Functional characterization of an insulin-responsive glucose transporter (GLUT4) from fish adipose tissue

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Capilla, Encarnación, Mónica Díaz, Amaya Albalat, Isabel Navarro, Jeffrey E. Pessin, Konrad Keller, and Josep V. Planas. Functional characterization of an insulin-responsive glucose transporter (GLUT4) from fish adipose tissue. Ann J Physiol Endocrinol Metab 287: E348–E357, 2004. First published April 27, 2004; 10.1152/ajpendo.00538.2003.—Glucose transport across the plasma membrane is mediated by a family of glucose transporter proteins (GLUTs), several of which have been identified in mammalian, avian, and, more recently, in fish species. Here, we report on the cloning of a salmon GLUT from adipose tissue with a high sequence homology to mammalian GLUT4 that has been named okGLUT4. Kinetic analysis of glucose transport following expression in Xenopus oocytes demonstrated a 7.6 ± 1.4 mM $K_m$ for 2-deoxyglucose (2-DG) transport measured under zero-trans conditions and 14.4 ± 1.5 mM by equilibrium exchange of 3-O-methylglucose. Transport of 2-DG by okGLUT4-injected oocytes was stereospecific and was competed by D-glucose, D-mannose, and, to a lesser extent, D-galactose and D-fructose. In addition, 2-DG uptake was inhibited by cytochalasin B and ethylidene glucose. Moreover, insulin stimulated glucose uptake in Xenopus oocytes expressing okGLUT4 and in isolated trout adipocytes, which contain the native form of okGLUT4. Despite differences in protein motifs important for insulin-stimulated translocation of mammalian GLUT4, okGLUT4 was able to translocate to the plasma membrane from intracellular localization sites in response to insulin when expressed in 3T3-L1 adipocytes. These data demonstrate that okGLUT4 is a structural and functional fish homolog of mammalian GLUT4 but with a lower affinity for glucose, which could in part explain the lower ability of fish to clear a glucose load.

Before the identification of putative GLUT proteins in fish, their existence was suggested by studies on the characteristics of glucose transport in different cellular systems. In erythrocytes from different fish species, glucose uptake was shown to be saturable, stereospecific, and inhibitable by cytochalasin B (41, 46, 48, 56). Moreover, Rodnick et al. (39) reported that 2-deoxyglucose (2-DG) uptake in cardiac muscle of American eels was significantly increased by anoxia and contractile activity. In addition, Soengas and Moon (42) demonstrated activation of glucose transport in isolated enterocytes of the black bullhead (Ictalurus melas) by glucagon, glucagon-like peptide, dexamethasone, and isoproterenol. In other studies, a protein with a molecular weight similar to that of GLUT1 was detected in the membranes of erythrocytes of Pacific hagfish (56) and, more recently, in the heart and brain of tilapia with the use of a polyclonal antibody against mammalian GLUT1 (55). Nevertheless, none of these studies was able to attribute the kinetic and biochemical properties of facilitated glucose transport to a specific facilitative GLUT.

Because more than one GLUT isoform may be expressed in a single cell type, a system would be needed in which they could be individually expressed in a functional form. In the past decade, several authors have demonstrated the effectiveness of the Xenopus oocyte system for this purpose, particularly because of the presence of the necessary cellular machinery to express heterologous proteins (i.e., GLUTs) in a correct manner and because of the low levels of endogenous glucose transport activity (5, 13, 14, 18). Thus the biochemical properties of mammalian GLUTs have been well characterized, and it has been demonstrated that they are stereospecific, with differences in substrate specificity, and inhibited by cytochalasin B, the well-known facilitative GLUT inhibitor. Among the different GLUTs identified in fish, only the GLUT1-like transporter (OnmyGLUT1) from rainbow trout has been analyzed functionally to date by expressing it in Xenopus oocytes (43). OnmyGLUT1 shows similar biochemical properties to the mammalian GLUT1 isoforms, indicating that it is indeed a GLUT1 homolog.

Glucose homeostasis in mammals is maintained primarily by the action of insulin. In its target tissues, skeletal muscle and adipose tissue, insulin stimulates glucose uptake mostly through the GLUT4 isoform by promoting its translocation from intracellular storage compartments to the plasma membrane (17). Thomas-Delloye et al. (46) have demonstrated in...
avian skeletal muscle the existence of a protein that immuno-reacts with a mammalian GLUT4 antibody and that changes its intracellular localization in response to insulin. In fish, the hypoglycemic effects of insulin have been well described (24), but the mechanisms by which insulin regulates glucose plasma levels are not completely understood. Our initial studies demonstrated the presence of a structural GLUT4 homolog, named btGLUT, in insulin-sensitive tissues (i.e., skeletal muscle and adipose tissue) of brown trout and suggested that this transporter might be important for the hypoglycemic action of insulin in fish (36). More recently, we (7) have shown that the expression of btGLUT mRNA in trout red muscle is regulated in vivo by the plasma levels of insulin, suggesting that btGLUT may be not only a structural homolog of GLUT4 but a functional homolog as well. However, there is no information to date regarding the functional characterization of the fish GLUT4 homolog, including the possibility that it translocates to the plasma membrane in response to insulin, similarly to what is known in mammals.

In the present study, we report on the cloning and functional characterization of a fish GLUT from adipose tissue (okGLUT4) with high sequence homology to mammalian GLUT4. Using the X. laevis oocyte system to express heterologous proteins, we have investigated the kinetic and biochemical characteristics of okGLUT4. We demonstrate that it is a functional GLUT with an affinity for glucose more similar to mammalian GLUT4 than to GLUT1 and that it is stereospecific and inhibitable by cytochalasin B. Furthermore, we show that, in okGLUT4-expressing Xenopus oocytes, glucose transport can be stimulated by insulin similarly to that mediated by rat GLUT4 in the same system. In addition, we show that insulin stimulates glucose transport in trout adipocytes and, more importantly, that insulin stimulates the translocation to the plasma membrane of an okGLUT4-enhanced green fluorescent protein (eGFP) fusion protein in 3T3-L1 adipocytes. Our results indicate that okGLUT4 is a functional fish homolog of mammalian GLUT4.

**MATERIALS AND METHODS**

**Materials.** The radiolabeled glucose analogs 2-deoxy-[6-3H]glucose (2-[6-3H]DG; specific activity 26.2 Ci/mmole) and 3-O-methyl-[6-3H]glucose (3-[6-3H]OMG; specific activity 2.5 Ci/mmole) were purchased from Amersham Buchler (Braunschweig, Germany). Porcine insulin was obtained from Lilly (Indianapolis, IN). Restriction endonuclease from New England Biolabs (Beverly, MA). Dulbecco’s modified Eagle’s medium (DMEM) and supplemented solutions for 3T3-L1 cell culture were from Gibco (Invitrogen, Carlsbad, CA), and calf and fetal bovine serum were from HyClone (Logan, UT). Unless otherwise indicated, all other reagents used were purchased from Sigma (Deisenhofen, Germany).

A peptide antibody against the salmon adipose GLUT was custom made (Sigma-Genosys, Cambridge, UK) by immunizing rabbits with the peptide HSTELDYGEGSLL corresponding to the last 15 amino acids of the carboxyl terminus of okGLUT4. The peptide was conjugated to keyhole limpet hemocyanin through the amino terminus by adding a cysteine residue. The final bleed, obtained 1 wk after the last of six immunizations, was used in the experiments, as well as the preimmunization bleed, used as control. The rat GLUT4 antibody was a kind gift from Dr. Antonio Zorzano (University of Barcelona) and has been previously well characterized (27).

**Cloning of a salmon adipose glucose transporter.** A salmon (O. kisutch) adipose tissue cDNA library (Lambda ZAP II, Stratagene, La Jolla, CA; kindly donated by Drs. Dianne Baker and Penny Swanson) was screened at high stringency using a 1.2-kb cDNA fragment (nucleotides 1–1196; Genbank acc. no. AF247395) of btGLUT as a probe (36). After three rounds of screening by plate hybridization, eight positive clones were purified to homogeneity and in vivo excised, and the resulting plasmids were digested with EcoRI to determine the insert size. Six of the clones were ~3.3 kb in length, whereas the other two clones were <3 kb. From the sequencing data, all eight clones were found to be identical and represented the same cDNA, except for the two shorter ones, which were truncated in the 5’ region. Two of the six full-length clones were sequenced in their entirety with the BigDye terminator cycle sequencing kit (PE Biosystems) and an ABI Prism 360 sequencer. Sequence compilations, comparisons, and features were obtained using the Wisconsin package version 9.0 (Genetics Computer Group).

**Isolation of Xenopus oocytes.** Adult X. laevis females were obtained from the African Xenopus Facility (Knysna, Republic of South Africa). The frogs were anesthetized by immersion in 0.1% ethylene glycol monophenyl ether for 20–30 min. Lobes of ovaries were surgically removed and rinsed with modified Barth’s solution [MBS; in mM: 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.82 MgSO4, 0.41 CaCl2, 0.33 Ca(NO3)2, 10 HEPES (pH 7.5)] and supplemented with 2.5 mM sodium pyruvate, 0.1% bovine serum albumin (BSA), and 100 μg/ml gentamicin (Bio Whittaker)]. Stage V and VI oocytes (11) were manually dissected, and follicle cells were removed by incubation in Ca2+-free MBS containing 2 mg/ml collagenase (type I) for 30 min at room temperature. Oocytes were then washed six times with phosphate-buffered saline (PBS) and maintained in MBS at 18°C for all subsequent procedures.

**cRNA preparation and microinjection into Xenopus oocytes.** A 1.7-kb BamHI fragment of okGLUT4, comprising the entire open reading frame, was subcloned into the BglII site of the SP64T expression vector, which is flanked by fragments of the 5’- and 3’-untranslated regions of the Xenopus β-globin mRNA (20). Then, the XbaI-okGLUT4 and the SalI-rat GLUT4 (from a pSPgT) linearized plasmids were transcribed using the SP6 RNA polymerase in the presence of cap analog (mMESSAGE mMACHINE; Ambion, Austin, TX). The amount of transcribed RNA was calculated by counting the radioactivity of incorporated thio-[35S]UTP and also by measuring the 260-nm absorbance. After an overnight incubation period, healthy oocytes were selected for injection with 25 ng of okGLUT4- or rat GLUT4-capped RNA or with the same volume (50 nl) of diethyl pyrocarbonate-treated water. Oocytes were maintained at 18°C in MBS supplemented with BSA and gentamicin with the medium changed every 2 days. Three days after injection, healthy oocytes were selected for glucose transport experiments.

**Culture and transient transfection of 3T3-L1 cells.** Murine 3T3-L1 preadipocytes were purchased from the American Type Tissue Culture repository. Cells were cultured at 37°C and 8% CO2 in DMEM containing 25 mM glucose, 10% bovine calf serum, and 1% penicillin-streptomycin. At confluence, cells were induced to differentiate into adipocytes in DMEM containing 25 mM glucose, 10% FBS, 1 μg/ml insulin, 0.25 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. After 4 days, the medium was changed to DMEM, 25 mM glucose, 10% FBS, and 1 μg/ml insulin and maintained for 4 additional days. Fully differentiated 3T3-L1 adipocytes (between 8 and 11 days after initiation of differentiation) were transiently transfected by electroporation as described previously (23). Briefly, the adipocytes were put into suspension by mild trypsinization and electroporated with 50 μg of either the rat or the salmon (described below) egFP-tagged plasmids under low-voltage conditions (160, 1950 μF). After electroporation, the cells were seeded on collagen-coated glass coverslips that were placed in six-well plates and allowed to recover overnight in complete medium before use.

The pcDNA3-okGLUT4-eGFP construct was prepared by subcloning the full-length salmon GLUT4 cDNA in frame into the EcoRI-
Not sites of the pcDNA3 vector (Invitrogen) to generate a carboxy-terminal eGFP fusion protein.

**Adipocyte isolation.** Rainbow trout (O. mykiss) adipocytes were prepared by the method of Rodbell (38) with some minor modifications. Fish (250 g) were anesthetized in 3-aminobenzoic acid ethyl ester (0.1 g/L Sigma, St. Louis, MO) dissolved in fresh water and were killed by a blow on the head, and the mesenteric adipose tissue was removed. Fat tissue was cut into small pieces and incubated in polypropylene tubes with isosmotic Krebs buffer (pH 7.4, 280 mosM) containing collagenase type II (0.3 mg/mL) and 1% BSA without glucose for 60 min in a water bath under gentle shaking at 15°C. The cell suspension was filtered through a double layer of nylon cloth and then washed three times by flotation. Finally, cells were carefully resuspended in Krebs buffer containing 2% BSA.

**Glucose transport measurements.** The kinetics for glucose transport in okGLUT4-expressing Xenopus oocytes were determined under equilibrium exchange and zero-trans conditions. For the equilibrium exchange experiments, groups of 100 oocytes were incubated for 18 h in MBS containing 3-OMG at various concentrations (4, 10, 30, 50, and 100 mM). For the assay, groups of 10 oocytes were incubated in plastic vials with 0.5 ml of MBS containing the appropriate sugar concentration. To start the reaction, 10 μL of MBS containing 3 μCi of 3-[^3H]OMG were added and mixed. After the incubation period (2, 5, 10, or 30 min) the reaction was stopped by rapid withdrawal of the oocytes four times with 3 ml of ice-cold PBS containing 0.1 mM phloretin (Carl Roth, Karlsruhe, Germany), a potent transport inhibitor (21). Oocytes were individually transferred into scintillation vials and dissolved with 0.5 ml of 1% SDS for 1 h at 50°C and counted before the addition of scintillation liquid. The zero-trans kinetics were carried out in groups of 10 oocytes incubated in 0.5 ml of MBS with different 2-DG/2-[^3H]DG concentrations (0.4, 0.5, 0.7, 1, 2, 5, 7.5, 10, 15, 20, and 25 mM), with 1–3 μCi per assay. After the required time (2, 5, 10, or 30 min), the oocytes were washed and the counts per oocyte determined as described for the equilibrium exchange conditions.

Substrate specificity in okGLUT4-expressing Xenopus oocytes was determined by incubating groups of 10 oocytes in MBS with different hexoses (α- and β-glucose, α- and β-galactose, and α- and β-mannose) at a concentration of 50 mM and β-fructose at 25, 50, and 100 mM) for 30 s before the addition of 2-[^3H]DG (50 μM final concentration and 1 μCi per assay). The reaction was stopped after 30 min, and the radioactivity on each individual oocyte was determined as described above. In addition, the effect of cytochalasin B and ethylidene glucose on 2-DG transport in okGLUT4-expressing Xenopus oocytes was determined as described for the hexose competition, with the only difference being that the preincubation time with the inhibitors was 15 min. Finally, the effect of insulin on 2-DG uptake was determined by incubating groups of 10–15 oocytes expressing either okGLUT4, or rat GLUT4 for comparison, for 4 h in the presence or absence of insulin (1 μM) in MBS before the 2-[^3H]DG uptake was begun.

In mature trout adipocytes, glucose transport was determined by a modification of the method by Olefsky (31). Trout adipocytes (2.5 × 10^5 cells/mL) were incubated in Krebs buffer in the presence or absence of insulin (100 nM) for 30 min at 15°C in a shaking water bath. Subsequently, 2-[^3H]DG (0.8 μCi) was added, and transport was stopped after 2 h with cytochalasin B. The transport assay was terminated by transferring a 200-μl aliquot of the cell suspension into small polyethylene microcentrifuge tubes containing 150 μl of di-nonyl phthalate. Cells and buffer were separated by centrifugation at 16,000 g for 2 min. The upper phase, which contains the adipocytes, was collected and subjected to liquid scintillation counting. Nonspecific uptake was measured in cells pretreated with cytochalasin B, and all uptake data were subtracted with these values to correct for extracellularly trapped isotope and cell-associated radioactivity.

**Membrane preparation and immunoblot analysis.** For total membrane preparations from Xenopus oocytes, groups of 30 oocytes collected 3 days after the microinjection were washed with homogenization buffer (10 mM HEPES (pH 7.9), 83 mM NaCl, 1 mM MgCl_2_, 1 mM EDTA, 0.5 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL pepstatin), kept on ice, and homogenized with a Teflon-glass homogenizer for 1 min at maximum speed. The homogenates were centrifuged twice at 1,000 g for 10 min at 4°C to pellet all yolk protein, and the supernatant was further centrifuged at 165,000 g for 1 h at 4°C. Pellets were then resuspended in the same buffer. Total membranes from trout adipose tissue were obtained by homogenizing the tissue in a buffer containing 25 mM HEPES, 4 mM EDTA, 250 mM sucrose, 0.2 mM PMSF, 25 mM benzamidine, 1 U/mL aprotinin, 1 μM leupeptin, and 1 μM pepstatin and centrifuging the homogenate at 5,000 g for 5 min at 4°C. Subsequently, the supernatant was centrifuged at 200,000 g for 1.5 h at 4°C, and the resulting pellet was resuspended in homogenization buffer. Protein concentration was determined by the Bradford method (3).

Total membrane samples were subjected to SDS-PAGE in a 10% running gel and the proteins then transferred to an Immobilon membrane (Millipore). Immunoblots were performed using the okGLUT4 antibody or the rat GLUT4 antibody at 1:500 and 1:5,000 dilutions, respectively, in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk for 2 h at room temperature. The secondary anti-rabbit antibody (Transduction Laboratories) was used at a 1:5,000 dilution in the same buffer, and the detection of the luminescence was done using an enhanced chemiluminescence kit (Amersham). Specificity of the okGLUT4 antibody was tested by using preimmune serum at a 1:500 dilution and by blocking overnight the anti-okGLUT4 antibody with an excess (50- to 200-fold) of the immunization peptide.

**Immunofluorescence microscopy.** Healthy Xenopus oocytes injected with water or with okGLUT4 or rat GLUT4 cRNA were selected 3 days after injection for scanning microscopy analyses. The oocytes were rinsed in MBS without BSA and then fixed overnight in MBS containing 4% paraformaldehyde at 4°C. The fixed oocytes were then washed three times with PBS and kept in MBS at 4°C until the day of assay. The oocytes were cut into two halves along the equator with a scalpel, and the protocol for immunodetection was followed as described previously by Olsowski et al. (32). The okGLUT4 and the rat GLUT4 primary antibodies (the same as for Western blot analyses) were used at 50 and 20 μg/mL, respectively. The secondary FITC-conjugated goat anti-rabbit IgG (Sigma, Deisenhofen, Germany) was used at a 1:500 dilution. Fluorescence images were obtained with a confocal laser scanning microscope (LSM 510; Carl Zeiss Jena, Jena, Germany) by scanning the fluorescence from the cut surface of each oocyte through a depth of 10 μm and under the same conditions of instrumental amplification to allow comparisons among different samples.

Differentiated 3T3-L1 adipocytes expressing okGLUT4-eGFP or rat GLUT4-eGFP were serum starved in DMEM for 2 h before the incubation with or without insulin (100 nM) for 30 min. Cells were then fixed with 4% paraformaldehyde for 15 min at room temperature and blocked with a solution containing 1% BSA and 5% donkey serum for another 15 min at room temperature. The coverslips were then washed three times with PBS and mounted on Vectashield medium (Vector Laboratories). The fluorescence was analyzed the next day by confocal immunofluorescence microscopy using a Nikon Eclipse E600 microscope.

**Calculations and statistical analyses.** For all glucose transport measurements, background values obtained by averaging the uptake into water-injected oocytes were subtracted from the uptake observed in eRNA-injected oocytes. K_m values are presented as means ± SE of calculations from three independent experiments. Linearizations and K_m value determinations were performed using the Prism 3.0 package. All the data for 2-DG uptake are presented as means ± SE (10–20 oocytes/group from 1–5 independent experiments). Data for 2-DG uptake in trout adipocytes are presented as means ± SE of five independent experiments, each performed on triplicate. Insulin-stimulated translocation in 3T3-L1 adipocytes is expressed as a percentage...
(means ± SE) of cells showing a plasma membrane ring, obtained by counting 50 cells per situation in six independent experiments. Statistical analyses were assessed by a one-way analysis of variance, followed by the Fisher protected least significant different test or by the unpaired Student’s t-test. Differences were considered statistically significant when *P* < 0.05.

**RESULTS**

Cloning of salmon adipose GLUT. Using a btGLUT cDNA fragment as a probe, several identical clones resulted from the screening of a salmon adipose tissue cDNA library. The complete sequence obtained was 3137 nucleotides long and contained a 237-nucleotide 5′-untranslated region, a 1518-nucleotide open reading frame, and a 1382-nucleotide 3′-untranslated region, which included the poly(A) tail (GenBank acc. no. AF502957). The deduced amino acid sequence encoded for a protein of 505 amino acids, which is 95% identical to btGLUT (36) and 72 and 75% homologous to OnmyGLUT1 and CyGLUT1, respectively, the two known isoforms of GLUT1 in fish (44, 45). In relation to mammalian sequences, the salmon adipose GLUT is 79–79.5% similar to GLUT4 isoforms and 72–73% similar to GLUT1 isoforms. Therefore, the salmon adipose tissue glucose transporter was named okGLUT4 and appears to be homologous to mammalian GLUT4 and fish muscle btGLUT. An alignment of the amino acid sequences of okGLUT4, btGLUT, and rat GLUT4 is presented in Fig. 1. The Kyte and Doolite analysis of the protein sequence revealed the expected conformation of 12 hydrophobic transmembrane domains with four significant hydrophilic regions, including the amino and carboxyl termini (29, 34). Furthermore, okGLUT4 showed the predicted glycosylation site (Asn50), as well as other motifs characteristic of a functional GLUT. In addition, okGLUT4 presents similar motifs to those found only in mammalian GLUT4 isoforms, such as the Phe-Gln-Gln on its amino terminus and the Tyr502, and the acidic cluster (Thr498-Glu-Leu-Glu-Tyr-Leu-Gly-Pro505) on the carboxyl terminus, which are important for the intracellular trafficking of the transporter. However, okGLUT4 lacks the Leu499-Leu500 sequence, which is characteristic of mammalian GLUT4 isoforms.

**Kinetic characterization of okGLUT4 in Xenopus oocytes.** As reported previously, the *Xenopus* oocyte is an efficient system for studying the heterologous expression of facilitative GLUTs (5, 13, 14, 18); thus we chose this model to study the functional properties of okGLUT4. In preliminary experiments, we demonstrated the functionality of the oocyte system by measuring the ability of okGLUT4-expressing oocytes to take up glucose (data not shown). To assess the kinetic characteristics of okGLUT4, glucose analog uptake was measured under both equilibrium exchange and zero-trans conditions. The *Km* for 3-OMG (a nonmetabolizable glucose analog) was determined under equilibrium exchange conditions at sugar concentrations from 4 to 100 mM. Figure 2, A and B, shows the results from a representative experiment. With the assumption that the process follows first-order kinetics, as expected for equilibrium exchange (12), transport data from the accumulation curves were linearized by logarithmic transformation [using the equation ln(1 − R/Req), where R is the radioactivity per oocyte at time *t* and Req is the radioactivity per oocyte after full equilibration of the oocyte water space] and plotted against time for each 3-OMG concentration (Fig. 2A). In Fig. 2B, the negative reciprocals of the slopes shown in Fig. 2A were then replotted with respect to substrate concentration, and the value for the half-saturation constant (Km) was obtained from the negative value of the x intercept in the linear regression analysis. The Km value corresponded to 14.44 ± 1.54 mM obtained in 3 independent determinations. To further evaluate the kinetic characteristics of the fish glucose transporter, we also calculated the Km value for 2-DG under zero-trans conditions. In Fig. 2C, the Lineweaver-Burk plot from a representative experiment is presented. The inset shows the data obtained by Lineweaver-Burk linearization in three independent experiments. The *Km* values ranged from 5.03 to 9.68 mM, yielding an average value of 7.63 ± 1.37 mM.

**Biochemical properties of okGLUT4 in Xenopus oocytes.** In *Xenopus* oocytes expressing okGLUT4, the basal 2-DG uptake (control, at 30 min with 50 μM 2-DG final concentration) varied among the various experiments depending on the batch of oocytes and was in the range of 1.09 to 3.78 pmol oocyte−1·min−1. We then tested the effects of different sugars on 2-DG transport by oocytes expressing okGLUT4. The data illustrated in Fig. 3 show the effects of 50 mM D- and L-glucose, D- and L-galactose, D- and L-mannose and three different concentrations of D-fructose (100, 50, and 25 mM) on 2-DG transport. D-Glucose, D-mannose, and, to a lesser extent, D-galactose inhibited (*P* < 0.05) the rate of 2-DG uptake by okGLUT4-expressing oocytes. D-Fructose also showed competition with 2-DG in a concentration-dependent manner, whereas none of the L-isomers had any effect on 2-DG transport, thus demonstrating the stereospecificity of the carrier system.

The increased 2-DG uptake observed in okGLUT4-injected oocytes was clearly inhibited in a concentration-dependent manner by two well-known glucose transporter inhibitors: ethylidene glucose (Fig. 4A), an extracellular inhibitor, and cytochalasin B (Fig. 4B), an intracellular inhibitor specific to facilitative GLUTs.

**Effects of insulin on glucose uptake.** We have investigated the effect of insulin (1 μM) on 2-DG uptake in okGLUT4-expressing *Xenopus* oocytes and also, for comparison, in rat GLUT4-expressing oocytes (Fig. 5A). After an incubation period of 4 h, insulin caused a significant increase in 2-DG uptake in okGLUT4 (*P* < 0.0001)- and rat GLUT4 (*P* < 0.05) -expressing oocytes. To determine whether insulin can stimulate glucose uptake in isolated mature trout adipocytes, we incubated the adipocytes in the presence or absence of insulin (100 nM) for 30 min and measured the amount of 2-DG taken up. As shown in Fig. 6A, insulin caused a significant increase (*P* < 0.0001) of the uptake of 2-DG in mature trout adipocytes. Furthermore, preincubation with the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (15 min; 1 μM) completely blocked the stimulatory effects of insulin on glucose uptake in mature trout adipocytes (control: 32.54 ± 0.74; insulin: 40.57 ± 1; insulin + wortmannin: 32.48 ± 2.27 pmol glucose/105 cells; *P* < 0.05).

**Immunodetection of okGLUT4.** Immunoblot analysis of total membranes from *Xenopus* oocytes injected with water or either okGLUT4 or rat GLUT4 cRNA is shown in Fig. 5B. With the use of an anti-okGLUT4 polyclonal antibody, a band of ~50 kDa was detected in okGLUT4-expressing oocytes but not in water-injected oocytes (Fig. 5B). Furthermore, the okGLUT4
A polyclonal antibody revealed the presence of the native ok-GLUT4 protein in trout adipose tissue (Fig. 6B), which appeared to be of the same size as that detected in okGLUT4-expressing oocytes. The size of okGLUT4 was also very similar to that of rat GLUT4 expressed in *Xenopus* oocytes, as detected with a rat GLUT4-specific antibody (Fig. 5B). Interestingly, the okGLUT4 antibody did not immunoreact with rat GLUT4, and the rat GLUT4 antibody did not immunoreact with okGLUT4, indicating that these two antibodies are species specific. Specificity of the 50-kDa band was confirmed by the use of preimmune serum and by the ability of an excess of immunizing peptide to block okGLUT4 immunoreactivity (data not shown).

As a first approach to investigating whether insulin might promote the translocation of okGLUT4 to the plasma membrane from intracellular storage compartments, we analyzed the immunolocalization of okGLUT4, in parallel with rat GLUT4, by confocal laser scanning microscopy in *Xenopus* oocytes that had been previously incubated in the absence or presence of insulin. As shown in Fig. 5C, okGLUT4 and rat GLUT4 were correctly expressed in *Xenopus* oocytes, mostly at the plasma membrane, whereas no immunofluorescence was detected in oocytes injected with water. No differences could be observed between basal and insulin-stimulated oocytes, either in okGLUT4- or in rat GLUT4-expressing oocytes (data not shown).

Fig. 1. Amino acid alignment of salmon GLUT (okGLUT4) with brown trout GLUT (btGLUT4, GenBank acc. no. AF247395) and rat GLUT4 (SwissProt acc. no. P19357). Amino acids are represented by single-letter code, and motifs important for trafficking in mammalian GLUT4 are shown in boldface.

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<th>rat GLUT4</th>
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As a second approach to investigate whether okGLUT4 can translocate to the plasma membrane in response to insulin, we expressed an okGLUT4-eGFP fusion protein, and a rat GLUT4-eGFP for comparison, in 3T3-L1 adipocytes. Representative images of basal and insulin-stimulated 3T3-L1 adipocytes transfected with okGLUT4-eGFP or rat GLUT4-eGFP cDNAs are shown in Fig. 7A. The percent translocation of okGLUT4 and rat GLUT4 obtained by counting positive cells on each condition (considering positive those cells showing a plasma membrane ring) is presented in Fig. 7B. In basal conditions, fewer than 10% of cells were positive for the rat GLUT4 and fewer than 15% for okGLUT4, whereas a high percentage of cells with translocation (78%–84%) was found for both species after insulin stimulation. These results clearly indicate that insulin stimulates the translocation of okGLUT4 to the plasma membrane.

DISCUSSION

To date, different facilitative GLUTs have been cloned in fish (19, 33, 36, 44, 45), which are related in sequence to three different mammalian GLUTs (GLUT1, GLUT2, and GLUT4). However, very little information is available regarding the functional properties of these GLUTs in fish. In this study, we report on the functional properties of a facilitative GLUT cloned from a salmon adipose tissue cDNA library, which we have named okGLUT4 due to its high homology with mammalian GLUT4 isoforms. The fact that the amino acid sequence of okGLUT4 is 95% identical to that of btGLUT4, the GLUT4 homolog that we previously characterized from brown trout muscle (7, 36), indicates that okGLUT4 and btGLUT4 correspond most likely to the same isoform of transporter in adipose tissue and muscle, respectively, from two phylogenetically related fish species. In addition to being expressed in adipose tissue and muscle, the fish GLUT4 homolog is known to be expressed in other tissues, including kidney, gill, and heart (36).
In the present study, we have investigated the function of okGLUT4 by expressing it in Xenopus oocytes and have shown that okGLUT4 has transport properties typical of a functional GLUT. The affinity constant \( (K_m) \) for okGLUT4 was in the range of 5.03 to 9.68 mM (average 7.6/11006 1.4 mM). Interestingly, the \( K_m \) values of 2-DG for okGLUT4 are lower than those reported for rainbow trout GLUT1 (OnmyGLUT1) expressed in Xenopus oocytes [8.3 to 14.9 mM; (43)] and for glucose uptake in American eel erythrocytes [10.4/11006 0.6 mM; (41)], which probably, as in mammals, express exclusively GLUT1. Therefore, our data indicate that okGLUT4 has a higher affinity for glucose than does OnmyGLUT1, which suggests that okGLUT4 and OnmyGLUT1, the fish GLUT4 and GLUT1 homologs, are functionally different. Of particular interest is the observation that the \( K_m \) values of okGLUT4 and OnmyGLUT1 are higher than those of their mammalian counterparts [4.6 and 6.9 mM for human GLUT4 and GLUT1, respectively; (5)]. In addition, the relative differences in affinity for glucose between okGLUT4 and OnmyGLUT1, when calculated by the zero-trans method, are similar to those between mammalian GLUT4 and GLUT1. The above-mentioned differences in affinity between okGLUT4 and mammalian GLUT isoforms were confirmed by equilibrium exchange experiments. In fact, the \( K_m \) value (14.4 ± 1.5 mM) of okGLUT4 for 3-OMG uptake is higher than the two independently reported \( K_m \) values for rat GLUT4: 1.8 (18) and 4.3 mM (30), but significantly lower than those for human and rat GLUT1: 21.3 (18) and 26.2 mM (30), respectively. From these results, we can conclude that the kinetic characteristics of okGLUT4 are more similar to GLUT4 than to GLUT1 iso-

![Fig. 4. Inhibition of 2-DG uptake in okGLUT4-injected Xenopus oocytes by ethylidene glucose (A) and cytochalasin B (B). Measurements were determined as described in MATERIALS AND METHODS. Results are expressed as % uptake over basal value (control, 100%) obtained in oocytes incubated without inhibitor. Values obtained in water-injected oocytes were subtracted as background. Values are means ± SE of 20–40 oocytes. Different letters indicate significant differences among groups at \( P < 0.05 \).

![Fig. 5. Effects of insulin in okGLUT4- and rat GLUT4-expressing Xenopus oocytes. A: effects of insulin (1 \( \mu \)M) on 2-DG uptake in okGLUT4- and rat GLUT4-expressing Xenopus oocytes. Results are expressed as % uptake over basal value (control, 100%) obtained in oocytes incubated without hormone. Values obtained in water-injected oocytes were subtracted as background. Values are means ± SE of 2–5 experiments (10–30 oocytes per experiment). *Significant differences vs. control, \( P < 0.0001 \) for okGLUT4, \( P < 0.05 \) for rat GLUT4. B: immunodetection of okGLUT4 and rat GLUT4 in water-injected okGLUT4- and rat GLUT4-expressing oocytes with an anti-okGLUT4 polyclonal antibody (left) and an anti-rat GLUT4 polyclonal antibody (right). Total membranes were prepared from oocytes injected with water, okGLUT4, or rat GLUT4 cRNA. Fifty micrograms of each sample were immunoblotted using specific antibodies for okGLUT4 (left) or rat GLUT4 (right) at 1:500 and 1:5,000 dilutions, respectively. C: effects of insulin (1 \( \mu \)M) on immunolocalization of okGLUT4 and rat GLUT4 in Xenopus oocytes. okGLUT4- and rat GLUT4-expressing oocytes were incubated in the absence or presence of insulin (1 \( \mu \)M) for 4 h, and 5–7 oocytes per group were fixed, cut, and analyzed for microscopy as described in MATERIALS AND METHODS. Representative confocal laser scanning microscopy images of 3 independent experiments from oocytes of the different groups are shown.](http://ajpendo.physiology.org/10.1152/ajpendo.00174.2004)
forms. Furthermore, our results support the notion that fish GLUTs have a lower affinity for glucose than mammalian GLUTs. We postulate that the lower affinity of fish GLUTs underlies the well-known decreased ability of fish to clear a glucose load, which has led to the belief that fish, compared with mammals, are relatively glucose intolerant (8, 25).

As reported previously for mammalian facilitative GLUTs (5, 14), okGLUT4 shows the expected D- and L-stereoselectivity with respect to sugar transport. Like mammalian GLUT1, GLUT2, and GLUT3, okGLUT4 transports primarily D-glucose and D-mannose, as well as D-galactose albeit with lower affinity. In addition, okGLUT4 is able to transport D-fructose but only at high concentrations, a capacity shared only by the mammalian GLUT2 isofrom. Unfortunately, no comparison with regard to substrate specificity can be made between okGLUT4 and mammalian GLUT4 because of the low levels of expression of the latter in the experiments in which it was expressed in Xenopus oocytes (5, 14).

In okGLUT4-expressing oocytes, cytochalasin B and ethylidene glucose, the known intracellular and extracellular inhibitors of glucose transport, respectively, blocked 2-DG uptake in a concentration-dependent manner. Cytochalasin B is an inhibitor known to bind near the 11th transmembrane domain of each GLUT isofrom with different binding constants (5, 6, 40) and to inhibit glucose transport in human erythrocytes by competing with glucose for the carrier at the inner surface of the cell membrane (1, 2, 9). In fish erythrocytes, cytochalasin B has also been shown to inhibit glucose transport (41, 47, 48, 56), and its use in photoaffinity labeling experiments followed by SDS-PAGE allowed the identification of a protein similar in mass to GLUT1 in the Pacific hagfish (56). On the other hand, few studies have reported glucose uptake inhibition in mammals by the exofacial inhibitor ethylidene glucose (28, 53), and the present study constitutes the first report on the effects of this inhibitor in nonmammalian vertebrates. Overall, these results confirm once more the similar biochemical properties of okGLUT4 to the well-characterized mammalian GLUTs.

An important question regarding the functional characterization of okGLUT4 as a putative GLUT4 homolog is whether its ability to transport glucose can be modulated by insulin. In mammals, insulin is known to stimulate glucose uptake in muscle and adipose tissue, its two major target tissues, by increasing the number of GLUT molecules (mostly GLUT4) at the plasma membrane (17). In addition, insulin has been shown to stimulate basal glucose uptake by intact Xenopus oocytes and by oocytes that express mammalian GLUT4 (16, 26). In our study, we have seen that insulin stimulated glucose uptake by Xenopus oocytes expressing okGLUT4 similarly to those expressing the rat GLUT4 isofrom, which suggests that okGLUT4 is an insulin-responsive GLUT in fish. However, insulin has also been reported in one study to stimulate glucose uptake by Xenopus oocytes expressing mammalian GLUT1 and GLUT2 isoforms (49), whereas another study reported no effects of insulin on glucose uptake in GLUT1-expressing Xenopus oocytes (26). This controversy has been attributed to

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**Fig. 6.** Effects of insulin on glucose uptake in trout adipose tissue. A: effects of insulin on 2-DG uptake by isolated trout adipocytes were assessed by incubating them in the absence or presence of insulin (100 nM) for 30 min. Results are expressed as % uptake over basal value (control, 100%) obtained in trout adipocytes incubated without hormone. Values are means ± SE of 5 independent experiments, each performed in triplicate. *Significant difference between the control and insulin groups, P < 0.0001. B: immunodetection of native okGLUT4 in trout adipose tissue. Total membranes (50 μg) were immunoblotted using a specific antibody for okGLUT4 at a 1:500 dilution.

**Fig. 7.** Effects of insulin on okGLUT4-enhanced green fluorescent protein (eGFP) and rat GLUT4-eGFP fusion protein localization in 3T3-L1 adipocytes. Cells were electroporated with okGLUT4-eGFP or rat GLUT4-eGFP, incubated in the absence or presence of insulin (100 nM, 30 min), and immunofluorescence was detected as described in MATERIAL AND METHODS. A: representative images of 6 independent experiments are shown. B: quantification of positive cells showing translocation (those with a plasma membrane ring). Values are means ± SE of 6 independent experiments. *Significant differences between control and insulin-treated groups, P < 0.0001.
the possibility that the responsiveness to insulin of a given GLUT depends on the cell type in which the protein is expressed (49). To further investigate the nature of the stimulatory effect of insulin, we have also examined its effects on the cellular localization of okGLUT4 by confocal immunofluorescence in okGLUT4-expressing Xenopus oocytes. Despite the fact that okGLUT4 was correctly expressed in Xenopus oocytes and that a protein of ~50 kDa could be immunodetected, no changes in the localization of okGLUT4 or rat GLUT4 (used as a control) were detected as a result of insulin treatment. The lack of correlation between the effects of insulin on glucose uptake and on the localization of okGLUT4 in Xenopus oocytes is evidence for the fact that the Xenopus oocyte is not an appropriate system to study the regulation of GLUT4 translocation.

Therefore, we investigated the effects of insulin on the cellular localization of okGLUT4 by expressing an okGLUT4-eGFP fusion protein in 3T3-L1 adipocytes. The results obtained in the present study clearly demonstrate for the first time that the fish GLUT4 translocates to the plasma membrane in response to insulin similarly to rat GLUT4, suggesting that okGLUT4 is a functional homolog of mammalian GLUT4. A comparison of the amino acid sequences of both transporters reveals the presence in okGLUT4 of protein motifs similar, albeit not identical, to NH₂- and COOH-terminal motifs (e.g., Phe⁵-Gln⁶-Gln⁷, Thr⁴⁹⁶-Glu-Leu-Glu-Tyr-Leu-Gly-Pro⁵⁰⁵) shown to be important for the subcellular localization of GLUT4 in mammals (52). Interestingly, okGLUT4 lacks the Leu⁴⁹⁹-Leu⁵⁰⁰ motif, which is suggested to be important for endocytosis of mammalian GLUT4. Consequently, okGLUT4 is an insulin-responsive GLUT4 protein with specific amino acid substitutions in important protein motifs and may prove to be a useful tool with which to study the amino acids that are critical for the intracellular trafficking of GLUT4.

At the present time, we do not know the functional role of okGLUT4 in mediating hormone-regulated glucose uptake in fish adipose tissue. However, our results on the stimulation of glucose uptake by insulin in mature trout adipocytes, in accordance with the reported presence of specific insulin receptors (37), and on the immunodetection of okGLUT4 in fish adipose tissue would suggest that insulin could stimulate glucose uptake by increasing the number of okGLUT4 molecules at the plasma membrane. The observation that the effects of insulin on glucose uptake in trout adipocytes are completely blocked as a result of the inhibition of PI3K, a key molecule involved in the insulin-regulated translocation of GLUT4 (4), further supports the idea that insulin may cause the translocation of okGLUT4 to the plasma membrane through the activation of PI3K and, consequently, stimulate glucose uptake. Current efforts in our laboratory are underway to examine whether insulin stimulates the translocation of the okGLUT4 protein to the plasma membrane in fish adipocytes.

In conclusion, our results indicate that okGLUT4 is a structural and functional fish homolog of mammalian GLUT4. Interestingly, our results also indicate that okGLUT4 is a fish insulin-responsive GLUT that differs from mammalian GLUT4 in its lower affinity for glucose and in carboxyl termini protein motifs that are known to be important for GLUT4 trafficking.

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REFERENCES


21. Krupka RM. Inhibition of sugar transport in erythrocytes by fluorodini-

22. McVie-Wylie AJ, Lamson DR, and Chen YT. Molecular cloning of a novel member of the GLUT family of transporters, SLC2a10 (GLUT10), localized on chromosome 20q13.1: a candidate gene for NIDDM suscepti-


31. Olefsky JM. Effect of dexamethasone on insulin binding, glucose trans-


46. Tiithonen K, Ninikmaa M, and Lappiavaara J. Glucose transport in carp erythrocytes: individual variation and effects of osmotic swelling, extra-


51. Watson RT and Pessin JE. Subcellular compartmentalization and traf-


