The High-Affinity Sulfonylurea Receptor: Distribution, Glycosylation, Purification, and Immunoprecipitation of Two Forms from Endocrine and Neuroendocrine Cell Lines†

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ABSTRACT: The high-affinity sulfonylurea receptor, a novel member of the ATP-binding cassette superfamily, is one component of the ATP-sensitive K^+ channel. The protein is critical for regulation of insulin secretion from pancreatic β-cells, and mutations in the receptor have been linked to familial hyperinsulinemia, a disorder characterized by unregulated insulin release despite severe hypoglycemia. The sulfonylurea receptor is present in membranes from a number of endocrine and neuroendocrine cell lines, including HIT-Tl5, RINm5f, αTC-6, AtT-20, and GH_3 cells. Two forms of the receptor are present in RINm5f and αTC-6 cells, with apparent SDS gel molecular masses of 140 and 150 kDa. The two forms have equally high affinity, $K_D \approx 3$ nM, for an iodinated derivative of glyburide, an anti-diabetic sulfonylurea. The receptor is a glycoprotein; treatment of RINm5f or αTC-6 cells with tunicamycin reduces the 140 and 150 kDa species to a single ∼137 kDa protein. The 140 and 150 kDa receptors bind differentially to concanavalin A and wheat germ agglutinin, and lectin-affinity chromatography is ideal for the initial stages of receptor purification. After lectin-affinity chromatography, the same methods can be applied for purifying the 150 kDa form as for the 140 kDa receptor. A transiently expressed receptor with a histidine-tagged carboxy-terminus was purified by Ni-agarose chromatography, and this variant was used to demonstrate that the 140 kDa polypeptide is full length. Anti-peptide antibodies directed against the amino-terminus of the receptor and antibodies against the nucleotide binding folds immunoprecipitate both receptor forms. The results indicate the 140 and 150 kDa receptors are differentially glycosylated forms of the same polypeptide chain.

The sulfonylureas, particularly glyburide (glibenclamide) and tolbutamide, are drugs used in the treatment of non-insulin dependent diabetes mellitus because they prompt insulin release from pancreatic β-cells (Ashcroft & Ashcroft, 1992). This action is through inhibition of an ATP-sensitive potassium conductance, I_{KATP},[1] which sets the β-cell resting membrane potential. I_{KATP} is normally modulated by changes in the cytosolic ATP/ADP ratio brought about by changes in plasma glucose levels. Elevated glucose leads to inhibition of I_{KATP}, membrane depolarization, and activation of voltage-gated Ca^{2+} channels. The resultant increase in cytosolic Ca^{2+} triggers insulin release.

The high-affinity receptor for sulfonylureas is a member of the ATP-binding cassette superfamily (Aguilar-Bryan *et al*., 1995) with multiple transmembrane-spanning domains and two potential nucleotide binding folds. Cloning and expression studies have demonstrated the sulfonylurea receptor is an integral component of the β-cell ATP-sensitive potassium channel. Co-expression of an inactive member of the small inward rectifier family, designated $K_{IR}6.2$, with the sulfonylurea receptor, designated SUR1, reconstitutes a potassium conductance with the properties expected for the native β-cell ATP-sensitive K^+ channel (Inagaki *et al*., 1995). Mutations in SUR1 have been linked to familial hyperinsulinemia, a disease characterized by constitutive, unregulated insulin release in the face of severe hypoglycemia (Thomas *et al*., 1995). ATP-sensitive K^+ channels have been found predominantly in pancreatic islets (Ashcroft & Ashcroft, 1992) and brain (Bernardi *et al*., 1988) and are relatively abundant in cell lines derived from islets. Two forms of the receptor have been described in αTC-6 cells, a pancreatic glucagon secreting α cell line (Rajan *et al*., 1993), and in insulinoma and islet cells (Ozanne *et al*., 1995). We show that these two forms of SUR1 are present in several neuroendocrine- and islet-derived cell lines, that both forms bind an iodinated derivative of glyburide with high affinity, and that the two forms are the result of differential glycosylation.

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[1] Abbreviations: I_{KATP}, ATP-sensitive K^+ channel conductance; SUR1, high-affinity β-cell sulfonylurea receptor; $K_{IR}6.2$, member of inward rectifier K^+ channel family; K_{ATP}, ATP sensitive K^+ channel; HIT, hamster insulin-secreting tumor; RIN, rat insulinoma; αTC, α (glucagon-secreting) tissue culture; AtT, anterior pituitary tumor; GH, growth hormone secreting; NBF, nucleotide binding fold; PHHI, persistent hyperinsulinemic hypoglycemia of infancy; cDNA, complementary DNA; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; WGA, wheat germ agglutinin; MAP, multiple antigenic peptide; K_D, dissociation constant; MW, molecular weight; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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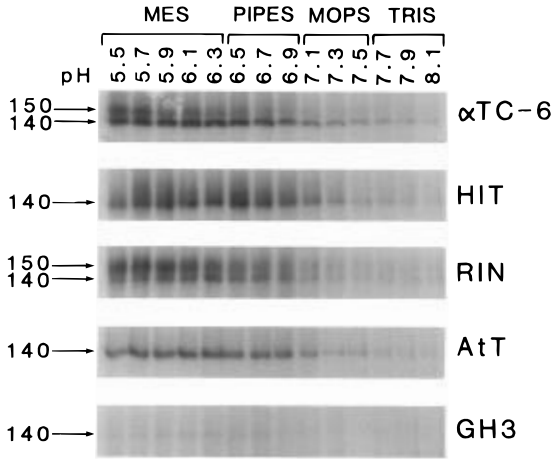

FIGURE 1: High-affinity sulfonylurea receptors in αTC-6, HIT-T15, RINm5f, AtT-20, and GH_3 cells photolabeled as a function of pH. Membranes were prepared in four different buffers: 10 mM MES (pH 6.0), PIPES (pH 6.5), MOPS (pH 7.1), or Tris (pH 7.7) plus 0.1 M NaCl, 2 mM EDTA, and were resuspended in the same buffers at the pH values indicated in the figure. Aliquots were incubated with 10 nM [^{125}I]iodoglyburide and photolabeled at 312 nm in a UV cross-linker at 1.5 J/cm^2. Samples were solubilized in 2× SDS sample buffer at pH 9, heated for 3 min at 100 °C, and separated by electrophoresis on an SDS 7.5% acrylamide gel, and an autoradiogram was prepared.

EXPERIMENTAL PROCEDURES

Chemicals. 5-[^{125}I]Iodo-2-hydroxyglyburide (^{125}I-iodoglyburide) was synthesized as described previously (Aguilar-Bryan *et al*., 1990).

Maintenance and Isolation of Cells. HIT-T15 (passage 65−75; CRL1777), RINm5f, GH3 (CCL82.1), and COSm6 cells were provided by the Tissue Culture Core of the Diabetes Research Center at Baylor College of Medicine. AtT-20 (CCL89) cells were from the American Type Culture Collection. αTC-6 cells, from a glucagon-secreting mouse glucagonoma, were provided by Dr. K. Hamaguchi (Jackson Laboratory, Bar Harbor, ME). HIT-T15, RINm5f, and αTC-6 cells were seeded in roller bottles in DMEM + 10% fetal bovine serum (Aguilar-Bryan *et al*., 1992). AtT-20 and GH3 cells were grown in Ham's F-10 medium plus 10% fetal bovine serum and seeded in roller bottles or 150 mm petri dishes. Cells were maintained in 5% CO_2 at 37 °C and fed three times prior to isolation. Cells were removed from roller bottles with PBS containing 2 mM EDTA, pelleted by centrifugation at 900*g* for 10 min, and washed once with PBS prior to preparation of membranes. COSm6 cells were plated at 50−60% confluence on 100 mm Petri dishes 1 day prior to transient transfection.

Preparation of Membranes, Receptor Photolabeling, and Filtration Binding Assays. Cell membranes were prepared as described previously (Aguilar-Bryan *et al*., 1990, 1992; Nelson *et al*., 1992). Membranes were photolabeled by incubating with 1−10 nM [^{125}I]iodoglyburide for 30 min at room temperature followed by UV-crosslinking, as optimized in Nelson *et al*. (1992) (see legend to Figure 1). Filtration binding assays were performed as described in Aguilar-Bryan *et al.* (1990).

Whole-Cell Photolabeling. Cells were incubated at room temperature in the dark for 30 min with 1−10 nM [^{125}I]-iodoglyburide in phosphate-buffered saline supplemented with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM glucose. Cells were irradiated as described for membranes, washed 3 times with phosphate-buffered saline, solubilized in SDS sample buffer (Laemmli, 1970), separated by gel electrophoresis, and visualized by autoradiography.

Gel Electrophoresis and Autoradiography. Proteins were separated by electrophoresis on SDS polyacrylamide gels (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue R, destained in H_2O/HOAc/MeOH (5:1:4), vacuum dried at 80 °C, and prepared for autoradiography.

Receptor Purification. Membrane isolation was followed by photolabeling approximately 20−25 mg of membrane proteins with [^{125}I]iodoglyburide, as above. The labeled membranes were mixed with approximately 200−300 mg of unlabeled membrane protein and solubilized with digitonin. 20% digitonin was prepared just prior to use by boiling in deionized water and added to the membranes to 1%. All subsequent steps were performed at room temperature in the presence of a cocktail of protease inhibitors (0.1 mM PMSF, 0.1 mM phenanthroline, and 0.1 mM iodoacetamide). Membranes were solubilized for 15 min and then ultracentrifuged for 1 h at 100 000*g*. The supernatant was divided into 4 mL aliquots which were cycled twice over a 1 mL concanavalin A-Sepharose (Sigma, St. Louis, MO) column equilibrated with 25 mM Tris (pH 7.5), 0.1 M NaCl, 2 mM EDTA, 1% digitonin. The column was washed with 8 mL of the equilibrating buffer and eluted with 4 mL of the equilibrating buffer containing 0.5 M methyl α-D-mannopyranoside. The eluted protein was stored at this stage at −80 °C. The eluates from three columns were combined and cycled twice over a 1 mL column of Reactive Green 19-agarose (Sigma) equilibrated with 50 mM HEPES (pH 8.5), 2 mM EDTA, 0.2% digitonin. After being washed with 8 mL of the equilibrating buffer and 8 mL of the equilibrating buffer plus 0.4 M NaCl, the protein was eluted with 4 mL of 1.5 M NaCl in the equilibrating buffer. Two eluates were pooled, diluted 1:1 with the HEPES equilibrating buffer to reduce the ionic strength, and then cycled twice over a 1 mL phenylboronate-10 Sepharose (Amicon, Beverly, MA) column. The phenylboronate column was washed with 8 mL of the HEPES buffer, followed by 2 mL of 0.1 M Tris (pH 7.5), 2 mM EDTA, 0.1% digitonin. Protein was eluted with 4 mL of 0.1 M Tris (pH 7.5), 2 mM EDTA, 0.1% SDS. Pooled samples from two phenylboronate-10 columns were concentrated to 0.5 mL using Amicon 100 000 MW cutoff filters pretreated with 5% Tween-20. This material was loaded onto a single 5 cm wide lane of a 5.5% polyacrylamide SDS gel, separated by gel electrophoresis, stained with Coomassie Blue, and destained. The 140 kDa band was excised with a razor blade, electroeluted into a 14 000 MW cutoff dialysis bag, and concentrated by Amicon filtration. A typical yield was 8−16 pmol (1−2 μg of purified receptor).

Wheat germ agglutinin (WGA; Sigma)-Sepharose was used instead of concanavalin A-Sepharose for the purification of the 150 kDa receptor. The receptor was eluted with 0.3 M N-acetylglucosamine. All other manipulations are as described for the purification of the 140 kDa protein.

Deglycosylation and V8 Protease Digestion. Purified, radiolabeled 140 kDa receptor was made 1% in *n*-octyl glucoside and incubated with endoglycosidase F/N glycosidase (Boehringer-Mannheim, Indianapolis, IN; 6×10^{-3}

units/μL) at 37 °C for 1 h). Native and deglycosylated receptors were incubated with V8 protease (Sigma; 0.1 mg/mL) at 37 °C for 30 min.

Antibodies and Immunoprecipitation. Multiple antigenic peptides (MAPs; Posnett *et al*., 1988) were synthesized based on residues 2−9 and 11−21 of the sulfonylurea receptor sequence. Polyclonal antibodies were produced in rabbits by standard methods (Harlow & Lane, 1988). Interdermal injections of 1 mg of antigen were spaced 2−3 weeks apart and contained complete (first injection) or incomplete Freund's adjuvant. Antibodies against the two nucleotide binding domains were generated by expression of receptor cDNA fragments as fusions with the maltose-binding protein. Residues 696−894 and 1358−1536 from the rat sulfonylurea receptor were cloned in frame into the pMAL-c2 expression vector (New England BioLabs, Beverly, MA) and expressed in *Escherichia coli*. The fusion proteins were purified by electrophoresis and electroelution. Amounts of 200 μg were used for injection into rabbits as described above.

Cell membranes were incubated with 1 nM [^{125}I]iodoglyburide for 30 min, photolabeled, solubilized with 1% digitonin, and centrifuged at 100 000*g* for 1 h at 4 °C. Soluble protein (0.5 mL) was incubated for 1 h at room temperature with 25 μL of the immune serum. Protein A-Sepharose (Sigma) was added, and after a further 1 h incubation the beads were washed 3 times with 25 mM Tris (pH 7.5), 0.1 M NaCl, 2 mM EDTA, and 0.1% digitonin, and then heated to 90 °C for 5 min in the presence of pH 9 sample buffer. Following electrophoresis on 6−8% polyacrylamide SDS gels autoradiograms were prepared.

Construction and Transient Expression of a C-Terminal Histidine-Tagged Rat Sulfonylurea Receptor. The RIN receptor was cleaved with *Eco*RI and then partially cleaved with *Hin*dIII and ligated with two complementary synthetic oligonucleotides, 5′-AGCTTCATCACCATCACCATCACTG-3′ and 5′-AATTCAGTGATGGTGATGGTGATGA-3′, which specify six histidine residues followed by a stop codon. Candidate clones were sequenced to verify the presence of the insert in the appropriate reading frame. COSm6 cells were transfected with this construct in the vector pECE (Ellis *et al.*, 1986) as described previously for the HIT cell cDNA sequence (Aguilar-Bryan *et al*., 1995). After whole-cell photolabeling the cells were washed with phosphate-buffered saline and then solubilized in 1% Triton X-100, 10 mM HEPES, pH 7.4, 0.3 M NaCl, 2 mM dithiothreitol, 1 μg of leupeptin mL, 1.5 μM pepstatin A, 100 nM benzamidine. The lysate was centrifuged in a microfuge for 10 min, and the supernatant was chromatographed on 200 μL of Ni-agarose (Qiagen Inc., Chatsworth, CA) equilibrated with the lysis buffer. The sample was incubated with the resin for 4 h at 4 °C, centrifuged for 2 min in a microfuge, and then washed 2 times with 10 mL of lysis buffer and a third time with 500 μL of lysis buffer. The bound receptor was eluted with 500 μl of 0.2 M imidazole, pH 6.3, in half-strength lysis buffer, diluted with SDS sample buffer, separated on an 8% polyacrylamide gel, and visualized by autoradiography.

RESULTS

Evidence for Two Forms of the Sulfonylurea Receptor That Are Differentially Glycosylated. High-affinity sulfonylurea receptors were identified by photolabeling in islet (HIT-T15, RINm5f, αTC-6) and anterior pituitary (AtT-20 and GH_3) cell lines (Figure 1). Filtration binding assays confirmed the presence of high-affinity receptors in these cell lines. Two forms of the receptor at 140 and 150 kDa are visible in RINm5F cells, as was previously reported for αTC-6 cells (Rajan *et al*., 1993). The estimated molecular weights of these proteins vary somewhat depending on the percentage of acrylamide and bisacrylamide in the separating gel. We have adopted 140 and 150 kDa as estimated apparent molecular masses with the understanding that these values range from ∼140 to ∼170 kDa. The data in Figure 1 show that the pH sensitivity of binding and photolabeling for the two forms are similar.

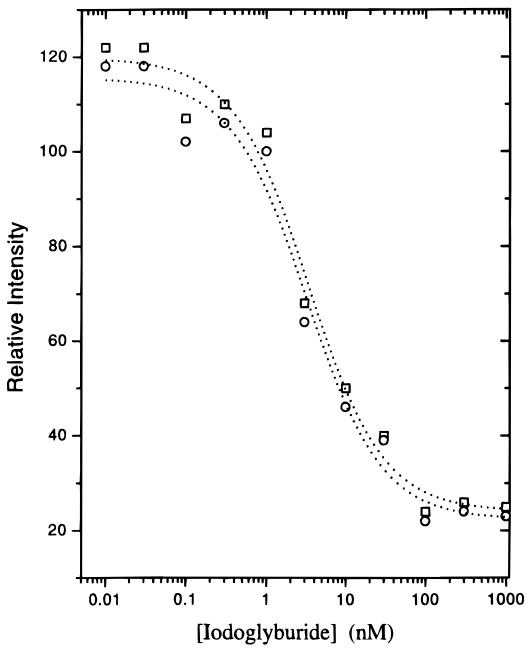

FIGURE 2: Displacement of [^{125}I]iodoglyburide with unlabeled iodoglyburide, as assayed by photolabeling of the sulfonylurea receptors in RINm5f membranes. Receptors were photolabeled at pH 6.8 in PIPES buffer as described in Figure 1. The autoradiographs were scanned electronically, and the relative densities were plotted. The dotted lines are for a single-site binding model; the calculated K_i values are 3.2 and 3.3 nM for the 140 kDa (○) and 150 kDa (□) species, respectively.

Photolabeling displacement experiments (Nelson *et al*., 1992; Rajan *et al*., 1993) indicate both forms have approximately equal dissociation constants. A quantitative comparison of iodoglyburide binding of the two forms from RINm5f cells is shown in Figure 2. Autoradiographs were prepared and scanned to determine the relative intensities associated with each band. The data, plotted against the concentration of competing unlabeled iodoglyburide, indicate that displacement for the two forms is equivalent, with K_Ds, estimated from half-maximal values, of 3.3 and 3.2 nM for the 140 and 150 kDa species, respectively. The range of dissociation constants (K_D) for [^{125}I]iodoglyburide in all five cell lines is 3−10 nM as determined by filtration binding assays. The B_{MAX} values, determined at pH 7.5, range from 0.15 (GH_3) to 3.1 (αTC-6) pmol/mg of membrane protein, with HIT-T15 and AtT-20 cells at ∼1.0−1.6 pmol/mg of membrane protein and RINm5f cells at ∼0.4 pmol/mg of membrane protein.

Tunicamycin treatment of RINm5f cells eliminates the 150 kDa band and slightly reduces the apparent molecular mass of the lower, 140 kDa species as shown in Figure 3. The

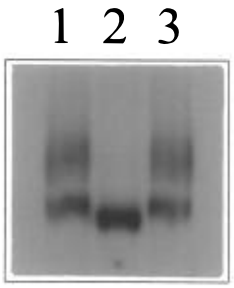

FIGURE 3: Tunicamycin blocks glycosylation of the sulfonylurea receptor. RINm5f cells were grown for 48 h in tunicamycin (1 μg/mL) and then incubated with [^{125}I]iodoglyburide. The cells were photolabeled as described in the Experimental Procedures. Bands were visualized by autoradiography after separation on an 8% polyacrylamide gel. Lanes 1 and 3 are control, untreated RINm5f cells; lane 2 is tunicamycin-treated cells.

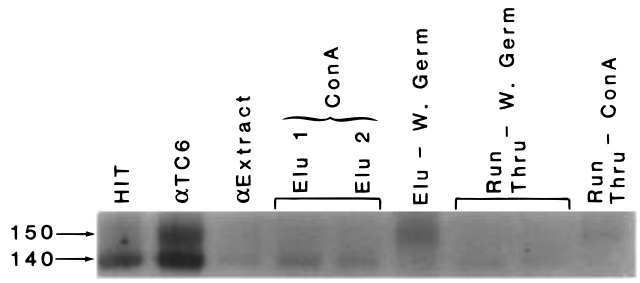

FIGURE 4: Separation of the 140 and 150 kDa species by concanavalin A- and wheat germ agglutinin-Sepharose chromatography. αTC-6 membranes were prepared and radiolabeled as described in the Experimental Procedures. Membranes were solubilized with 1% digitonin. A 100 000*g*, 60 min supernatant was prepared and chromatographed over concanavalin A-Sepharose. The unretained protein from this column, containing the 150 kDa receptor, was loaded onto a WGA-Sepharose column. Each form was eluted using the appropriate sugar. Elu, protein in eluates; run thru, unretained protein.

estimated mobility shift for the lower band is approximately 3 kDa; treatment of the purified 140 kDa receptor with endoglycosidase F/N glycosidase produces a comparable mobility shift (see Figure 5). Similar tunicamycin and endoglycosidase experiments from all cell lines yield equivalent results, suggesting that the 140 kDa species is the same in each cell line, and, where found, that the 150 kDa species are identical in each cell line. We do not know what regulates the relative amounts of the two receptor forms.

Solubilization of Sulfonylurea Receptors. The ability of a variety of detergents to solubilize the sulfonylurea receptors was assessed prior to receptor purification. In initial experiments, the membrane-bound HIT-T15 cell receptor was photolabeled with [^{125}I]iodoglyburide, and the minimum detergent concentration needed to solubilize at least 50% of the 140 kDa photolabeled protein was established. Ten detergents were tested, *n*-octyl glucoside, CHAPS, Triton X-100, Nonidet P-40, Lubrol PX, sodium cholate, sodium deoxycholate, Tween 80, digitonin, and SDS. Maximal solubilization was generally reached at 0.5−1.0% detergent concentrations, with the exception of Tween 80, which did not solubilize the receptor, and SDS, which required concentrations of 0.1−0.2%. Using minimum detergent concentrations which maximally solubilized the sulfonylurea receptor, we determined whether we could first solubilize and then photolabel the protein. CHAPS, Triton X-100, Nonidet P-40, and digitonin were able to solubilize the receptor with the retention of displaceable binding activity. Digitonin was the best at solubilization, with the receptor retaining 30% of binding activity. The rationale for these experiments was based on our original, qualitative experience with receptor purification (Aguilar-Bryan *et al*., 1990). The retention of binding activity made the protein less prone to loss by irreversible binding to column beds. 1% digitonin was chosen for receptor solubilization; this detergent concentration was reduced in subsequent purification steps without receptor loss, as described in the Experimental Procedures. In subsequent experiments, we show that digitonin is a suitable detergent for the solubilization and purification of both the 140 and l50 kDa forms of the receptor.

Separation of the Two Forms of the Sulfonylurea Receptor by Lectin Chromatography. The two species of the receptor can be separated on the basis of their differential glycosylation. The 140 kDa species binds to concanavalin A and can be eluted with methyl α-D-mannopyranoside, while the 150 kDa form binds to wheat germ agglutinin (WGA) and can be eluted with *N*-acetylglucosamine (Figure 4). Bernardi *et al*. (1988) have reported partial purification of a 150 kDa brain sulfonylurea receptor using WGA, and the brain receptor is apparently equivalent to the 150 kDa form described in this paper.

Purification of the Two Forms of the Sulfonylurea Receptor. The strategy used to purify the 140 kDa species from HIT-T15 cell membranes is described in the Experimental Procedures and summarized in Table 1. A crude receptor fraction is obtained by chromatography of the digitonin extracts on concanavalin A-Sepharose. This step is critical and removes a 140 kDa, non-glycosylated contaminant protein that co-purifies with the 140 kDa sulfonylurea receptor through several chromatographic steps. The contaminant protein has no sequence similarity to the cloned receptor described in Aguilar-Bryan *et al* (1995). Two additional chromatographic steps on Reactive-Green-19-agarose and phenylboronate-10 Sepharose enrich the receptor sufficiently that a 140 kDa band is visible on Coomassie Blue stained gels and can be retrieved by electroelution. Typical yields from 20 roller bottles, 200−300 mg of membrane protein, were 1−2 μg of electrophoretically pure protein. The 150 kDa species can be purified using the same strategy if WGA is substituted for concanavalin A in the first chromatographic step. Both receptor forms can be purified from αTC-6, or RINm5f membranes by passing the Concanavalin A-Sepharose flow-through fraction over WGA-Sepharose.

The radiochemical purity of the purified 140 kDa receptor is shown in Figure 5 along with the results of digestion with V8 protease before and after digestion with endoglycosidase F/N glycosidase F. Deglycosylation of the 140 kDa receptor decreases the apparent molecular size by ∼3 kDa (compare lane 2 with 1 and 3). Cleavage with V8 protease yields two major radiolabeled polypeptides of 66 and 49 kDa that also show a 3 kDa size shift following endoglycosidase treatment (compare lane 5 with lanes 4 and 6).

The sequences of the N-termini of all of these major photolabeled fragments were the same, as described previously (Aguilar-Bryan *et al.*, 1995). The tenth residue in the receptor could not be identified in the glycosylated polypeptides but was an aspartate in the deglycosylated form. This observation and the fact that residue 12 is a serine suggests

Table 1: Purification of the 140 kDa, High-Affinity HIT Cell Sulfonylurea Receptor[a]

step	total volume (mL)	total protein (mg)	receptor (pmol)	receptor (pmol/mg)	purification *n*-fold (*n*)	yield (%)
crude membrane	90	200	320	1.6	1	100
supernatant	90	150	240	1.6	1	75
concanavalin A-Sepharose	48	10.2	80	7.8	4.9	25
Green 19-agarose	16	1.80	56	31.1	19.5	18
phenylboronate-agarose	4	0.56	45	80.4	50.4	14
SDS-page/electroelute	0.2	~0.002	11	5600	3500	3

[a] The table summarizes the yields and *n*-fold purification in the scheme developed for receptor purification. The amounts of receptor, yields, and *n*-fold purification reported after each step are based on the cpm in the 140 kDa band after electrophoresis (the band was excised and counted in a γ-counter), relative to the amount of protein from that sample loaded on a gel lane (as determined using the Bio-Rad protein assay). The HIT cell membrane starting material contains approximately 1.6 pmol of receptor per mg of membrane protein (Aguilar-Bryan *et al*., 1992).

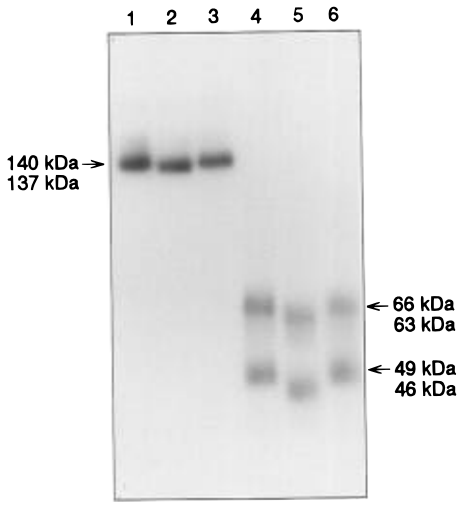

FIGURE 5: V8 and endoglycosidase cleavage of the hamster 140 kDa sulfonylurea receptor. The radiolabeled 140 kDa receptor (lanes 1 and 3) was cleaved with endoglycosidase F/N glycosidase F, increasing the mobility of the protein by approximately 3 kDa (lane 2). Partial V8 protease digestion (lanes 4 and 6) yields radiolabeled fragments that increase mobility with endoglycosidase treatment (lane 5).

N-linked glycosylation of the receptor at the asparagine residue at position 10. It is not clear whether this is the only glycosylation site in SUR1. A comparison with the open reading frame determined from the cDNA sequence with the N-terminus found by Edman degradation indicates the terminal met has been removed in the mature receptor (Aguilar-Bryan *et al*., 1995).

Edman degradation of the 150 kDa receptor from either αTC-6 or RINm5f cells did not yield sequence, suggesting that the amino terminus is either blocked or protected by the additional glycosylation. V8 digestion of the deglycosylated 150 kDa polypeptide, however, gave fragments of 63 and 46 kDa with electrophoretic mobilities indistinguishable from those derived from the deglycosylated 140 kDa protein (data not shown). Thus the 140 and 150 kDa forms appear closely related, or identical in peptide sequence, possibly differing only in the extent of glycosylation at residue 10.

Expression of Sulfonylurea Receptor cDNAs Produces the Glycosylated 140 kDa Protein. Figure 6 (left side) compares the mobility of the HIT-T15 cell receptor expressed in COSm6 cells (Aguilar-Bryan *et al.*, 1995) with native RIN sulfonylurea receptors before and after tunicamycin treatment. Expression in COSm6 cells produces only the 140 kDa receptor (lane 3), which shows about a 3 kDa decrease in size following tunicamycin treatment (results not shown).

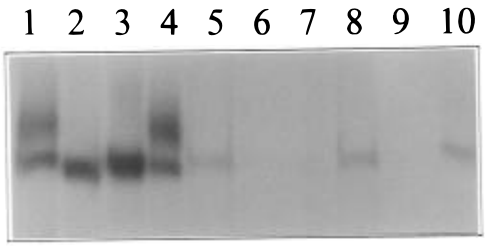

FIGURE 6: COSm6 cells transfected with a histidine-tagged RINm5f cell sulfonylurea receptor cDNA expresses a full-length 140 kDa polypeptide. Receptors from untreated RINm5f cells (lanes 1 and 4), receptors from tunicamycin treated RINm5f cells (lane 2), and the HIT cell receptor expressed in COSm6 cells (lane 3) are provided as markers. Lanes 5−7 show the behavior of the unmodified RINm5f receptor on a Ni-agarose column. Lane 5 is the loaded sample, lane 6 is the flow-through fraction, and lane 7 is the 0.2 M histidine eluate. Lanes 8−10 show the behavior of the histidine-tagged RIN cell receptor using the same elution protocol. The RIN receptor, histidine-tagged at its C-terminus, is adsorbed to Ni-agarose, elutes with 0.2 M histidine, and has the same apparent molecular weight as the other native receptors.

The receptor sizes estimated from SDS gels are approximately 40 kDa lower than expected based on the receptor cDNA sequences (177 209 daltons for the HIT cell and 177 102 daltons for the RIN cell receptors). To eliminate the possibility that the 40 kDa difference is accounted for by proteolysis, we constructed an expression vector containing the rat receptor-tagged 18 amino acids from its C-terminal end with six histidine residues as described in the Experimental Procedures. COSm6 cells were transfected with this construct, grown overnight, and then photolabeled. The receptor was solubilized with 1% Triton X-100, applied to a Ni-agarose column, and then eluted with histidine as shown in Figure 6. The results show that the histidine-tagged receptor can bind drug and photolabel, and that the labeled receptor binds to Ni-agarose and is eluted with histidine. The estimated size of the full length, tagged receptor, 140 kDa, is equivalent to the receptor identified in RINm5f cells, indicating there is a discrepancy between the actual molecular mass of the receptor and that estimated by SDS gel electrophoresis. The receptor expressed in COSm6 cells is retained on concanavalin A-Sepharose but not WGA-Sepharose (data not shown), consistent with the 140 kDa form of glycosylation.

Nucleotide-Binding Fold and Amino Terminal Antibodies Immunoprecipitate the Receptors. Polyclonal antibodies were produced against two peptides, residues 2−9 and 11−21 from the consensus N-terminal sequence described previously (Aguilar-Bryan *et al*., 1995). These antibodies were originally used to immunoprecipitate photolabeled receptors to verify that the chemical sequence was from the

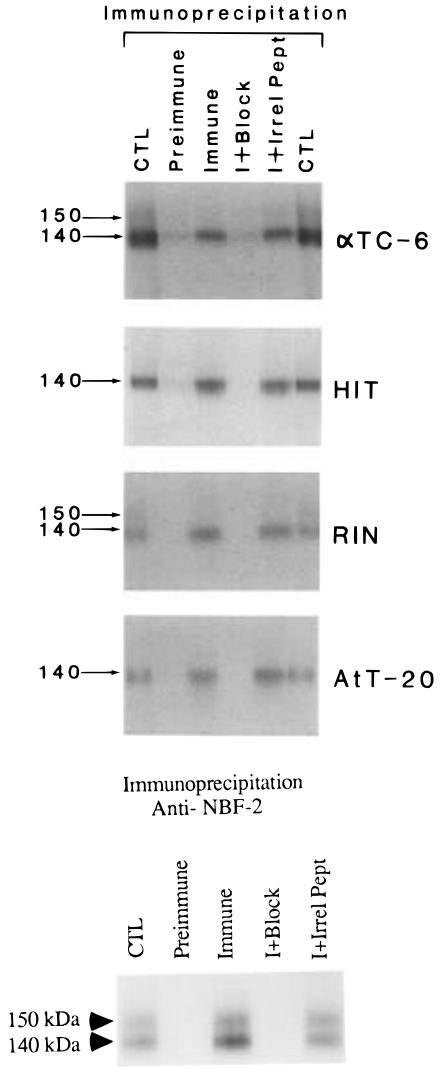

FIGURE 7: (Top panel) Immunoprecipitation of the sulfonylurea receptor with antibodies directed against the amino terminus. Membranes were prepared from αTC-6, HIT-T15, RINm5f, and AtT-20 cells, photolabeled with [^{125}I]iodoglyburide, and protein solubilized as described in the Experimental Procedures. Aliquots were mixed with rabbit serum and antibodies collected with protein A-Sepharose. Samples were separated by electrophoresis on an SDS polyacrylamide gel, and autoradiograms were prepared. Soluble protein (control; CTL) was incubated with preimmune serum, immune serum, immune serum + MAP 11−21 (I + Blocking peptide), and immune serum + an irrelevant peptide (I + Irrel Pept). (Bottom panel) Immunoprecipitation of the sulfonylurea receptor from RINm5f cells with polyclonal antibodies directed against NBF-2. CTL: radiolabeled 140 and 150 kDa receptors from solubilized RINm5f cell membranes. Immunoprecipitation was with Preimmune serum, Immune serum, Immune serum + NBF-2 fusion protein (I + Block), and Immune serum + irrelevant peptide (I + Irrel Pept).

receptor and not a co-purifying polypeptide. Antibodies against residues 11−21 (Figure 7, top panel) efficiently precipitate the 140 kDa receptor from all cell lines and appear to be somewhat selective for this form, although longer exposures show precipitation of the 150 kDa species. This difference may result from the more extensive glycosylation of the 150 kDa receptor at Asn_{10}, but we cannot rule out N-terminal sequence differences. Results using antibodies against residues 2−9 were identical to those against residues 11−21 (data not shown).

Polyclonal antibodies generated against the nucleotide binding folds, specifically residues 696−894 and 1358−1536 in the rat receptor sequence, were expressed as fusions with the maltose-binding protein in *E. coli*. These antibodies gave equivalent results and, as illustrated in Figure 7 (bottom panel) for antibodies against NBF-2, specifically immunoprecipitate both the 140 and 150 kDa receptors from RINm5f cells. These results further support the notion that both receptor forms have similar, if not identical, polypeptide sequences.

DISCUSSION

Cloning of the high-affinity sulfonylurea receptor has demonstrated it is a member of the ATP-binding cassette superfamily with multiple transmembrane spanning domains and two potential nucleotide-binding folds. Expression of cloned sulfonylurea receptors in COSm6 cells generates appropriately sized proteins (140 kDa as assayed by SDS gel electrophoresis) that bind sulfonylureas with nM affinities (Aguilar-Bryan *et al*., 1995). Expression of SUR1 does not generate K^+ channel activity detectable by single-channel methods or $^{86}Rb^+$ efflux assays, but co-expression with a silent member of the small inward rectifier family, $K_{IR}6.2$, reconstitutes I_{KATP} (Inagaki *et al*., 1995). This finding demonstrates that the receptor is required for channel activity and implies these two polypeptides form a heteromultimeric channel with an unknown stoichiometry. Truncations of the sulfonylurea receptor that result in destruction of the second nucleotide-binding fold have been linked to persistent hyperinsulinemic hypoglycemia of infancy (PHHI; Thomas *et al*., 1995). This autosomal recessive disorder of newborns, also referred to as familial hyperinsulinemia or nesidioblastosis, is characterized by continuous, unregulated secretion of insulin despite severe hypoglycemia. $K_{IR}6.2$ has no obvious nucleotide-binding motif, while SUR1 has two potential NBFs, indicating the receptor is the ATP sensor for I_{KATP}. This is further supported by the following findings: (1) Channels reconstituted using SUR2 and $K_{IR}6.2$ have altered nucleotide sensitivity relative to those reconstituted with SUR1 (Inagaki *et al.*, 1996); (2) a point mutation in the Walker A site of NBF1 in SUR1 is causative for PHHI (Thomas *et al*., 1996); and (3) a PHHI point mutation in NBF2 of SUR1 allows inhibition by ATP, but not activation by MgADP (Nichols *et al*., 1996). The linkage between PHHI and a component of I_{KATP} suggests this potassium channel is a crucial regulator of insulin secretion.

The molecular masses of the HIT-T15 and RINm5f receptors are calculated to be 177 209 and 177 102 daltons, respectively, based on the cDNA sequence (Aguilar-Bryan *et al*., 1995). The 140 kDa size estimate from SDS gel electrophoresis of the high-mobility (140 kDa) form is an SDS gel anomaly and is not due to proteolytic cleavage since insertion of a histidine tag at the C-terminus allows purification of the full-length receptor with an apparent mobility of 140 kDa. The conclusion that the receptor is actually 177 kDa agrees with a recent study using Triton X-100-solubilized sulfonylurea receptor from the MIN6 cell line (Skeer *et al*., 1994) which reports molecular masses of 166 (±1) and 182 (±5) kDa by gel filtration of the photolabeled and unlabeled receptor, respectively, and a calculated target size of 250 (±30) kDa using radiation inactivation of the membrane-bound protein.

Two forms of SUR1, the functional significance of which is not yet clear, can be identified in a number of cell lines.

Preliminary results with membranes isolated from adult human pancreatic β-cells indicate the 140 and 150 kDa species are present in about the ratio seen in RINm5f cells. The present results suggest that the 140 and 150 kDa polypeptides bind sulfonylureas with equal affinity and that they arise from differential glycosylation of a single protein. This conclusion is based on the finding of a single human gene for the β-cell high-affinity receptor, the precipitation of both forms of the receptor by anti-NBF and anti-N-terminal antibodies, and the finding of a single polypeptide at ~137 kDa following either endoglycosidase F/N glycosidase treatment, or growth of cell lines in tunicamycin. The small apparent molecular weight shift of the 140 kDa form, ~3 kDa following growth in tunicamycin, or digestion with endoglycosidases, is consistent with the identification by chemical sequencing of an N-linked glycosylation site at asparagine 10 (Aguilar-Bryan *et al*., 1995). We cannot rule out the use of additional glycosylation sites in the protein. Glycosylation is not required for drug binding and photolabeling since the 137 kDa species is easily detectable after tunicamycin, or endoglycosidase treatment using radiolabeled iodoglyburide.

The two receptor isoforms can be separated on lectin-affinity columns. Specifically, the 140 kDa species is bound to concanavalin A-Sepharose indicating it contains mannose as the terminal residues of the added oligosaccharide. The 150 kDa species is bound to wheat germ agglutinin-agarose, indicating that it contains *N*-acetylneuraminic acid (sialic acid) in the added oligosaccharide. We speculate that the 140 and 150 kDa forms are, respectively, intermediate and mature species in the normal glycosylation pathway. The mannose containing 140 kDa receptor could represent any of three stages in glycosylation: the receptor after transfer of the oligosaccharide from dolichol lipid, a "core" glycosylated stage after partial "trimming" of mannose residues, or a high-mannose stage after addition of additional mannose residues to the core region. We suggest that the 150 kDa *N*-acetylneuraminic acid-containing species is the mature glycoprotein after addition of complex *N*-acetylglucosamine−galactose−*N*-acetylneuraminic acid-containing sugar chains. It is not clear what fraction(s) of these two species are in the plasma membrane versus internal membranes. Ozanne *et al*. (1995) have argued that most of the 140 and some of the 150 kDa species are in internal membranes, while only the 150 kDa species is in the plasma membrane. We note that <10% of the receptor in HIT-T15 cells are the 150 kDa species, while ~50% of the RINm5f cell receptors are the 150 kDa species. This differential in receptor forms in different cell types could provide a means to determine if only one of the glycosylated receptors forms functionally active K^+ channels, and may provide insight into the existence and function of multiple isoforms.

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REFERENCES

Aguilar-Bryan, L., Nelson, D. A., Vu, Q. A., Humphrey, M. B., & Boyd, A. E., III (1990) *J. Biol. Chem. 265*, 8218−8224.

Aguilar-Bryan, L., Nichols, C. G., Rajan, A. S., Parker, C., & Bryan, J. (1992) *J. Biol. Chem. 267*, 14934−14940.

Aguilar-Bryan, L., Nichols, C. G., Wechsler, S. W., Clement, J. P., Boyd, A. E., Gonzalez, G., Herrera-Sosa, H., Nguy, K., Bryan, J., & Nelson, D. A. (1995) *Science 268*, 423−426.

Ashcroft, S. J H., & Ashcroft, F. M. (1992) *Biochim. Biophys. Acta 1175*, 45−59.

Bernardi, H., Fosset, M., & Lazdunski, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9816−9820.

Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., & Rutter, W. J. (1986) *Cell 45*, 721−732.

Harlow, E., & Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Inagaki, N., Gonoi, T., Clement, J. P., Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S., & Bryan, J. (1995) *Science 270*, 1166−1170.

Inagaki, N., Gonoi, T., Clement, J. P., Wang, C. Z., Aguilar-Bryan, L., Bryan, J., & Seino, S. (1996) *Neuron 16*, 1011−1017.

Laemmli, U. K. (1970) *Nature (London) 227*, 680−685.

Nelson, D. A., Aguilar-Bryan, L., & Bryan, J. (1992) *J. Biol. Chem. 267*, 14928−14933.

Nichols, C. G., Shyng, S.-L., Nestorowicz, A., Glaser, B., Clement, J. P., Gonzalez, L., Aguilar-Bryan, L., Permutt, M. A., & Bryan, J. (1996) *Science* 272, 1785−1787.

Ozanne, S. E., Guest, P. C., Hutton, J. C., & Hales, C. N. (1995) *Diabetologia 38*, 277−82.

Posnett, D. N., McGrath, H., & Tam, J. P. (1988) *J. Biol. Chem. 263*, 1719−1725.

Rajan, A. S., Aguilar-Bryan, L., Nelson, D. A., Nichols, C. G., Wechsler, S. W., Lechago, J., & Bryan, J. (1993) *J. Biol. Chem. 268*, 15221−15228.

Skeer, J. M., Degano, P., Coles, B., Potier, M., Ashcroft, F. M., & Ashcroft, S. J.H. (1994) *FEBS Lett. 338*, 98−102.

Thomas, P. M., Cote, G. J., Wohllk, N., Haddad, B., Mathew, P. M., Rabl, W., Aguilar-Bryan, L., Gagel, R. F., & Bryan, J. (1995) *Science 268*, 426−429.

Thomas, P. M., Wohllk, N., Huang, E., Kuhnle, U., Rabl, W., Gagel, R. F., & Cote, G. J. (1996) *Am. J. Hum. Genet. 59*, 510−518.

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