RESEARCH COMMUNICATION

Biochemical and immunocytochemical localization of the 'GLUT5 glucose transporter' in human skeletal muscle

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Using biochemical and immunocytochemical techniques, we have assessed both the protein expression and the cellular localization of the GLUT5 transporter in human skeletal muscle. Human muscle membranes, prepared by subcellular fractionation, were subjected to SDS/PAGE and Western-blot analyses using antiserum raised against a specific C-terminal amino acid sequence of the human GLUT5 transporter. GLUT5 was detected as a discrete 49 kDa protein band in a plasma-membrane-enriched fraction prepared from either soleus or gracilis muscle. In contrast, GLUT5 protein was not detectable to any significant extent in fractions which were devoid of muscle plasma membranes (mean GLUT5 abundance in intracellular fractions from three muscle preparations amounted to ~10% of that in the plasma-membrane-enriched fraction). Immunofluorescence studies using cryostat sections of human triceps muscle supported the biochemical observations and revealed that GLUT5 antibody selectivity labelled the plasma membrane of muscle cells. This immuno-labelling was significantly suppressed after tissue incubation with antiserum in the presence of a 14-amino-acid synthetic peptide corresponding to a specific C-terminus sequence of human GLUT5. These results indicate that human skeletal muscle expresses the GLUT5 transporter and that it is specifically localized to the plasma membrane, where it may participate in regulating hexose transfer across the sarcolemma.

INTRODUCTION

Skeletal muscle represents the principal site of glucose disposal in the fed state (DeFronzo *et al.*, 1981; Baron *et al.*, 1988). The rate of glucose utilization, however, is heavily dependent on the transfer of glucose across the sarcolemma, which represents the rate-limiting step in muscle glucose metabolism (Klip & Paquet, 1990). The transmembrane transport of glucose in mammalian cells is mediated by a family of structurally related facilitative glucose transporters that have been designed GLUT1 to GLUT5 (based on the chronological order in which they were identified) (for reviews see Bell *et al.*, 1990; Thorens *et al.*, 1990). Biochemical and immunocytochemical studies have demonstrated that in rat skeletal muscle GLUT1 (the erythrocyte/Hep G2/rat brain transporter) exists and is predominantly localized to the plasma membrane (PM) (Handberg *et al.*, 1992; Marette *et al.*, 1992b), whereas GLUT4 (the insulin-responsive glucose transporter of muscle and fat) is found largely in the intracellular compartment, but also to a significant extent in the PM (Douen *et al.*, 1990; Hirshman *et al.*, 1990; Marette *et al.*, 1992b). The presence of other glucose transporter isoforms in skeletal muscle (especially human skeletal muscle) has not been comprehensively addressed at the molecular level. The GLUT5 facilitative transporter was recently shown to be expressed and localized to the brush-border membrane (BBM) of human jejunum, where it may act in concert with the Na⁺-dependent glucose co-transporter in facilitating uptake of dietary sugars (Davidson *et al.*, 1992). Here we present novel biochemical and immunocytochemical evidence that the jejunal-type GLUT5 transporter is also present in human skeletal muscle and that it is exclusively found in the muscle PM. The possible functional role of GLUT5 and the implications for muscle hexose metabolism are discussed.

MATERIALS AND METHODS

Human skeletal muscle

Human soleus muscle (20–30 g) was obtained from patients undergoing elective limb amputation (Kings Cross Hospital, Dundee). Excised muscle was routinely obtained from a site with no evidence of necrosis; only skeletal muscle that was judged as being well perfused, oxygenated and which subsequently appeared histologically normal was used for subcellular fractionation. Skeletal muscle obtained at amputation surgery was immediately frozen in liquid N₂ and stored at –80 °C before fractionation. Biopsy samples of gracilis muscle (1–4 g) were obtained from patients undergoing testing for malignant hyperthermia (a disorder of intracellular Ca²⁺ handling, at the Toronto Hospital, Toronto). Cross-sections of triceps muscle were obtained from the Neuropathology Laboratory, Hospital For Sick Children (Toronto) from muscle biopsies taken from 10–12-year-old boys with suspected structural myopathy that subsequently were diagnosed as normal. All procedures used to procure human skeletal muscle met the guidelines of the local ethics committee.

Subcellular fractionation of human skeletal muscle

The subcellular fractionation method used for isolating PM and intracellular membranes from human skeletal muscle was based on a well-established procedure previously described for rat skeletal muscle, with some minor modifications (Klip *et al.*, 1987; Ahmed *et al.*, 1990b). The exact fractionation details have previously been reported (Ahmed *et al.*, 1990a). Briefly, the procedure involves Polytron homogenization of 20–30 g of skeletal muscle, followed by a series of differential centrifugations in low-ionic-strength sucrose buffers to isolate crude muscle

Abbreviations used: PM, plasma membrane; BBM, brush-border membranes; PBS, phosphate-buffered saline; CB, cytochalasin B.

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membranes. Further fractionation of the crude membranes is achieved by discontinuous sucrose-gradient [25%, 30% and 35% (w/w) sucrose] ultracentrifugation. Membranes on each sucrose layer are washed and collected by high-speed centrifugation. Human muscle membranes isolated on the 25%-sucrose layer are enriched in 5'-nucleotidase and K⁺-stimulated *p*-nitrophenyl phosphatase activities (PM markers) by 12–15-fold and depleted in Ca²⁺-ATPase activity by 1.6-fold relative to the starting muscle homogenate (Ahmed *et al.*, 1990a). The enrichment in PM marker-enzyme activities of the human muscle 25%-sucrose fraction resembles that obtained by the same procedures with rat skeletal muscle (Klip *et al.*, 1987; Marette *et al.*, 1992a). The PM fraction obtained by fractionating rat skeletal muscle has also been extensively characterized immunologically by using antibodies to specific PM proteins (Marette *et al.*, 1992a) suggesting that human muscle membranes isolated in the cognate sucrose fraction were predominantly of PM origin: we therefore denoted this fraction as the PM. The human 30%-sucrose fraction is considered to be an intermediate fraction in which PM and membranes of intracellular origin are not as completely resolved, on the basis of results from the rat muscle preparation (Ahmed *et al.*, 1990a; Douen *et al.*, 1991; Marette *et al.*, 1992a). The human 35%-sucrose fraction may predominantly contain intracellular membranes, since it showed no detectable enrichment of PM markers or of sarcoplasmic Ca²⁺-ATPase activity, which is predominantly recovered in the gradient pellet-associated membrane fraction (A. Ahmed & M. J. Rennie, unpublished work). A more detailed characterization of this fraction awaits further investigation, but the equivalent 35%-sucrose fraction obtained from rat muscle is highly enriched with an intracellular complement of GLUT4 glucose transporters (Douen *et al.*, 1990) and is thus termed the intracellular membrane fraction. In response to insulin treatment there is a marked decrease in rat GLUT4 abundance in this intracellular-membrane compartment, with an concomitant increase in GLUT4 content in the PM. GLUT4 translocation from the intracellular membrane to the PM is thought to constitute the basis by which rat skeletal muscle stimulates glucose uptake in response to insulin action (Klip *et al.*, 1987; Hirshman *et al.*, 1990; Douen *et al.*, 1991).

A mini preparation for isolating skeletal-muscle membranes

In an attempt to scale down the requirement for large quantities of skeletal muscle for fractionation studies, we have recently initiated studies to develop a fractionation procedure for isolating skeletal-muscle membranes from surgical muscle biopsies (where tissue availability is often limited to less than 1 g). Briefly, 0.5–1 g of human gracilis muscle (obtained during elective limb surgery) was homogenized. Crude membranes were treated with a high-salt (600 mM-KCl) buffer, centrifuged and resuspended in buffer containing 100 mM-KCl before loading on to a discontinuous sucrose gradient [25%, 30%, 35% and 40% (w/w) sucrose]. Membranes recovered from each sucrose gradient (after ultracentrifugation for 16 h at 190 000 *g*) were removed, washed, resuspended in KCl-containing buffer and stored at –20 °C until required for Western-blot analyses.

Anti-GLUT5 antiserum

Isoform-specific antiserum to GLUT5 was purchased from East Acres Biologicals, Southbridge, MA, U.S.A. (1:500 dilution) raised against a specific peptide sequence (KEELKEL-PPVTSEQ) of the C-terminus of human GLUT5. We performed additional tests to assess the specificity of this antiserum. Since the last 25 C-terminal amino acid residues of human GLUT4 and rat GLUT4 are identical (Burant *et al.*, 1991), we used rat tissue fractions rich in GLUT4 to rule out cross-reactivity of the

GLUT5 antibody with GLUT4. Western-blot analyses revealed that anti-GLUT5 antiserum did not immunoreact with rat GLUT4 present in intracellular membrane fractions prepared from either rat adipocytes or rat skeletal muscle, underscoring the isoform specificity of the antibody used in the present study.

Western-blot analyses

As a positive control for GLUT5 reactivity, human BBM were prepared from jejunal biopsies (obtained with a Crosby capsule in children from infant to adolescent age that were tested negative for coeliac disease) by some modifications of a previously reported procedure (Shirazi-Beechey *et al.*, 1990). BBM (1 µg) and muscle membrane fractions (20 µg each from soleus muscle or 4 µg each from gracilis muscle) were subjected to SDS/PAGE on precast 10% polyacrylamide gels (Novex, San Diego, CA, U.S.A.) as described by Laemmli (1970), electrophoretically transferred to PVDF (polyvinylidene difluoride) sheets (0.2 µm pore size) and blocked with 3% BSA/Tris/saline/Tween 20 for 1 h at room temperature. These were incubated overnight at 4 °C with rabbit anti-GLUT5 antibody with or without GLUT5 peptide (100 µg/ml). ¹²⁵I-labelled Protein A (1 µCi/ml) was used for detecting anti-GLUT5. The PVDF membranes were washed three times in Tris/saline/Tween 20 for 15 min, air-dried and autoradiographed on to Kodak XAR-5 film.

Immunocytochemistry

Frozen cross-sections (7 µm thickness) of normal human triceps muscle were air-dried for 30 min, subsequently fixed in cold acetone and then blocked for 1 h with 5% (v/v) goat serum/phosphate-buffered saline (PBS; 140 mM-NaCl/10 mM-Na₂HPO₄, pH 7.4) at room temperature. Muscle sections were then either treated with anti-GLUT5 antibody (at a dilution of 1:100 with PBS) or with anti-GLUT5 antibody solution pre-absorbed with GLUT5 peptide (500 µg/ml, overnight at 4 °C with mixing by rotation). Sections were serially washed (3 × 10 min) in PBS and incubated with fluorescein isothiocyanate-labelled anti-rabbit IgG for 1 h at room temperature and washed again (3 × 10 min) before mounting in 50% (v/v) glycerol/1% *n*-propyl gallate, viewing under a Nikon inverted fluorescence microscope (magnification × 1000) and photographed on Tri-X Pan (Kodak, ISO 400) film.

RESULTS

GLUT5 glucose-transporter expression and localization in human skeletal-muscle membranes prepared from soleus muscle

To assess whether the GLUT5 transporter was expressed in human skeletal muscle, we performed SDS/PAGE and Western-blot analyses of isolated muscle membranes, using anti-GLUT5 antiserum. Fig. 1 shows a representative immunoblot obtained from three individual preparations of human soleus muscle. On SDS gels a single discrete protein band of 49 kDa that was present in the PM-enriched fraction (lane 2) reacted with anti-GLUT5 antiserum. A very minor reaction could also be observed for a protein band of the same molecular size in the 30%-sucrose fraction (lane 3), which probably arose owing to the small amount of PM present in this fraction. By contrast with the PM fraction, we were unable to detect any GLUT5 reaction in the 35%-sucrose fraction or in membranes isolated in the gradient pellet or in crude membranes (lanes 4–6). Laser scanning densitometry confirmed that GLUT5 abundance in PM-depleted fractions was negligible, on the basis of results from three individual human muscle preparations. Values for GLUT5 abundance in membranes in the 30%- and 35%-sucrose fractions, in the gradient pellet and in crude membranes, expressed

Fig. 1. Western blot of GLUT5 protein in muscle membranes isolated from human soleus muscle

Lanes 1–6 represent membrane samples incubated with anti-GLUT5 antiserum. Lanes 7–12 represent the same samples incubated with anti-GLUT5 antiserum pre-absorbed with 100 μ g of GLUT5 peptide/ml. Samples (20 μ g) of all muscle membranes were loaded. Lanes 1 and 7, 1 μ g of jejunal BBM (positive control); lanes 2 and 8, PM (25%-sucrose fraction); lanes 3 and 9, membranes isolated from 30%-sucrose fraction; lanes 4 and 10, 35%-sucrose fraction; lanes 5 and 11, membranes isolated in the sucrose-gradient pellet; lanes 6 and 12, crude membranes.

Fig. 2. Immunoblot of GLUT5 in muscle membranes isolated from human gracilis muscle by a small-scale high-salt fractionation procedure

SDS/PAGE and Western blots were performed as described in the Materials and methods section; 4 μ g samples of all muscle membranes were loaded. Lane 1, PM (25%-sucrose fraction); lane 2, 30%-sucrose fraction; lane 3, 35%-sucrose fraction; lane 4, 40%-sucrose fraction; lane 5, membranes isolated in sucrose-gradient pellet; lane 6, crude membranes.

relative to the PM fraction (normalized to 1.0) were: 30%-sucrose fraction, 0.24 ± 0.17 ; 35%-sucrose fraction, 0.1 ± 0.005 ; membranes in gradient pellet, 0.01 ± 0.005 ; crude membranes, 0.006 ± 0.006 (means $\pm$ S.E.M.).

To assess the possibility that the signal observed in the PM fraction was non-specific, we immunoblotted purified BBM prepared from human jejunal biopsies with anti-GLUT5 antiserum as a positive control. In BBM a broad but strong GLUT5 signal with a front corresponding to a molecular size of 49 kDa was observed (Fig. 1, lane 1), which ran adjacent to a protein band detected in the muscle PM fraction (lane 2). When BBM and the muscle PM fractions were immunoblotted under identical conditions but with pre-absorbed anti-GLUT5 antiserum, the bands previously observed were protected from detection (Fig. 1, lanes 7 and 8). Lanes 3–6 and 9–12 represent membranes isolated from the 30%-sucrose fraction, the 35%-sucrose fraction, sucrose-gradient pellet and crude membranes. These showed no reactivity with anti-GLUT5 antiserum. This provides strong evidence for the existence of GLUT5 in human skeletal muscle.

GLUT5 localization in muscle membranes from gracilis muscle prepared by a mini-preparation

The intriguing finding that GLUT5 was specifically found in a PM-enriched fraction led us to test whether muscle membranes prepared from a different muscle type and by an alternative fractionation procedure would yield similar results. Muscle

membranes prepared from about 1 g of skeletal muscle in a high-ionic-strength buffer were immunoblotted for GLUT5 after SDS/PAGE. Fig. 2 shows a Western blot from a single muscle preparation. As observed with membranes prepared from soleus muscle, anti-GLUT5 antiserum reacted strongly with a protein band in a PM-enriched fraction corresponding to a molecular size of 49 kDa. These results furthermore indicated that the GLUT5 immunoreactive band is an integral membrane protein that is not extracted by high-salt treatment.

Immunocytochemical localization of GLUT5 in human skeletal muscle

To rule out the possibility that the observed biochemical localization of GLUT5 in the muscle PM fraction could be attributed to membranes of non-muscle cell types, we performed immunocytochemical studies using cryo-sections of human triceps muscle. Fig. 3(a) depicts GLUT5 immunofluorescence in a cross-section of triceps muscle and shows that the anti-GLUT5 antibody specifically labels the plasma membrane of muscle fibres (Fig. 3b depicts the same field under phase contrast). This specific PM labelling was noticeably depressed when muscle sections were incubated with pre-absorbed serum (Figs. 3c and 3d for the same field under phase contrast), suggesting that the GLUT5 transporter was localized in the PM. In separate experiments, as a negative control, we preincubated muscle sections with PBS alone (i.e. without primary antibody), followed by incubation with the fluorescent secondary antibody, and observed no fluorescent labelling of the muscle sections (results not shown).

DISCUSSION

Skeletal muscle represents a major site of disposal in the post-prandial state. The influx of glucose into skeletal muscle has been shown, in animal and cell studies, to be a process that is sensitive to hormones (insulin and insulin-like growth factor 1) (Rennie *et al.*, 1985; Bilan *et al.*, 1992), surrounding glucose concentrations (Koivisto *et al.*, 1991), muscle contraction (Douen *et al.*, 1990) and oxygenation status (Bashan *et al.*, 1992). The ability of skeletal-muscle cells to regulate glucose uptake under such circumstances is thought to arise largely through subcellular redistribution of glucose transporters (e.g. GLUT4) in response to acute stimuli (e.g. insulin) or by regulating transporter biosynthesis (e.g. GLUT1 and GLUT4) in response to more chronic stimuli (e.g. long-term insulin treatment, hypoxia etc.) requiring long-term adaptive measures by skeletal muscle (Klip & Marette, 1992; Wertheimer *et al.*, 1991).

In contrast, our knowledge of the subcellular distribution of glucose transporters in human skeletal muscle and their regulation in response to physiological and pathological stimuli is at present very limited. Major reasons for this are due to difficulties in obtaining sufficient quantities of human skeletal muscle for study, and technical problems encountered in fractionating relatively small amounts of muscle biopsy material. Some information, however, is available. Pederson *et al.* (1990) and Garvey *et al.* (1991) were able to demonstrate that the GLUT4 glucose transporter is present in skeletal muscle of lean and NIDDM (non-insulin-dependent diabetes mellitus) patients. These observations were based on studies with unfractionated membranes of human skeletal muscle obtained by biopsy, and can therefore only be used to assess the total content of GLUT4. Such an approach does not convey information about the subcellular distribution of GLUT4 and may not be sensitive in detecting a particular protein isotype, should it reside in a discrete cellular compartment in skeletal muscle. The latter

Fig. 3. Immunofluorescence localization of human GLUT5 in triceps muscle

Muscle sections were incubated with (a) rabbit anti-GLUT5 antiserum or (c) pre-absorbed rabbit anti-GLUT5 antiserum (with 500 μ g of GLUT5 peptide/ml), followed by incubation with fluorescein isothiocyanate-conjugated rabbit IgG as described in the Materials and methods section. Phase-contrast pictures of the respective sections are shown in (b) and (d). Magnification $\times 1000$.

appears especially true for GLUT5, which could not be detected in crude human muscle membranes.

The present work reports the results of the use of two different fractionation procedures utilizing large and small quantities of human skeletal muscle to isolate PM and intracellular muscle membranes of relatively high enrichment. PM prepared by either fractionation procedure apparently contain the GLUT5 glucose transporter. A separate immunocytochemical approach also localizes GLUT5 to the PM in a third distinct muscle type, the triceps muscle, a finding that complements and strengthens the biochemical observations. Our finding that GLUT5 protein is present in human skeletal muscle is consistent with the observation that low levels of GLUT5 mRNA could be detected in human skeletal muscle (Kayano *et al.*, 1990). The latter study, however, did not provide information about the half-life of the mRNA or the efficiency with which it may be translated, and hence whether the protein product would be expressed to any significant extent in skeletal muscle. Those authors speculated that the GLUT5 protein might be regulated by insulin. If GLUT5 transporter activity is acutely influenced by insulin, then its unique localization within the PM of skeletal muscle suggests that it may play an important functional role in regulating hexose delivery during circumstances when circulating insulin levels are elevated (e.g. after a meal).

In rat skeletal muscle GLUT1 has been shown to be present in the PM by both biochemical and immunocytochemical studies (Handberg *et al.*, 1992; Marette *et al.*, 1992b). The observation that GLUT1 is restricted to the PM has led to the belief that

GLUT1 may be involved in maintaining normal basal glucose uptake, whereas intracellular GLUT4 provides a regulatable component that can be recruited to the PM in response to acute stimuli such as insulin (Cushman & Wardzala, 1980; Douen *et al.*, 1990; Hirshman *et al.*, 1990). The issue of whether human skeletal muscle expresses GLUT1 mRNA remains questionable (Flier *et al.*, 1987; Bell *et al.*, 1990), but if the protein product is not present, then basal glucose uptake may be maintained by a different glucose transporter that may share certain similarities with the rat muscle GLUT1. In this respect GLUT5 in human skeletal muscle may be an appropriate candidate. If GLUT5 shares functional similarities to GLUT1, then its abundance in the PM would have widespread implications for glucose metabolism in pathological circumstances such as diabetes, hypoxia, muscle injury and sepsis, conditions during which GLUT1 biosynthesis is normally elevated in rat skeletal muscle (Block *et al.*, 1991; Zeller *et al.*, 1991; Dimitrakoudis *et al.*, 1992; Bashan *et al.*, 1992). Whether GLUT5 expression is affected under such situations is unknown, but such possibilities could be examined by investigating GLUT5 content in the PM fraction of skeletal muscle obtained from NIDDM patients and from patients who have suffered muscle injuries.

Another plausible hypothesis, which as yet remains untested, is that GLUT5 may be a transporter of wide substrate specificity that may be responsible for mediating uptake of other hexoses, as has been suggested by Davidson *et al.* (1992). Human skeletal muscle has been shown to take fructose from the blood, and this requirement appears to increase after exercise (Ahlborg &

Bjorkman, 1990). The identity of a specific fructose transporter in human skeletal muscle remains at present unknown, but we have performed preliminary studies showing that, in addition to D-glucose, PM prepared from human skeletal muscle display D-fructose-protectable cytochalasin B (CB) binding (the binding assay gives a quantitative measure of the total number of membrane glucose transporters; Klip *et al.*, 1987). D-Glucose-protectable CB binding in the PM fraction was 6.32 pmol/mg of protein; 30% of the D-glucose-protectable CB binding in this fraction was also protected by D-fructose (1.85 pmol/mg of protein; values are means of two preparations each in quintuplicate). Importantly, D-fructose did not confer any measurable protection against CB binding in muscle membrane fractions depleted of plasma membrane (i.e. the 35%-sucrose fraction) or in erythrocyte membranes (which do not contain detectable levels of GLUT5 protein, but only GLUT1). The observed pattern of D-fructose-protectable CB binding correlates well with the subcellular distribution of GLUT5 in human skeletal muscle, but we are unable as yet to attribute this fructose protection of CB binding to GLUT5. However, it is noteworthy that Gould *et al.* (1991) have shown that human GLUT2 (but not GLUT1) expressed in *Xenopus* oocytes displays D-fructose-inhibitable 2-deoxyglucose uptake. The ability of GLUT5 to transport other hexoses was not addressed in that study, but, considering that GLUT2 and GLUT5 are both abundantly expressed in the small intestine, where they are likely to encounter a much broader spectrum of dietary sugars, such a possibility cannot be excluded. Although the substrate specificity of GLUT5 at present remains speculative, Burant *et al.* (1992) have recently suggested that the GLUT5 transporter expressed in *Xenopus* oocytes is a fructose transporter, on the basis of observations that it transports fructose in preference to glucose. Future work in our laboratories will attempt to assess whether GLUT5 can also transport other hexoses in skeletal muscle, an issue that may be resolved by a more comprehensive analyses using GLUT5 expression and transfection systems.

In conclusion, the present results provide strong evidence for the existence of the GLUT5 facilitative hexose transporter in human skeletal muscle and its specific localization in the muscle PM. The exact functional role of GLUT5 in skeletal muscle is at present unknown, but the experimental protocols described will aid in identifying how its expression is regulated in skeletal muscle of patients suffering from impaired hexose uptake.

We are grateful to Mr. A. Jain, F.R.C.S., and the staff of the orthopaedic theatre, Kings Cross Hospital, Dundee, and to Dr. B. A. Britt, Toronto Hospital, Toronto, for providing us with muscle samples. We also thank Dr. P. Sherman (Hospital For Sick Children, Toronto) for providing us with small-bowel biopsies. This work was supported by grants from the M.R.C. (U.K.) and M.R.C. (Canada). A.A. was supported by the Scottish Home and Health Department, A.G. by the Canadian Diabetes Association, Y.M. by The Hospital For Sick Children Research Foundation, and A.M. by M.R.C. (Canada). H.S.H. is an International Human Frontier Science Program Research Fellow.

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