Developmental expression and cellular localization of glucose transporter molecules during mouse preimplantation development

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Summary

Two general mechanisms mediate glucose transport, one is a sodium-coupled glucose transporter found in the apical border of intestinal and kidney epithelia, while the other is a sodium-independent transport system. Of the latter, several facilitated transporters have been identified, including GLUT1 (erythrocyte/brain), GLUT2 (liver) and GLUT4 (adipose/muscle) isoforms. In this study, we used Western-blot analysis and high resolution immunoelectron microscopy (IEM) to investigate the stage-related expression and cellular localization of GLUT1, 2 and 4. The Western blot results demonstrate that GLUT1 is detectable in the oocyte and throughout preimplantation development. GLUT2 isoforms were not detectable until the blastocyst stage, while the GLUT4 isoform was undetectable in the oocyte through blastocyst stages. The present findings confirm previous studies at the molecular level which demonstrated that mRNAs encoding the same GLUT isoforms are detectable at corresponding developmental stages. GLUT1 and GLUT2 display different cellular distributions at the blastocyst stage as shown by IEM studies. GLUT1 has a widespread distribution in both trophectoderm and inner cell mass cells, while GLUT2 is located on trophectoderm membranes facing the blastocyst cavity. This observation suggests a different functional significance for these isoforms during mouse preimplantation development.

Key words: mouse embryo, glucose transporters, preimplantation development

Introduction

Two general mechanisms are involved in glucose transport across the plasma membrane of mammalian cells. A sodium-coupled glucose transporter has been described in the apical membrane of absorptive epithelial cells, such as in kidney and intestine (Esposito, 1984; Hediger et al., 1987). This transporter is inhibited by phlorizin, and indirectly by ouabain. The other glucose transport system, which is widely distributed, is mediated by a facilitated diffusion carrier that catalyzes glucose movement down its concentration gradient (Wheeler et al., 1985). This system is pharmacologically distinct from the sodium-coupled transporter, since phlorizin is without effect while phloretin and cytochalasin B are specific inhibitors.

In the mouse preimplantation embryo, Gardner and Leese (1988) concluded that the sodium-coupled transport system does not seem to play a role since glucose uptake was not inhibited by phlorizin, and continued in sodium-free media. Studies by Benos (1981) in rabbit blastocysts also showed that glucose transport was sodium-independent. However, the participation of a Na+/glucose cotransport system in the induction of polarity across blastomeres of compacted 8-cell stage mouse embryos has been suggested recently (Wiley et al., 1991). At this stage, a solute transporter has been detected in the apical membrane of polar cells, with structural and immunological similarities to a renal Na+/glucose cotransport system.

A number of recent studies have used molecular biology techniques to demonstrate the existence of a family of facilitated glucose transporters (GLUT) (reviewed by Bell et al., 1990; Kasanicki and Pilch, 1990; Thorens et al., 1990). These transporters exhibit considerable homology in their primary sequence, but display a marked tissue-specific pattern of expression (Table 1). In adult tissues, GLUT1 (erythrocyte/brain)
Muscle, brain and liver membranes were prepared using bovine serum albumin (BSA; Sigma) in CO2 in humidified air. Both culture media contained 0.4% animals and embryo recovery tissues were homogenized in 4 volumes of 0.3 M sucrose, 3 methods described previously (Thorens et al., 1988). Briefly, buffered human tubal fluid (HTF) medium (Quinn et al., 1985). Embryos were flushed from the reproductive tract using HEPES-gonadotropin (PMSG; Sigma Chemicals Co, St. Louis, MO) was taken as evidence that mating had occurred. Embryos at different stages of development (2-cell, 8-cell and blastocyst) were injected with 5 IU pregnant mare's serum (hCG; Sigma). A single female was placed with a single caged male (CD-I; Charles River Breeding Laboratories) over-night. The presence of a vaginal plug the following morning was taken as evidence that mating had occurred. Embryos at different stages of development (2-cell, 8-cell and blastocyst) were flushed from the reproductive tract using HTF bicarbonate-buffered medium (Quinn et al., 1985). Embryos were cultured for at least one hour in HTF bicarbonate-buffered medium at 37°C in an atmosphere of 5% CO2 in humidified air. Both culture media contained 0.4% bovine serum albumin (BSA; Sigma).

**Table 1. Human glucose transporters**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Size (Number of amino acids)</th>
<th>Major sites of expression</th>
<th>Chromosome location</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) facilitative glucose transporters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) GLUT 1 (Erythrocyte, HepG2, brain)</td>
<td>492</td>
<td>Fetal tissues, brain, kidney, colon</td>
<td>1</td>
</tr>
<tr>
<td>(2) GLUT 2 (Liver)</td>
<td>524</td>
<td>Liver, β-cell, kidney, small intestine</td>
<td>3</td>
</tr>
<tr>
<td>(3) GLUT 3 (Fetal muscle)</td>
<td>496</td>
<td>Many tissues including brain, placenta and kidney</td>
<td>12</td>
</tr>
<tr>
<td>(4) GLUT 4 (Muscle/adipocyte, insulin-regulatable)</td>
<td>509</td>
<td>Skeletal muscle, heart, adipocytes</td>
<td>17</td>
</tr>
<tr>
<td>(5) GLUT 5 (Small intestine)</td>
<td>501</td>
<td>Small intestine</td>
<td>1</td>
</tr>
<tr>
<td>b) Na+/glucose co-transporter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) SGLT1 (Na+/glucose co-transporter)</td>
<td>664</td>
<td>Small intestine</td>
<td>22</td>
</tr>
</tbody>
</table>

Modified from Gould and Bell, 1990 and Hogan et al 1991

and GLUT3 (brain) isoforms appear to be responsible for basal glucose uptake. The GLUT2 isoform mediates glucose transport primarily in hepatocytes and pancreatic β-cells. GLUT4 is responsible for insulin-stimulated glucose uptake in muscle and adipose tissue, and GLUT5 is expressed in the small intestine. Of the five isoforms, GLUT4 is the major insulin-regulatable transporter in insulin-target tissues. Although GLUT1 expression is also modulated by insulin in muscle and fat cells, the effect is less significant because GLUT4 is the primary and most abundant transporter expressed in these cells (Walker et al., 1989; de Herreros and Birnbaum, 1989; Kasanacki and Pilch, 1990).

Expression of insulin receptors coincides with the switch to glucose as the preferred energy substrate at the 8-cell stage onwards in the mouse preimplantation embryo (Biggers and Borland, 1976). At this time, embryos cultured in vitro can first respond to exogenous insulin with accelerated rates of DNA, RNA and protein synthesis (Heyner et al., 1989; Rao et al., 1990). The present study was undertaken to examine the stage-related expression of glucose transporter proteins in the preimplantation mouse embryo.

**Materials and methods**

**Animals and embryo recovery**

Female mice (CD-1; 6 to 8 weeks old; Charles River Breeding Laboratopres) were injected with 5 IU pregnant mare's serum gonadotropin (PMSG; Sigma Chemicals Co., St. Louis, MO) and 48 hours later with 5 IU human Chorionic Gonadotropin (hCG; Sigma). A single female was placed with a single caged male (CD-1; Charles River Breeding Laboratories) overnight. The presence of a vaginal plug the following morning was taken as evidence that mating had occurred. Embryos at different stages of development (2-cell, 8-cell and blastocyst) were flushed from the reproductive tract using HEPES-buffered human tubal fluid (HTF) medium (Quinn et al., 1985). Embryos were cultured for at least one hour in HTF bicarbonate-buffered medium at 37°C in an atmosphere of 5% CO2 in humidified air. Both culture media contained 0.4% bovine serum albumin (BSA; Sigma).

**Tissues**

Muscle, brain and liver membranes were prepared using methods described previously (Thorens et al., 1988). Briefly, tissues were homogenized in 4 volumes of 0.3 M sucrose, 3 mM dithiothreitol (DTT), 0.26 U/ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). Homogenates were centrifuged for 10 minutes at 8000 rpm/min and the supernatant was centrifuged for 20 minutes at the same speed. The cytosol was centrifuged for 40 minutes at 45000 rpm/min and the pellet resuspended in 0.25 M sucrose, 50 mM Tris- HCl (pH 7.4), 100 mM KCl, 5 mM MgCl2. Protein determination was performed using Bradford protein assay (Bradford, 1976) with BSA as a standard.

**Antibodies**

Polyclonal anti-peptide antibodies were raised in rabbits against the carboxyl-terminal 16 amino-acids of the rat GLUT1 and GLUT4 (Kahn et al., 1991), and against the carboxyl terminus of the mouse GLUT2 (Suze et al., 1989). Controls included preimmune sera (GLUT1 and GLUT4) and undiluted antibody adsorbed by minced liver (GLUT2). Briefly, mouse liver was minced over ice and mixed with an equal volume of ice cold BWW medium (Gibco, Grand Island, NY) containing 0.5% polyvinylpyrrolidone (Sigma) and 0.05% sodium azide. After repeated washes the supernatant was removed and an equal volume of antibody was added to the pellet. After 40 minutes incubation on ice, the mixture was centrifuged to recover the adsorbed antibody. The specificity of the antisera to GLUT1 and GLUT4 was checked by immunoblotting from target tissues (brain and muscle), and from Xenopus oocyte extracts following microinjection with in vitro transcripts specific for GLUT1 and GLUT4 (Baldini et al., 1991). In the case of GLUT2, preabsorption of the antibody with the immunizing peptide completely blocked the reactivity of the antisera to GLUT2 as detected by Western blotting of total liver lysate.

**Western blot analysis**

Embryos at the desired developmental stage were flushed from the reproductive tract and cultured as described above (see Animals and embryo recovery). They were washed extensively, resuspended in Laemmli (1970) sample buffer containing 5% β-mercaptoethanol (Sigma), and boiled for 3 minutes. Groups of approximately 200 embryos were subjected to 12% SDS-PAGE electrophoresis using a minigel system (BioRad, Richmond, CA). The proteins were electro-transferred to nitrocellulose membranes overnight at 30 V, 4°C. Following transfer, Western blot analysis was carried out using two different methods: (1) Nitrocellulose membranes were washed for 10 minutes in distilled water; for 20 minutes in Tns-buffered saline (TBS: 20 mM Tris pH 7.4, 150 mM NaCl), 0.1% Tween-20 (Sigma); and for 30 minutes in TBS, 5% nonfat dry milk, 0.2% Nonidet P-40 (Sigma), at 37°C. Membranes were incubated with anti-GLUT1 antibody or the
preimmune serum diluted 1:200 for 2 hours at room temperature in TBS, 5% nonfat dry milk, 0.2% Nonidet P-40. The membranes were finally washed for 20 minutes in TBS, 0.1% Tween-20 and incubated in a 0.25 μCi/ml 125I-Protein A (Amersham) for 30 minutes. Filters were washed again and exposed for autoradiography using Kodak X-Omat AR film, at -20°C. Filters were placed in the washing buffer (50 mL TBS, 150 mM NaCl, pH 10.2, 5 mM sodium azide) for 6 hours and for 1 hour in the blocking buffer (washing buffer, 0.05% Tween-20, 5% nonfat dry milk) at room temperature. They were then incubated overnight at room temperature with anti-GLUT2 antibody (1:500), the adsorbed anti-GLUT2 (1:500), anti-GLUT4 antibody (1:1000), and the corresponding preimmune serum (1:1000). All antibodies were diluted in washing buffer, containing 0.05% Tween-20. The following morning, the filters were washed for 30 minutes in washing buffer, 0.05% Tween-20, prior to incubation with a 1:20000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) at room temperature. The filters were finally washed for 1 minute in washing buffer, containing 0.05% Tween-20, and incubated for 1 minute in the enhanced chemiluminescence reagents (ECL Western blotting detection system, Amersham). Exposure time to Kodak X-Omat AR films was 5 to 30 seconds depending on the antibody used.

High resolution immunoelectron microscopy

Blastocyst-stage embryos were flushed from the reproductive tract and immobilized on a lawn of 3T3 fibroblasts, using the methods described by Heyner et al. (1989). Embryos were fixed in 1% glutaraldehyde plus 0.2% picric acid in phosphate-buffered saline (PBS). Fixation was followed by dehydration in graded ethanol and embedding in LR White resin (London Resin Co Ltd.). Sections were floated on wetting/blocking solution (1% ovalbumin in PBS, pH 7.4) for 60 minutes at 24°C and then incubated with appropriately diluted IgG for 48 hours at 4°C in a humidified chamber. Sections were washed 4X in 1% ovalbumin in 10 mM sodium phosphate buffer, pH 8.0, and then incubated for 60 minutes with gold-labelled protein A. Sections were washed 4X with 10 mM Tris-HCl-buffered normal saline and deionized water prior to staining for 5 minutes in neutralized 2% aqueous uranyl acetate. The specificity of the immunoelectron microscopy staining of GLUT1 and GLUT2 proteins was assessed. Omission of immune IgG or substitution of pre- or non-immune IgG resulted in a 98% decrease in the number of cell-associated gold particles. Preincubation of immune antipeptide IgG to GLUT1 or GLUT2 with a 10-fold molar excess of its corresponding peptide decreased the number of gold particles by 92% (GLUT1) and 97% (GLUT2). Preincubation of GLUT antipeptide IgG with GLUT2 or GLUT4 peptide did not affect the number of gold particles observed. These results indicated that the gold-labelled protein A represented specific association of the labelled particle with specifically bound IgG.

Results

Western blot analysis

Western blot analysis detected GLUT1 (erythrocyte/brain) isoform at all stages of preimplantation development and in oocytes as well as on the positive control, the brain membrane preparation (Fig. 1A). At the blastocyst-stage, this protein had a relative molecular mass of approximately 43×10^3 Mr, which is in keeping with published reports (Thorens et al., 1990). In oocytes and cleavage-stage embryos, two bands of approximately 34 and 20×10^3 Mr were strongly revealed whereas the 43×10^3 Mr band was weakly detected. The preimmune serum failed to reveal any bands or proteins in the brain tissue (Fig. 1A), oocytes, cleavage-stage and blastocyst-stage embryos (data not shown).

The antibody directed against GLUT2 (the liver glucose transporter) did not react with oocytes or cleavage-stage embryos but detected a protein of 59×10^3 Mr in blastocyst-stage embryos (Fig. 1B). The positive control, liver, also showed a band at the same position. When embryos were probed with anti-GLUT2 antibody, bands other than the 59×10^3 Mr protein were also detected. No signal was observed when filters were probed with previously absorbed antibody.

The analysis of GLUT4 (the muscle/adipocyte isoform) revealed a band around 50×10^3 Mr when muscle was the target tissue but no reactivity with oocytes, cleavage-stage, or blastocyst-stage embryos (Fig. 1C). Although the 50×10^3 Mr protein was not detected at any preimplantation stage, a band of approximately 59×10^3 Mr, which is most likely non-specific, was found in oocytes and blastocyst-stage embryos.

High resolution immunoelectron microscopy

The GLUT1 isofrom was readily detectable in trophoderm and inner cell mass cells of the mouse blastocyst, and was associated with intracellular membranes as well as plasma membranes of all cell types (Fig. 2). In contrast, the GLUT2 isoform was restricted to membranes of the trophectoderm that faced the blastocyst cavity, and was not present on the apical surface facing the zona pellucida. A small amount of GLUT2 was found in intracellular vesicles of the trophectoderm. Inner cell mass membranes exhibited localization on both apical and basal surfaces as well as on intracellular vesicles (Fig. 3). GLUT4 isoform was not detectable on any developmental stages of mouse embryogenesis that were studied (Fig. 4A), although immunostaining revealed reactivity with control target adipocyte tissue (Fig. 4B). Immunoelectron microscopic staining of mouse embryos with other GLUT4 antibodies, as described by Smith et al. (1991), also failed to detect GLUT4 proteins. Differences seen in the extent of labelling, i.e. in the number of gold protein A particles, with the GLUT1 and 2 antibodies does not necessarily correlate with the actual number of each transporter isoform in the blastocyst. Unknown differences in the affinity of the antibodies along with other factors preclude these comparisons. A variety of controls (described in Materials and methods) demonstrated the specificity of the immunoelectron microscopic staining.

Discussion

At a physiological concentration (1 mM), glucose uptake by mouse preimplantation embryo is carrier-mediated and is detectable from the 2-cell stage onwards. This transport is mediated by facilitated
diffusion, and the sodium-coupled glucose transport system does not seem to play a role (Gardner and Leese, 1988). However, studies in mouse blastocysts using higher concentrations of glucose (5.6 mM) have shown a significant decrease in glucose uptake when embryos were cultured in sodium-free media (Manejwala et al., 1989). Moreover, Wiley et al. (1991) have shown the presence of an antigen that is similar to a renal Na⁺/glucose cotransporter at the cell surface of preimplantation mouse embryos. This observation seems to agree with the hypothesis that solute transport systems that become apically restricted during compaction might participate in the early events in the establishment of apical-basal cell polarity across the mouse blastomere (Wiley and Obasaju, 1988, 1989). Further investigation will be needed to resolve reports regarding the involvement of the Na⁺/glucose cotransporter in glucose uptake during mouse preimplantation development.

Recent studies have shown that facilitative transport of glucose across the plasma membrane is mediated by glucose transporter proteins, a group of structurally related proteins that possess distinct tissue distributions (reviewed by Gould and Bell, 1990; Thorens et al., 1990). In this study, we examined the expression of three isoforms, GLUT1, GLUT2 and GLUT4 at different stages of development in mouse preimplantation embryos. Western blot analysis detected GLUT1 throughout preimplantation development stages as well as in oocytes. The 34 and 20×10³ Mr bands seen in oocytes and in cleavage-stage embryos might represent early embryonic isoforms of GLUT1 and/or a difference in glycosylation of the protein at these stages. High resolution immunoelectron microscopy revealed a widespread distribution of this isoform in the cells at the blastocyst-stage. This observation is not surprising since the low Kₘ GLUT1 is known to be responsible for basal glucose uptake in most fetal and adult tissues and is also induced in hepatoma and insulinoma cells (Unger, 1991), where there is an increased demand for glucose in response to rapid cellular proliferation.

We did not detect the GLUT2 protein until the blastocyst-stage, although studies using the reverse transcriptase polymerase chain reaction (RT-PCR), have detected the mRNA for this isoform at the late 8-cell stage (Hogan et al., 1991). The bands other than the 59×10³ Mr bands present on the Western blots may be non-specific, given the polyclonal nature of the antibody. They may also represent embryonic versions of GLUT2 with different glycosylation patterns. Several pieces of evidence suggest that GLUT2 is required for normal glucose sensing by pancreatic b-cells (Thorens et al., 1990): first, it is localized to the microvillar
membranes in β-islet cells, second; its $K_m$ (15-20 mM) for glucose is in the concentration range that stimulates insulin release; and third, its expression is drastically reduced in insulinomas that are no longer normally responsive to glucose. From studies in glucose-intolerant Zucker fatty rats, a model for non-insulin-dependent diabetes (reviewed by Unger, 1991), it seems that decreased glucose uptake by pancreatic β-cells and the resulting insulinopenia is due to a decrease in GLUT2 mRNA transcription and GLUT2 protein synthesis in these cells.

At the blastocyst-stage, the first two cell lineages are established and the first intraembryonic fluid is formed. The inner cell mass will give rise to the embryo proper and the trophectoderm to extraembryonic tissues. It is tempting to speculate that the expression of the GLUT2 isoform at this stage might reflect an early homeostatic role for this transporter. This idea is supported by the observation of the GLUT2 distribution, which in high resolution immuno-electron microscopy studies, was found in the cells facing the blastocoel cavity and not in those facing the zona pellucida. Further studies examining the expression of GLUT2 protein in environments with varying levels of glucose are needed in order to establish the role of GLUT2 as a "glucose sensor" in the mouse preimplantation embryo.

Although a number of studies have established the anabolic and proliferative effects of insulin on early mouse embryos (Harvey and Kaye, 1988, 1990; Heyner et al., 1989; Rao et al., 1990; Gardner and Kaye, 1991), the effects of insulin on glucose uptake by preimplantation mammalian embryos are not entirely clear. Functional studies in mouse (Gardner and Leese, 1988) and in rabbit blastocysts (Robinson et al., 1990) have led to the conclusion that glucose uptake was not affected by physiological levels of insulin. In contrast, Gardner and Kaye (1984) described an increase of approximately 2-fold in the glucose uptake by preimplantation mouse embryos in the presence of pharmacological concentrations of insulin (1 µg/ml). A similar 2-fold increase in GLUT1 translocation to the plasma membrane has been reported in insulin-treated fat cells (Zorzano et al., 1989).

Since insulin receptor expression (Mattson et al., 1988; Heyner et al., 1989) as well as mRNA encoding the insulin receptor (Schultz et al., 1990) have been detected in mouse preimplantation embryos, glucose uptake could potentially be influenced by insulin.

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Fig. 2. Immunoelectron microscopic localization of GLUT1 on mouse embryo blastocyst. Mouse embryos were prepared for immunoelectron microscopy and stained with GLUT1 antipeptide antibody (50 µg/ml) as described in Materials and methods. Antibody binding was detected with gold-labelled protein A (arrowheads) GLUT1 was found on cells of the trophectoderm and inner cell mass. GLUT1 was randomly distributed along apical, basolateral, and intercellular portions of the plasma membrane of these cells. Approximately 50% of the particles were found in intracellular subplasma membrane vesicles. tro, trophectoderm, m, mitochondrion, zp, zona pellucida. Bar, 0.5 μm.
However, in the present study, using Western blotting and high resolution immunoelectron microscopy, expression of the major insulin-regulatable GLUT4 isoform has not been demonstrated at any stage of preimplantation development. Similarly, previous studies of mRNA expression and cellular detection by immunofluorescence microscopy did not provide evidence of GLUT4 expression during this developmental period in the mouse (Hogan et al., 1991). Moreover, RT-PCR experiments did not show any expression of GLUT4 mRNA in "muscle primordia" cells in post-implantation mouse embryos, at 9.5 days post-cotum (Hogan et al., 1991). Taken together these results suggest that GLUT4 isoform may not be expressed until fetal β-cell differentiation, and any effect of exogenous insulin during preimplantation development must be via the GLUT1 isoform.

In insulin-responsive tissues such as adipose and muscle tissues, insulin causes an increase in glucose uptake by mediating the translocation of GLUT4 from intracytoplasmic pools to the plasma membrane (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Charron et al., 1989). More specifically, studies in the rat white adipocyte (Smith et al., 1991) suggest that insulin may cause the movement of GLUT4 from subplasma membrane vesicles to the plasma membrane, in addition to inducing a change in GLUT4 conformation. Additionally, evidence provided by experimental models of insulinopenia (fasting and diabetic rats) suggests that insulin also controls GLUT4 expression at the pretranslational level (Gould and Bell, 1990). When cultured at a physiological concentration of glucose, 40% of blastocyst-stage embryos from streptozotocin-induced diabetic rats were degenerate and showed impaired glucose uptake (Brison and Leese, 1990). However, these authors showed that in those blastocysts that developed normally, there was no impairment of glucose uptake, supporting the hypothesis that glucose uptake is insulin-insensitive.

Besides the importance of insulin plasma levels in the regulation of glucose uptake, it is now well established that insulin is more effective biologically when it is delivered in pulses rather than continuously (see review by Lefèvre et al., 1987). The insulin found in the mouse preimplantation embryo is of maternal origin (Heyner et al., 1989), and the rapid pulsatile fluctu-
tions of its level in blood, due to the oscillatory pattern of its secretion, might disappear after diffusion in the reproductive tract. Therefore, the absence of GLUT4 expression in our studies might be related to an insufficient level of insulin or to an inadequate cyclic pattern of its concentration during preimplantation. Establishing the ontology of GLUT4 expression during postimplantation development will provide more insight into this question.

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References


Fig. 4. Immunoelectron microscopic localization of GLUT4 on mouse embryo blastocyst (A) and rat adipocyte (B). Mouse embryos were prepared for immunoelectron microscopy as described in Materials and methods, rat adipocytes, incubated for 30 minutes with 10 ng/ml insulin, were prepared as described previously (Smith et al., 1991). Both were stained with GLUT4 antipeptide antibody (50 μg/ml) as described in Materials and methods. Antibody binding was detected with gold-labelled protein A (arrowheads) No significant labelling of GLUT4 was detected in mouse blastocysts. In contrast, labelling of GLUT4 in the plasma membrane of insulin-treated rat adipocytes was extensive. m, mitochondrion; zp, zona pellucida. Bar, 0.5 μm.


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