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Two insulin-responsive glucose transporter isoforms and the insulin receptor are developmentally expressed in rabbit preimplantation embryos

Anne Navarrete Santos, Sarah Tonack, Michaela Kirstein, Silke Kietz and Bernd Fischer

Department of Anatomy and Cell Biology, Martin Luther University Faculty of Medicine, Grosse Steinstrasse 52, D-06108 Halle (Saale), Germany

Correspondence should be addressed to B Fischer; Email: bernd.fischer@medizin.uni-halle.de

Abstract

Glucose is the most important energy substrate for mammalian blastocysts. Its uptake is mediated by glucose transporters (GLUT). In muscle and adipocyte cells insulin stimulates glucose uptake by activation of the insulin receptor (IR) pathway and translocation of GLUT4. GLUT4 is expressed in bovine preimplantation embryos. A new insulin-responsive isoform, GLUT8, was recently described in mouse blastocysts. Thus, potentially, two insulin-responsive isoforms are expressed in early embryos. The mechanism of insulin action on embryonic cells, however, is still not clear. In the present study expression of IR, GLUT1, 2, 3, 4, 5 and 8 was studied in rabbit preimplantation embryos using RT-PCR, Western blotting and immunohistochemistry. The rabbit mRNA sequences for the complete coding region of IR, GLUT4 and a partial GLUT8 sequence were determined by RACE-PCR and sequencing. GLUT4 was expressed in 3-day-old morulae and in 4- and 6-day-old blastocysts. IR and GLUT8 transcripts were detectable only in blastocysts. Blastocysts also expressed GLUT1 and 3, but not GLUT2 and 5. Transcript numbers of GLUT4 and 8 were higher in trophoblast than in embryoblast cells. Translation of IR, GLUT4 and 8 proteins in blastocysts was confirmed by Western blotting. GLUT4 was localized mainly in the membrane and in the perinuclear region in trophoblast cells while in embryoblast cells its localization was predominantly in the perinuclear cytoplasm. The possible function(s) of two insulin-responsive isoforms, GLUT4 and GLUT8, in rabbit preimplantation embryos needs further investigation. It may not necessarily be linked to insulin-stimulated glucose transport.

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Introduction

Insulin stimulates cell proliferation and differentiation in preimplantation embryos. Although these embryos do not synthesize insulin (Telford *et al.* 1990*a*,*b*, Kaye 1997, Lighten *et al.* 1997) they have access to maternal insulin *in vivo* via oviduct and uterine fluid (Heyner *et al.* 1989, Chi *et al.* 2000). The presence of insulin receptors (IR) and insulin growth factor-I (IGF-I) receptors (IGF-IR) in embryos has been described in several species (see Kaye 1997 for review). IR and IGF-IR are expressed in human (Lighten *et al.* 1997) and bovine (Schultz *et al.* 1992, Watson *et al.* 1992, Schultz & Heyner 1993, Yaseen *et al.* 2001) oocytes and throughout preimplantation embryo development. In mouse embryos expression starts at the 8-cell stage (Harvey & Kaye 1988, 1990, Rappolee *et al.* 1992, Schultz & Heyner 1993, Markham & Kaye 2003). Rabbit embryos bind insulin and IGF-I from the morula stage onwards on embryoblast and trophoblast cells.

Insulin binds to its cell surface located receptor. The mature receptor is composed of two extracellular α and two transmembrane β subunits which are disulfide-linked to an $\alpha_2\beta_2$ heterotetrameric structure (Lee & Pilch 1994, Czech & Corvera 1999). Following insulin binding to the extracellular α-subunit tyrosine kinase domain, the β subunits undergo a series of intramolecular transautophosphorylation reactions, resulting in tyrosine autophosphorylation at multiple sites. This activates a series of intracellular signaling cascades which coordinately initiate the appropriate cellular response. Insulin and IGF-I act as a survival factor by decreasing apoptosis and increasing cell proliferation (Herrler *et al.* 1998, Spanos *et al.* 2000). Another important mechanism is the increase of glucose transport via the insulin-dependent translocation of the facilitative glucose transporter 4 (GLUT4) from intracellular storage vesicles (Rea & James 1997) to the plasma membrane in insulin target tissues, primarily striated muscle and adipose tissue (Pessin *et al.* 1999, Patki *et al.* 2001). Currently, the GLUT family comprises 13 members, GLUT1–12 and HMIT (H^+ coupled myo-inositol-transporter) (Joost *et al.* 2002, Stuart Wood & Trayhurn 2003). Each GLUT isoform consists

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of 12 helical transmembrane-spanning domains, an extracellular glycosylated hydrophilic segment, an intracellular loop and intracellular located amino- and carboxyl-terminals (Mueckler *et al.* 1985, Cope *et al.* 1994).

The expression pattern of glucose transporters in preimplantation embryos has been studied in the mouse (Hogan *et al.* 1991, Aghayan *et al.* 1992, Morita *et al.* 1992, Chi *et al.* 1993, Pantaleon *et al.* 1997), rabbit (Robinson *et al.* 1990), bovine (Lequarre *et al.* 1997, Wrenzycki *et al.* 1998, 2003, Navarrete Santos *et al.* 2000, Augustin *et al.* 2001) and human (Dan-Goor *et al.* 1997). In mice, GLUT1 was found in all preimplantation stages. GLUT3 was detected from the eight-cell stage onwards (Pantaleon *et al.* 1997). While expression of the recently described insulin responsive GLUT8 was shown in mouse blastocysts (Carayannopoulos *et al.* 2000), the other insulin-sensitive isoform, GLUT4, was not found in mice preimplantation or early postimplantation stages (Hogan *et al.* 1991, Aghayan *et al.* 1992). In preimplantation embryos of other species, however, GLUT4 has been detected (bovine: Navarrete Santos *et al.* 2000, Augustin *et al.* 2001, rat: Korgun *et al.* 2001). Therefore, the question arose whether and which compounds of the insulin–GLUT signaling cascade are expressed in embryos and which function they may execute during preimplantation development in mammals. Here we show that IR and both insulin-responsive isoforms, GLUT4 and GLUT8, are expressed in rabbit preimplantation embryos in a developmentally regulated manner. Rabbit blastocysts also express GLUT1 and GLUT3, but not GLUT2 and GLUT5.

Materials and Methods

The TRIzol reagent, Superscript II RT-kit, dNTPs and Taq polymerase were purchased from Invitrogen (Karlsruhe, Germany), restriction enzymes were from New England Biolabs (Frankfurt, Germany), and T7 RNA polymerase, random primer, RNAse inhibitor and DNAse I were from Roche Diagnostics (Mannheim, Germany).

Embryo recovery

Embryos were collected from sexually mature rabbits (hybrid strain Zika) which had been stimulated by 100 I.U. follicle-stimulating hormone (Ovagen, Immuno-Chemical Products, Auckland, New Zealand). Mating, embryo recovery and embryo culture were performed as described before (Kietz & Fischer 2003). Morulae were flushed on day 3 and blastocysts on days 4 and 6 post coitum; they were washed three times in PBS, pooled and randomly divided among the experimental groups. For molecular analysis embryos were stored until use at −80 °C in TRIzol reagent and in RIPA buffer for RNA and protein isolation respectively.

In order to investigate spatial expression of GLUT4 and GLUT8 in trophoblast (TE) and embryoblast cells (inner cell mass, ICM), blastocyst coverings were mechanically removed and the embryonic disks were microdissected from the trophoblast under a stereomicroscope. Isolated ICM and TE from each blastocyst (total number 11 blastocysts) were stored separately at −80 °C. For immunohistochemistry embryos were fixed overnight in Bouin fixative containing 75% (v:v) aqueous-saturated picric acid solution, 20% (v:v) formalin, and 5% (v:v) acetic acid and/or in 4% (v:v) paraformaldehyde/PBS overnight. Bouin-fixed embryos were dehydrated and embedded in paraffin. Sections (5 μm) were prepared with an ultramicrotome. Paraformaldehyde-fixed embryos were dehydrated and stored in methanol at −20 °C until whole mount staining.

RNA extraction

Preparation of total RNA from whole embryos was performed by using 1 ml TRIzol reagent according to the previously described protocol (Koerber *et al.* 1998). Total RNA from tissues was extracted as described by Chomczynski and Sacchi (1987). RNA was treated with DNAse for 1 h. The amount of total RNA was determined spectrophotometrically at 260 nm. The mRNA extraction from separated trophoblast and embryoblast was performed with DYNABEADS (Dynal, Oslo, Norway) in order to collect sufficient amounts from the small number of cells.

Cloning of rabbit IR, GLUT4 and GLUT8 sequences

Rabbit IR, GLUT4 and GLUT8 cDNA sequences were determined by PCR amplification with degenerated primers derived from human sequences (IR accession no. (acc.) X02160.1, GLUT4 acc. M91463, GLUT8 acc. Y17801, primers shown in Table 1) and 3′ RACE-PCR (3/5 RACE kit, Roche Diagnostics) using specific rabbit primers (Table 1) on reverse transcribed rabbit skeletal muscle and liver mRNA for GLUT4 and IR, and GLUT8 respectively. The amplified PCR products were purified by separation in a preparative 1.8% agarose gel and extracted by a Gel Extraction kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Fragments were cloned into the pGEM-T vector and transformed into competent *E. coli* XL1blue cells. Recombinant plasmid DNA was analyzed by restriction analysis and sequenced using the ABI Prism Ready Reaction Dyedeoxy Terminator Sequencing kit (Amersham Biotech, Freiburg, Germany) and T3 and T7 sequencing primers in an ABI 373 automated sequencer. The sequenced cDNA was screened for homology in the GenBank EMBL using the BLASTN search modus and for amino acids using the BLASTP search modus.

Sequence alignment

Sequence alignment of rabbit IR and GLUT4 protein sequences was performed by CLUSTAL W (1.82) multiple sequence alignment.

RT-PCR of IR, GLUT1–5 and 8 mRNA

Primers used for RT-PCR are listed in Table 1. One microgram total RNA was reverse transcribed in a volume of

Table 1 Specific rabbit (rab) and human (hu) primers used for RT-PCR analysis, RACE-PCR and sequencing.

Gene	Name	5′-Sequence-3′
GLUT1	rabGLUT1 p1	GCTGATGATGAACCTGCTGG
	rabGLUT1 p2	GGTTGATGAGCAGGAAGCG
GLUT2	rabGLUT2 p1	CAGCTCTTTACCAACTCCAG
	rabGLUT2 p2	GCTCACATAACTCATCCAGG
GLUT3	rabGLUT3 p1	TCTGGAAATCATCTTGGGGTTCT
	rabGLUT3 p2	CCGTAGAGTAATAGAACACAGC
GLUT4	rabGLUT4 p1	GGCGGCATGATTTCCTCC
	rabGLUT4 p2	GAAGGGCAGCAGGATCAGCT
	rabGLUT4 p3	GGTGCTCCCTGCCCTCCT
	rabGLUT4 p4	ATCGCCCTCTTCCTTCCCA
	huGLUT4 p1	GAAGGTGATTGAACAGAGC
	huGLUT4 p2	GATGATGTAGAGGTAGCG
	huGLUT4 p3	ATGCCGTCGGGCTTCCAAC
GLUT5	rabGLUT5 p1	GCAGCAAAGTCGCCAGGTCG
	rabGLUT5 p2	GTAGATGGCGTTCACTCCCGA
GLUT8	rabGLUT8 p1	CACGTCAAGGGTGTGGCT
	rabGLUT8 p2	CAGGGACGCAGAACAAAGTG
	huGLUT8 p1	GCTCCTCATGTCAGAGATCT
	huGLUT8 p2	TCCCCTTGGTTTCAGGGAC
Insulin receptor	rabInsR p1	GCTGGTGGTGATGGAGTTG
	rabInsR p2	TCTCTCTGGACAGTTGTCGG
	rabInsR p3	AGATCTCATGCGTCACGGG
	rabInsR p4	AGCAGGTGTTGAAGTTTGTC
	rabInsR p5	AGAGGCCTCGGAAGTCGG
	hu InsR p1	GGACCATGCCTGAAGCCAAG
	hu InsR p2	TGAATTGCCAGCACATGCG
	hu InsR p7	CTGAGATGGAGACCGTACTG
	hu InsR p8	TTAGGAAGGATTGGACCGAG
	hu InsR p9	ATCGACTGGTCCCGTATCC
	hu InsR p12	CACCTGTACCCCGGAGAG
Cytokeratin 18	rabCytoker p1	CAGATTGAGGAGAGCACCAC
	rabCytoker p2	AGTCCTCGCCATCTTCCAGC
β-actin	rabActin p1	CTACAATGAGCTGCGTGTGG
	rabActin p2	TAGCTCTTCTCCAGG GAGGA

20 μl containing 0.5 mM dNTPs, 10 mM dithiothreitol (DTT), 200 units superscript II, 20 units RNAse inhibitor, 1 μl random primer and 2 μl reverse transcriptase buffer at 42 °C for 1 h, followed by an incubation at 90 °C for 5 min. As a control for DNA contamination, 1 μg RNA was PCR amplified without reverse transcription reaction. This control reaction was performed for each primer combination and in all PCR amplifications. PCR amplification was carried out with 1 μl cDNA in a 50 μl volume containing 5 μl dNTP, 2.5 units Taq polymerase, employing the primer combinations listed in Table 1. Resulting PCR products were separated by electrophoresis on 1.8% agarose gel and stained with ethidium bromide.

Semiquantitative RT-PCR on the separated trophoblast and embryoblast tissue

Trophoblast and embryoblast mRNA were directly transcribed into cDNA with the RNA-PCR Core kit (Perkin-Elmer Roche, Boston, USA). Reverse transcription reaction was performed in a thermocycler (Biometra, Göttingen, Germany) under the following conditions: 10 min at 25 °C, 1 h at 42 °C, 5 min at 99 °C. Afterwards 40 μl H_2O were added. The quality of tissue separation was controlled by cytokeratin 18 PCR (see Fig. 5A). Only embryos with clearly higher transcript numbers of cytokeratin 18 in the separated trophoblast and low expression in embryoblast tissues were used for semiquantitative PCR on GLUTs. To equalize for different RNA amounts of individual embryos, first a PCR on β-actin was performed. This housekeeping gene was used as an internal standard for GLUT transcript numbers (Kietz & Fischer 2003). All PCR reactions were carried out in 50 μl volume containing 2 μl cDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U Taq polymerase (Life Technologies, Eggenstein, Germany) and 150 ng of the primer combination, rabActinp1/p2 and rabGLUT1p1/p2, rabGLUT3p1/p2, rabGLUT4p1/p2 and rabGLUT8p1/p2 (Table 1) for GLUT1, GLUT3, GLUT4 and GLUT8 respectively. The amplification profile was as follows: 5 min at 94 °C, 28 (β-actin), 32 (GLUT1, GLUT3, GLUT8) and 35 (GLUT4, cytokeratin 18) cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C, and a final extension period for 5 min at 72 °C. Gels were photographed and the product bands were quantified by densitometric analysis employing the software BIO-Profile 1D (LTF-Labortechnik, Wasserburg, Germany). The relative amount of GLUT1, GLUT3, GLUT4 and GLUT8 mRNA was calculated as a ratio of the specific product and the housekeeping gene band volume (β-actin). All PCR reactions were performed three times.

The statistical analysis was performed using paired *t*-test (SigmaPlot 4.0, Jandel Corporation (San Rafael, CA, USA), mathematical and statistical analysis). The data are expressed as means ± S.E.M.

Protein preparation and immunoblotting

Blastocysts were washed three times with PBS after culture and transferred to a 1.5 ml Eppendorf tube. They were homogenized in 100 μl cold RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) with a protease inhibitor cocktail (Sigma, St Louis, MO, USA). The samples were centrifuged at 5000 ***g*** for 10 min. The supernatant was stored at −80 °C until use. The total protein content was determined using the BIO-RAD Protein Assay (Bio-RAD, München, Germany). Twenty micrograms embryonic protein and 40 μg of reference tissues were heated at 70 °C for 5 min before solubilizing in Laemmli buffer containing 200 mM DTT and electrophoresed on 8% SDS-PAGE. Proteins were electrotransferred to nitrocellulose membranes. Membranes were blocked for 1 h in Tris-buffered saline containing 0.1% Triton (TBST) with 5% BSA (for detection of IR) or 5% non-fatty milk powder (for GLUT4 and GLUT8) at room temperature. Blots were incubated in TBST containing 5% BSA with monoclonal mouse anti-IR (β-subunit, Ab-4, Oncogene Research Products Calbiochem, Darmstadt, Germany) at a dilution of 1:200, in 5% nonfat milk powder/TBST with monoclonal mouse anti-GLUT4 antibody (1:6000, DPC Biermann, Bad Nauenheim, Germany) or rabbit anti-GLUT8 antibody (1:400, Alpha Diagnostics International, San Antonio, TX, USA) overnight at 4 °C.

Blots were subjected to three 20-min washes in TBST and incubated for 1 h with goat anti-mouse IgG (1:25 000) or goat anti-rabbit IgG (1:20 000) conjugated to horseradish peroxidase (Dianova, Hamburg, Germany) in 5% BSA/TBST or 5% non-fatty milk powder/TBST at room temperature. Afterwards, the immunoreactive signals were visualized by enhanced chemiluminescence detection (ECL Plus, Amersham Biotech). Apparent molecular weights were determined by comparison with standard molecular weight markers (high range marker, Promega Corp., Mannheim, Germany).

Immunohistochemistry (IHC)

The GLUT4 antigen was localized on embryonic sections and whole blastocysts. Bouin-fixed, paraffin-embedded

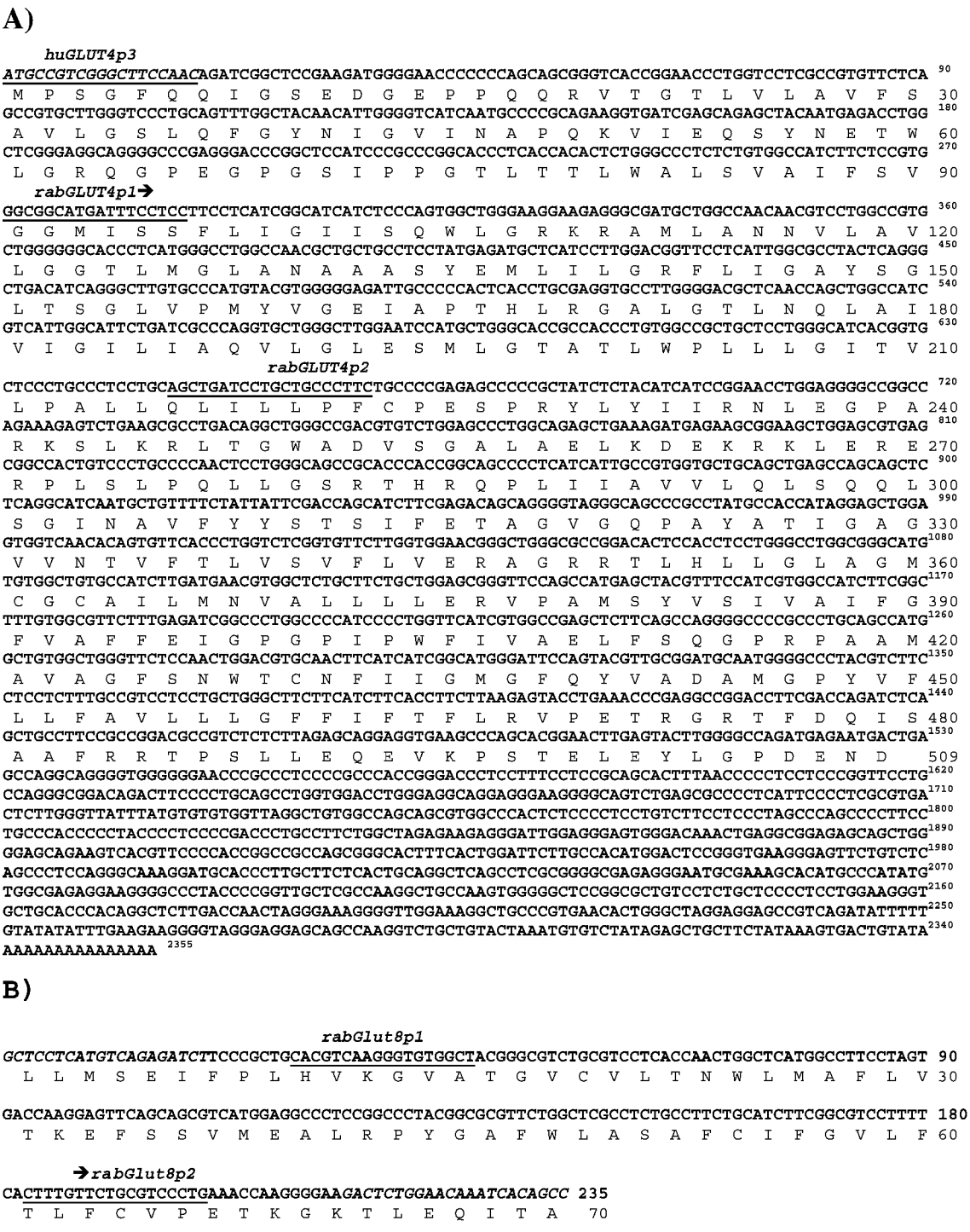

Figure 1 Continued.

C)

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Human_Glut4   MPSGFQQIGS--EDGEPPQQRVTGTLVLAVFSAVLGSLQFGYNIGVINAPQKVIEQSYNETWLGRQGPEGPSSIPPGTLTTLWALSVAIFSVGGMISSFL  98
Horse_Glut4   MPSGFQQIGS--EDGEPPQQRVTGTLVLAVFSAVLGSLQFGYNIGVINAPQKVIEQSYNETWLGRQGPEGPSSIPPGTLTTLWALSVAIFSVGGMISSFL  98
Rabbit_Glut4  MPSGFQQIGS--EDGEPPQQRVTGTLVLAVFSAVLGSLQFGYNIGVINAPQKVIEQSYNETWLGRQGPEGPGSIPPGTLTTLWALSVAIFSVGGMISSFL  98
Mouse_Glut4   MPSGFQQIGSDVKDGEPPRQRVTGTLVLAVFSAVLGSLQFGYNIGVINAPQKVIEQSYNATWLGRQGPGGPDSIPQGTLTTLWALSVAIFSVGGMISSFL  100
Rat_Glut4     MPSGFQQIGS--EDGEPPQQRVTGTLVLAVFSAVLGSLQFGYNIGVINAPQKVIEQSYNATWLGRQGPGGPDSIPQGTLTTLWALSVAIFSVGGMISSFL  98
Consensus     **********  :*****:****************************************** ******** **.*** ************************

Human_Glut4   IGIISQWLGRKRAMLVNNVLAVLGGSLMGLANAAASYEMLILGRFLIGAYSGLTSGLVPMYVGEIAPTHLRGALGTLNQLAIVIGILIAQVLGLESLLGT  198
Horse_Glut4   IGIISQWLGRKRAMLVNNALAVLGGSLMGLADTAASYEMLILGRFLIGAYSGLTSGLVPMYVGEIAPTHLRGALGTLNQLAIVIGILIAQVLGLESMLGT  198
Rabbit_Glut4  IGIISQWLGRKRAMLANNVLAVLGGTLMGLANAAASYEMLILGRFLIGAYSGLTSGLVPMYVGEIAPTHLRGALGTLNQLAIVIGILIAQVLGLESMLGT  198
Mouse_Glut4   IGIISQWLGRKRAMLANNVLAVLGGALMGLANAVASYEILILGRFLIGAYSGLTSGLVPMYVGEIAPTHLRGALGTLNRLAIVIGILVAQVLGLESMLGT  200
Rat_Glut4     IGIISQWLGRKRAMLANNVLAVLGGALMGLANAAASYEILILGRFLIGAYSGLTSGLVPMYVGEIAPTHLRGALGTLNQLAIVIGILVAQVLGLESMLGT  198
Consensus     ***************.**.******:*****:::.****:****************************************:********:********:***

Human_Glut4   ASLWPLLLGLTVLPALLQLVLLPFCPESPRYLYIIQNLEGPARKSLKRLTGWADVSGVLAELKDEKRKLERERPLSLLQLLGSRTHRQPLIIAVVLQLSQ  298
Horse_Glut4   ATLWPLLLGITVLPALLQLVLLPFCPESPRYLYISRNLEGPARKSLKRLTGWADVSGVLAELKEEKRKLERERPLSLLQLLGSRVHRQPLVIAVVLQLSQ  298
Rabbit_Glut4  ATLWPLLLGITVLPALLQLILLPFCPESPRYLYIIRNLEGPARKSLKRLTGWADVSGALAELKDEKRKLERERPLSLPQLLGSRTHRQPLIIAVVLQLSQ  298
Mouse_Glut4   ATLWPLLLALTVLPALLQLILLPFCPESPRYLYIIRNLEGPARKSLKPLTGWADVSDALAELKDEKRKLERERPMSLLQLLGSRTHRQPLIIAVVLQLSQ  300
Rat_Glut4     ATLWPLLLAITVLPALLQLLLLPFCPESPRYLYIIRNLEGPARKSLKRLTGWADVSDALAELKDEKRKLERERPLSLLQLLGSRTHRQPLIIAVVLQLSQ  298
Consensus     *:******.:*********:********** :*********** ********..*****:**********:** ******.*****:*********

Human_Glut4   QLSGINAVFYYSTSIFETAGVGQPAYATIGAGVVNTVFTLVSVLLVERAGRRTLHLLGLAGMCGCAILMTVALLLLERVPAMSYVSIVAIFGFVAFFEIG  398
Horse_Glut4   QLSGINAVFYYSTSIFEKAGVGQPAYATIGAGVVNTVFTLVSVFLVERAGRRTLHLLGLAGMCGCAILMTVALLLLERVPAMSYVSIVAIFGFVAFFEIG  398
Rabbit_Glut4  QLSGINAVFYYSTSIFETAGVGQPAYATIGAGVVNTVFTLVSVFLVERAGRRTLHLLGLAGMCGCAILMNVALLLLERVPAMSYVSIVAIFGFVAFFEIG  398
Mouse_Glut4   QLSGINAVFYYSTSIFESAGVGQPAYATIGAGVVNTVFTLVSVLLVERAGRRTLHLLGLAGMCGCAILMTVALLLLERVPAMSYVSIVAIFGFVAFFEIG  400
Rat_Glut4     QLSGINAVFYYSTSIFELAGVEQPAYATIGAGVVNTVFTLVSVLLVERAGRRTLHLLGLAGMCGCAILMTVALLLLERVPSMSYVSIVAIFGFVAFFEIG  398
Consensus     ***************** *** ********************:*********************.**********:******************

Human_Glut4   PGPIPWFIVAELFSQGPRPAAMAVAGFSNWTSNFIIGMGFQYVAEAMGPYVFLLFAVLLLGFFIFTFLRVPETRGRTFDQISAAFHRTPSLLEQEVKPST  498
Horse_Glut4   PGPIPWFIVAELFSQGPRPAAMAVAGFSNWTCNFIIGMGFQYVADAMGPYVFLLFAVLLLGFFIFTFLRVPETRGRTFDQISAAFHRTPSLLEQEVKPST  498
Rabbit_Glut4  PGPIPWFIVAELFSQGPRPAAMAVAGFSNWTCNFIIGMGFQYVADAMGPYVFLLFAVLLLGFFIFTFLRVPETRGRTFDQISAAFRRTPSLLEQEVKPST  498
Mouse_Glut4   PGPIPWF-VAELFSQGPRPAAMAVAGFSNWTCNFIVGMGFQYVADRMGPYVFLLFAVLLLGFFIFTFLKVPETRGRTFDQISAAFRRTPSLLEQEVKPST  499
Rat_Glut4     PGPIPWFIVAELFSQGPRPAAMAVAGFSNWTCNFIVGMGFQYVADAMGPYVFLLFAVLLLGFFIFTFLRVPETRGRTFDQISATFRRTPSLLEQEVKPST  498
Consensus     ******* ***********************.***:********: **********************:**************:*:**************

Human_Glut4    ELEYLGPDEND    509
Horse_Glut4    ELEYLGPDEXD    509
Rabbit_Glut4   ELEYLGPDEND    509
Mouse_Glut4    ELEYLGPDEND    510
Rat_Glut4      ELEYLGPDEND    509
Consensus      ********** *
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Figure 1 Rabbit GLUT4 (A) and GLUT8 (B) cDNA and the predicted protein sequences. The localizations of the specific rabbit PCR primers (rabGLUT4p1 and rabGLUT4p2) used in this study are underlined. The single open reading frame is translated into a 509 amino acid protein (A). Interspecies comparisons of the GLUT4 protein sequences (C) show that the rabbit GLUT4 is a member of the GLUT family. The multiple sequence alignments of GLUT4 protein for mouse (accession number AB008453), rat (accession number D28561), rabbit, human (accession number M20747) and horse (accession number AF531753) were performed by CLUSTAL W (1.82) multiple sequence alignment. The consensus is displayed by the following symbols denoting the degree of conservation observed in each column: (*) means that the residues or nucleotides in that column are identical in all sequences in the alignment, (:) means that conserved substitutions have been observed, (.) means that semi-conserved substitutions have occurred.

day 6 rabbit blastocysts were sectioned at 5 μm. Sections were mounted on silanized slides, deparaffinized in xylene and rehydrated through a series of graded alcohols. Various antigen retrieval methods were tested on the paraffin-embedded rabbit tissues for IR antigen detection. With the monoclonal mouse anti-IR (β-subunit, Ab-4, Oncogene Research Products Calbiochem) we could not detect any immunoreactions on fixed tissues (rabbit liver, muscle) or embryo sections. For whole mount-IHC the paraformaldehyde-fixed blastocysts were rehydrated through a series of graded alcohols. The neozona was removed mechanically before peroxidase blocking. Endogenous peroxidase was quenched by treatment with 3% hydrogen peroxide in methanol for 30 min. Non-specific antibody binding was blocked with 10% normal goat or donkey serum in PBS at room temperature for 1 h and incubated with the primary antisera overnight at 4 °C in a humidified chamber. GLUT4 antibody (mouse anti-GLUT4 antibody 1:2500, DPC Biermann) was diluted in PBS with 1% BSA. Sections and whole blastocysts were rinsed with PBST and incubated with the peroxidase-labeled secondary antisera (DAKO EnVision + /HRP-goat-anti mouse IgG, DAKO, Hamburg, Germany) for conventional light microscopy. The antigen was visualized with the diaminobenzidine (DAB, WAK-Chemie Medikal, Bad Soden, Germany) substrate. The development of DAB was stopped in water after 5 min. Sections were counterstained with hematoxylin, dehydrated and cleared in xylene. The slides were mounted in DPX and examined under bright-field microscopy with an AH-3 microscope (Olympus, Hamburg, Germany). For whole mount confocal microscopy the GLUT4 protein was localized by immunofluorescence detection with the secondary antibody fluorescein (FITC)-conjugated AffinPure donkey-anti-mouse IgG (1:300, Jackson ImmunoResearch Lab. Ltd, Cambridgeshire, UK). The nuclei were counterstained with 7-amino-actinomycin. Whole blastocysts were scanned and examined by confocal laser microscopy with Leica TCS SP (Bensheim, Germany). The specificity of immunostaining was demonstrated by the absence of signals in sections and whole embryos incubated with control mouse IgG (DAKO) or in sections processed after omission of the primary antibody. Only reactions with negative controls were included in the study. At least 5 embryos, pooled from different donor animals, were examined per group.

IHC reactions of the same specimens were repeated three times.

Accession numbers

The GeneBank accession numbers for rabIR, rabGLUT2, rabGLUT4 and rabGLUT8 were AY339877, CB814983, AY339876 and BF146289 respectively.

Results

Cloning and sequencing of rabbit GLUT4, GLUT8 and IR mRNA

The rabbit GLUT4 and IR cDNA obtained by RACE-PCR were 2355 bp and 4026 bp long respectively, covering the complete coding region (Figs 1A, 2A). The partial rabbit GLUT8 cDNA sequence with a length of 230 bp (Fig. 1B, rabGLUT8 – accession number BF146289) was amplified from rabbit liver tissues. All sequences show a high homology to human and other mammalian sequences at both the DNA and protein level (Table 2).

The new rabbit cDNA rabGLUT4 (accession number AY339876) belongs to the facilitative glucose transporter family and is closely related to mammalian GLUT4 (Fig. 1C). The GLUT4 5′ coding region and the putative start of translation were amplified by human huGLUT4p3 and rabGLUT4p4, whereas the 3′ terminus was determined by 3′-RACE with specific rabGLUT4p3 and a poly(A) specific anchor primer (primers listed in Table 1). The resulting cDNA contains an open reading frame of 1527 nucleotides encoding a protein of 509 amino acids (aa) with a calculated molecular mass of 54.84 kDa and an estimated pI of 7.77. The TGA stop codon is followed by 825 bases of 3′ non-coding region. The rabGLUT4 3′-flanking region shows partial homology with the horse, bovine, mouse and human GLUT4 genes and is specific for rabGLUT4. The specific rabbit primers rabGLUT4p1 and rabGLUT4p2 (Table 2, Fig. 1A) were designed for PCR amplification on rabbit embryos yielding a PCR product of 398 bp. The primer design avoided genomic amplification due to two bridged introns.

The new rabbit IR cDNA (accession number AY339877) sequence contains the complete region for the α- and β-subunits of the receptor (Fig. 2). The insulin receptor sequence is highly conserved in mammalian species (Fig. 2). The insulin binding region and the amino acids phosphorylated by tyrosine kinase are identical in the rabbit and human sequences. The complete coding region was cloned. Analysis of a 4026 bp cDNA revealed an open reading frame coding for a propolypeptide of 1341 aa with a molecular mass of 152.4 kDa. The protein sequence between rabbit and human IR differed only in 44 aa. In the α-subunit the amino acids involved in insulin binding are present. In the β-subunit the tyrosine residues representing the autophosphorylation consensus sequence were identical to the human ones (Ottensmeyer *et al.* 2000) (Fig. 2). These molecular features prove that the *rabIR* gene product is a member of the IR family and represents the insulin receptor from *Oryctolagus cuniculus*. The identity of the rabbit IR to the receptor molecule is higher than with other members of the IR family such as IGF-IR.

Expression of GLUT isoforms and IR in rabbit embryos

The glucose transporter isoforms 1, 3, 4, 8 and IR are expressed in expanded rabbit blastocysts (day 6) (Figs 3 and 4). While transcripts of the GLUT4 gene were detectable in rabbit morulae and blastocysts, IR and GLUT8 were not found at the morula stage. First transcripts for IR and GLUT8 mRNA were detectable in early blastocysts recovered on day 3 and in day 4 blastocysts respectively (Fig. 4A). GLUT4 and 8 transcript numbers were significantly higher in the trophoblast than in the embryoblast ($P < 0.05$). Such cell lineage-specific effects were not found for GLUT1 and 3 (Fig. 5B). The GLUT isoforms 2 and 5 were not found in day 6 blastocysts (Fig. 3, rabGLUT2 – accession number CB814983).

Translation of rabGLUT4, GLUT8 and rabIR protein was shown by Western blotting in day 6 blastocysts with bands of the expected molecular weights (Fig. 4B, C, D). Molecular weight and signal specificity of the antibody were verified by employing target tissues such as rabbit heart muscle for GLUT4, GLUT8 and liver for IR in the same blot (Fig. 4B, C, D). GLUT4 immunoreactivity was confirmed by IHC on day 6 blastocysts (Fig. 6). Antibodies for GLUT8 and IR, however, were not immunoreactive on rabbit embryo and target tissues (liver, heart muscle).

Localization of GLUT4

GLUT4 protein was localized in both the embryoblast and trophoblast of day 6 blastocysts (Fig. 6). Immunohistochemical staining revealed a different subcellular localization of the protein. Whereas the outer trophoblast layer showed a membrane and perinuclear staining, the extraembryonic endoderm exhibited an intense staining for cytoplasmic and perinuclear compartments. Confocal laser scans showed a closed localization of GLUT4 with nuclear membranes in the extraembryonic endoderm. In embryoblast cells, GLUT4 was localized mainly perinuclearly in the cytoplasm.

Discussion

IR and GLUT4 expression has been reported in various insulin-sensitive tissues of eukaryotic organisms. The present study is the first report on the simultaneous expression of IR, GLUT4 and GLUT8 in preimplantation embryos. All three genes are expressed in rabbit blastocysts. The presence of small amounts of IR and GLUT8 mRNA at the morula stage cannot be excluded. However, the sensitive RT-PCR approach used in this study, based on specific primers for transcript detection, was negative. Due to the lack of appropriate antibodies, we could not verify translation of all three genes into protein. It is

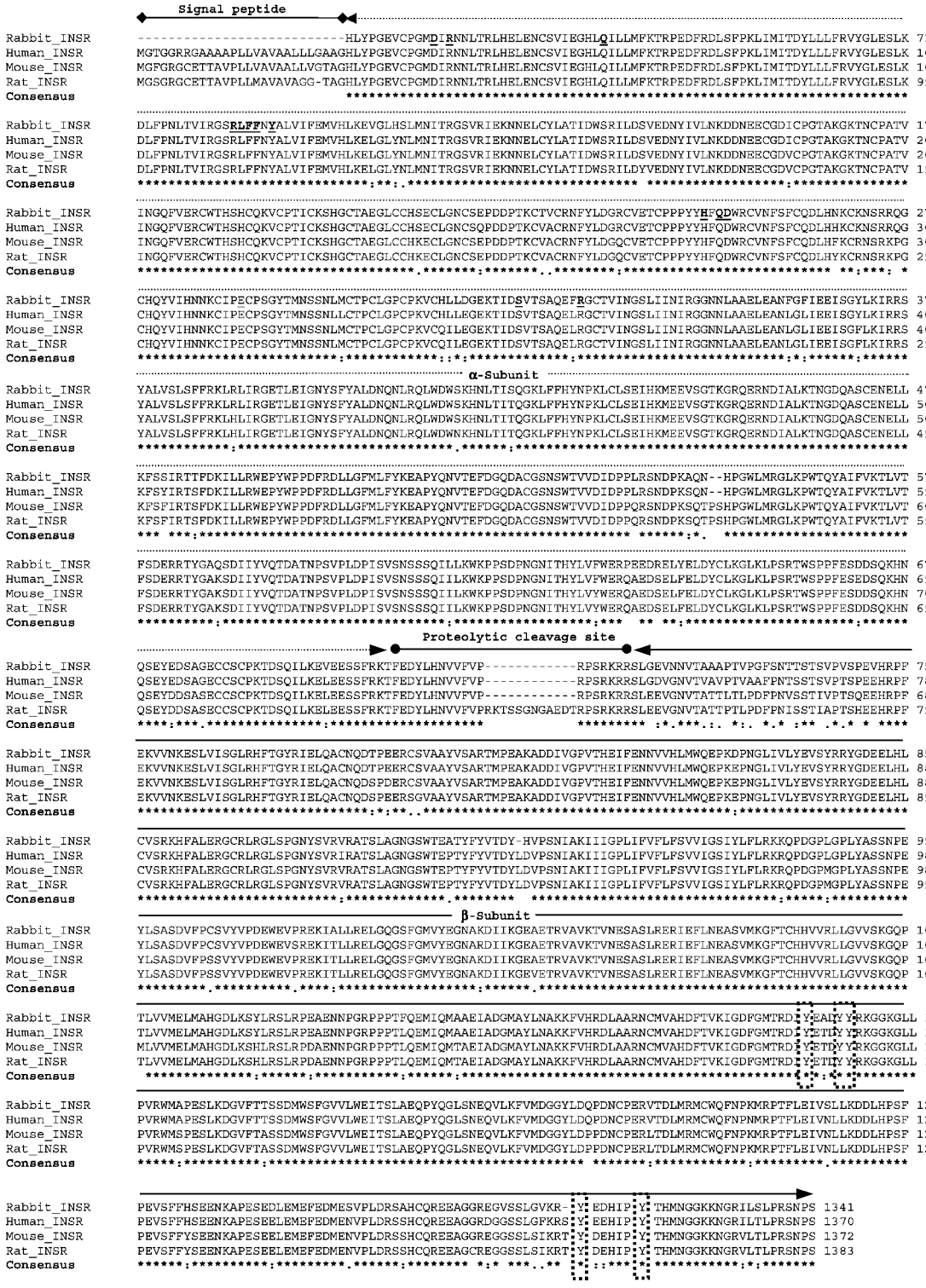

Figure 2 Deduced amino acid sequence of rabIR cDNA. The single open reading frame of rabIR cDNA is translated into a 1341 amino acid protein. Interspecies comparisons of the rabbit IR protein sequence show that the rabbit cDNA and protein sequences belong to the IR family. The multiple sequence alignments of rabbit IR protein with mouse, rat, rabbit and human (accession number X02160) sequences were performed by CLUSTAL W (1.82) multiple sequence alignment. The regions for the signal peptide, the α- and β-subunit and the proteolytic cleavage site are marked with lines and arrows. The consensus is displayed by the following symbols denoting the degree of conservation observed in each column: (*) means that the residues or nucleotides in that column are identical in all sequences in the alignment, (:) means that conserved substitutions have been observed, (.) means that semi-conserved substitutions have occurred. IR amino acids found to be involved in insulin binding are underlined in the rabbit sequence. Conserved tyrosine residues for autophosphorylation are framed in boxes in the β-subunit. INSR, insulin receptor.

Table 2 Homology of rabbit GLUT4 with GLUT4 of four other mammalian species and of rabbit IR with IR of three other mammalian species.

	cDNA			Protein		
Species	Accession number	Identity (identical bases/abs. number)	Identity (%)	Accession number	Identity (identical aa/abs. number)	Identity (%)
Homo sapiens GLUT4	M91463	1424/1549	91	M20747	494/509	97
Equus caballus GLUT4	AF531753	1394/1531	91	AF531753	492/509	96
Mus musculus GLUT4	AB008453	1341/1531	87	AB008453	490/509	96
Rattus norvegicus GLUT4	X14771	1339/1531	87	D28561	491/509	96
Homo sapiens IR	X02160.1	3595/4031	89	X02160	1287/1343	95
Rattus norvegicus IR	NM01701.1	1852/2144	86	NM010658	1260/1345	93
Mus musculus IR	NM105681.1	1855/2156	85	NM017071	1256/1357	92

aa, amino acids, abs., absolute (total).

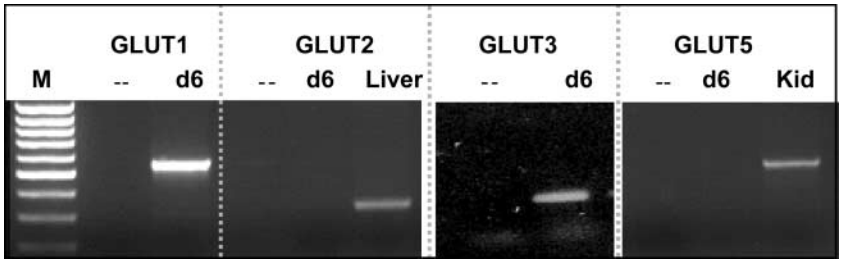

Figure 3 Messenger RNA of GLUT1, 2, 3 and 5 in expanded rabbit blastocysts. The total RNA from 10 pooled 6-day-old (d6) rabbit blastocysts was reverse transcribed and amplified with specific primers for glucose transporter 1 (GLUT1, 517 bp), glucose transporter 2 (GLUT2, 323 bp), glucose transporter 3 (GLUT3, 367 bp) and glucose transporter 5 (GLUT5, 523 bp). Resulting PCR fragments were resolved in 1.8 % agarose gel. In case of GLUT2 and GLUT5 the PCR conditions were controlled on rabbit liver (Liver) or kidney (Kid) cDNA as positive controls. For each primer combination a PCR control without cDNA template (- -) was performed.

reasonable to speculate, however, that the rabbit blastocyst does express the full complement of the IR-GLUT signal cascade. Since morulae do transcribe these genes or only in low copy numbers, the expression of IR signaling components seems to be under developmental control. The function and developmental impact of this cascade, for example its involvement in the mitogenic activity and/or energy metabolism of blastocysts, still needs to be uncovered.

Sequence analyses of the new rabbit genes prove that the rab IR encoded protein is a member of the IR family and that rabGLUT4 and 8 belong to the facilitative glucose transporters. The amino acid sequence responsible for insulin binding is almost identical with the human insulin receptor sequence. Insulin initiates its effects through interaction with the high-affinity cell surface glycoprotein receptors, consisting of two α (135 kDa) and two β (95 kDa) subunits each. The human insulin receptor is expressed in two isoforms, A and B, which are generated by alternate splicing (Ebina *et al.* 1985, Ullrich *et al.* 1985, Seino & Bell 1989). The two mature receptor proteins differ in the absence or presence of 12 aa in the C terminus of the extracellular α subunit. This insertion is encoded by the 36 nucleotide exon 11 of the receptor gene (Seino *et al.* 1989). The A and B isoforms have different tissue distributions (Moller *et al.* 1989, Seino & Bell 1989) and functional properties (Yamaguchi *et al.* 1991, 1993, Kosaki *et al.* 1995). The α subunit in the rab IR was found to be highly homologous with the α subunits of human IR isoform A without insertion of exon 11. A more detailed analysis regarding the presence of two IR isoforms in rabbit tissues was not performed in the present study.

The IR protein-tyrosine kinase has been implicated as the mediator of most, if not all, effects of insulin. IR deficiency in $IR^{-/-}$ mice caused a number of major metabolic alterations and led to the death of the newborns within one week after birth (Accili *et al.* 1996). At birth, mouse $IR^{-/-}$ mutant pups could not be distinguished from other littermates, contrary to human patients with mutations in IR, which usually have a severe intrauterine growth retardation (Lamothe *et al.* 1998).

During embryo development insulin exerts a number of important actions. (i) It accelerates the uptake of amino acids and proteins by preimplantation embryos (mouse: Dunglison & Kaye 1993; pig: Lewis *et al.* 1992), (ii) it promotes blastocyst formation and increases the number of embryonic cells (Matsui *et al.* 1995, Herrler *et al.* 1998, Augustin *et al.* 2003), in some species specifically of ICM cells (mouse: Harvey & Kaye 1990, Gardner & Kaye 1991, Smith *et al.* 1993, bovine: Sirisathien *et al.* 2003), and (iii) it prevents apoptosis (rabbit: Herrler *et al.* 1998, bovine: Augustin *et al.* 2003). The mechanism of insulin action in mammalian embryos is still unclear. Also, the effects on glucose transport and metabolism need further consideration. For mouse blastocysts it has been shown that insulin

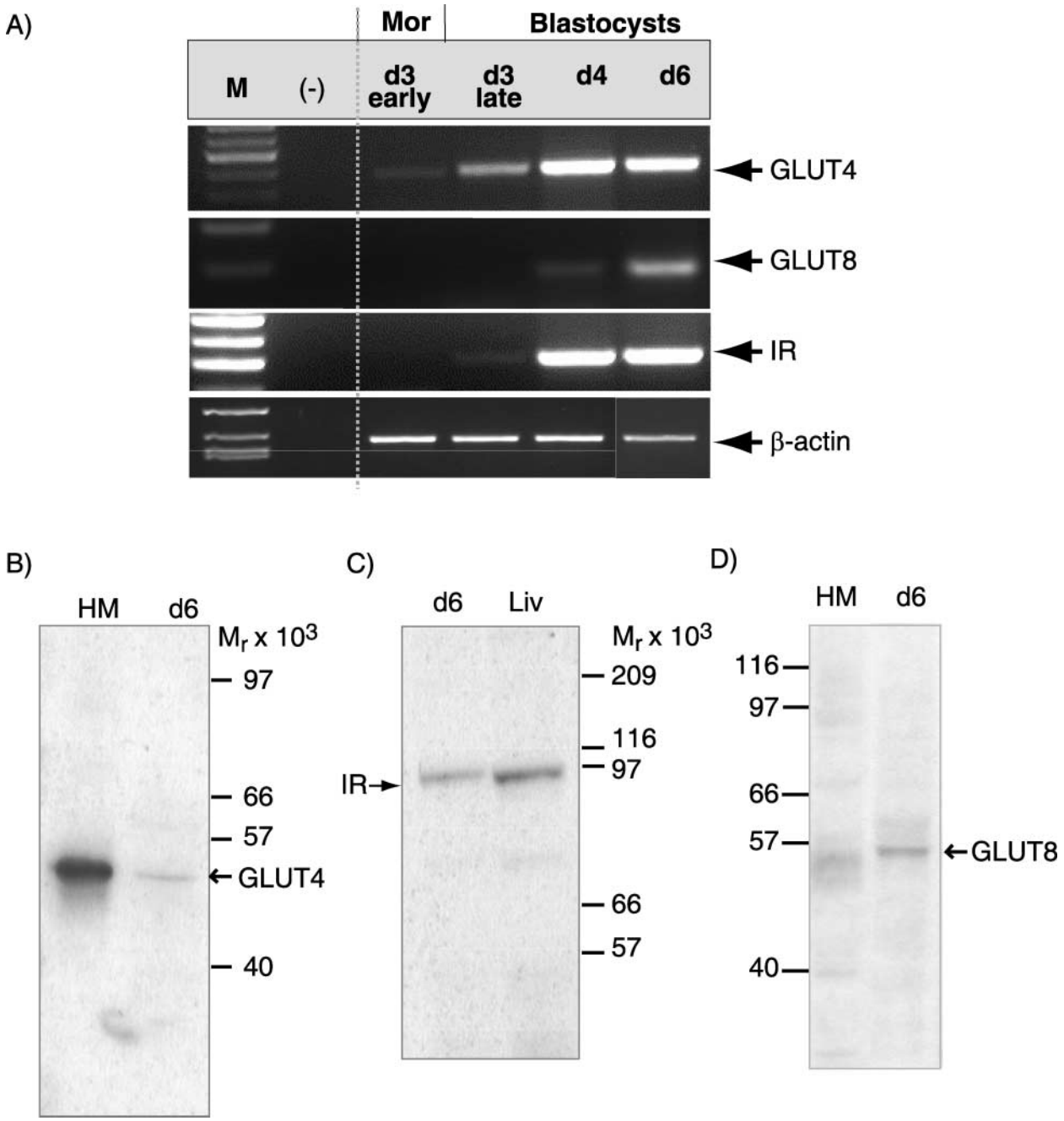

Figure 4 Messenger RNA and protein expression of GLUT4, GLUT8 and IR in rabbit preimplantation embryos. For RT-PCR (A) total RNA from 25 early day-3 morulae (d3 early), 15 day-3 late (d3 late), 15 day-4 (d4) and 5 day-6 blastocysts (d6) were reverse transcribed and amplified with specific rabbit primers for glucose transporter 4 (GLUT4, 398 bp), glucose transporter 8 (GLUT8, 170 bp), insulin receptor (IR, 497 bp) and β-actin (450 bp). The protein expression was analyzed by Western blotting in (B) (C) and (D) for GLUT4, IR and GLUT8 respectively. Total protein amount of 10 pooled rabbit day-6 blastocysts was isolated and 20 μg protein were resolved on 8% SDS-PAGE and immunoblotted with anti-GLUT4 (B), anti-β-subunit IR (C) and anti-GLUT8 (D). As controls for GLUT4, GLUT8 and IR protein, 40 μg total protein extracts of heart muscle (HM in B, D) and liver (Liv in C) were added to the blot.

and IGF-I act via IR to increase glucose uptake (Gardner & Leese 1988, Harvey & Kaye 1991, Pantaleon & Kaye 1996, Carayannopoulos *et al.* 2000). Compared with IGF-I, glucose uptake, measured by uptake of 3-o-methyl-D-glucose, was clearly more stimulated by the IGF-IR/IGF-I system than by insulin (Pantaleon & Kaye 1996). An insulin-dependent increase in glucose uptake via translocation of GLUT4, as described for differentiated myocytes and adipocytes, has not yet been shown in preimplantation embryos. First screening studies of glucose transporter isoform expression in mammalian preimplantation embryos, performed in mice, failed to prove

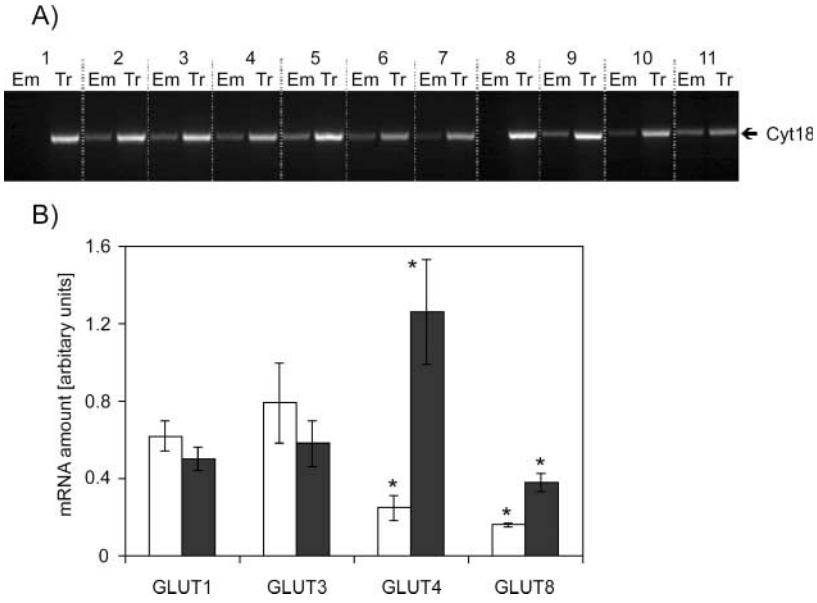

Figure 5 Relative amounts of GLUT1, GLUT3, GLUT4 and GLUT8 mRNA in the trophoblast and embryoblast of rabbit blastocysts. (A) The relative amounts of GLUT1, 3, 4 and 8 transcripts were quantified by semiquantitative RT-PCR in isolated trophoblast (Tr) and embryoblast (Em) of 11 day-6 (d6) blastocysts (1–11) as described in Materials and Methods. The housekeeping gene, β-actin, was used as an internal standard. The tissue separation was controlled by PCR amplification of cytokeratin 18 (cyt 18) as a specific trophoblast marker. In all studied individuals, cytokeratin 18 mRNA was clearly higher in trophoblast than in embryoblast cells, proving the stable and reliable separation of both cell lineages. (B) The expression of GLUT4 and GLUT8 mRNA was significantly higher in the trophoblast (solid bars) than in the embryoblast (open bars; $^{*}P < 0.05$), while no cell-lineage effects were found for GLUT1 and GLUT3.

GLUT4 expression (Hogan *et al.* 1991, Aghayan *et al.* 1992). The expression of the insulin responsive GLUT4 isoform has been shown for the first time in bovine blastocysts (Navarrete Santos *et al.* 2000). During bovine preimplantation development GLUT4 was expressed in 8-day-old *in vitro*-derived blastocysts and in day 14 elongated *in vivo*-grown blastocysts (Augustin *et al.* 2001). Another isoform demonstrated to be insulin responsive in blastocysts, GLUT8, has recently been described in mouse blastocysts (Carayannopoulos *et al.* 2000). GLUT8 was found to change its intracellular localization and to be involved in increased glucose uptake after insulin treatment in murine blastocysts (Carayannopoulos *et al.* 2000). Inhibition of GLUT8 translation and translocation enhanced the rate of apoptosis in mouse blastocysts (Pinto *et al.* 2002). Functional studies in other species expressing this isoform (bovine, rabbit) are needed to clarify the exact role of GLUT8 for embryo development. It is remarkable and may be indicative of different functions of GLUT isoforms in different species that mice and rabbits express GLUT8 only at the blastocyst stage (Carayannopoulos *et al.* 2000, present study) while during bovine embryogenesis GLUT8 mRNA is present from oocytes throughout preimplantation development (Augustin *et al.* 2001).

In mouse blastocysts, the high affinity isoform 3, localized in the outer apical cell membrane of the trophoblast cells, mediates glucose transport from the uterine fluid into the blastocyst (Pantaleon *et al.* 1997). GLUT1, situated at the basal and basolateral trophoblast cell membranes, accomplishes the supply of the ICM (Pantaleon & Kaye 1998). In the present study, both isoforms were also found in the rabbit, pointing to a similar mechanism for glucose uptake and supply as in mice. The experimental proof for GLUT2 expression in mammalian preimplantation embryos is controversial. Transcripts were reported for mice 8-cell/compacted morulae (Schultz *et al.* 1992) and blastocysts (Harvey & Kaye 1991). The protein was found in blastocysts (Aghayan *et al.* 1992). However, in the present study, as in others (mouse: Morita *et al.* 1992, Tonack *et al.* 2004, cattle: Augustin *et al.* 2001), GLUT2 expression could not be verified in rabbit blastocysts.

The diversity of glucose transporter expression in mammalian embryos presumably reflects the importance of glucose as the major metabolic energy substrate. Diverse glucose transporters have evolved to allow an efficient, stage- and cell-specific uptake and utilization. Glucose concentration in human serum is maintained around 5 (4.4 to 6.6) mM. The early human embryo is exposed to lower (3.15 mM; Gardner *et al.* 1996) or almost the same (Casslen & Nilsson 1984) glucose concentrations *in utero* as those present in serum. The oxygen level in this organ, however, is significantly lower than in blood (Fischer & Bavister 1993). This specific constellation and its physiological implications may have led to the more complex furnishing with glucose transporters in embryos than in differentiated muscle, fat or neuronal cells. Considering glucose uptake by preimplantation embryos, which is in a pmol per embryo per hour range (rabbit: Robinson *et al.* 1990, rat: Brison & Leese 1994, mouse: Martin & Leese 1999, bovine: Donnay & Leese 1999), and assuming a

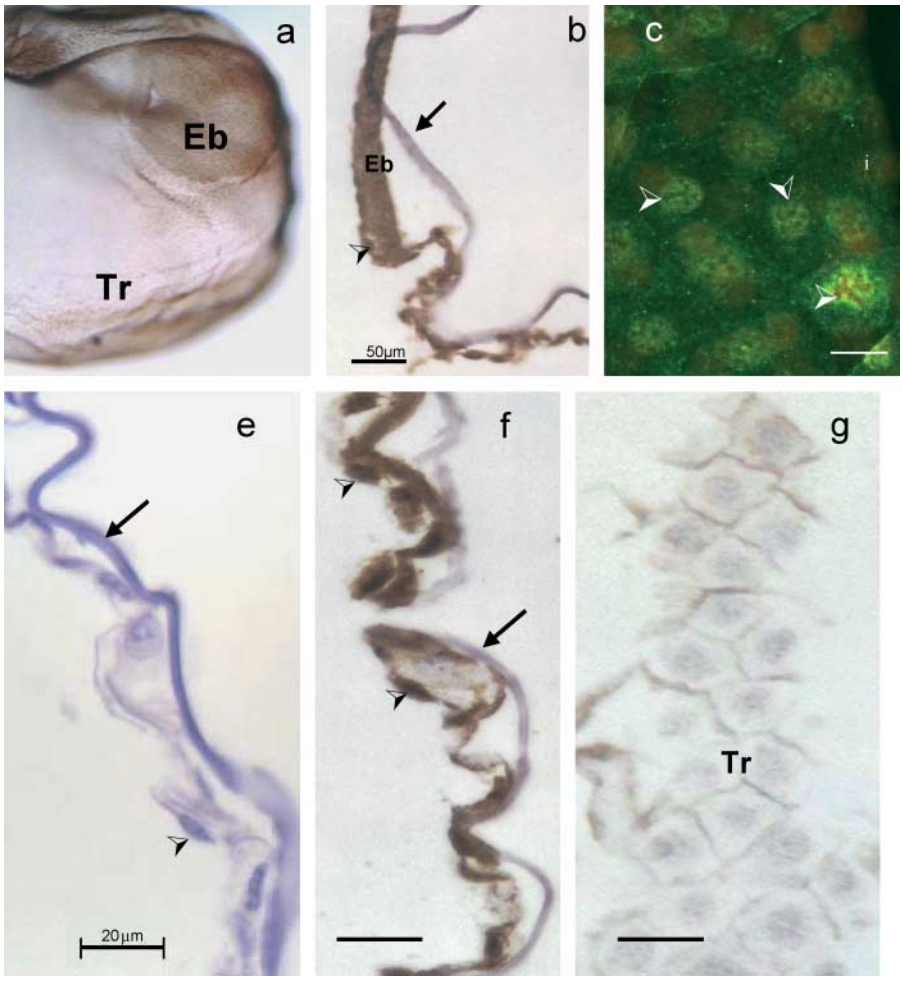

Figure 6 Localization of GLUT4 in day-6 (d6) blastocysts. Whole mount IHC (a) of a 6-day-old blastocyst shows positive staining for GLUT4 in embryoblast (Eb) and trophoblast (Tr) cells. The GLUT4 protein was visualized by peroxidase-DAB reaction (brown color in a, b, f, g). The nuclei were counterstained with hematoxylin (e, f, g). The subcellular localization was investigated by conventional light microscopy on paraffin sections (b, e, f, g) and whole mount confocal microscopy (c) with a fluorescence detection for GLUT4 (green color) and nuclear counterstaining with 7-aminoactinomycin (in red, c). The embryoblast cells (Eb) revealed an intense cytoplasmic staining for GLUT4 (b), whereas the outer trophoblast layer (Tr) was stained in the cytoplasm (f) and cell membranes (g). The extraembryonic endoderm cells show an intense cytoplasmic and perinuclear staining (e, f). Nuclei are marked with an arrowhead in b, c, (embryoblast cells) and e, f (extraembryonic endoderm cells). The neozona is marked with an arrow. The bar in (b) = 50 µm, and bars in c, d, e, f and g = 20 µm.

relatively constant replenishment of intraluminal glucose stores by fresh transudates, then a sufficient supply of this nutrient can be postulated under physiological conditions *in utero*, even for multiovulatory species (H J Leese, personal communication).

Different results have been reported on the developmental implications of an altered glucose supply for embryos. In mice, glucose deprivation affected trophoblast cells more than the ICM. In deprived blastocysts cell numbers in the trophoblast, but not in the ICM, were statistically significantly decreased (Leppens-Luisier *et al.* 2001). Also high glucose concentrations are reported to exert detrimental effects on embryo development. Blastocysts from diabetic rats showed an impaired growth of both cell types with cell numbers being more affected in the ICM than in the trophoblast (Dufrasnes *et al.* 1993). Blastocysts from diabetic mice have lower intraembryonic glucose concentrations (Moley *et al.* 1998) and an increase in the rate of apoptosis (Chi *et al.* 2000).

GLUT4 has been localized in the cytoplasm of trophoblast, embryoblast and extraembryonic endoderm cells in close association with membranes and nuclei. A GLUT4 shuttling is well investigated in insulin responsive adipocytes and myocytes (for review see Zorzano *et al.* 1998, Watson & Pessin 2001). In these cells GLUT4 is associated with cytoplasmic vesicles (so-called GLUT4 storage vesicles, GSV; Rea & James 1997) in several morphologically distinct localizations. Ultrastuctural studies have shown that GLUT4 is present in tubulovesicular structures distinct from lysosomes. As in rabbit blastocysts in the present study, GLUT4 has also been found in the perinuclear compartment which is in close vicinity to the trans-Golgi network (Hudson *et al.* 1992, Jhun *et al.* 1992, Lee *et al.* 1999). Insulin increases the rate of GLUT4 translocation

from the cytoplasm to the cell membrane so that the proportion of GLUT4 at the cell surface increases from <10% in the absence of insulin to 35 to 50% in its presence (adipocytes: Bogan *et al.* 2001). In the rabbit blastocyst, the cells in the outer trophoblast layer showed a comparable subcellular localization of GLUT4 as insulin sensitive tissues. The membrane localization of GLUT4 in trophoblast cells can be regarded as good evidence that the transporter is active in rabbit blastocysts. The more intense staining of the embryoblast and the extraembryonic endoderm may indicate another functional state or a different function of GLUT4. The perinuclear localization and the association of GLUT4 with the nuclear membranes, not described in adult tissues so far, support the view of a function of GLUT4 other than glucose transport in embryonic cells. Recently, a nuclear localization has been described for GLUT1 in mouse oocytes and early cleavage stages (Pantaleon *et al.* 2001) stressing the need for a more detailed analysis of potential functions of the various glucose transporter isoforms during early embryogenesis.

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