GLUT1 Is Not The Primary Binding Receptor But Is Associated With Cell-Cell Transmission Of HTLV-1

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running title: Role of GLUT1 in Cell-cell Spread of HTLV-1

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Abstract

GLUT1 has recently been suggested to be a binding receptor for HTLV-I. We used a novel, short-term assay to define the role of GLUT1 in cell-cell transmission. Although increasing cell surface levels of GLUT1 enhanced HTLV-I transfer, efficient virus spread correlated largely with HSPG expression on target cells. Moreover, since activated CD4+ T cells and fetal cord blood that are susceptible to HTLV-1 infection expressed undetectable levels of surface GLUT1, these results indicate that GLUT1 and HSPGs are important for efficient cell-cell transmission of HTLV-1 but raise concerns on the role of GLUT1 as the HTLV-1 primary binding receptor.
The human T-cell leukemia virus type I (HTLV-1) (20) is the causative agent of adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (3, 18, 24). Efficient transmission of HTLV-1 is believed to require contact between cells. Cell-free HTLV-1 virions are very poorly infectious. Formation of a virological synapse (VS), polarization of the cytoskeleton, and passage of HTLV-1 virions has been observed during contact between infected and uninfected T cells (5). Although the precise mechanism of HTLV-1 virion transfer remains elusive, several recent studies have suggested that the glucose transporter-1 (GLUT1) can function as a receptor for HTLV (2, 14) as well as participate in Env-mediated cell-cell fusion (2, 7). However, it is still unclear whether GLUT1 is important for cell-cell transmission of HTLV-1. To address this question, we have adapted methods to express and detect GLUT1 on the surface of target cells that express low levels of endogenous cell surface GLUT1. COS-7 cells were transfected with an expression vector encoding GLUT1 and an extracellular HA-tag (HA-GLUT1). A mutant form of glucose transporter-6, which also contains HA-tag (HA-GLUT6M) and the parental vector (pCIS) were used as controls (1, 12). Both HA-GLUT1 and HA-GLUT6M were expressed at high level on the cell surface (Fig. 1A, top row). Increase in cell surface GLUT expression was verified by another approach using a photoaffinity label technique (Bis-glucose Photolabel; Bio-LC-ATB-BMPA) (4) performed as recently described (9). These studies revealed that, while the endogenous level of cell surface GLUT1 was below the detectable level of this assay (Fig. 1B, lane 2), cells transfected with HA-GLUT1 vector expressed high levels of GLUT1 on the cell surface (Fig. 1B, lane 4). In addition,
significant amount of endogenous intracellular GLUT1 could be detected in the total cell lysate from both the non-transfected and transfected cells by standard Western blotting (Fig. 1B, lanes 5 and 6).

We next determined the effect of GLUT1 overexpression on the cell-cell transmission of HTLV-1 virions. The HTLV-1 producing CD4+ T cell line HUT-102 (21) was co-cultured with target cells transfected with pCIS, HA-GLUT1, or HA-GLUT6M expression vectors. After 2 days of co-culturing, the target cells were immunostained with anti-HA antibody, anti-HTLV-1 Env antibody (NIH AIDS Research and Reference Reagent Program; #309) and anti-human CD4 antibody (BD Biosciences) and analyzed by flow cytometric analysis. Virus producing cells HUT-102 were excluded from the analysis based on CD4 expression and cell size. Target cells expressing GLUT1 had a higher percentage of HTLV-1 productive infection (46.7%) compared to the control (GLUT6M expressing) cells (4%) (Fig. 2A, middle and right panels). These results are specific for HTLV-1 since we did not observe increased cell-cell transmission when co-culture assay was performed with another T-lymphotropic enveloped virus (HHV-6) (data not shown). As expected from previous studies, exposure of the COS-7 cells to cell-free HTLV-1 did not result in detectable levels of infection (data not shown).

To confirm that GLUT1 expression enhanced productive cell-cell transmission of HTLV-1, levels of proviral DNA and viral mRNA in into COS-7 cells were examined as previously described (23). Real time PCR analysis revealed that the level of HTLV-1 tax mRNA expression in GLUT1 transfected cells was approximately 3.5 times higher than those transfected with the parental vector (Fig. 2B, left panel). Similarly, an increase of more than 2-fold in the proviral DNA load was observed in GLUT1-expressing cells.
(data not shown). When similar studies were performed following selecting of HA-positive GLUT1 expressing cells, the proviral load of GLUT1 expressing cells was nearly 4-fold higher than the level in the cells expressing the control GLUT6M (Fig. 2B, right panel). These results indicate that GLUT1 facilitates the productive cell-cell transmission of HTLV-1.

Although it has been reported that GLUT1 is involved in both binding and fusion of HTLV-1 (13, 14), other studies have demonstrated that the majority of binding of HTLV-1 to both adherent cell lines and primary T cells involves heparan sulfate proteoglycans (HSPGs), a type of glycosaminoglycan (GAG) (8, 11, 19). To examine the contribution of GLUT1 and HSPGs to HTLV-1 Env binding to COS cells, we determine the level of binding of soluble HTLV-1 SU to cells expressing high levels of GLUT1 in the presence or absence of HSPGs. COS-7 cells, transfected with the plasmids described in Figure 1A, were either enzymatically treated to remove HSPGs or left untreated as recently described (9). COS-7 cells transfected with the parental vector bound significant levels of HTLV-1 SU (Fig. 1A, middle row, left panel). This is consistent with previous reports that all vertebrate cell lines bind soluble HTLV-1 SU (6, 10, 14, 17, 22), Overexpression of GLUT1 in the presence of HSPGs did not increase the binding of HTLV-1 SU compared to controls (Fig. 1A, middle row). Furthermore, removal of HSPGs eliminated more than 95% of the binding of HTLV-1 SU despite the overexpression of GLUT1 (Fig. 1A, bottom row, middle and left panels). Enzymatic removal of HSPGs also dramatically reduced the binding of HTLV-1 virions to COS-7 cells (data not shown). These results are consistent with previous reports that cell surface expression of HSPGs is critical for efficient binding of HTLV-1 Env proteins, and
suggest that the majority of the increase in cell-cell transmission into COS-7 cells following GLUT1 overexpression involves enhancement of a step in entry other than Env-mediated binding to the target cell.

We next directly examined the relative contributions of GLUT1 and HSPGs during the cell-cell transmission HTLV-1. The role of HSPGs could not be evaluated by enzymatic removal from COS-7 cells, since the HSPGs were restored to the cell surface during the time of the co-culture (unpublished observations). Therefore, we determined the effect of GLUT1 expression in the cell-cell transmission in the presence and absence of HSPGs using cell lines carrying mutations in enzymes required for synthesis of HSPGs. Like COS-7 cells, no evidence of infection was observed following exposure of CHO-K1 cells to cell-free HTLV-1 virions. HTLV-1 producing (HUT-102 cells) were co-cultivated with either parental CHO-K1 and one of two mutant CHO-K1 cell lines: 2241 (which expresses low level of proteoglycans) and 2244 (negative for HSPGs). For cells transfected with the control vector (pCIS), the percentage of cells expressing viral proteins was higher in the parental CHO-K1 than in the mutant cell lines (Figure 3). This observation indicates that HSPGs play a role in the cell-cell transmission of HTLV-1 and suggests that other GAG may also function to enhance HTLV-1 transmission. As expected from the results shown in Figure 2, overexpressing of GLUT1 in CHO-K1 cells dramatically increased the spread of HTLV-1, as judged by the number of target cells expressing HTLV-1 Env. In the cells expressing lower levels of proteoglycans, GLUT1 also increased Env expression in the target cells, but the level was lower than that observed in the cells expressing wild-type levels of HSPGs. These experiments indicate that both GLUT1 and HSPGs play a role in the cell-cell transmission of HTLV-1.
It has previously been reported that freshly isolated, quiescent CD4\(^+\) and CD8\(^+\) T cells do not bind soluble HTLV-1 SU (15, 16). The lack of specific antibodies that recognize extracellular domains of the proteins has hampered investigations of cell surface GLUT1 expression on quiescent T cells. Recently, two independent monoclonal antibodies that recognize extracellular domains of GLUT1 (MAB1418; R&D systems and GT15-M; Alpha Diagnostics) have become commercially available. We confirmed specificity of these antibodies in recognizing cell surface expression of GLUT1 using COS-7 cells transfected with the control vector, HA-tagged-GLUT1, GLUT6M or wild-type GLUT1 vector (Fig. 4A, top row and data not shown). We used these anti-GLUT1 antibodies to examine the cellular distribution of this glucose transporter in PBMCs. These studies revealed that unstimulated CD8\(^+\) T cells expressed high levels of GLUT1 on the cell surface (Figure 4B, lower left and middle panels). In contrast, unstimulated CD4\(^+\) T cells did not express GLUT1 on the cell surface at levels above background (Figure 4B, upper left and middle panels). Consistent with what we have recently reported (9), CD4\(^+\) T cells stimulated with PHA for forty-eight hours expressed very low levels of GLUT1 on the cell surface, while activated CD8\(^+\) T cells continued to express high levels of GLUT1 (Fig 4B, right panels). We also examined cell surface expression on populations of quiescent CD4\(^+\) and CD8\(^+\) T cells freshly isolated from cord blood lymphocytes. Naïve CD8\(^+\) T cells isolated from cord blood lymphocytes expressed high levels of cell surface GLUT1, while very low levels were observed on CD4\(^+\) T cells (Fig. 4C, left panels). These cells expressed low levels of CD69, a marker of T cell activation, confirming that they were still phenotypically naïve (Fig. 4C, right panels). We have now examined expression of GLUT-1 on than 10 samples of unactivated CD8\(^+\) T cells isolated.
from cord blood lymphocytes and more than 20 samples isolated from adult peripheral blood. In all of these samples, the CD8+ T cells expressed high levels of cell surface GLUT-1.

These studies reveal that naïve CD8+ T cells, which do not bind significant levels of HTLV-1 SU, express high levels of GLUT1. Conversely, activated CD4+ T cells, which bind high levels of HTLV-1 SU, express low or undetectable levels of GLUT1 (Figure 4B) (9). Although our observation differ from those reported by Jin et al. (7) which demonstrated GLUT1 staining on primary CD4+ T cells, the specificity of antibodies used for cell surface GLUT1 detection may, in part, contribute to these variations. Taken together, these observations further support the hypothesis that GLUT1 is not responsible for the majority of binding of HTLV-1.

The observations reported in this manuscript suggest that, although GLUT1 enhances HTLV-1 cell-cell transmission, efficient virus spread also requires the presence of HSPGs. The precise contributions of each of these molecules during HTLV-1 infection have yet to be determined.
References


Figure legends

FIG. 1. Overexpression of GLUT1 in the absence of HSPGs does not increase HTLV-1 SU binding to the cell surface. (A and B) COS-7 cells, transfected twenty four hours previously with pCIS, HA-GLUT1 or HA-GLUT6M, were examined for expression of GLUT. (A) Top row: Levels of GLUT1 and GLUT6M expression on the cell surface determined by flow cytometry using antibodies directed the HA tag (HA.11; Berkeley Antibody Co.). Middle and bottom rows: COS-7 cells, transfected with plasmids indicated, were either treated with HS lyase (bottom row) or left untreated (middle row), then analyzed for binding of a soluble form of HTLV-1 SU. Percent positive staining compared to isotype control staining are shown. (B) Cell surface and total levels of GLUT1 expression determined following photoaffinity labeling. COS-7 cells transfected with with HA-GLUT1 or pCIS were exposed to UV light in the presence of a biotinylated photoaffinity label, lysed, and analyzed by immunoprecipitation followed by Western blot analysis as recently described (8). Arrow indicates the size of the expected band for GLUT1.

FIG. 2. Increasing levels of GLUT1 on the surface of COS-7 cells increases productive cell-cell transmission of HTLV-1. (A and B) COS-7 cells, transfected with HA-GLUT1 or control vectors (pCIS,HA-GLUT6M), were co-culturing with HUT-102. Two days later, the percentage of target cells infected with HTLV-1 was determined by staining the cells with antibodies directed against CD4, HA and HTLV-1 SU. (A) Expression of HTLV-1 Env expression levels on target cells expressing HA-GLUT. Cells from the co-culture were stained, and the target cells analyzed by gating on the CD4 negative population. Data shown here is a representative figure from more than ten experiments.
performed. (B) Quantitative real-time PCR analysis of the level of HTLV-1-specific mRNA and DNA in target cells. Left panel: Target cells were isolated by negative selection with MACS beads (Miltenyi, CA) for CD4, RNA isolated, and the amount of HTLV-1 tax mRNA quantified by real-time RT-PCR following calculation of normalized values. Result is from three independent experiments. Statistical analyses were performed using paired-T test. Right panel: Target cells were isolated by positive selection for HA by FLOW, DNA isolated, and HTLV-1 proviral load was determined by real-time PCR.

FIG. 3. Contribution of GLUT1 and HSPGs cell surface expression to cell-cell transmission of HTLV-1. CHO-K1 cells (HSPGs positive; ATCC CCL-61), 2241 cells (low levels of proteoglycans; ATCC pgsB-618) and 2244 cells (HSPGs negative; ATCC pgsD-677) were transfected with pCIS, HA-GLUT1 or HA-GLUT6M, then co-culturing with HUT-102, and the percentage of target cells infected with HTLV-1 was determined 3 days later as described on the legend of figure 2A. Transfection efficiencies of HA-GLUT1 as determined by HA expression were 34.5%, 37% and 32.4% for CHO-K1, 2241 and 2244, respectively. HA-GLUT6M transfection efficiencies were determined to be 18.9%, 54.0% and 43.6% for CHO-K1, 2241 and 2244, respectively.

FIG. 4. Cell surface GLUT1 expression in primary CD4$^+$ and CD8$^+$ T cells. Percent positive staining compared to isotype control staining are indicated for all histograms. (A) Specificity of antibody directed against GLUT1. Top row: COS-7 cells were analyzed by flow cytometry twenty four hours after transfection using an antibody directed against an extracellular epitope of GLUT1 (R&D System). Results shown are representative from three independent experiments. Similar results were obtained with an independently derived α-GLUT1 antibody (Alpha Diagnostic; data not shown). Bottom
Flow cytometry analysis of same samples using an antibody directed against HA tag. Representative data from two experiments are shown. (B) Cell surface GLUT1 expression on quiescent and PHA-stimulated human T cells. Peripheral blood samples were obtained after informed consent as part of a clinical protocol reviewed and approved by the National Institutes of Health institutional review panel. The samples were analyzed either immediately following isolation (left and middle panels) or after forty eight hours of culturing with PHA (right panels). The cells were triple stained with α-GLUT1 (R & D systems), α-CD8 and α-CD4 antibodies. The cells were then gated on either the CD4+ or CD8+ population, and the level of staining of GLUT1 determined.

Left panels: Level of cell-surface GLUT1 expression on non-stimulated PBMC using the α-GLUT1 antibody from Alpha diagnostic. Middle and right panels: Level of cell-surface GLUT1 expression on non-stimulated and PHA stimulated PBMC using the α-GLUT1 antibody from R&D Diagnostic. (C) Expression of GLUT1 on naïve cord blood T lymphocytes. CD4+ and CD8+ T cells were isolated from cord blood lymphocytes by negative selection with MACS beads, then immediately analyzed for GLUT1 expression (left panels) using the α-GLUT1 antibody (R & D systems). As a control to confirm that the cells remained phenotypically naïve, cells were also stained with an antibody directed against CD69, a marker of T cell activation (right panels). Data shown is a representative experiment out of eight performed.
Figure 1

A

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<th>HA-GLUT1</th>
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HA

untreated

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HTLV SU binding

- Isotype control
- Anti-HA Ab or soluble HTLV SU

B

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<tr>
<td>Total cell lysate</td>
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GLUT1

1 2 3 4 5 6
Figure 2

A

HTLV-1 Env

pCIS

2.6%

1%<

HA-GLUT1

5.2%

23.6%

1%<

HA-GLUT6M

1%

2.3%

55.1%

HA

B

Normalized Tax mRNA expression

Transfected: pCIS HA-GLUT1 HA-GLUT6M HA-GLUT1

P<0.0001

HTLV-1 DNA load (copies/100 cells)

0 1 2 3 4 5 12 13 14 15 16 (%)
Figure 3

% positive for HTLV-1 Env

[Bar graph showing data for CHO-K1, 2241, and 2244 with different categories: pCIS, HA-GLUT1, HA-GLUT6M.]