Functional expression of rat GLUT 1 glucose transporter in *Dictyostelium discoideum*

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To facilitate expression of the rat GLUT 1 glucose transporter cDNA in *Dictyostelium discoideum*, we mutated the 5' end of the coding sequence such that the codons for the first ten amino acids conformed to preferred *Dictyostelium* codon usage. As determined by Western-blot analysis, a population of *Dictyostelium* transformed with the mutated cDNA expressed non-glycosylated GLUT 1 protein. Cell lines expressing GLUT 1 transport radiolabelled 2-deoxy-D-glucose at a rate 6–10 times that of cell lines transformed with vector alone. The initial rate of inward transport of 2-deoxy-D-glucose was stimulated several-fold by the presence of unlabelled glucose in the *Dictyostelium* cytoplasm, exemplifying the *trans*-activation of GLUT 1 trans-

port characteristic of GLUT 1 present in erythrocyte membranes. The K_m and K_i values for 2-deoxy-D-glucose, D-glucose, D-mannose and D-galactose were 3.7 mM, 2.6 mM, 11 mM and 30 mM respectively, similar to the values for GLUT 1 expressed in mammalian cells. L-Glucose and L-galactose, which are not transported by GLUT 1, do not inhibit uptake of 2-deoxy-D-glucose in *Dictyostelium* expressing GLUT 1. Thus, even though GLUT 1 expressed in *Dictyostelium* is not N-glycosylated, it transports hexoses normally; this is the first example of functional expression of a mammalian transport protein in this lower eukaryote.

INTRODUCTION

Most mammalian cells utilize facilitated diffusion glucose transporters that move glucose across the plasma membrane down its chemical concentration gradient. Seven facilitated diffusion glucose transport proteins, designated GLUT 1 to GLUT 7, have been cloned and sequenced; all have homologous amino acid sequences with 12 predicted membrane-spanning α -helices and intracellular N- and C-termini. Each is found in specific types of cells, and each has unique kinetic and other properties that enable cells that express it to carry out important physiological functions [1–4].

GLUT 1 is expressed by many types of cells, and is particularly abundant in erythrocytes; its K_m for glucose is between 1 and 2 mM. It is abundant in hepatocytes and in kidney tubule cells that have relatively high rates of glucose oxidation [5,6]. Expression of GLUT 1 is elevated by several oncogenes in cultured tumour cells, accounting for the enhanced rate of glucose uptake characteristic of many tumour cell lines [7,8]. GLUT 2 is found on the basolateral plasma membrane of hepatocytes and of small intestine and proximal kidney absorptive epithelial cells [5,6] and is the major glucose transporter in the β -cells of the islets of Langerhans [9]. The K_m of GLUT 2 is ~ 17 mM [10]. GLUT 4, which also has a K_m less than 2 mM [11], is found only in adipose and muscle cells. In resting cells GLUT 4 is localized to endosome-like vesicles just under the plasma membrane; in response to insulin these vesicles fuse with the plasma membrane [12–14].

In order to study the function of mammalian glucose transporters, they have been expressed in several types of cells, including bacteria [15], cultured mammalian cells [16,17] and *Xenopus* oocytes [11,18]. Bacteria usually do not fold eukaryotic membrane proteins properly nor insert them functionally into

the plasma membrane; expression of eukaryotic membrane proteins in bacteria is often toxic, resulting in low levels of expression [19,20]. Cultured mammalian cells, which express endogenous GLUT 1 or other glucose transporters, generally have high levels of endogenous transporter activity; this may complicate interpretation of experiments in which exogenous transporters are expressed. *Xenopus* oocytes, which have little or no endogenous glucose uptake activity, require microinjections of individual eggs that have an average life span of 2–3 days [11]. However, both GLUT 1 and GLUT 4 display similar kinetic behaviour when expressed in the frog oocyte membrane as compared with their native membrane environments [11].

The soil amoebae *Dictyostelium discoideum* is a eukaryotic organism that is useful as a model system for studying many cellular processes. The morphology, structure and behaviour of the amoebae are similar to those of motile mammalian cells, especially leucocytes, and *Dictyostelium* normally grows by phagocytosis of bacterial cells [21]. The cells have a small haploid genome which can be manipulated easily with molecular genetic techniques including gene targeting and antisense RNA mutagenesis. Cells grow by binary fission in broth axenic media, making it easy to isolate large quantities of cells for biochemical analyses [22]. When grown in broth medium *D. discoideum* exhibits a very low level of uptake of radiolabelled 2-deoxy-D-glucose (DOG) and D-glucose, an unpublished observation made in our laboratory several years ago by Dr. Donald Schneider and confirmed in this paper. Given these attributes, *Dictyostelium* presented an attractive alternative for expression of mammalian glucose transport proteins. As we describe here, rat brain GLUT 1 can be functionally expressed in stably transfected *Dictyostelium* cell lines; the kinetic properties of glucose transport are similar to those of GLUT 1 normally present in erythrocytes.

Abbreviations used: DOG, 2-deoxy-D-glucose; [^{14}C]DOG, 2-deoxy-D-[1- ^{14}C]glucose.

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MATERIALS AND METHODS

Materials

Geneticin was purchased from Gibco BRL. The vector pBSII SK⁺ and the Sequenase 2.0 DNA sequencing kit were obtained from Stratagene. [¹²⁵I]-labelled Protein A and 2-deoxy-D-[1-¹⁴C]glucose ([¹⁴C]DOG) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ecocint-H scintillation fluid was purchased from National Diagnostics. N-Glycosidase-F was purchased from Boehringer-Mannheim.

Cell culture

Dictyostelium Ax₄ cells were grown in suspension in HL-5 growth medium which contained, per litre, 5 g Difco yeast extract, 10 g Difco proteose peptone, 10 g glucose, 0.35 g KH₂PO₄, and 0.35 g Na₂HPO₄ with pH adjusted to 6.3–6.5. Cells were grown at room temperature on a gyrotory shaker.

Construction of a *Dictyostelium* cloning vector containing GLUT 1

A GLUT 1 cDNA was generated in which the codons for the first 10 amino acids were consistent with *Dictyostelium* codon usage [23]. First, rat brain GLUT 1 cDNA [24] was subcloned into the *Dictyostelium* cloning vector pDNEOII [25]. The portion corresponding to bp 192–906 (indicated by the hatched area in Figure 1) was then mutagenized by PCR amplification. The 5' sense primer (Primer 1): 5'-CCCGGG-CCCGGGAAAAAT-GGAACCATCATCAAAAAAAGTTACTGGTCGTCTTAT-GTTGGCCGTG-3', corresponded to bp 192–256, encoding amino acids 1–16. This primer created two tandem *Sma*I sites (underlined) and achieved similarity to a typical *Dictyostelium* gene by placing multiple A residues 5' of the ATG start codon (bold) and altering the first ten codons to conform to *Dictyostelium* preferred codon usage for the amino acids present in

Figure 1 Schematic representation of GLUT 1-pAD-80 DNA

Synthetic PCR primers (see the Materials and methods section) were used to amplify a 715 bp portion of rat GLUT 1 (hatched area) which had been previously subcloned into the *Dictyostelium* cloning vector pDNEO II. Multiple As were inserted just upstream of the initiator ATG, and mutations, indicated by asterisks, were made in the first ten codons after the ATG to achieve co-incidence with *Dictyostelium* preferred codon usage. The amplified PCR product was digested with *Sma*I and *Hind*III and ligated into the polylinker region of PBS II SK⁺ for sequencing. The wild-type GLUT 1 cDNA in pDNEO II was digested with *Hind*III and *Xho*I and ligated into the PCR-PBS II SK⁺ construct. Using *Spe*I and *Xho*I, the full-length mutated rat GLUT 1 cDNA was released from PBS II SK⁺ and ligated into the *Dictyostelium* cloning vector pAD-80, the cloning site of which is flanked by the AT-rich actin 15 promoter (A15P) and the 2 H3 terminator (2H3T).

wild-type GLUT 1 cDNA. The 3' antisense primer (Primer 2): 5'-CCCTCGAAGCTTTTTCAGCACACTCTTGCCCG-3', representing bp 874–906, contained a *Hind*III site at bp 895–900 (underlined; see Figure 1). PCR amplification was performed according to the manufacturers' instructions for 25 cycles: 45 s at 95 °C; 45 s at 60 °C; 2 min at 72 °C with a final extension at 72 °C for 8 min. The PCR product was digested with *Sma*I and *Hind*III, ligated into the polylinker of the vector pBSII SK⁺, and sequenced to confirm the fidelity of the amplification. Next, the wild-type GLUT 1-pDNEOII plasmid was digested with *Hind*III (bp 896) and *Xho*I (in the polylinker at the 3' end of the pDNEOII vector) and the resulting 994 bp fragment (clear area, Figure 1) was ligated to the mutagenized PCR product in the pBSII SK⁺ vector. The full-length modified GLUT 1 cDNA was subcloned, using restriction enzymes *Spe*I and *Xho*I, into the *Dictyostelium* cloning vector pAD-80, kindly provided by Dr. Arturo de Lozanne, Duke University, Durham, NC, U.S.A.; we refer to this DNA as GLUT 1-pAD-80.

Generation of a stable *Dictyostelium* cell line expressing transfected rat GLUT 1

A population of *Dictyostelium* Ax₄ amoebae was transformed with the GLUT 1-pAD-80 cDNA using a calcium phosphate method [22]. Transformants were selected in medium containing 5 µg/ml geneticin. Clonal lines were isolated by plating at limiting dilution and were screened by Western-blot analysis. A representative clone was selected for subsequent transport assays.

Immunoblot analysis

Rat brain membranes were prepared according to the method described by Thorens et al. [26] with the following modifications suggested by Dr. Maureen Charron: minced brain tissue was homogenized in 7 vols. of 0.32 M sucrose containing protease inhibitors and centrifuged at 2000 g for 12 min. The supernatant was centrifuged for 20 min at 9649 g in the SS-34 rotor. The resultant supernatant, consisting primarily of cytosol, was then centrifuged for 1 h at 112000 g in a Beckman Ti 50.2 rotor and the pellet was resuspended in 0.25 M sucrose, 50 mM Tris/HCl (pH 7.5), 25 mM KCl, 5 mM MgCl₂. Membranes were dissolved in sample buffer containing SDS [27] followed by sonication for 1 min at maximum power using a Heat Systems Ultrasonics instrument. Similarly, *Dictyostelium* cells, both wild-type and GLUT 1-transformed, were dissolved in gel sample buffer followed by sonication.

Lysates were treated with N-glycosidase-F as follows: 400 µg of protein in a lysate was brought to 10 mM EDTA and 1% (v/v) Nonidet P-40 and to pH 8.5 with Tris base in a total reaction volume of 100 µl. An aliquot (200 units/ml) of N-glycosidase-F was added to each sample and incubated at 37 °C for 18 h. Samples containing 100 µg of protein from undigested and N-glycosidase-F-digested lysates were resolved by SDS/10%-PAGE and transferred to a nitrocellulose filter [26]. The filter was incubated for 1 h at room temperature with a 1:100 dilution of a polyclonal rabbit anti-peptide serum raised against the C-terminus of GLUT 1 [26]. Antibody binding was detected after incubation with [¹²⁵I]-labelled Protein A by autoradiography using Kodak XAR film at –70 °C for 7 days.

Transport measurements

DOG transport assays were performed on *Dictyostelium* transformed with the GLUT 1-pAD-80 plasmid (GLUT 1⁺), using parental pAD-80-transformed cells (GLUT 1[–]) as a negative control. *Dictyostelium* cells growing in HL-5 medium were

harvested by centrifugation for 3 min at 1171 g, then washed three times in phosphate buffer consisting of 2.5 mM KH_2PO_4 and 2.5 mM Na_2HPO_4 with a final pH of 6.5.

The cells were then resuspended in 50 ml of buffer in an Erlenmeyer flask and placed on a gyrotory shaker at room temperature for ~ 1 h prior to the assay. They were then centrifuged and resuspended in the same buffer at 4×10^8 cells/ml. Aliquots of 50 μl volume (2×10^7 cells) were used for each sample tested. Transport was initiated by addition of [^{14}C]DOG at a final concentration of 3 $\mu\text{Ci}/\text{ml}$ and 1 mM (see Figure 4) or 0.5 mM (see Figure 3); cells were kept in motion throughout the incubation to prevent aggregation. Assays were performed in duplicate or triplicate for periods of up to 60 min at room temperature. Reactions were stopped by dispensing 3–5 ml of ice-cold phosphate buffer into the reaction mixture, followed by immediate transfer to a Whatman GF/A filter in a 24-sample Brandel M-24 cell harvester. The filters were placed in vials containing 8 ml of scintillation fluid and ^{14}C radioactivity was measured in a Packard Minaxi Tri-Carb 4000 scintillation counter. A 10 μl aliquot of the labelled DOG mix was counted as well as a blank filter to assess the background.

K_t values for the hexoses tested were determined in transport reactions containing 1 mM [^{14}C]DOG and unlabelled hexoses at concentrations from 0 to 50 mM. Individual tubes containing 2×10^7 cells in phosphate buffer were incubated for 5 min. In all cases, the background of [^{14}C]DOG incorporated by mock-transfected cells (GLUT 1 $^-$) was subtracted from that incorporated by cells expressing GLUT 1 (GLUT 1 $^+$).

RESULTS

In our initial attempts to express rat GLUT 1 in *Dictyostelium*, we inserted the GLUT 1 cDNA into a standard *Dictyostelium* expression vector employing the actin 15 promoter. Transformants obtained by this protocol usually contain multiple, tandemly arrayed, vector DNAs stably inserted at a random site in the genome [28]. While abundant GLUT 1 mRNA was synthesized, no GLUT 1 protein was detected by Western-blot analysis. We suspected that the problem resided in mRNA

Figure 2 *Dictyostelium* transfected with the GLUT 1-pAD-80 plasmid expresses unglycosylated GLUT 1 protein

A Western-blot analysis, using a polyclonal rabbit antibody specific for the C-terminus of GLUT 1, was performed on 100 μg of protein from lysates of the indicated cells: lanes 1 and 2, wild-type *Dictyostelium*; lanes 3 and 4, GLUT 1-transformed *Dictyostelium*; lanes 5 and 6, rat brain membrane. Lanes 1, 3 and 5 contain undigested protein and lanes 2, 4 and 6 contain N-glycosidase-F-digested protein. The open arrow indicates the 46 kDa GLUT 1 protein recognized by the antibody in rat brain membranes. The closed arrow indicates the 38 kDa protein recognized by the antibody in *Dictyostelium* transformed with GLUT 1-pAD-80, as well as the 38 kDa species of GLUT 1 protein resulting from N-glycosidase-F digestion of rat brain membranes.

Figure 3 Kinetics of transport of [^{14}C]DOG into *Dictyostelium* cells expressing the GLUT 1 transporter

On the left *Dictyostelium* were grown in HL-5 medium containing 1% glucose, and on the right they were grown in HL-5 medium lacking glucose. *Dictyostelium* cells transformed with the pAD-80 vector with GLUT 1 ($\blacklozenge$) or without (control, $\bullet$) were used. 2×10^7 cells/ml were incubated with 0.5 mM [^{14}C]DOG at room temperature and uptake of [^{14}C]DOG was measured at the indicated times.

translation, since *Dictyostelium* genes are typically AT-rich and mRNAs generally contain several A residues just upstream of the ATG start codon. To facilitate expression of GLUT 1, we constructed a new vector, GLUT 1-pAD-80, containing four As just upstream of the ATG start codon. Also, we altered the first 10 amino acids to conform to *Dictyostelium* preferred codon usage (Figure 1). We used this plasmid to transform a population of Ax_4 *Dictyostelium* vegetative cells.

The Western blot in Figure 2 shows that *Dictyostelium* transformed with GLUT 1-pAD-80 express non-glycosylated GLUT 1; blots were probed with a polyclonal antibody against the C-terminus of GLUT 1 [26]. The antibody recognized a 46 kDa GLUT 1 protein in rat brain membranes (Figure 2, lane 5) that was converted into a 38 kDa species upon digestion with N-glycosidase-F (lane 6). The antibody recognized a 38 kDa protein in *Dictyostelium* transformed with GLUT 1-pAD-80 (Figure 2, lane 3) that was absent in lysates from wild-type *Dictyostelium* (lanes 1 and 2) and whose mobility was unaffected by digestion with N-glycosidase-F (lane 4). GLUT 1 in brain and erythrocytes contain a single complex N-linked oligosaccharide [29] that is absent in GLUT 1 produced in transfected *Dictyostelium* cells. Nonetheless, the transport assays in Figures 3 and 4 show that GLUT 1 expressed in transfected *Dictyostelium* is functional.

In the initial transport assays, *Dictyostelium* were grown in standard axenic medium containing 1% (w/v) glucose. Prior to the experiment, the cells were harvested, washed, and resuspended in a phosphate buffer, as detailed in the Materials and methods section. Transport was initiated by the addition of [^{14}C]DOG and was assayed at times up to 60 min. GLUT 1-transfected cells incorporated [^{14}C]DOG at a rate 6–10 times that of mock-transfected cells, and uptake was inhibited by the presence of a 10-fold excess of unlabelled DOG (results not shown). We performed nine uptake assays of 60 min duration. In all of these, uptake of radiolabelled DOG rose sharply for the first 10–20 min and decreased markedly between 20 and 30 min, after which uptake of [^{14}C]DOG resumed for the next 30 min but at a rate about one-third of that during the initial 10 min. An example of this unexpected biphasic uptake is shown in Figure 3 (left-hand side).

Figure 4 Inhibition of [^{14}C]DOG transport by unlabelled sugars

K_i values for the hexoses tested were determined in transport reactions containing 1 mM [^{14}C]DOG and unlabelled hexoses at concentrations from 0 to 50 mM. Individual tubes containing 2×10^7 cells in phosphate buffer were incubated for 5 min. In all cases, the background of [^{14}C]DOG incorporated by mock-transfected cells (GLUT 1 $^-$) was subtracted from that incorporated by cells expressing GLUT 1 (GLUT 1 $^+$). (C) shows a Lineweaver-Burk plot of data from a separate experiment in which increasing concentrations of unlabelled DOG were added to transport reactions containing 1 mM [^{14}C]DOG. We corrected the counts/min [^{14}C]DOG transported for the dilution of the specific activity of the [^{14}C]DOG by the unlabelled DOG. A least-squares fit was used to calculate the K_m value for DOG uptake.

While the kinetics of glucose transport are complex and somewhat controversial, the rate-limiting step in GLUT 1-mediated transport of glucose from outside the cell inward is thought to be a conformational change of the transporter that occurs following the release of the transported glucose to the cytosol: the cytosolic-facing glucose-binding site becomes inactivated and the glucose-binding site facing the cell exterior is regenerated [30]. *Dictyostelium* expressing GLUT 1 and growing in medium containing 1% (w/v) glucose might accumulate glucose in their cytosol; when placed in the transport assay the concentration of glucose in the cytosol will be greater than that in the surrounding medium. This would cause a net movement of glucose from inside the cell outward, generating a severalfold stimulation of the initial rate of inward transport of [^{14}C]DOG, referred to as 'glucose overshoot' and exemplified by the biphasic pattern of glucose uptake observed in Figure 3 (left-hand side). To address this situation, we propagated *Dictyostelium* in glucose-free medium, in which the doubling time was only slightly reduced. In these cells, uptake of [^{14}C]DOG by *Dictyostelium* transformed with GLUT 1-pAD-80 was linear for the first 20 min, plateaued at about 50 min, and was at least 10-fold above that of non-transfected cells (Figure 3, right-hand side).

The experiments in Figure 4 show that GLUT 1 expressed in

Table 1 K_i values, expressed in mM, for sugar transport by GLUT 1 expressed in different cells

	2-DOG	D-Glu	D-Man	D-Gal
Human erythrocytes*	4	7	25	40
<i>Xenopus</i> oocytes†	7.5	5	5	15
<i>D. discoideum</i> ‡	3.7 (2.6–5.2; 8)	2.6 (1.8–4.3; 10)	11 (9–12; 4)	30 (22–35; 5)

* Normal human erythrocytes [31].

† GLUT 1 expressed in *Xenopus* oocytes [35].

‡ Data obtained from experiments similar to those in Figure 4. In parentheses are the range of K_i values obtained in repeats of the experiment and, after the semicolon, the number of experiments.

Dictyostelium exhibits the same selectivity towards hexoses as does GLUT 1 in erythrocytes [31]. In these experiments, increasing concentrations of unlabelled sugars were added to transport assays containing 1 mM [^{14}C]DOG. [^{14}C]DOG was measured after 5 min, at which time uptake is still linear (Figure 3) and a reasonably strong signal can be obtained. When increasing concentrations of DOG were used, a Lineweaver-Burk plot of the transport data from one experiment showed that the apparent K_m for DOG was 1.9 mM (Figure 4C). As judged by the concentrations of D-glucose, D-mannose and D-galactose required to inhibit the initial rate of uptake of [^{14}C]DOG by 50%, the K_d values for these sugars were, in this experiment, 2.6 mM, 11 mM and 30 mM respectively. L-Glucose and L-galactose, which are not transported by GLUT 1, do not inhibit uptake of DOG in *Dictyostelium* expressing GLUT 1. Data from a number of experiments on several lines of transfected *Dictyostelium* cells are tabulated in Table 1. While there is some variation between experiments, the K_d or K_i values for these sugars were similar to the K_m values for GLUT 1 expressed in erythrocytes, and also to those for GLUT 1 expressed in frog oocytes.

DISCUSSION

This study demonstrates that GLUT 1 cDNA from rat brain can be functionally expressed in the cellular slime mould *Dictyostelium discoideum*. To our knowledge, this is the first example of a mammalian membrane protein functionally expressed in *Dictyostelium*. While GLUT 1 in *Dictyostelium* is not glycosylated, it exhibits kinetic parameters and hexose selectivity similar to those of GLUT 1 present normally in human erythrocytes and expressed by recombinant techniques in *Xenopus* oocytes, in which GLUT 1 is heterogeneously glycosylated [18,32]. Studies in which mutations were made in the glycosylation site of GLUT 1 expressed in Chinese hamster ovary (CHO) cells indicated that N-linked glycosylation was important both for proper membrane targeting and protein stability [33]. However, when purified human erythrocyte GLUT 1 was treated with N-glycosidase-F and then reconstituted in phospholipid bilayers, deglycosylated GLUT 1 bound and transported hexoses normally [32,34]. At this moment we have no estimate of the fraction of newly made GLUT 1 that folds properly in the *Dictyostelium* or mammalian endoplasmic reticulum and appears in functional form on the plasma membrane.

GLUT 1 expressed on the plasma membrane of *Dictyostelium* cells exhibits many properties of GLUT 1 found in human erythrocytes [31]. In particular, the phenomenon of glucose overshoot, observed in DOG uptake assays performed on cells

grown in medium containing glucose, but not on cells grown in medium lacking glucose, is a strong indication that the kinetic properties of the transporter are normal. Similarly, Table 1 shows that the apparent affinities of *Dictyostelium*-expressed GLUT 1 for the four D-hexoses tested are similar to those of GLUT 1 expressed in *Xenopus* oocytes, a popular expression system for GLUT 1 [35], as well as those of GLUT 1 in human erythrocyte membranes [31].

Several mammalian and plant membrane proteins have been expressed in yeast cells. In several cases, such as the plant plasma membrane H⁺-ATPase [36], the human AE1 anion exchanger [37], Na⁺,K⁺-ATPase α -subunits, the gastric H⁺,K⁺-ATPase β -subunit [38], and the M1 subtype of the human muscarinic receptor [39], the proteins accumulate in apparently functional form in the endoplasmic reticulum or other internal cell membranes. In particular, the plant ATPase synthesized in yeast is fully active; it hydrolyses ATP, pumps protons, and the reaction cycle involves a phosphorylated intermediate. Unlike the situation in plants, however, most of the plant ATPase is not expressed in the yeast plasma membrane [36]. The human AE1 anion exchanger, expressed in yeast, is not glycosylated, yet is fully functional once purified from internal membranes and reconstituted [37]. In contrast, the human β_2 -adrenergic receptor together with the mammalian G_s α -subunit were functionally expressed on the surface of *Saccharomyces cerevisiae* cells and could partially activate the yeast pheromone response pathway [40]. Similarly, a vertebrate inwardly rectifying K⁺ channel can be functionally expressed on the yeast plasma membrane [41].

Thus, GLUT 1 is one of the few mammalian membrane transport proteins that can be functionally expressed on the plasma membrane of a eukaryotic micro-organism. *Dictyostelium* provides the advantage of a consistently low background of endogenous glucose transport along with the relative ease of establishing and handling stable cell lines. This system will be useful in determining the amino acids in GLUT 1 responsible for binding glucose and transporting it into the cell.

While this paper was being revised, we learned that the human m2 muscarinic receptor could be functionally expressed in *D. discoideum* [42].

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