence of MgADP or MgATP at 37°C, the efficiency of photoaffinity labeling with 8-azido-[γ-^{32}P]ATP was increased significantly (Fig. 4). This result suggests that the conformational change in NBF1 transduced by MgADP binding to NBF2 could stabilize ATP binding at NBF1. Mg^{2+} binding to NBF2 might also transduce a conformational change in NBF1 to some extent, becuase Mg^{2+} alone weakly stabilizes 8-azido-ATP binding at NBF1 (Fig. 2) and because photoaffinity labeling was increased significantly in the presence of Mg^{2+} at 0°C (Fig. 4).

In conclusion, the two NBFs of SUR1 show strong cooperativity in nucleotide binding. Indeed, K_{ATP} channel activation may be induced primarily by the cooperative interaction of ATP binding at NBF1 and MgADP binding at NBF2. The intracellular concentration of MgADP is the primary factor in the determination of the active state of SUR1, although ATP is required for the action of SUR1. Such cooperative interaction of the two NBFs is also important in the function of other ABC proteins, such as MDR1 and CFTR, and thus deserve investigation as an appropriate target for various therapeutic drugs. Variations in the cooperativity of the two NBFs in ABC proteins could account for the differences in function of the various ABC proteins as transporters, ion channels, or ion-channel regulators.

Cooperative nucleotide binding of NBFs of PHHI mutant SUR1

PHHI is an autosomal recessive disorder of childhood characterized by severe, recurrent, and fasting hypoglycemia associated with inappropriate hypersecretion of insulin. Linkage analysis has mapped PHHI to chromosome 11p14-15.1 and mutations of *Sur1* or *Kir6.2* gene are responsible for PHHI (7-15,21-23). Recently, two mutations (R1420C and R1436Q) of SUR1 were identified in Japanese PHHI patients.

To examine the causal relationship of mutations to PHHI directly, the author conducted functional studies. When R1436Q SUR1 alone was expressed in COS-7 cells, the expression level of SUR1 protein in the crude membrane fraction was less than 1/10 that of wild-type SUR1. This result suggests instability of the protein in the cell, or defective transport to the membrane fraction, and may account for the absence of functional K_{ATP} channels in cells coexpressing R1436Q SUR1 and Kir6.2. Single channel function of K_{ATP} constituted with

this mutant SUR1 needs to be further investigated by electrophysiological studies using patch clamp technique.

Impairment of K_{ATP} channel activity with R1420C SUR1 was significant under metabolic inhibition. The author showed that stabilization of 8-azido-[α-^{32}P]ATP binding to NBF1 by MgADP or MgATP was impaired in R1420C SUR1 (Fig. 7). This may explain impaired K_{ATP} (R1420C SUR1) channel activation in COS-7 cells by metabolic inhibition. K_{ATP} channels are under complex regulation by intracellular ATP and ADP. Inhibition of the K_{ATP} channel by ATP appeared to be mediated by direct interaction of ATP with the pore-forming subunit Kir6.2, whereas activation is conferred by the regulatory subunit, SUR1 (16,24). According to stabilization effects experiment, K_{ATP} channel activation is induced when SUR1 binds ATP at NBF1 and MgADP at NBF2 cooperatively. At high intracellular MgADP concentrations, most SUR1 is in this condition. When the intracellular MgADP concentration decreases, MgADP dissociates from NBF2, leading to closure of K_{ATP} channels. MgADP dissociation from NBF2 further leads to instability of ATP binding at NBF1, allowing the release of ATP. ATP binding at NBF1 is required for the channel activating effect of SUR1. Most of the SUR1 mutants identified in PHHI patients have exhibited a reduced response to MgADP stimulation, when reconstituted channel activities were measurable (8,14). This may also be the case for the R1420C mutation. Direct interaction of mutant SUR1 and adenine nucleotides has not previously been assessed. The data presented herein constitute the first direct biochemical evidence of defective nucleotide interaction with SUR1 linked to impaired K_{ATP} channel activity.

Failure of R1420C SUR1 to stabilize ATP binding at NBF1 by MgADP could be due to

impaired MgADP binding to NBF2. Or alternatively, it may be due to impaired cooperative interaction between two NBFs (possibly conformational change of SUR1).

Electrophysiological study has suggested that impairment of K_{ATP} channel function was modest with the R1420C mutation, whereas no channel activities were observed for the R1436Q mutation. This parallels clinical disease severities of these patients: patients with the R1420C mutation achieved seemingly spontaneous remission after a few months of medical therapy, whereas patients who had R1436Q mutation required partial pancreatectomy.

In conclusion, the author present herein the first direct biochemical evidence that the SUR1 mutant identified in PHHI patients impairs cooperative nucleotide binding, thereby leading to dysfunction of K_{ATP} channel activity and dysregulation of insulin secretion.

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Chapter 3

ATP binding properties of SUR1

In chapter 2, the author revealed that MgADP and MgATP stabilized binding of prebound 8-azido-ATP to SUR1, and that mutations in the Walker A and B motifs of NBF2 abolished this stabilizing effect of MgADP and MgATP. These biochemical results suggest that SUR1 binds 8-azido-ATP strongly at NBF1 and binds MgADP at NBF2 and that the two NBFs of SUR1 cooperate in nucleotide binding. However, the author has not been able to detect nucleotide binding of NBF2 directly due to the high-affinity ATP binding to NBF1.

The ATP binding/hydrolysis properties of the NBF are best studied with MDR1 among the ABC proteins, and its two NBFs are proposed to be equivalent in function (1). The covalent modification of the cysteine residue in the Walker A motif of either NBF has been shown to be sufficient to inactivate the ATPase activity (2-5). Mutation of the Walker A lysine residue of either NBF, thought to interact with the β- and γ- phosphates of ATP, abolishes the ATPase activity of MDR1 and its ability to confer multidrug resistance (6-8). Both NBFs of MDR1 can hydrolyze nucleotides (9-11), and mild trypsin digestion showed that two NBFs were labeled in equal proportion with 8-azido-ATP after hydrolysis in the presence of orthovanadate (12).

The author tried to separate the two NBFs of SUR1 by mild digestion with proteases, because mild trypsin digestion of MDR1 or CFTR produces two large polypeptides corresponding to the N- and C- terminal halves (13-16). SUR1 photoaffinity labeled with 8-

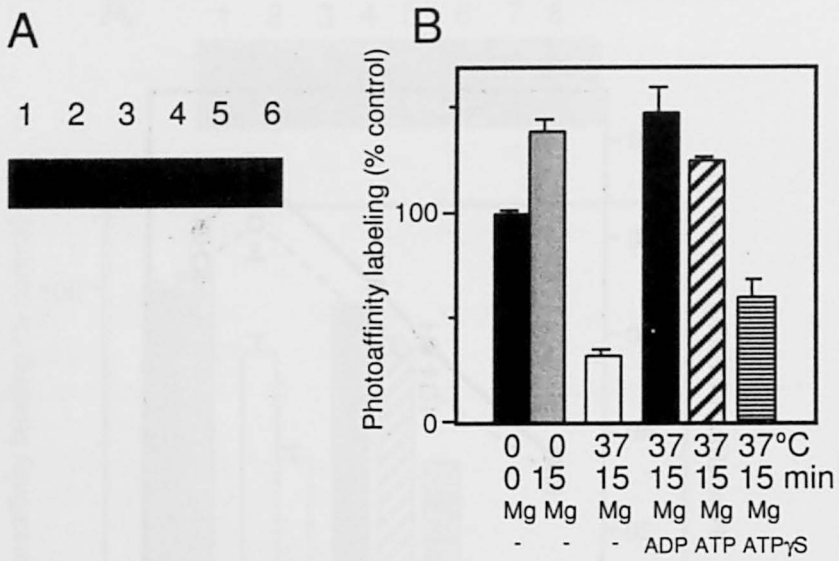

Fig. 4. **Cooperative binding of MgADP and 8-azido-[γ-^{32}P]ATP.** Membrane proteins from COS-7 cells expressing SUR1 were preincubated with 10 μM 8-azido-[γ-^{32}P]ATP. A, After free 8-azido-[γ-^{32}P]ATP was removed, proteins were photoaffinity-labeled immediately (lane 1), or after incubation with 3 mM $MgSO_4$ for 15 min at 0°C (lane 2), with $MgSO_4$ for 15 min at 37°C (lane 3), with $MgSO_4$ and 1 mM ADP (lane 4), with $MgSO_4$ and 1 mM ATP (lane 5), and with $MgSO_4$ and 1 mM ATP-γS (lane 6) for 15 min at 37°C as described for Fig. 1. B, Photoaffinity labeling is expressed as percentage of control (photoaffinity-labeled immediately) as those shown in A, lane 1.

the Walker A and B motifs, K1385M and D1506N, abolished the stabilizing effects of MgADP on 8-azido-ATP binding, although they had almost no effect on 8-azido-ATP (10 μM) binding overall (Fig. 5). Mutations at the corresponding sites in other ABC proteins reduce MgATP binding at low concentrations (< 10 μM) but not at high concentrations (~1 mM), and abolish ATP hydrolysis (3,6). The results suggest that SUR1 binds 8-azido-ATP strongly at NBF1, and that MgADP, by binding at NBF2, stabilizes prebound 8-azido-ATP binding at NBF1. Because MgADP had maximal effect on wild-type SUR1, it is unlikely that ATP hydrolysis is required to stabilize 8-azido-ATP binding. MgADP binding to NBF2 more likely induces a conformational change at NBF2 that transduces another conformational

(15,000 x g, 5 min, 2°C). Pellets were resuspended in 8 µl of TEM buffer and irradiated for 5 min (at 254 nm, 5.5 mW/cm^2) on ice. Samples were electrophoresed on a 12% SDS-polyacrylamide gel and autoradiographed. When membranes were incubated with 5 µM 8-azido-[α-^{32}P]ATP in the absence of Mg^{2+}, the reactions were stopped by the addition of 500 µl of ice-cold TE buffer (40 mM Tris-Cl (pH 7.5), 0.1 mM EGTA). After centrifugation, pellets were resusupended in 8 µl of TE buffer and UV-irradiated as described above.

Photoaffinity labeling of a low-affinity ATP binding site. Membranes were incubated with 50 or 100 µM 8-azido-[α-^{32}P]ATP in the presence or absence of indicated concentrations of ATP or ADP in 5 µl TEM buffer containing 2 mM ouabain for 10 min on ice. Reactions were done on ice to prevent hydrolysis. Proteins were UV-irradiated as described above, and free ligands were removed after centrifugation.

Preparation of antibodies against NBF1 and NBF2. NBF1 (amino acids 695-934) or NBF2 (amino acids 1361-1582) of hamster SUR1 was fused with maltose-binding protein in a pMALc2 vector (New England Biolabs. Inc.), and fusion proteins were expressed in *Escherichia coli* strain BL21. The fusion proteins were purified with amylose resin (New England Biolabs. Inc.), and rabbit polyclonal antibodies were raised against the purified proteins.

Limited trypsin digestion and immunoprecipitation of tryptic fragments of SUR1. Photoaffinity-labeled membranes were resusupended in TE buffer containing 2.5 µg/ml trypsin and 250 mM sucrose to 10 µg membrane proteins/µl, and incubated for 60 min at 37°C. 100 µl of RIPA buffer (20 mM Tris-Cl (pH 7.5), 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) containing 100 µg/ml (*p*-amidinophenyl)methanesulfonyl fluoride was added to

the samples to terminate proteolysis, and membrane proteins were solubilized for 30 min at 4°C. After centrifugation for 15 min at 15,000 x g, tryptic fragments were immunoprecipitated from the supernatant with the antibody raised against NBF1, NBF2, or C-terminal 21 amino acids of rat SUR1.

RESULTS

Limited tryptic digestion of SUR1

When SUR1 was photoaffinity-labeled with 5 μM 8-azido-[α-^{32}P]ATP and digested with 2.5 μg/ml trypsin, two photoaffinity labeled fragments of about 100- and 35-kDa were produced (Fig. 1A). The amount of 100-kDa fragment decreased and the amount of 35-kDa fragment concomitantly increased with increasing incubation time with trypsin. When SUR1 was digested with trypsin under the same condition, the anti-C terminus antibody recognized the 100- and the 65-kDa fragments, but not the 35-kDa fragment, in immunoblotting (Fig. 1B). These results suggest that the 100-kDa fragment is further digested to the 35-kDa fragment, which contains the high-affinity ATP binding site, and the 65-kDa fragment, which contains the C terminus, as diagramed in Fig. 2.

Antibodies against NBF1 and NBF2 of SUR1

To determine which NBF the 35-kDa fragment contained, polyclonal antibodies were prepared against NBF1 or NBF2 fused with maltose-binding protein. These antibodies

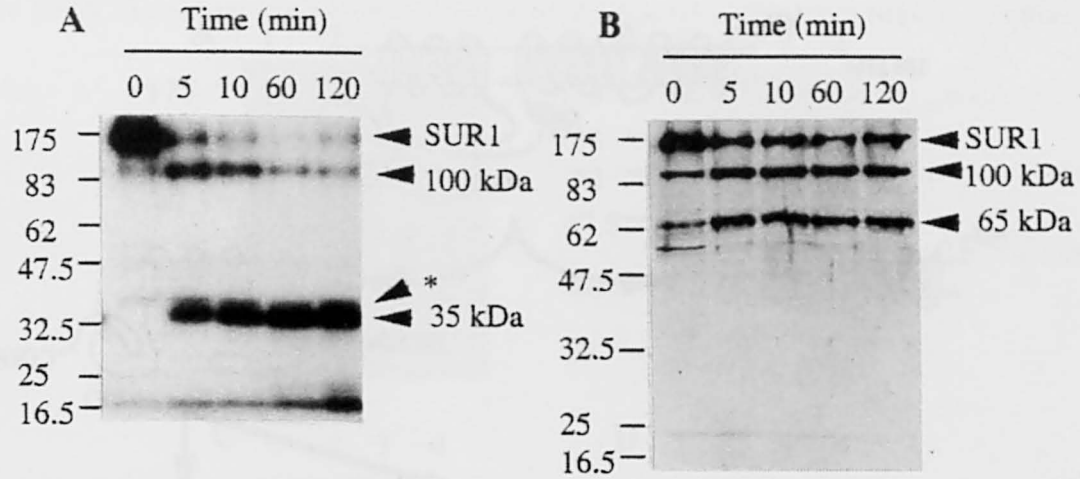

Fig. 1. **Limited digestion of SUR1 with trypsin.** A, autoradiogram of membrane proteins (25 μg) from COS-7 cells expressing SUR1 photoaffinity-labeled with 5 μM 8-azido-[γ-^{32}P] ATP as described under "Materials and Methods." Proteins were digested with 2.5 μg/ml trypsin at 37°C for indicated periods and separated by 12% SDS-polyacrylamide gel electrophoresis. B, immunoblotting of membrane proteins (20 μg) from COS-7 cells expressing SUR1 with anti-C terminus antibody. Proteins were digested and separated as described for A. The band indicated by an asterisk is a nonspecific one, because membranes from untransfected COS-7 cells produce the same band (data not shown).

recognized the NBF1 and NBF2 of SUR1 expressed in *E. coli*, respectively, but scarcely recognized those of MDR1 (Fig. 3). The photoaffinity-labeled 35-kDa fragment was precipitated with the anti-NBF1 antibody, but not with the anti-NBF2 or anti-C terminus antibody (Fig. 4, lanes 1-3). These results indicate that the 35-kDa tryptic fragment contains NBF1, and support the possibility that the 100-kDa fragment is digested to the 35-kDa fragment containing NBF1 and the 65-kDa fragment containing the C terminus (Fig. 2).

Interaction of the NBFs of SUR1 with 8-azido-[α-^{32}P] ATP

The author has suggested that NBF1 of SUR1 binds 8-azido-ATP strongly even in the absence of Mg^{2+}, and that NBF2 binds MgADP in the previous chapters. To investigate the

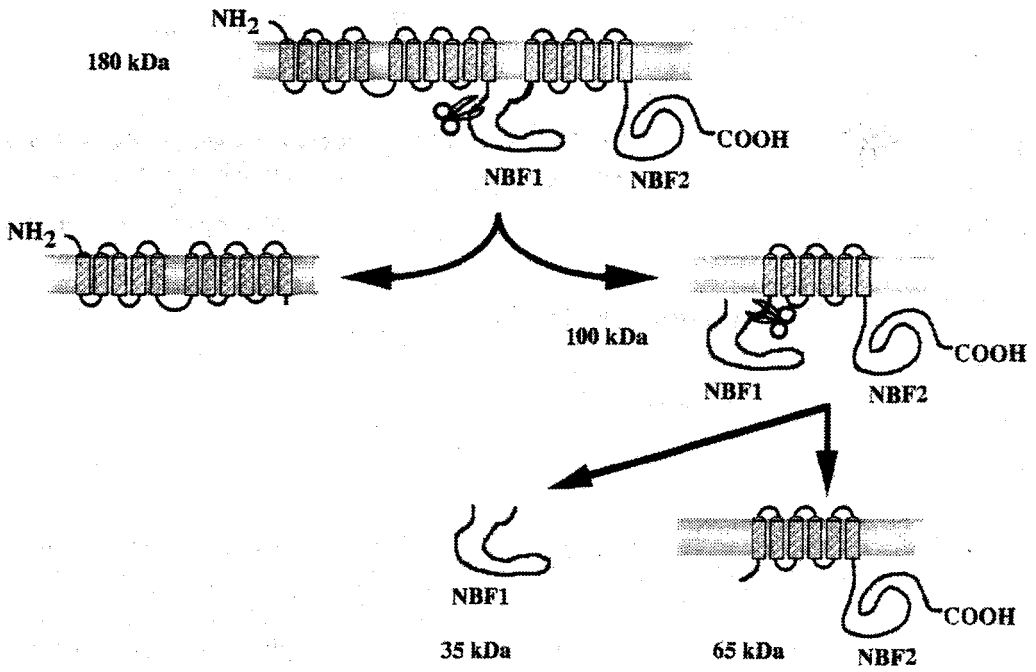

Fig. 2. **Predicted diagram of limited trypsin digestion of SUR1.** Mild trypsin digestion of SUR1 (180-kDa) first produces a 100-kDa fragment containing NBF1, NBF2, and C terminus, then produces a 35-kDa fragment containing NBF1 and a 65-kDa fragment containing NBF2 and C terminus.

ATP binding properties of the NBFs of SUR1, SUR1 was photoaffinity-labeled with 5 or 100 μM 8-azido-[α-^{32}P]ATP in the presence or absence of Mg^{2+}. Proteins were then immunoprecipitated with the anti-NBF1, anti-NBF2, or anti-C terminus antibody after mild trypsin digestion (Fig. 4). The labeled 35-kDa fragment was precipitated with the anti-NBF1 antibody when SUR1 was photoaffinity-labeled by 5 or 100 μM 8-azido-[α-^{32}P] ATP either in the presence or absence of Mg^{2+}. In contrast, the labeled 65-kDa fragment was precipitated with the anti-NBF2 and anti-C terminus antibodies only when SUR1 was incubated with 100 μM 8-azido-[α-^{32}P] ATP in the presence of Mg^{2+} and UV-irradiated without removing free ligands. When membranes were washed with buffer before UV irradiation, the 65-kDa fragment was not labeled with 100 μM 8-azido-[α-^{32}P]ATP with Mg^{2+} (data not shown).

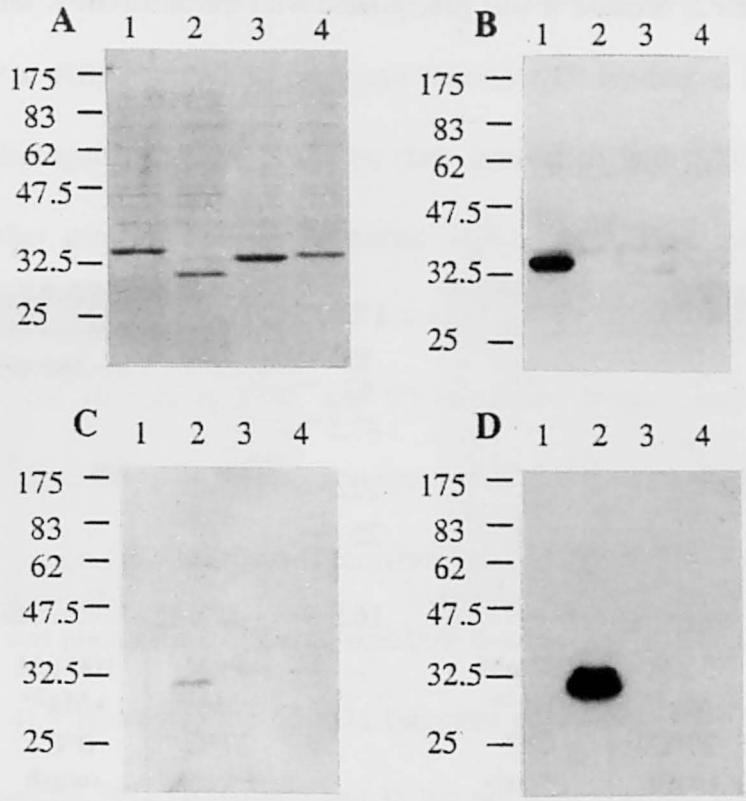

Fig. 3. **Specific recognition of NBF1 and NBF2 of SUR1 with polyclonal antibodies.** A, Coomassie Brilliant Blue staining of cell lysates (0.2 μg) of *E. coli.* BL21(DE3) strains expressing SUR1 NBF1 (amino acids 695-934) (lane 1), SUR1 NBF2 (amino acids 1361-1582) (lane 2), MDR1 NBF1 (amino acids 409-632) (lane 3), or MDR1 NBF2 (amino acids 1052-1280) (lane 4). Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis. B, immunoblotting with anti-SUR1 NBF1 antibody. C, immunoblotting with anti-SUR1 NBF2 antibody. D, immunoblotting with anti-SUR1 C terminus antibody. In B-D, cell lysates (50 ng) of *E. coli.* were separated as described for A.

These results indicate that NBF1 of SUR1 binds 8-azido-ATP strongly even in the absence of Mg^{2+} and that NBF2 binds 8-azido-ATP weakly only in the presence of Mg^{2+}. Less labeling of NBF1 with 100 μM 8-azido-[α-^{32}P]ATP on ice (lane 4) compared to that with 5 μM at 37°C suggests that high-affinity 8-azido-ATP binding to NBF1 is temperature-dependent and that incubation at 37°C is necessary. It also indicates that the 100-kDa tryptic fragment contains

both NBF1 and NBF2, because it was precipitated with the anti-NBF1, anti-NBF2, or anti-C terminus antibody.

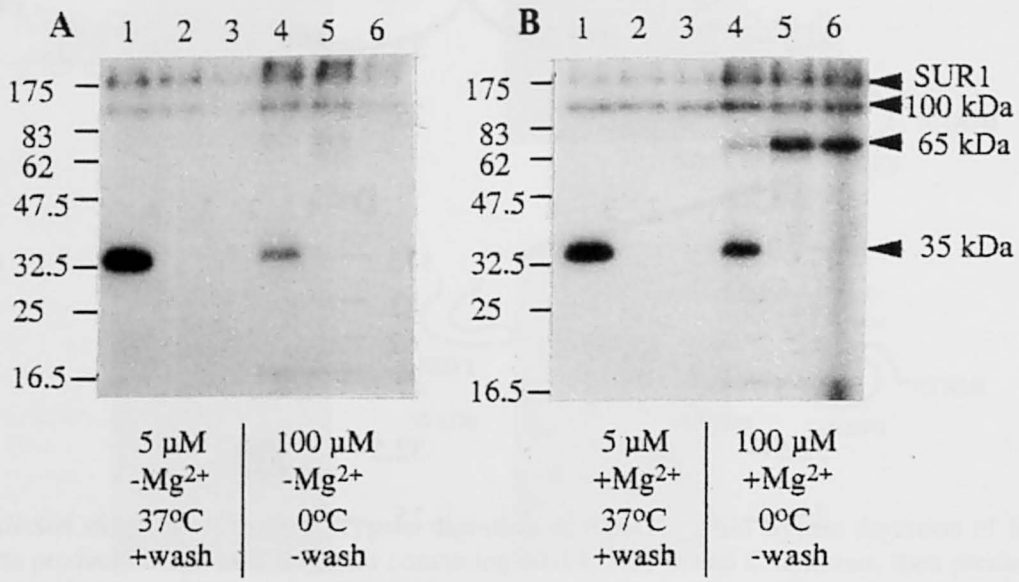

Fig. 4. **Limited tryptic digestion of SUR1 after photoaffinity labeling.** Membrane proteins (20 μg) from COS-7 cells expressing SUR1 were incubated with 5 μM 8-azido-[α-^{32}P] ATP for 10 min at 37°C and UV-irradiated after washing with buffer (lanes 1-3), or were incubated with 100 μM 8-azido-[α-^{32}P] ATP for 10 min on ice and UV-irradiated before washing with buffer (lanes 4-6). Reactions with 8-azido-ATP were done in the absence (A) or presence (B) of Mg^{2+}. Membrane proteins were digested with 2.5 μg/ml trypsin for 60 min at 37°C and solubilized with RIPA buffer. The tryptic fragments were immunoprecipitated with anti-NBF1 (lanes 1 and 4), anti-NBF2 (lanes 2 and 5), and anti-C terminus (lanes 3 and 6) antibodies and separated by 12% SDS-polyacrylamide gel electrophoresis. Undigested SUR1, a tryptic 100-kDa fragment contains both NBF1 and NBF2, a 65-kDa fragment containing NBF2, and a 35-kDa fragment containing NBF1 are indicated. Experiments were done in triplicate.

Interaction of the NBFs of SUR1 with 8-azido-[γ-^{32}P] ATP

Among the eukaryotic ABC superfamily proteins, MDR1, MRP1, CFTR, and ABCR (the rod photoreceptor-specific ABC transporter responsible for Stargardt's disease) have been reported to have ATPase activity (9,18-22). In chapter 2, the author has proposed that NBF2

of SUR1 has ATPase activity, and that MgADP, either by direct binding to NBF2 or hydrolysis of bound MgATP at NBF2, stabilizes prebound 8-azido-ATP binding at NBF1. Because it was possible to distinguish the two NBFs by their interaction with ATP by limited trypsin digestion, the author tried to determine whether SUR1 has ATPase activity. Membranes expressing SUR1 were incubated with 50 μM 8-azido-[α-^{32}P]ATP or 8-azido-[γ-^{32}P]ATP in the presence of Mg^{2+} for 10 min at 37°C, and UV-irradiated without removing free ligands. SUR1 was mildly digested with trypsin, and the tryptic fragments were immunoprecipitated with the anti-NBF1, anti-NBF2, or anti-C terminus antibody (Fig. 5). The 35-kDa fragment containing NBF1 was photoaffinity-labeled with both 8-azido-[α-^{32}P]ATP (lane 1) and 8-azido-[γ-^{32}P]ATP (lane 4). However, the 65-kDa fragment containing NBF2 was photoaffinity-labeled with 8-azido-[α-^{32}P]ATP (lanes 2 and 3), but not with 8-azido-[γ-^{32}P]ATP (lanes 5 and 6). These results suggest that γ-phosphate dissociates from 8-azido-ATP bound at NBF2 and that NBF2 of SUR1 may have ATPase activity. In contrast to NBF2, NBF1 appears to have little or no ATPase activity under the conditions examined.

To demonstrate loss of the terminal phosphate from 8-azido-[γ-^{32}P]ATP bound at NBF2, the author tried to show photoaffinity labeling of NBF2 with 8-azido-[γ-^{32}P]ATP at time 0. Membranes were incubated with 50 μM 8-azido-[γ-^{32}P]ATP in the presence of Mg^{2+} on ice, and photoaffinity-labeled without removing free ligands. The 65-kDa tryptic fragment containing NBF2 was not, however, photoaffinity-labeled with 8-azido-[γ-^{32}P]ATP even when the reaction with trypsin was done on ice, although the same fragment was photoaffinity-labeled with 8-azido-[α-^{32}P]ATP (data not shown).

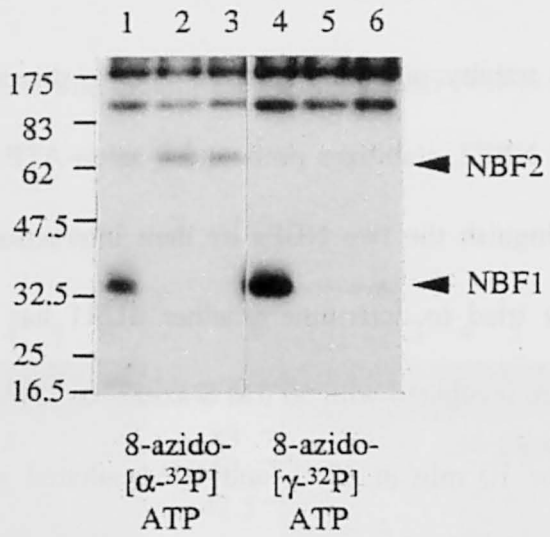

Fig. 5. **Photoaffinity labeling of NBFs with 8-azido-[α-^{32}P]ATP and 8-azido-[γ-^{32}P]ATP.** Membrane proteins (20 μg) from COS-7 cells expressing SUR1 were incubated with 50 μM 8-azido-[α-^{32}P]ATP (lanes 1-3) or 8-azido-[γ-^{32}P]ATP (lanes 4-6) in the presence of Mg^{2+} for 10 min at 37°C, followed by UV irradiation without removing free ligands. Membrane proteins were mildly digested with trypsin and tryptic fragments were immunoprecipitated by anti-NBF1 (lanes 1 and 4), anti-NBF2 (lanes 2 and 5), or anti-C terminus antibody (lanes 3 and 6). Experiments were done in triplicate.

Affinity to nucleotides of NBFs of SUR1 to ATP and ADP

The author suggested that NBF1 of SUR1 is a high-affinity 8-azido-ATP binding site and NBF2 is a low-affinity one. However, it is not clear whether it is applicable for ATP and ADP. It is important to know the exact affintiy to ATP and ADP of NBFs of SUR1, because K_{ATP} channels are sensors of intracellular ATP and ADP concentrations. This will lead us to know which nucleotide (ATP or ADP) binds to both NBFs in a physiological condition. When SUR1 was photoaffinity-labeled with 50 μM 8-azido-[α-^{32}P] ATP in the presence of cold ATP or ADP, photoaffinity labeling of both NBFs of SUR1 was inhibited by ATP and ADP in a concentration-dependent manner (Fig. 6), indicating that NBFs can bind not only ATP but also ADP. The *Ki* values for ATP or ADP of NBFs were obtained (Table 1). The affinity of NBF1 for ATP was significantly higher than that for ADP and the affinity of NBF1 for

nucleotides was higher than that of NBF2.

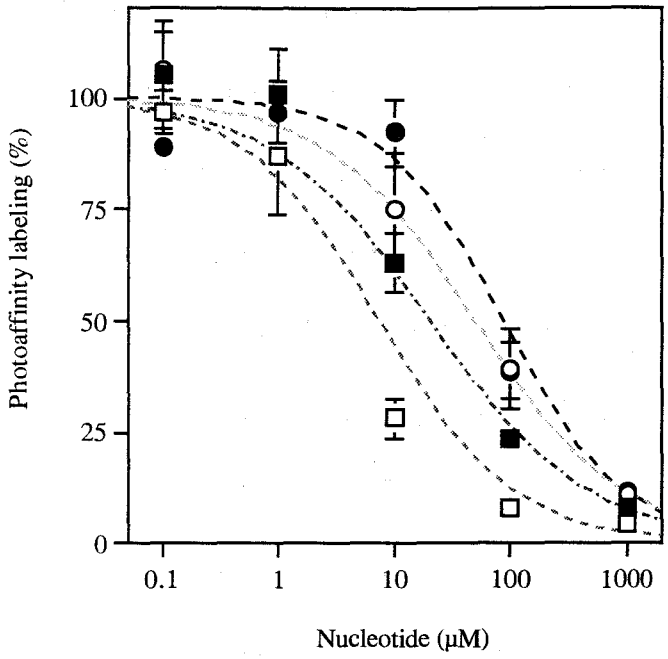

Fig. 6. **Inhibition of photoaffnity labeling of NBFs of SUR1 by nucleotides.** Membrane proteins (20 μg) from COS-7 cells expressing SUR1 were incubated with 50 μM 8-azido-[α-^{32}P]ATP in the presence of the increasing concentration of ATP or ADP for 10 min at 0°C followed by UV irradiation before washing with buffer. Membrane proteins were mildly digested with trypsin and tryptic fragments were immunoprecipitated by anti-NBF1 or anti-NBF2 antibody as described in Fig. 4. Relative photoaffnity labeling of NBF1 in the presence of ATP (open squares) or ADP (closed squares) and that of NBF2 in the presence of ATP (open circles) or ADP (closed circles) were expressed as a percentage of that in the absence of cold nucleotides. The data were fit using the Hill equation. Experiments were done in triplicate and the average values are represented with SEM.

Table. 1. **Affinities for ATP and ADP of NBFs of SUR1**

Ki values	NBF1	NBF2
ATP	4.4 ± 3.7	60 ± 26
ADP	26 ± 8.6	100 ± 26

DISCUSSION

In this chapter, the author has determined that NBF1 of SUR1 binds 8-azido-ATP strongly in a Mg^{2+}-independent manner, and that NBF2 binds 8-azido-ATP weakly in a Mg^{2+}-dependent manner. The author has also demonstrated that NBF1 has no or very little ATPase activity, whereas NBF2 of SUR1 may have ATPase activity. These characteristics of the two NBFs of SUR1 are quite different from those of MDR1: MDR1 has no high-affinity binding site for ATP; both NBFs of MDR1 hydrolyze ATP; and the roles of the two NBFs in drug transport are assumed to be equivalent in MDR1 (11). After hydrolysis, MDR1 is thought to form the metastable state MDR1·MgADP·Pi, and the phosphate ion is released before MgADP. When ATP is hydrolyzed in the presence of orthovanadate, orthovanadate binds to this intermediate in place of the released Pi to form a stable inhibitory complex, MDR1·MgADP·Vi (1). Therefore, when MDR1 is incubated with 8-azido-[α-^{32}P]ATP in the presence of orthovanadate, MDR1 traps 8-azido-[α-^{32}P]ADP and is specifically photoaffinity-labeled (1,5,17).

Photoaffinity labeling of CFTR with 8-azido-[α-^{32}P]ATP has also been reported recently (16). NBF1 of CFTR was preferentially labeled with 5 μM 8-azido-[α-^{32}P]ATP in the absence of orthovanadate. Because this high-affinity labeling of NBF1 is Mg^{2+}- and temperature-dependent, Szabó et al. predicted that the nucleotide occlusion occurs in the ATP hydrolysis cycle of CFTR (16). The high-affinity labeling of NBF1 of SUR1 is also temperature-dependent (Fig. 4), but does not require Mg^{2+} (17). Accordingly, a structural change, a nucleotide occlusion, might also occur in NBF1 of SUR1, but it would not be a step

in the ATP hydrolysis cycle in the case of SUR1 because Mg^{2+} is not required. The addition of orthovanadate increases photoaffinity labeling and resulted in the labeling of both NBFs of CFTR (16), but had no effects on photoaffinity labeling of SUR1 (17). These results suggest that the catalytic mechanism of NBFs of SUR1 may be different from that of the NBFs of MDR1 and CFTR. This is in agreement with experiments demonstrating that orthovanadate does not influence K_{ATP} channel activity (23,24).

To examine if NBF2 of SUR1 has ATPase activity, phoaffinity labeling of SUR1 with 8-azido-[γ-^{32}P]ATP was investigated. However, we could not confirm that NBF2 of SUR1 has ATPase activity, because the 65-kDa fragment containing NBF2 was not photoaffinity-labeled with 8-azido-[γ-^{32}P]ATP even at time 0. This is probably because 8-azido-[γ-^{32}P]ATP bound at NBF2 is hydrolyzed and the terminal phosphate is released during trypsin digestion.

In CFTR, ATP hydrolysis is important in channel regulation. Electrophysiological studies have suggested that both NBFs of CFTR hydrolyze ATP. ATP hydrolysis at NBF1 is predicted to be involved in channel opening, whereas that at NBF2 is predicted to be involved in channel closing (25,26). Orthovanadate, which is assumed to trap ADP at an NBF, causes extreme stabilization of the open conformation of the CFTR channel (27,28). MgADP, produced by hydrolysis of bound MgATP at NBF2 of SUR1, is thought to stabilize ATP binding at NBF1, and K_{ATP} channel activation may be induced primarily by the cooperative interaction of ATP binding at NBF1 and MgADP binding at NBF2. Accordingly, ATP hydrolysis at NBF2 could modulate K_{ATP} channel regulation. Because NBF1 was photoaffinity-labeled with 8-azido-[α-^{32}P]ATP and with 8-azido-[γ-^{32}P]ATP, ATP is not hydrolyzed at NBF1 under the conditions examined in this study. If NBF1 of SUR1 showed

ATPase activity in some other conditions, such as by binding pharmacological agents or unknown endogenous ligands, the activity of the K_{ATP} channel would also be affected.

The author proposes the model of the activation mechansims of K_{ATP} channels by nucleotide through SUR1 based on this study (Fig. 7). Channel activation is induced when SUR1 binds MgATP at NBF1 and MgADP at NBF2 (state **I**). When the cellular metabolism is stimulated and intracellular MgATP concentration increases and MgADP concentration concomitantly decreases, MgADP dissociates from NBF2 and MgATP binds to NBF2 instead (state **I** to **III** through state **II**). MgADP interacts with NBF2 either by direct binding (state **II** to **I**) or hydrolysis of bound MgATP (state **III** to **I** by hydrolysis). States **I** and **II** are of equilibrium, and most of the SUR1 subunits will be in state **I** at high MgADP concentrations and in state **II** or **III** at low MgADP concentrations. MgADP dissociation from NBF2 leads to instability of MgATP binding at NBF1, allowing the release of MgATP from NBF1. Because the intracellular concentrations of nucleotides are high enough under physiological conditions, state **II** would be transient and nucleotides (MgATP or MgADP) would bind to NBF2 to stabilize MgATP binding at NBF1 before the release of MgATP from NBF1. Consequently, SUR1 is in either states **I**, **II**, or **III** under physiological conditions. As shown in chapter 2, the amount of the photoaffinity-labeled SUR1 (in state **I**) was directly proportional to the concentration of MgADP from 10 μM to 1 mM (Fig. 3 in chapter 2). These results suggest that intracellular concentration of MgADP is the primary factor determining the alteration between the active state (state **I**) and the inactive states (state **II** and **III**) of SUR1.

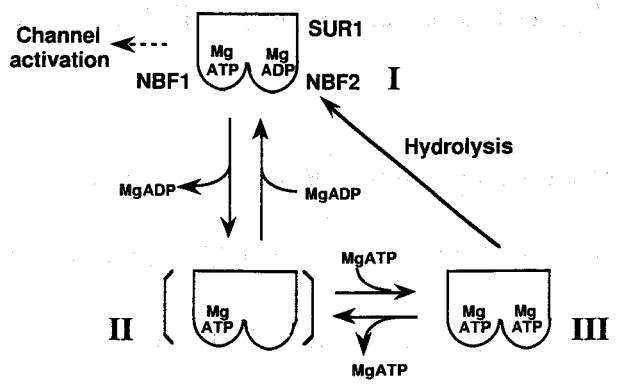

Fig. 7. **Model for nucleotide activation of the K_{ATP} channel by SUR1 subunit.** State **I** is the 'active state' in which SUR1 potentiates channel activity. State **II** is transient, and states **II** and **III** are 'inactive states' in which SUR1 cannot potentiate channel activity.

In summary, the author has analyzed properties of the two NBFs of SUR1 and demonstrated that NBF1 binds 8-azido-ATP strongly even in the absence of Mg^{2+} and that NBF2 binds 8-azido-ATP weakly in a Mg^{2+}-dependent manner. And the author has showed the affinity to ATP and ADP of NBFs of SUR1. NBF1 of SUR1 is a high-affinity ATP binding site and NBF2 is a low-affinity binding site for ATP and ADP. NBF2 of SUR1 may have ATPase activity, whereas NBF1 have no or little ATPase activity. Because ATP binding and hydrolysis are important in the regulation of the K_{ATP} channel, this study provides evidence of a critical part of the molecular mechanism of pancreatic β-cell K_{ATP} channels.

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CONCLUSIONS

The research presented in this thesis focuses on mechanism of ATP-sensitive potassium channel (K_{ATP} channel) regulation by sulfonylurea receptor1 (SUR1). For that, the author has focused on the interaction with nucleotides of two nucleotide-binding folds (NBFs) of SUR1. The results described in each chapter can be summarized as follows.

In **Chapter 1**, the author demonstrated that NBF1 of SUR1 is a high-affinity ATP binding site. Pancreatic β-cell K_{ATP} channels, composed of SUR1 and Kir6.2 subunits, serve as sensors for intracellular nucleotides and regulate glucose-induced insulin secretion. Ueda et al. have reported that SUR1 has a high-affinity ATP binding site. To learn more about the interaction of SUR1 with nucleotides, the author examined the effect of *N*-ethylmaleimide (NEM) modification. Photoaffinity labeling of SUR1 with 5 μM 8-azido-[α-^{32}P]ATP or 8-azido-[γ-^{32}P]ATP is inhibited by NEM with K_i of 1.8 μM and 2.4 μM, and Hill coefficients of 0.94 and 1.1, respectively. However, when the cysteine residue in the Walker A motif of the NBF1 of SUR1 is replaced with serine (C717S), photoaffinity labeling is not inhibited by 100 μM NEM. Takada et al. have reported that NEM modification of the cystine residue in the Walker A motif of NBF1 of MDR1, a multidrug transporter, reduces 8-azido-ATP binding to MDR1. These results suggest that NBF1 of SUR1 has a NEM-sensitive structure similar to that of NBF1 of MDR1 and confirm NBF1 as the high-affinity ATP binding site on SUR1.

In **Chapter 2**, the author demonstrated the strong cooperativity in nucleotide binding of the two NBFs of SUR1. The cooperative interaction of the two NBFs is important in the function of other ABC superfamily proteins, such as MDR1 and CFTR. To investigate the cooperative interaction of two NBFs of SUR1, the author investigated the effects of nucleotide binding at NBF2 on stabilizing prebound 8-azido-ATP binding at NBF1. The author showed that MgATP and MgADP, but not MgATP-γS, stabilize binding of prebound 8-azido-[α-^{32}P]ATP to SUR1. Mutations in the Walker A and B motifs of NBF2 abolish this stabilizing effect of MgADP. These results suggest that SUR1 binds 8-azido-ATP strongly at NBF1 and that MgADP, either by direct binding to NBF2 or by hydrolysis of bound MgATP at NBF2, stabilizes prebound 8-azido-ATP binding at NBF1.

To elucidate the molecular etiology of persistent hyperinsulinemic hypoglycemia of infancy (PHHI), the effect of mutations, identified in Japanese PHHI patients, on the cooperative nucleotide binding was examined. Western blot analysis after transient expression in COS-7 cells revealed that R1420C mutation, unlike R1436Q mutation, does not severely affect SUR1 expression. R1420C mutation does not affect high-affinity ATP binding to NBF1, either. However, it impairs stabilization of ATP binding to NBF1 by MgATP or MgADP. This is the first direct biochemical evidence that the cooperativity of nucleotide binding to SUR1 is impaired by mutation found in PHHI, and suggests this defect may account for impaired K_{ATP} (R1420C SUR1) channel function.

In **Chapter 3**, the author demonstrated that NBF1 of SUR1 is a Mg^{2+}-independent high-affinity nucleotide binding site and NBF2 is a Mg^{2+}-dependent low-affinity one. To

distinguish the ATP binding properties of the two NBFs of SUR1, the author prepared antibodies against NBF1 and NBF2, and the tryptic fragment of SUR1 was immunoprecipitated after photoaffinity labeling with 8-azido-[^{32}P]ATP. The 35-kDa fragment was strongly labeled with 5 μM 8-azido-[^{32}P]ATP even in the absence of Mg^{2+} and was immunoprecipitated by the antibody against NBF1. The 65-kDa fragment labeled with 100 μM 8-azido-[α-^{32}P]ATP in the presence of Mg^{2+} was immunoprecipitated with anti-NBF2 and anti-C terminus antibodies. These results indicate that NBF1 of SUR1 binds 8-azido-ATP strongly in a Mg^{2+}-independent manner, and that NBF2 binds 8-azido-ATP weakly in a Mg^{2+}-dependent manner. Furthermore, the 65-kDa tryptic fragment was not photoaffinity-labeled with 8-azido-[γ-^{32}P]ATP at 37°C, whereas the 35-kDa tryptic fragment was, suggesting that NBF2 of SUR1 may have ATPase activity and that NBF1 has none or little. The inhibition of photoaffinity labeling by ATP or ADP showed that NBF1 of SUR1 has a high-affinity to nucleotides, especially to ATP compared with NBF2.

From these results, the author presents the model that SUR1 binding MgATP at NBF1 and MgADP at NBF2 activates K_{ATP} channel and that SUR1 regulates K_{ATP} channel activity by monitoring the intracellular MgADP concentration.

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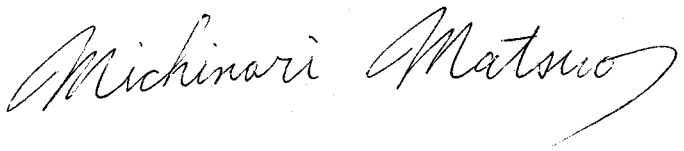

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