Original Articles

Peptide-Based Radioimmunoassay Specific for GLUT1 Glucose Transporter

KATSUNORI TSUKUDA, TOMOICHIRO ASANO, JIANN-LIANG LIN, HIDEKI KATAGIRI, HISAMITSU ISHIHARA, FUMIMARO TAKAKU, AND YOSHITOMO OKA

A radioimmunoassay for the GLUT1 glucose transporter was developed with a synthesized peptide based on the sequence of the cDNA for GLUT1. A peptide corresponding to the COOH-terminal domain of the GLUT1 glucose transporter (Thr-Pro-Glu-Glu-Leu-Phe-His-Pro-Leu-Gly-Ala-Asp-Ser-Gln-Val) was synthesized and conjugated to keyhole limpet hemocyanin through the NH_2-terminal of the peptide. An antibody was raised against this complex and affinity purified with the immobilized peptide. A second peptide, with tyrosine residue added to the NH_2-terminal of the above peptide, was synthesized and used as a standard and iodinated for preparation of the radioactive ligand. The assay is highly reproducible, sensitive, and specific for the COOH-terminal domain of the GLUT1 glucose transporter. It has no cross-reactivity with the other glucose-transporter isoforms GLUT2 and GLUT4. Furthermore, the results obtained with this radioimmunoassay on the number of glucose transporters in human erythrocytes were in good agreement with previous studies based on cytochalasin B binding, suggesting that this radioimmunoassay is able to quantify the number of glucose transporters. The assay is completed within 4 h and can be used for simultaneous measurement of GLUT1 in many samples. In addition, it can be applied to the measurement of GLUT1 in several types of tissue. *Diabetes* 40:315–18, 1991

Glucose transport into mammalian cells is mediated by a stereo-specific carrier called glucose transporter and is regulated by many factors (1–3). The facilitated glucose transporter, as opposed to the Na^+-glucose cotransporter, is in many tissues, such as the brain, liver, muscle, and adipose tissues, and has been demonstrated to consist of a structurally related family of proteins (4–14). To measure the amount of facilitated glucose transporter, Scatchard analysis of D-glucose–inhibitable cytochalasin B binding (15–17) or Western blotting with an antibody against glucose transporter was used. However, these methods require time-consuming procedures and are not convenient for the simultaneous handling of many samples. In addition, the cytochalasin B–binding assay cannot distinguish the glucose-transporter isoforms from each other. Western blotting reveals only the relative number of glucose transporters with an isoform-specific antibody. Theoretically, it is possible to calculate the actual number of transporters with the Western-blotting technique if several concentrations of a standard were applied on the same blot. However, this is cumbersome, and a standard is sometimes unavailable. In contrast, radioimmunoassay (RIA) can handle many samples and also allows quantitative analysis. However, RIA for glucose transporters requires a quantity of purified glucose transporter adequate for use as an unlabeled standard, which would be difficult to obtain in many laboratories.

To overcome these difficulties, we took advantage of recent advances in molecular cloning and developed a peptide-based RIA for the glucose-transporter isoform GLUT1. A synthesized peptide corresponding to a domain specific for GLUT1 was used to prepare an antibody and also used as a standard in this RIA.

RESEARCH DESIGN AND METHODS

Preparation of anti-peptide antibody against the COOH-terminal domain of the GLUT1 (HepG2-type) glucose transporter and purification of antibody have been described previously (18). A chemically synthesized short peptide corresponding to the COOH-terminal 15 amino acid residues of the GLUT1 glucose transporter (Thr-Pro-Glu-Glu-Leu-Phe-His-Pro-Leu-Gly-Ala-Asp-Ser-Gln-Val) was conjugated to keyhole limpet hemocyanin through the NH_2-terminal end of

From the Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Tokyo, Japan.

Address correspondence and reprint requests to Yoshitomo Oka, MD, Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1, Bunkyo-ku, Tokyo 113, Japan.

Received for publication 19 June 1990 and accepted in revised form 1 November 1990.

the short peptide with glutaraldehyde and injected with Freund's complete adjuvant into rabbits. Subsequent immunization was performed by injecting the conjugated complex emulsified with Freund's incomplete adjuvant. The antibody was affinity purified with immobilized peptide that was coupled to activated agarose beads (Affi-Gel 10, Bio-Rad, Richmond, CA) through the NH_2-terminal of the short peptide. For iodination, a tyrosine residue was added to the NH_2-terminal of the peptide described above. The newly synthesized peptide, composed of 16 amino acid residues, was also used as the standard in the RIA. Iodination of the peptide was performed by the chloramine-T method (19). A 0.1-μg peptide was incubated with 0.5 μg chloramine-T and 7.4 mBq ^{125}I-labeled Na for 2 min at 22°C, and the reaction was terminated with the addition of 1 μg $Na_2O_5S_2$. The labeled peptide was purified with Sep-Pak cartridge C-18 (Waters, Milford, MA). The Sep-Pak cartridge was prewashed with 5 ml of methanol and subsequently with 20 ml of 0.3 M sodium phosphate buffer (pH 7.4). The labeled reaction mixture (0.25 ml) was applied to the cartridge, and the cartridge was extensively washed with 20 ml of 0.3 M sodium phosphate buffer and subsequently with 50 ml of distilled water. The labeled peptide was then eluted with 2 ml of methanol.

Human erythrocyte membranes were prepared from frozen human erythrocytes. The frozen erythrocytes were thawed in phosphate-buffered saline (PBS; 10 mM) and centrifuged at 13,000 × *g* for 15 min at 4°C. The pellet was suspended in PBS and recentrifuged at 13,000 × *g* for 15 min. The pellet was then solubilized with 0.5% Triton X-100 in PBS containing 1 mM phenylmethylsulfonyl fluoride, followed by centrifugation at 13,000 × *g* for 15 min. Solubilization of the membranes was required for the immunoassay because the antibody cannot bind to the COOH-terminal domain if the resealed membranes are right-side-out "ghosts," in which the COOH-terminal domain of the glucose transporter resides inside the ghost (20,21). Membranes from Chinese hamster ovary (CHO) cells and various tissues were prepared as previously described and solubilized as described above (22). Protein concentration was determined by incubation of 20 μl solubilized sample with 400 μl BCA protein assay reagent (Pierce, Rockford, IL) for 30 min at 37°C and subsequent measurement of optical density at 564-nm wavelength.

Varying concentrations of sample and unlabeled peptide, 0.1 μg antibody, and ^{125}I-labeled peptide (~30,000 counts/min, corresponding to a peptide of ~70 pg) were added sequentially in a final volume of 200 μl PBS (pH 7.4) containing 0.1% bovine serum albumin and 0.1% Triton X-100. The mixture was incubated for 45 min at 22°C and was further incubated for 30 min with 3 μl of protein A Cellulofine (Seikagaku Kogyo, Tokyo) at 22°C. When the incubation period with the antibody was extended to 60 min, there were no significant effects on the results. The mixture was then centrifuged at 13,000 × *g* for 1 min and washed three times with PBS, and the radioactivity in the pellet was determined.

RESULTS

The standard curve of the RIA for the peptide is shown in Fig. 1. The binding of the labeled peptide to antibody was >95% inhibited at a concentration of 5 × 10^{-4} μg/μl, whereas the binding was not inhibited by unlabeled peptide at concentrations of <5 × 10^{-6} μg/μl under the assay condition that was used. Furthermore, the assay was highly reproducible with respect to both intra- and interassay variations. Our intra-assay coefficient of variation for single quadruplicates was ~5% in the functional portion (15–85% displacement) of the standard curve (Fig. 1*A*). In addition, the standard curves for three separate assays conducted 3 wk apart were similar (Fig. 1*B*). The apparent K_d for the binding of the peptide to antibody was 8 × 10^{-9} M under the assay conditions. Although Triton X-100 did not have any significant effects on the standard curve at concentrations of 0.05–0.5% (data not shown), concentrations of antibody did affect the standard curve. Approximately 15–20, 20–30, and 35–45% of the labeled peptide was bound to the antibody at 0.05, 0.1, and 0.2 μg antibody/200 μl assay mixture, respectively. Considering both the sensitivity and accuracy of the assay, we used 0.1 μg antibody in 200 μl assay mixture containing 0.1% Triton X-100 and 0.1% bovine serum albumin in this system. These assay conditions were used in the experiments shown in Fig. 1, *A* and *B*, and all the following experiments.

Next, we studied whether the GLUT1 glucose transporter could compete with the peptide for binding to the antibody in this assay system. As shown in Fig. 2, in solubilized human erythrocyte ghosts, the GLUT1 glucose transporter inhibited the binding of the labeled peptide to the antibody at concentrations >10^{-1} μg/μl. The displacement curve produced by human erythrocytes was parallel to that by the peptide, and 1 μg of solubilized human erythrocyte membrane was

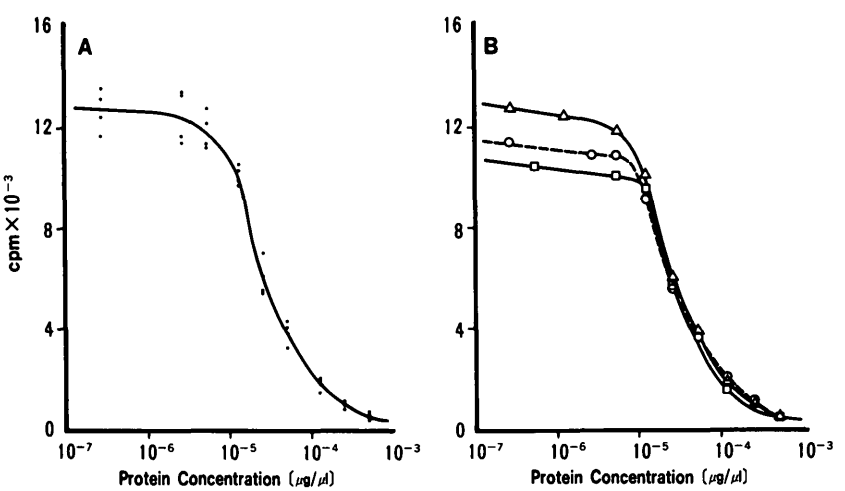

FIG. 1. Standard curves for peptide-based radioimmunoassay for GLUT1. *A*: single standard curve run in quadruplicates. *B*: standard curves obtained from 3 separate experiments performed 3 wk apart.

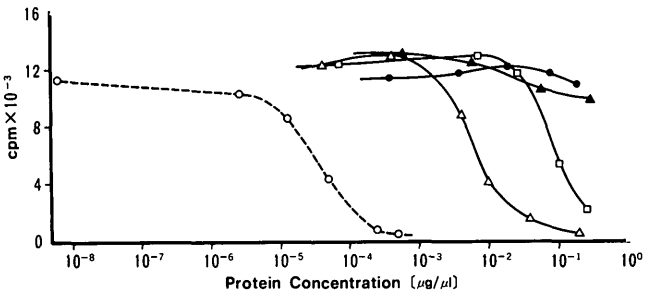

FIG. 2. Specificity of radioimmunoassay for COOH-terminal domain of GLUT1 glucose transporter. Standard curve (○), human erythrocyte "ghost" (△), Chinese hamster ovary (CHO) cells (●), clone B CHO cells expressing large amount of normal GLUT1 glucose transporter (□), and clone DC37 CHO cells expressing large amount of COOH-terminal deletion mutant of GLUT1 glucose transporter (▲).

estimated to contain an amount of glucose transporter equivalent to 2.8×10^{-3} μg peptide. Considering the molecular weight difference between the peptide and human erythrocyte glucose transporter, we estimated that ~7% of the solubilized human erythrocyte membrane is glucose transporter, assuming that the binding affinity of the antibody for the peptide is the same as that for the glucose transporter (see DISCUSSION).

The specificity of RIA for the COOH-terminal domain of the GLUT1 glucose transporter was demonstrated by measuring the number of transporters in CHO cells expressing a large amount of COOH-terminal deletion mutant of the GLUT1 glucose transporter, which has previously been called clone DC37 (23). This deletion mutant lacks 37 COOH-terminal amino acid residues. Solubilized membranes from CHO cells expressing a large amount of normal GLUT1 glucose transporter, previously called clone B (22), significantly inhibited the binding of the labeled peptide (Fig. 2) and were estimated to contain 0.094 pmol glucose transporter/μg membrane. In contrast, no significant amount of GLUT1 glucose transporter was detected in as much as 100 μg solubilized membranes from control CHO cells or clone DC37 in 200 μl assay mixture. This, despite the amount of COOH-terminal deletion mutant of the glucose transporter in clone DC37 being similar to that of normal glucose transporter expressed in clone B (23).

The specificity of RIA for GLUT1 glucose transporter was further investigated. Because the COOH-terminal domains of GLUT1, GLUT2, and GLUT4 glucose transporters are different, it would be expected that this assay system could recognize only GLUT1 but not GLUT2 or GLUT4 glucose transporters. Rat liver membranes or low-density microsome fractions from isolated rat adipocytes, which are rich in GLUT2 (7,8,24) and GLUT 4 (10–14) glucose transporter, respectively, did not significantly inhibit the binding of the labeled peptide to the antibody (Fig. 3). In contrast, not only human erythrocytes but also rat brain membranes, which are rich in GLUT1 glucose transporter (4–6), significantly inhibited the binding of the labeled peptide.

DISCUSSION

Because cDNA cloning has revealed the entire sequence of the GLUT1 glucose transporter (4–6), we are now able to synthesize a good amount of purified short peptide corresponding to a given domain of the glucose transporter. This has enabled us to develop an RIA with a peptide as a standard instead of the glucose-transporter molecule itself. To quantify the amount of glucose transporter in this RIA, the binding properties of the antibody need to be identical or at least similar between the synthesized peptide and the glucose-transporter molecule. This appears to be the case for the RIA described herein for the GLUT1 glucose transporter.

A synthesized peptide corresponding to 15 amino acid residues of the COOH-terminal of the glucose transporter was conjugated to keyhole limpet hemocyanin through the NH_2-terminal of the peptide such that the COOH-terminal end of the peptide would be the epitope for preparing the antibody. Furthermore, the antibody was affinity purified with the peptide that was conjugated to agarose beads through its NH_2-terminal. Thus, the purified antibody recognizes the COOH-terminal of the glucose transporter and the COOH-terminal of the peptide. In addition, a second peptide with a tyrosine residue added to the NH_2-terminal of the above peptide was synthesized. Thus, the iodination of a second peptide is not expected to affect binding to the purified antibody. These experimental designs for preparation of the antibody and peptides make it possible to quantify the number of GLUT1 transporters in this RIA. In fact, the estimated fraction of erythrocyte membranes composed of GLUT1 glucose transporter is 7%. This number is in the range of previous results (5%) obtained from the erythrocyte membranes depleted of peripheral proteins, based on cytochalasin B binding (15,16) or immunological assay, with the purified glucose transporter as a standard (25). The figure of 7% is slightly higher than previously reported results, but this may be due to selective solubilization of glucose transporters from erythrocyte membranes compared with other erythrocyte membrane proteins.

If we assume that 7% of solubilized erythrocyte membrane is glucose transporter, the standard curve (inhibition by the unlabeled peptide of the labeled peptide binding to the antibody) and the inhibition curve produced by the erythrocyte glucose transporter (inhibition by the glucose transporter of the labeled peptide binding to the antibody) are completely identical on a molar basis (Figs. 2 and 3). This result strongly indicates that the binding affinity of the antibody is the same for the peptide and the glucose transporter, although experiments that use purified glucose transporter are needed to obtain a definitive answer.

The peptide-based RIA described herein for the GLUT1 glucose transporter overcomes not only difficulties in obtaining enough purified glucose transporters but also the

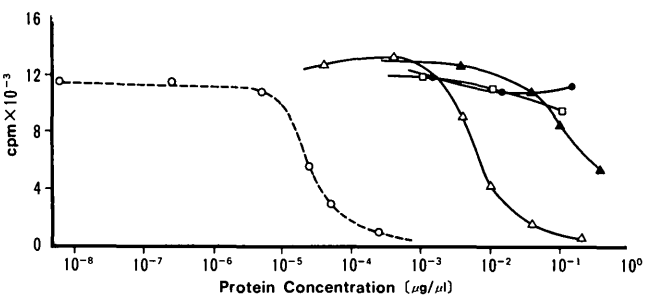

FIG. 3. Radioimmunoassay for GLUT1 glucose transporter in human erythrocyte, rat brain, liver, and adipocyte. Standard curve (○), human erythrocyte "ghost" (△), rat brain (▲), rat liver (□), and low-density microsome fractions from isolated rat adipocytes (●).

problem of cross-reactivity among the glucose-transporter isoforms. Although the cytochalasin B–binding assay can also quantify the number of glucose transporters (15–17), it cannot distinguish glucose-transporter isoforms. Because glucose-transporter isoforms GLUT1, GLUT2, and GLUT4 share 54–65% amino acid identity and some domains of these isoforms are completely identical (4–8,10–14), antibodies raised against one glucose-transporter isoform may recognize other isoforms. However, the COOH-terminal domains of these isoforms are different, and in fact, an antibody raised against this domain of GLUT1 does not react with other glucose-transporter isoforms (Fig. 3). Thus, the peptide-based RIA has an advantage over the RIA with the whole protein as a standard in measuring the amount of the protein isoform. On the other hand, the peptide-based RIA cannot detect the protein if a mutation exists in the domain corresponding to the synthesized peptide. The COOH-terminal deletion mutant of the glucose transporter is such a case (Fig. 2). However, this in turn implies that the peptide-based RIA may be useful for screening mutations of a given domain of the protein from many samples when the mutations alter the reactivity of the antibody.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid for developmental scientific research from the Ministry of Education, Science, and Culture of Japan.

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