A Structural Basis for the Acute Effects of HIV Protease Inhibitors on GLUT4 Intrinsic Activity

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Running Title: Peptide Inhibitors of GLUT4
Summary

HIV protease inhibitors (PIs) act as reversible noncompetitive inhibitors of GLUT4 with binding affinities in the low micromolar range and are known to contribute to alterations in glucose homeostasis during treatment of HIV infection. As aspartyl protease inhibitors, these compounds all possess a core peptidomimetic structure together with flanking hydrophobic moieties. To determine the molecular basis for GLUT4 inhibition, a family of related oligopeptides containing structural elements found in PIs was screened for their ability to inhibit 2-deoxyglucose transport in primary rat adipocytes. The peptide oxybenzylcarbonyl-His-Phe-Phe-o-ethyl ester (zHFFe) was identified as a potent inhibitor of zero-trans glucose flux with a $K_i$ of 26 µM. Similar to PIs, transport inhibition by this peptide was acute, noncompetitive, and reversible. Within a *Xenopus* oocyte expression system, zHFFe acutely and reversibly inhibited GLUT4-mediated glucose uptake, whereas GLUT1 activity was unaffected at concentrations as high as 1 mM. The related photoactivatable peptide zHFF-p-benzoylphenylalanine-[125I]-Tyr-o-ethyl ester selectively labeled GLUT4 in rat adipocytes and indinavir effectively protected against photolabeling. Furthermore, GLUT4 bound to a peptide affinity column containing the zHFF sequence and was eluted by indinavir. These data establish a structural basis for PI effects on GLUT4 activity and support direct binding of PIs to the transport protein as the mechanism for acute inhibition of insulin-stimulated glucose uptake.
Introduction

The clinical use of HIV protease inhibitors (PIs) has led to dramatic improvements in HIV-related morbidity and mortality (1). However, it is now recognized that these drugs directly contribute to several metabolic changes that significantly increase the risk of developing diabetes mellitus and cardiovascular disease (2,3). Insulin resistance appears early in the development of this dysmetabolic syndrome, and can occur in the absence of lipodystrophy or hyperlipidemia (4). While the complexity of drug regimens used during highly active antiretroviral therapy (HAART) has complicated efforts to characterize adverse effects that are the direct result of PI use, a definitive link to the development of insulin resistance has been established by both in vitro and in vivo studies (5-7).

Several mechanisms have been proposed to mediate PI-induced insulin resistance including changes in insulin signaling (8,9), SREBP processing (10), and adipokine secretion (11). Our previous studies establishing that the insulin responsive facilitative glucose transporter GLUT4 is acutely inhibited by PIs at pharmacologically relevant drug levels (12) have identified a direct molecular target for the cellular effects of these drugs. Several observations support the hypothesis that GLUT4 inhibition is produced by direct, noncovalent binding of PIs to a unique structural domain within the transport molecule: 1.) Inhibition of glucose transport by low micromolar concentrations of PIs is observed following maximal insulin stimulation with GLUT4 already translocated to the cell membrane. 2.) Inhibition is also observed in a heterologous Xenopus oocyte expression system that is not insulin responsive. 3.) In this same system, GLUT1 mediated transport is unaffected by millimolar concentrations of the PI indinavir. 4.) These effects are
observed on a time scale of seconds to minutes and would thus be incompatible with changes in gene or protein expression. 5.) The inhibitory effects *in vitro* and *in vivo* are readily reversible following removal of the drug.

Despite these data, without direct evidence that PIs bind to GLUT4, it remains possible that the effects of PIs on GLUT4 activity are indirect. For example, the drugs could interact with a unique regulatory molecule that either binds to GLUT4 or reversibly alters its structure such as by phosphorylation. Elucidation of the specific structural features of PIs that confer their ability to inhibit GLUT4 would not only facilitate efforts to define the molecular mechanism for this effect but could also provide a rationale way to design newer generations of PIs that retain their efficacy in treating HIV infection without producing insulin resistance.

We report here the identification of acute, potent, and isoform-selective peptide inhibitors of GLUT4 and provide evidence that this inhibition is due to direct binding of these compounds to the transporter protein.
Experimental Procedures:

*Materials*—*Xenopus laevis* frogs were purchased from Xenopus Express (Plant City, FL). Iodobeads, BCA reagent and aminolink beads were obtained from Pierce (Rockford, IL). Indinavir was acquired from Merck (Whitehouse City, NJ). Na$^{125}$I and [3H] 2-deoxyglucose were purchased from Amersham Biosciences (Piscataway, NJ) and American Radiolabeled (St. Louis, MO), respectively. Sep-Pak cartridges were obtained from Waters (Milford, MA). Dinonyl phthalate was purchased from VWR Scientific (Westchester, PA). z-His-Phe-Phe-Bpa-Tyr-o-Et was synthesized by Anaspec (San Jose, CA) and z-His-Phe-Phe-Phe-Lys was made by Genosys (The Woodlands, TX). All other peptides were obtained from Bachem (King of Prussia, PA). Affinity purified anti-GLUT4 antibody produced from partially purified GLUT4 was purchased from Chemicon (Temecula, CA). Polyclonal antibody directed toward the carboxy terminus of GLUT4 was a kind gift from Mike Mueckler (Washington University, St. Louis, MO). Unless otherwise specified, all other reagents were acquired from Sigma (St. Louis, MO).

2-Deoxyglucose Uptake Measurements in primary rat adipocytes—Adipocytes were prepared from rat epididymal fat pads as described previously (8). Adipocytes were stimulated with the addition of 1 μM insulin for 30 min at 37°C immediately prior to use. 2-Deoxyglucose uptake measurements were performed by adding 200 μl of the cell suspension to 200 μl of Krebs–Ringer bicarbonate Hepes buffer (120 mM NaCl, 4 mM KH$_2$PO$_4$, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 10 mM NaHCO$_3$, 200 nM adenosine, 30 mM Hepes, pH 7.4) containing 1% BSA together with the indicated concentration of peptide or indinavir in 5 ml plastic scintillation vials. After incubation the mixture for 1 min at
37°C, the assay was initiated with the addition of 20 µl of [3H]-2-deoxyglucose (final 2-DOG concentration of 50 to 200 µM; 0.5 µCi per assay). After 1 min at 37°C, the assay was quenched by adding cytochalasin B to a final concentration of 40 µM. The cells were then immediately separated from labeling mix by spinning the reaction through dinonylphthalate as described previously (8), and the intracellular radioactivity was quantified by liquid scintillation counting.

*Glucose transport activity in Xenopus laevis oocytes* - *X. laevis* oocytes were prepared and microinjected as described previously with 50 ng of Glut isoform mRNA synthesized *in vitro* (9). Measurement of [3H]-2-deoxyglucose uptake was performed on groups of 15-20 oocytes in Barth's saline at 22°C for 30 minutes. All assays were performed using 50 µM 2-DOG, 0.5 µCi/assay unless otherwise indicated. Peptides were added to the assay mixture 6 minutes prior to the initiation of uptake assays. Reactions were terminated by washing the oocytes with ice cold Barth’s saline containing 20 mM Phloretin. Each oocyte was then transferred to an individual scintillation vial, solubilized in 1% SDS and incorporated radioactivity was determined by liquid scintillation counting.

*Radioiodination of z-His-Phe-Phe-Bpa-Tyr-o-Et* - A 100 µM solution of z-His-Phe-Phe-Bpa-Tyr-o-Et in 0.1 M phosphate buffer (2.5 ml) was incubated with two Iodobeads and 100 µCi Na125I at room temperature with gentle agitation for 30 min. The beads were then removed and the solution was applied to a C18 Sep-Pak cartridge pre-equilibrated with distilled water. The cartridge was washed 3 times with 5 ml water and the peptide
was eluted with 1 ml DMSO. Yield and purity were assessed by HPLC analysis in comparison to a known amount of z-His-Phe-Phe-o-Et labeled with cold NaI. The amount of $^{125}$I labeled peptide was assessed by liquid scintillation counting and the efficiency of labeling was estimated from the yield of radiolabeled peptide divided by the total peptide concentration.

**Affinity labeling of primary rat adipocytes** - Primary rat adipocytes were incubated with 1-20 µM radiolabeled z-His-Phe-Phe-Bpa-$^{[125]$I]$\text{Tyr-o-Et}$ in KRB buffer at room temperature in a 60 mm Petri dish. The photolabel was activated by exposure to 254 nm UV light with three successive cycles of 30 seconds of light followed by 30 seconds of darkness (Total UV exposure 90 seconds). The adipocytes were then collected on ice into a 5 ml centrifuge tube containing PE200 tubing and spun at 200 rpm for 4 minutes. The layer below the floating adipocytes was removed via the PE tubing and the adipocytes were washed three times with 4 ml of ice-cold KRB buffer to remove any unreacted peptide. Samples were then stored at -20°C.

**Peptide affinity chromatography** - The peptide z-His-Phe-Phe-Phe-Lys was attached via amide linkage to aminolink agarose beads (Pierce, Rockford IL) according to the manufacturer’s specifications. Prior to each experiment, the column was equilibrated in phosphate buffered saline at room temperature. Purified primary rat adipocytes were lysed by rapid freeze thawing in phosphate buffered saline (PBS) containing 0.1% decylmaltoside and Sigma protease inhibitor cocktail, centrifuged at 4000 rpm for 30 minutes, and the supernatant fatty layer was carefully removed. The remaining cell
lysate was applied to the peptide column and which was then washed with 3 column volumes of PBS. The bound transporter was eluted with PBS containing 500 µM indinavir. The column effluent was collected in 0.5 ml fractions. Protein in each fraction was quantified by the BCA assay. SDS page was performed by loading 5 micrograms of protein onto a 10% acrylamide gel. Protein staining was assessed with Ruby red dye. GLUT4 content was assessed by Western blot analysis using polyclonal antibody directed toward the carboxy terminus of rat GLUT4.
Results.

Aromatic peptide effects on GLUT4 activity. All HIV protease inhibitors share a similar core structure that resembles the aromatic peptide backbone that acts as the native substrate for the HIV-1 protease (Figure 1). We therefore hypothesized that synthetic peptides containing this basic structure would serve as effective inhibitors of glucose transport. To test this hypothesis we screened a family of aromatic di and tripeptides for their affects on insulin stimulated glucose uptake in primary rat adipocytes, which predominately express the insulin responsive facilitative glucose transporter GLUT4 (13). With the addition of 200 µM of each peptide to primary rat adipocytes, four compounds were identified with significant inhibitory effects on zero-trans 2-deoxyglucose flux (Figure 2). Like PIs, all four of these peptides contain a highly aromatic core peptide structure flanked by hydrophobic moieties. None of the peptides with charged amino or carboxy terminus affected transport activity. The most potent inhibitor of glucose transport identified in this screen was z-His-Phe-Phe-o-Et (zHHFe), which is also a known substrate for the aspartyl protease pepsin (14).

Kinetic characterization of the inhibition of GLUT4 by z-His-Phe-Phe-o-Et. The ability of z-HFFe to impair glucose transport was further investigated by studying the concentration dependence for inhibition in both primary rat adipocytes and Xenopus oocytes heterologously expressing the rat GLUT4 transporter isoform. As the Dixon plots shown in Figure 3 demonstrate, this peptide acutely inhibited glucose transport in a concentration dependent manner in both cell types. The apparent binding affinities for the peptide differed considerably between the adipocytes and oocytes, with $K_i$s of 26 ± 2
and 205 ± 5 µM, respectively. This difference is comparable to that seen with indinavir-mediated inhibition of glucose uptake in these two cell types (5,12). The intercept on the negative x-axis is indicative of non-competitive inhibition under the kinetic conditions used, which is also identical to the inhibition pattern observed for indinavir in primary rat and 3T3-L1 adipocytes (12). Due to the rapidity of the inhibitory effects of zHFFe (occurring within minutes after adding the drug) in adipocytes following insulin stimulation, it is unlikely that zHFFe produces its effect through alterations in insulin signaling or GLUT4 translocation. This is further supported by the inhibitory effect of this peptide in the *Xenopus* oocyte system, in which cell surface expression of the glucose transporter is independent of insulin signaling (15).

*Isoform selectivity and reversibility of zHFFe effects on glucose transport*- One of the distinguishing features of PI-mediated effects on glucose transport is their selectivity for inhibition of GLUT4. In the *Xenopus* oocyte system, GLUT1 is resistant to inhibition by indinavir at millimolar concentrations, whereas GLUT4 is highly sensitive with a Kᵢ of approximately 50 µM (12). To determine the isoform selectivity of zHFFe, oocytes injected with mRNA for GLUTs 1-4 were tested for their ability to acutely and reversibly inhibit zero-trans 2-deoxyglucose transport. As shown in figure 4A, only GLUT4 was inhibited by 250 µM peptide, whereas concentrations of zHFFe up to 1 mM had no effect on GLUT1 or GLUT3. A small (32%) but statistically insignificant (P=0.087) reduction in GLUT2 activity was observed with 1 mM zHFFe. This compares favorably with the behavior of PIs, which have been shown to inhibit GLUT2 at high concentrations in *Xenopus* oocytes (16). As shown in Figure 4B, acute inhibition of GLUT4 was fully
reversible following removal of the peptide. Full restoration of transport activity was observed irrespective of the time in which the oocytes were exposed to drug (1-30 minutes, data not shown).

**Photolabeling with a reactive peptide derivative.** Having identified zHFFe as a peptide-based inhibitor of GLUT4 that shares inhibitory characteristics identical to those observed with the PI indinavir, a radiolabeled and photoreactive derivative of this peptide was synthesized and used to test the hypothesis that transport inhibition is due to direct binding of the drug to the transport protein. The peptide z-His-Phe-Phe-Bpa-Tyr-o-Et was found to effectively inhibit glucose transport in primary rat adipocytes with a binding affinity comparable to the parent zHFFe compound (Figure 5a). When the adipocytes were exposed to UV light to activate the Bpa residue, a concentration dependent and irreversible inhibition of transport activity by the photactivatable peptide was observed (Figure 5B). As shown in Figure 6A, UV light exposure resulted in the photolabeling of a 50 kDa protein that corresponded precisely with the molecular weight of GLUT4. The degree of labeling directly correlated with the time of exposure to the UV light and was specific for this protein with up to 3 minutes of light exposure (data not shown). Since a large radioactive signal was present at the bottom of the gel, reflecting unbound peptide, it was not possible to definitively exclude the additional modification of a small molecular weight protein <10 kDa. While initial attempts to immunoprecipitate the radiolabeled protein with polyclonal antibody directed toward the carboxy terminus of GLUT4 were unsuccessful, the radiolabeled protein was effectively immunoprecipitated by affinity purified polyclonal GLUT4 antibody recognizing epitopes throughout the
transporter Figure 6B. Moreover, indinavir effectively protected against photolabeling. Together, these observations support the conclusion that the photolabel is bound to the same GLUT4 site as PIs.

*Peptide affinity chromatography*- The relative ease in modifying the core zHFF epitope that confers selective binding to GLUT4 provided a means to further investigate the relationship between aromatic peptide and PI-mediated inhibition of glucose transport. The peptide z-His-Phe-Phe-Phe-Lys, which contains the zHFF epitope, was linked to an agarose resin to generate an affinity column which could bind GLUT4. As shown in Figure 7, when homogenates of primary rat adipocytes were applied to this resin, immunoreactive GLUT4 was found to bind to this column and could be selectively eluted with 500 μM indinavir. The native structure of GLUT4 appeared to be necessary for this interaction, since boiling of the adipocytes homogenate prior to loading the peptide column resulted in no immunodetectable GLUT4 binding to the column (data not shown).
Discussion

Given the crystal-structure based rationale design of PIs, which target an aspartyl protease that selectively cleaves Phe-Pro and Tyr-Pro bonds in a large polyprotein precursor of the developing HIV virus, it is not surprising that all of the currently available PIs contain some elementary aromatic peptidomimetic structure. The ability of these compounds to alter the intrinsic activity of facilitative glucose transporters, however, was an entirely unforeseen effect of these drugs. Because of the indisputable clinical utility of PIs in reducing HIV associated morbidity and mortality (17), PIs are likely to remain a cornerstone of highly active antiretroviral therapy. Continuing efforts are clearly needed to design newer generations of PIs that retain their clinical utility without producing their adverse effects on glucose disposal.

While millimolar concentrations of dipeptide metalloproteinase substrates have previously been reported to inhibit glucose uptake into rat adipocytes (18), the relatively high concentrations of peptides required to inhibit transport in these studies made it difficult to determine whether this effect was due to nonspecific cellular changes. The identification of high affinity, isoform-selective, reversible peptide based inhibitors of facilitative glucose transport proteins together with the successful photolabeling of GLUT4 with a derivative of this peptide identifies a direct molecular mechanism for the inhibition of GLUT4 by these peptides and PIs.

Since the peptidomimetic phenylalanine moiety is the only feature that is consistently shared between PIs and the newly identified peptide inhibitors, this structure is likely to be a primary determinant in conferring the ability to inhibit GLUT4 activity. The shared participation of this structural feature in the binding of PIs to both the HIV
protease and GLUT4 may present a challenge in new drug design. Attempts to change this core structure to prevent GLUT4 inactivation may render the candidate drug ineffective in inactivating the HIV protease. The recent development of atazanavir, a PI which does not appear to alter glucose uptake in 3T3-L1 adipocytes (19), provides optimism that the problem of GLUT4 inactivation by PIs can be circumvented. Reflecting the close correlation between the effects of PIs on GLUT4 activity in vitro and insulin sensitivity in vivo, atazanavir does not appear to contribute to changes in glucose homeostasis in HIV infected patients who have been treated with this drug (20). Atazanavir differs from the other available PIs in several respects. While atazanavir contains a similar peptide-like core, with two phenylalanine-like structures arranged in a unique C-2 axis of symmetry, an additional pyridine ring is attached to one of the phenylalanine residues (Figure 1). While the location of the GLUT4 domain involved in PI binding remains to be identified, it is possible that this additional ring structure sterically prevents access of the drug to this region of the protein.

Atazanavir also differs from other PIs by lacking the same degree of hydrophobicity in the flanking structural domains. While the mechanism by which these hydrophobic moieties contribute to GLUT4 inhibition remains to be determined, all of the peptide inhibitors identified in this study contain an oxobenzylcarbonyl group at the amino terminus of the peptide. It is possible that this hydrophobicity facilitates entry of the drugs into the cell, thereby allowing access to a GLUT4 binding domain on the cytosolic surface of the transporter. The involvement of a cytosolic binding domain is suggested by our observation that the affinity labeled transporter could not be immunoprecipitated using antibody directed toward the carboxy terminus. This could
either be due to direct steric interference of the affinity label or an inhibitor-induced change in the protein conformation that leads to masking of this epitope. Since the epitope was recognized in the denatured protein on Western blots, the latter appears more likely. Unmasking of the carboxy terminus of GLUT4 in response to insulin treatment has previously been reported in both primary rat adipocytes (21) and skeletal muscle (22). It has been postulated that this unmasking may contribute, together with GLUT4 translocation, to the activation of this transporter following insulin stimulation. The identification of peptide inhibitors thus raises the interesting possibility that an endogenous protein or peptide interacts at this same location to regulate GLUT4 intrinsic activity.

Although the current study supports the hypothesis that PIs directly bind to GLUT4 in vitro, it remains possible that these drugs also interact with other molecules in vivo to alter glucose homeostasis and/or contribute to the other adverse metabolic effects observed during PI containing HAART. Several recent studies have implied direct effects of PIs on glucose stimulated insulin release (23,24). While the differences observed in the ability of PIs and the peptide inhibitors to inhibit GLUT4 when measured in primary adipocytes versus oocytes may be merely reflective of differences in the assay procedures used, it is also possible that GLUT4 sensitivity is dependent upon the membrane environment of the transporter. If true, significant effects of zHFFe on GLUT2 activity in β-cells and/or hepatocytes may still occur at pharmacologically relevant drug levels. Future studies will be needed to determine whether these newly identified peptide-inhibitors or related proteins are capable of photoaffinity labeling
glucose transporters and/or other proteins in other tissues involved in glucoregulation, including skeletal muscle.

In addition to their utility in assisting the development of newer and safer PIs, the relative ease in designing and modifying peptide-based GLUT inhibitors provides a new approach to the development of reagents to study facilitative glucose transport. The unique selectivity of PIs and the newly identified peptide inhibitors of GLUT4 provide a facile way to acutely and reversibly induce insulin resistance in cell culture and whole animal model systems. With a more detailed understanding of the structural basis for PI binding to GLUT4, it may be possible to design additional reagents that selectively interact with other GLUT isoforms.
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Abbreviations Used:

2-DOG, 2-deoxyglucose; Bpa, benzoylphenylalanine; DMSO, dimethylsulfoxide; DMF, dimethylformamide; Et, ethyl ester; KRB, Krebs–Ringer bicarbonate Hepes buffer; PBS, phosphate buffered saline; PI, HIV protease inhibitor; SREBP, sterol response element binding protein; z, oxybenzylcarbonyl; zHHFe, oxybenzylcarbonyl-His-Phe-Phe-o-ethyl ester.
Figure Legends.

Figure 1. Structure Features of HIV Protease Inhibitors. The core peptidomimetic structure found within all HIV protease inhibitors is shown in the shaded regions. A) Amprenavir, B) Indinavir, C) Ritonavir, D) Lopinavir, E) Nelfinavir, and F) Saquinavir. G) Atazanavir

Figure 2. Inhibition of 2-Deoxyglucose uptake by aromatic peptides in primary rat adipocytes. Aromatic peptides (200 µM) were added 1 minute prior to measuring [3H]-2-deoxyglucose transport activity (50 µM, 1 min at 37°C). All assays were performed in triplicate and are expressed as a percent of a vehicle treated control ± S.E. Stars (*) indicate P<0.05 as determined by ANOVA.

Figure 3. Kinetic Analysis of GLUT4 inhibition by Z-His-Phe-Phe-0-Et. A. The uptake of [3H]-2-deoxyglucose (▲,50; ■,100; and ♦,200 µM) was measured in Xenopus oocytes 3 days after injecting GLUT4 mRNA. The peptide zHFFe was added 6 minutes prior to the start of each 30 minute assay performed at 22°C. B. [3H]-2-deoxyglucose uptake was measured in insulin-stimulated primary rat adipocytes one minute following the addition of z-HFFe. Assays were carried out for 1 minute at 37°C. The data are expressed as Dixon plots where the vertical axis represents the reciprocal of the transport velocity. All points represent the mean ± S.E. of 3 determinations.
Figure 4. Reversible, isoform selective inhibition of GLUT4 activity by z-His-Phe-Phe-O-Et. A, [3H]-2-deoxyglucose uptake (30 min at 22ºC) was measured three days following the injection of mRNA for each GLUT isoform into stage 5 Xenopus oocytes. The z-HFFe peptide (dissolved in DMF) was added 6 minutes prior to initiating the uptake assay. An equivalent volume of vehicle was added to the zero peptide control. Results represent the mean ± SEM of three independent experiments. Star (*) indicates P<0.05. B, Oocytes (15-20/group) were incubated for 60 seconds with 200 µM of z-His-Phe-Phe-O-Et (Peptide + Wash) or Bath’s saline (Control). Oocytes were then either washed with peptide-free Barth’s saline (Wash) or incubated in the continued presence of peptide (Peptide) during the 2-deoxyglucose uptake assay. Results represent the mean ± SE, Star (*) indicates P<0.01 versus control.

Figure 5. Inhibition of glucose uptake in rat adipocytes with z-His-Phe-Phe-Bpa-[125I]-Tyr-O-Et. A. The indicated concentrations of peptide were added to insulin stimulated primary rat adipocytes 1 minute prior to measuring 3H-2-deoxyglucose uptake (1 minute at 37ºC). HFF and CB represent positive controls in which 200 µM of the peptide z-HFFe or 20 µM cytochalasin b, respectively, were added to the assay mixture. B. Insulin-stimulated primary rat adipocytes were treated with the indicated concentrations of peptide and exposed to 254 nm light (black bars) or kept in the dark (grey bars) for 3 minutes at 37ºC. The adipocytes were then immediately washed three times with KRB buffer to remove unreacted peptide before measuring residual 2-deoxyglucose transport activity. All assays were performed in triplicate and the values
shown represent the mean ± SE. Stars (*) indicate P<0.05 compared to untreated control as determined by the Student’s t-test.

Figure 6. Photoaffinity labeling of GLUT4 with z-His-Phe-Phe-Bpa-[\(^{125}\)I]-Tyr-O-Et. A. SDS-PAGE analysis of primary rat adipocytes treated with 4 \(\mu\)M of photoactivated z-His-Phe-Phe-Bpa-[\(^{125}\)I]-Tyr-o-et in the presence or absence of 500 \(\mu\)M indinavir. Total cell homogenates (10 \(\mu\)g/lane) were analyzed by western blot analysis with polyclonal antibody directed toward the carboxy terminus of GLUT4 (Western) or autoradiography (Autorad) to determine incorporated \(^{125}\)I label. B. The total cellular homogenates from photolabeled rat adipocytes were immunoprecipitated using affinity purified polyclonal antibody directed toward partially purified GLUT4 and subjected to either western blot analysis using the GLUT4 carboxy-terminal antibody (Western) or autoradiography (Autorad). Each lane contains 20 \(\mu\)g of protein.

Figure 7. Peptide affinity chromatography of GLUT4. Primary rat adipocytes homogenized and passed over a 1 ml peptide affinity column containing the peptide z-His-Phe-Phe-Phe-Lys linked via the lysine residue to an agarose resin. Shown is a western blot of column fractions analyzed using polyclonal antibody directed toward the carboxy terminus of rat GLUT4. Lane 1 represents the adipocyte homogenate (5 \(\mu\)g) prior to loading onto the peptide column. Column wash fractions are shown in lanes 2-4 and protein eluted with buffer containing 500 \(\mu\)M indinavir is shown in lanes 5-8 (50 \(\mu\)l/lane). Rat GLUT4 heterologously expressed in *Xenopus* oocytes is shown in lane C as a positive control.
Figure 1

A

B

C

D

E

F

G
Figure 2

![Bar chart showing relative activity of various compounds.](http://www.jbc.org/Downloaded from)
Figure 3

A

\[ \frac{1}{V} \text{ (30 min/pmol)} \]

\[ \text{Peptide (µM)} \]

B

\[ \frac{1}{V} \text{ (min/pmol)} \]

\[ \text{Peptide (µM)} \]
Figure 4

A

2-DOG Uptake (pmol/oocyte/30 min)

|          | GLUT1 | GLUT2 | GLUT3 | GLUT4 |
|----------|-------|-------|-------|-------|
| 0        |       |       |       |       |
| 250      |       |       |       |       |
| 500      |       |       |       |       |
| 1000     |       |       |       |       |

B

2-DOG Uptake (pmol/oocyte/30 min)

- Control
- Peptide
- Wash

|          | GLUT1 | GLUT4 |
|----------|-------|-------|
|          |       |       |
| *        |       |       |
Figure 5

**A**

Bar chart showing 2DOG uptake (pmol/min) vs. Photolabel (µM). The x-axis represents different concentrations of Photolabel (0, 0.25, 1, 2, HFF, CB), and the y-axis represents the 2DOG uptake. The bars are labeled with asterisks indicating statistically significant differences.

**B**

Bar chart illustrating 2DOG uptake (pmol/min) compared between 'No Light' and 'Light' conditions. The x-axis represents different concentrations of Photolabel (0, 1, 5, 10, CB), and the y-axis represents the 2DOG uptake. Bars are colored black for 'No Light' and gray for 'Light', with asterisks indicating significant differences.
Figure 6

A

| Indinavir | - | + | - | + |
|-----------|---|---|---|---|
| MW        |   |   |   |   |
| 200       |   |   |   |   |
| 150       |   |   |   |   |
| 75        |   |   |   |   |
| 50        |   |   |   |   |
| 37        |   |   |   |   |
| 25        |   |   |   |   |
| Western   |   |   |   |   |
| Autorad   |   |   |   |   |

B

| Indinavir | - | + |
|-----------|---|---|
| Western   |   |   |
| Autorad   |   |   |
Figure 7
A structural basis for the acute effects of HIV protease inhibitors on GLUT4 intrinsic activity
Johann Hertel, Heidi Struthers, Christal Baird Horj and Paul W. Hruz

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