bility of selection-directed mutation is not eliminated. Adaptive mutation has been investigated in other systems that are not subject to the interpretations proposed here (*25*). Organisms sense their environment and make directed changes in many aspects of their metabolism. It would seem to be advantageous to direct mutability. We expect that organisms have exploited this possibility.

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Cloning of the β Cell High-Affinity Sulfonylurea Receptor: A Regulator of Insulin Secretion

Lydia Aguilar-Bryan,* Colin G. Nichols, Sérgio W. Wechsler, John P. Clement IV, A. E. Boyd III,† Gabriela González, Haydée Herrera-Sosa, Kimberly Nguy, Joseph Bryan,‡ Daniel A. Nelson‡§

Sulfonylureas are a class of drugs widely used to promote insulin secretion in the treatment of non–insulin-dependent diabetes mellitus. These drugs interact with the sulfonylurea receptor of pancreatic β cells and inhibit the conductance of adenosine triphosphate (ATP)–dependent potassium (K_{ATP}) channels. Cloning of complementary DNAs for the high-affinity sulfonylurea receptor indicates that it is a member of the ATP-binding cassette or traffic ATPase superfamily with multiple membrane-spanning domains and two nucleotide binding folds. The results suggest that the sulfonylurea receptor may sense changes in ATP and ADP concentration, affect K_{ATP} channel activity, and thereby modulate insulin release.

Sulfonylureas are oral hypoglycemics widely used in the treatment of non–insulin-dependent diabetes mellitus to stimulate insulin release from pancreatic islet β cells. The mechanism of stimulation is through inhibition of an ATP-dependent potassium channel, K_{ATP}, which sets the β cell resting membrane potential (*1*). A reduction of potassium outflow causes β cell depolarization and the activation of one or more L-type calcium channels (*2*). The resulting calcium influx triggers exocytosis (*3*). Sulfonylureas like tolbutamide or glyburide decrease K_{ATP} channel activity, which depolarizes the cell and prompts insulin release.

The K_{ATP} channels and sulfonylurea receptors (SURs) appear to be functionally linked, although it is not clear if they are a single entity (*4–6*). Biochemical studies indicate that SUR is a large membrane protein (140 to 170 kD) (*7*) that can bind sulfonylureas with high affinity (dissociation constant $K_d < 10$ nM) (*1*). The affinity of sulfonylureas for SUR is decreased by nucleotides and possibly phosphorylation (*8*). It has been proposed that the K_{ATP} channel contains a sulfonylurea binding domain, a binding site for potassium channel openers, and two or more nucleotide binding sites that can discriminate between ATP and ADP (*9*). One report has placed the nucleotide binding sites and the sulfonylurea binding site on the same large molecule with an ATP affinity label (*10*).

Glyburide (*11*) and an iodinated derivative of glyburide (*12*) can be cross-linked by photolabeling to a protein with an apparent molecular size of 140 kD. The pharmacological characteristics of the photolabeling (*12*), the low-nanomolar K_d, and the appropriate rank order displacement with other insulin-releasing sulfonylureas are expected from studies on glyburide-induced insulin release and inhibition of K_{ATP} channel activity. These results indicate that the 140-kD molecule is SUR. We used a radioiodinated derivative of glyburide (*12*) to purify hamster SUR (*13*) and obtain the NH_2-terminal amino acid sequence from

L. Aguilar-Bryan, S. W. Wechsler, J. P. Clement IV, A. E. Boyd III, G. González, H. Herrera-Sosa, K. Nguy, J. Bryan, D. A. Nelson, Departments of Cell Biology and Medicine, Baylor College of Medicine, Houston, TX 77030, USA.

C. G. Nichols, Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110, USA.

*To whom correspondence should be addressed.

†Deceased. This author contributed to the initiation of this work while Head of the Division of Endocrinology at Baylor College of Medicine.

‡These authors contributed equally to this work.

§Present address: Department of Biology, University of North Carolina, Charlotte, NC 28223, USA.

the intact molecule and from V8 protease fragments. The same peptide sequence, PLAFCGTE(N)HSAAYRVDQGVLNN-GC (*14*), was obtained from all radiolabeled peptides, including the intact receptor. The N in parentheses was identified as aspartic acid in deglycosylated peptides and as an asparagine within a consensus glycosylation site by complementary DNA (cDNA) sequencing. The size of the smallest labeled peptide was ~50 kD, which indicates that the site of labeling is near the NH_2 terminus of SUR. Two antipeptide antibodies were generated: one against PLAFCGTE, the second against HSAAYRVDQGV. Specific immunoprecipitation with these antibodies demonstrated that the sequence was derived from photolabeled SUR. Degenerate polymerase chain reaction (PCR) primers were designed on the basis of this sequence and were used to amplify a 67–base pair (bp) fragment from a random primed cDNA library constructed in λZAP II with polyadenylated mRNA isolated from a mouse glucagonoma–α cell line (αTC-6). The SUR and K_{ATP} channels are present in islet α cells and αTC-6 cells (*15*). The sequence of the 67-bp fragment (*16*) encoded the expected amino acids with codon degeneracy only in the primer regions. A minimally degenerate 47-residue oligonucleotide (*16*) was synthesized and used to rescreen the αTC-6 cell library. A 1.1-kb cDNA was cloned that encoded the 25 amino acids obtained by peptide sequencing of the SUR NH_2-terminus. This cDNA fragment was used to screen rat insulinoma (RINm5F) and hamster insulin-secreting tumor cell (HIT T15) λ phage libraries.

The open reading frames of hamster and rat SUR cDNAs (Fig. 1) encode proteins of 1582 amino acids with masses of 177,209 and 177,102 daltons, respectively, which are larger than expected from SDS–polyacrylamide gel electrophoresis. These two rodent sequences are about 98% identical. Mature hamster SUR, defined by peptide sequencing, begins with a proline. The adjacent amino acid, a methionine, is presumed to be the initiating methionine on the basis of the surrounding sequence, which resembles the consensus pattern for initiation, GCC(A/G)CCAUG(G) (*17*).

Northern (RNA) blot analysis of polyadenylated mRNA isolated from RIN, HIT, and αTC-6 cells showed that they all have an approximately 5000-nucleotide transcript (Fig. 2). These cells all express SUR and K_{ATP} channels. The same size transcript was present in total RNA from pancreas, brain, and heart.

To establish that the cloned cDNA encoded SUR, we transiently expressed the rat and hamster cDNAs in COSm6 cells. Untransfected COSm6 cells, or ones transfected with β-galactosidase, had no SUR detectable by photolabeling or filtration binding (Fig. 3) (*12*). Cells transfected with hamster or rat SUR cDNA expressed 140-kD photolabeled bands that comigrated with native SUR from HIT cells. Specific photolabeling was reduced by the addition of 1 μM iodoglyburide or glyburide. Competition experiments showed the correct rank order of displacement: glyburide > iodoglyburide > tolbutamide. Studies of iodoglyburide binding to membranes isolated from COSm6 cells transfected with hamster SUR cDNA revealed $K_d \approx 10$ nM. The maximum binding capacity B_{max} is about 145 pmol of SUR per milligram of membrane protein. This value of B_{max} is about 100 times that estimated for HIT cells (*5*). Membranes isolated from rat-SUR–transfected COSm6 cells gave $K_d \approx 2$ nM and $B_{max} \approx 9.4$ pmol of SUR per milligram of membrane protein. These transfected cell membranes do not appear to have the low-affinity receptors ($K_d \approx 15$ μM) previously observed in HIT and RIN cell membranes (*12*). We conclude that the cloned cDNAs encode functional rat and hamster SURs.

A Blast search (*18*) of the National Center for Biotechnology Information nucleotide database with the SUR sequence produces matches with several members of the P-glycoprotein multidrug resistance (MDR) protein family. A similar search with the amino acid sequence revealed similarities with cystic fibrosis transconductance regulators (CFTRs) and MDR proteins and indicated that SUR is a member of the ATP-binding cassette (ABC) or traffic adenosine triphosphatase (ATPase) superfamily with

```
        CHO
1       MPLAFCGTENHSAAYRVDQGVLNNGCFVDALNVVPHVFLLFITFPILFIGWGSQSSKVHIHHSTWLHFPGHNLRWIL
78      TFILLFVLVCEIAEGILSDGVTESRHLHLYMPAGMAFMAAITSVVYYHNIETSNFPKLLIALLIYWTLAFITKTIKF
155     VKFYDHAIGFSQLRFCLTGLLVILYGMLLLVEVNVIRVRRYIFFKTPREVKPPEDLQDLGVRFLQPFVNLLSKGTYW
232     WMNAFIKTAHKKPIDLRAIAKLPIAMRALTNYQRLCVAFDAQARKDTQSPQGARAIWRALCHAFGRRLILSSTFRIL
309     ADLLGFAGPLCIFGIVDHLGKENHVFQPKTQFLGVYFVSSQEFLGNAYVLAVLLFLALLLQRTFLQASYYVAIETGI
386     NLRGAIQTKIYNKIMHMSTSNLSMGEMTAGQICNLVAIDTNQLMWFFFLCPNLWTMPVQIIVGVILLYYILGVSALI
463     GAAVIILLAPVQYFVATKLSQAQRTTLEHSNERLKQTNEMLRGMKLLKLYAWESIFCSRVEVTRRKEMTSLRAFAVY
540     TSISIFMNTAIPIAAVLITFVGHVSFFKESDLSPSVAFASLSLFHILVTPLFLLSSVVRSTVKALVSVQKLSEFLSS
617     AEIREEQCAPREPAPQGQAGKYQAVPLKVVNRKRPAREEVRDLLGPLQRLAPSMDGDADNFCVQIIGGFFTWTPDGI
694     PTLSNITIRIPRGQLTMIVGQVGCGKSSLLLATLGEMQKVSGAVFWNSNLPDSEGRGFPQQPRAGDSSWLGYQEQRPR
771     GYASQKPWLLNATVEENITFESPFNPQRYKMVIEACSLQPDIDILPHGDQTQIGERGINLSGGQRPDQCGPEPSTSR
848     PMFVFLDDPFSALDVHLSDHLMQAGILELLRDDKRTVVLVTHKLQYLPHADWIIAMKDGTIQREGTLKDFQRSECQL
925     FEHWKTLMNRQDQELEKETVMERKASEPSQGLPRAMSSRDGLLLDEEEEEEEAAESEEDDNLSSVLHQRAKIPWRAC
1002    TKYLSSAGILLLSLLVFSQLLKHMVLVAIDYWLAKWTDSALVLSPAARNCSLSQECDLDQSVYAMVFTLLCSLGIVL
1079    CLVTSVTVEWTGLKVAKRLHRSLLNRIILAPMRFFETTPLGSILNRFSSDCNTIDQHIPSTLECLSRSTLLCVSALT
1156    VISYVTPVFLVALLPLAVVCYFIQKYFRVASRDLQQLDDTTQLPLVSHFAETVEGLTTIRAFRYEARFQQKLLEYTD
1233    SNNIASLFLTAANRWLEVCMEYIGACVVLIAAATSISNSLHRELSAGLVGLGLTYALMVSNYLNWMVRNLADMEIQL
1310    GAVKRIHALLKTEAESYEGLLAPSLIPKNWPDQGKIQIQNLSVRYDSSLKPVLKHVNTLISPGQKIGICGRTGSGKS
1387    SFSLAFFRMVDMFEGRIIIDGIDIAKLPLHTLRSRLSIILQDPVLFSGTIRFNLDPEKKCSDSTLWEALEIAQLKLV
1464    VKALPGGLDAIITEGGENFSQGQRQLFCLARAFVRKTSIFIMDEATASIDMATENILQKVVMTAFADRTVVTIAHRV
1541    HTILSADLVMVLKRGAILEFDKPETLLSQKDSVFASFVRADK
```

Fig. 1. Amino acid sequence of the hamster SUR. The amino acid sequence of five peptides determined by chemical methods are marked in bold. The predicted nucleotide binding domains are underlined, and the Walker "A" and "B" consensus sequences are boxed. Possible sites of phosphorylation by protein kinase A (open diamonds) and C (filled diamonds) are marked above the sequence. Potential transmembrane-spanning helices assigned by the algorithm of Eisenberg *et al.* (*29*) are highlighted. The NH_2-terminal glycosylation site is marked CHO. Abbreviations for the amino acid residues are in (*14*).

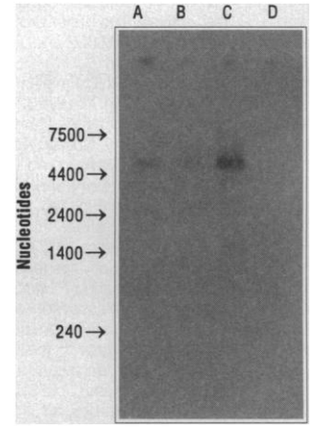

Fig. 2. Northern blot analysis of total RNA from α and β cell lines. About 10 μg of total RNA from (**A**) αTC-6 cells, (**B**) HIT T15 cells, (**C**) RINm5F cells, and (**D**) mouse liver was hybridized with a 2.2-kb Eco RI–Xho I fragment from the SUR cDNA by standard procedures. The estimated size of the major component is about 5000 nucleotides.

two putative nucleotide binding domains. The best match, 29% overall identity, is to a MDR-associated protein, termed MRP or "dvhuar" in the Protein Identification Resource database. This molecule was cloned from a small-cell lung-carcinoma cell line (H69AR) (*19*). A cluster analysis of dvhuar (*19*) indicates that it is most closely related to the leishmania P-glycoprotein–related molecule (Lei/PgpA) (*20*) and the human, bovine, mouse, and dogfish CFTRs. A similar analysis including SUR and *Xenopus* CFTR indicates that SUR is a member of this cluster.

The identification of two nucleotide binding folds (NBFs) extends beyond the Walker "A" and "B" consensus sequences (*21*). SUR has two domains with strong similarity to the 230– to 240–amino acid NBFs found in other members of the ABC superfamily (*22*). The SUR COOH-terminal fold, NBF-2, is the more highly conserved of the two, on the basis of similarity with other ATP-binding proteins and comparison of the rat and hamster SUR sequences. An alignment, found with the use of PILEUP (*18*), of 230 SUR amino acid residues, beginning with the second Walker "A" motif, with the corresponding regions of two CFTRs and two MDRs with the greatest similarity to SUR yielded a consensus sequence, defined as at least three identical residues out of five possible, at 165 of the 230 positions (~72%). The similarity of SUR to traffic ATPases raises the interesting possibility that SUR may transport some endogenous substance and that sulfonylureas might affect this transport.

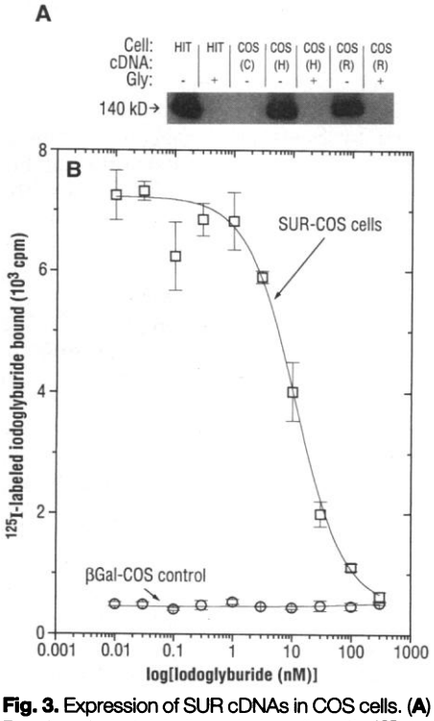

Fig. 3. Expression of SUR cDNAs in COS cells. (**A**) Results of photolabeling whole cells with ^{125}I-labeled iodoglyburide. HIT control cells or COSm6 cells transfected with β-galactosidase (C), hamster (H), or rat (R) SUR cDNA were photolabeled in the presence (+) or absence (−) of 1 μM glyburide as described (*30*). (**B**) Competition assay done with membranes isolated from COSm6 cells transfected either with β-galactosidase (β-Gal) or with hamster SUR cDNA subcloned into the pECE vector under control of the SV40 early promoter (*31*). Equivalent experiments with membranes from untransfected COSm6 cells gave the same result as those with membranes from cells transfected with β-galactosidase. cpm, counts per minute.

Phosphorylation has been suggested to regulate K_{ATP} channel conductance (*23*) and to change the affinity of SUR for various ligands. SUR has three potential protein kinase A (pKA) sites and 20 potential protein kinase C (pKC) sites (Fig. 1). There are nine potential phosphorylation sites in the two NBFs. Two pKA sites (positions 1447 and 1501) are in NBF-2, along with three pKC sites. One of the latter sites, Thr^{1381} in the second Walker "A" motif, would be expected to alter nucleotide binding if phosphorylated.

Preliminary efforts to assay SUR for K_{ATP} channel activity were carried out by the injection of *Xenopus* oocytes with about 50 ng of hamster SUR mRNA (*24*). Oocytes were assayed after 1 to 5 days with both two-electrode and patch-clamp methods, but no new K^+ currents were detected in the injected oocytes. Co-injection of SUR mRNA with mRNAs transcribed from cDNAs encoding three small inward rectifiers—ROMK1 (*25*), a brain homolog of IRK1 (*26*), and rcK_{ATP}-1, also termed CIR (*27*)—failed to confer sulfonylurea sensitivity on these K^+ channels. The results suggest either that recombinant SUR does not have intrinsic K^+ channel activity or that *Xenopus* oocytes are not an adequate background for its expression.

Hydrophobicity and hydrophobic moment data, plus the constraints that the NH_2-terminal glycosylation site be on the external face of the membrane and both NBFs be cytoplasmic, were used to generate a working model of SUR (Fig. 1). This model places the NH_2 terminus outside the cell with nine predicted transmembrane-spanning helices before the first NBF. Four predicted transmembrane-spanning helices separate the two NBFs. Thus, SUR differs from the canonical model for the traffic ATPase superfamily, which has a cytoplasmic NH_2 terminus and consists of two units with six transmembrane-spanning helices followed by a nucleotide binding domain (*22*). Dvhuar, the MRP with greatest similarity to SUR, is predicted to have eight transmembrane-spanning helices before the first NBF (*19*).

Mutations in SUR that truncate NBF-2 cause persistent hyperinsulinemic hypoglycemia of infancy (*28*). We hypothesize that destruction of NBF-2 blocks K_{ATP} channel activity and prevents SUR from sensing changes in nucleotide balance and thus modulating islet β cell membrane potential. This predicts that SUR is an integral part of the K_{ATP} channel, specifically that it functions as the ATP and ADP sensor.

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14. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K,

Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Mutations in the Sulfonylurea Receptor Gene in Familial Persistent Hyperinsulinemic Hypoglycemia of Infancy

Pamela M. Thomas,* Gilbert J. Cote, Nelson Wohllk, Bassem Haddad, P. M. Mathew, Wolfgang Rabl, Lydia Aguilar-Bryan, Robert F. Gagel, Joseph Bryan

Familial persistent hyperinsulinemic hypoglycemia of infancy (PHHI), an autosomal recessive disorder characterized by unregulated insulin secretion, is linked to chromosome 11p14-15.1. The newly cloned high-affinity sulfonylurea receptor (*SUR*) gene, a regulator of insulin secretion, was mapped to 11p15.1 by means of fluorescence in situ hybridization. Two separate *SUR* gene splice site mutations, which segregated with disease phenotype, were identified in affected individuals from nine different families. Both mutations resulted in aberrant processing of the RNA sequence and disruption of the putative second nucleotide binding domain of the SUR protein. Abnormal insulin secretion in PHHI appears to be caused by mutations in the *SUR* gene.

PHHI is an autosomal recessive disorder of glucose homeostasis characterized by unregulated secretion of insulin and profound hypoglycemia (*1*). The pathophysiology of this disease remains obscure, but in vitro studies have suggested a defect of glucose-regulated insulin secretion in pancreatic islet β cells (*1, 2*). The *PHHI* gene was assigned to chromosome 11p14-15.1 by linkage analysis (*3, 4*). Candidate genes for this disorder include those involved in the β cell glucose sensing mechanism and in insulin secretion. Localization of *PHHI* to chromosome 11p14-15.1 excluded previously mapped genes involved in β cell function, such as the *glucokinase*, *islet glucose transporter*, and *glucagon-like peptide-1 receptor* loci (*5*). We considered as a candidate the newly cloned high-affinity *SUR* gene, a member of the adenosine triphosphate (ATP)–binding cassette superfamily (*6*). The SUR protein is a putative subunit of the β cell ATP-sensitive potassium channel (K$_{ATP}$), a modulator of insulin secretion (*7*).

We used the fluorescence in situ hybridization (FISH) technique to localize the *SUR* gene on chromosome 11. Partial complementary DNA (cDNA) clones that constituted 3.8 kb of the coding sequence of the human homolog of *SUR* were obtained (*8*) and were used for the analysis. A specific hybridization signal was detected in 85% of metaphases at band 11p15.1 on both chromatids of the two chromosomes 11. No other hybridization sites were detected.

Genomic DNA samples from affected individuals from three families were analyzed by Southern (DNA) blot; human cDNA (approximately four-fifths of full length) was used as a probe. Because the restriction fragment patterns of affected and unaffected samples appeared to be similar, major insertions or deletions of the *SUR* locus were unlikely to have occurred in the affected individuals (*9*). We then used direct sequence analysis to screen for small deletions, insertions, or missense mutations. The first region evaluated was the putative second nucleotide binding fold (NBF-2) of the human *SUR* homolog (Fig. 1), which is the most highly conserved region of the *SUR* gene (*6*). In other superfamily members, NBF-1 and NBF-2 have functional importance in the control of channel activity through their interaction with cytosolic nucleotides (*10, 11*). In cystic fibrosis, an autosomal recessive disease caused by mutations in another ATP-binding cassette member [the cystic fibrosis transmembrane conductance regulator (CFTR)], the more frequent and severe disease alleles are located in the regions of the two NBFs (*12*).

Mutational analysis was done on samples from 16 affected progeny of nine consanguineous matings (*13*). Because of the consanguineous matings and the autosomal recessive inheritance pattern of PHHI, affected individuals were expected to be homozygous by descent at the disease gene locus (*14*). Sequencing of a cloned pancreatic cDNA product, isolated from an

P. M. Thomas, G. J. Cote, N. Wohllk, R. F. Gagel, Section of Endocrinology, Department of Medical Specialties, University of Texas M. D. Anderson Cancer Center, Box 15, Houston, TX 77030, USA.
B. Haddad, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA.
P. M. Mathew, Division of Specialty Pediatrics, Dhahran Health Center, c/o ARAMCO, Dhahran 31311, Saudi Arabia.
W. Rabl, Kinderklinik der Technischen Universität, Kölner Platz 1, 80804 München, Germany.
L. Aguilar-Bryan and J. Bryan, Departments of Cell Biology and Medicine, Baylor College of Medicine, Houston, TX 77030, USA.
*To whom correspondence should be addressed.