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HTLV ENVELOPES AND THEIR RECEPTOR GLUT1, THE UBIQUITOUS GLUCOSE TRANSPORTER: A NEW VISION ON HTLV INFECTION?

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1. ABSTRACT

We identified the ubiquitous glucose transporter GLUT1 as a receptor for Deltaretroviruses HTLV-1 and HTLV-2 envelopes (Env), mediating viral binding and entry. Here, we review the context and key observations that led us to this finding: functional modules of HTLV SU are similar to those of Gammaretrovirus Env which use multimembrane-spanning nutrient transporters as receptors; the HTLV Env receptor is an early marker of T lymphocyte activation; and HTLV Env inhibits glucose transport. We review several molecular, viral, cellular and physiological aspects of HTLV infection in relation to the *in vivo* and *in vitro* properties of GLUT1. Also, we examine the implications of HTLV-1 Env-GLUT1 interactions and altered glucose transport on the two major HTLV-1-induced diseases, adult T cell leukemia (ATL) and neurodegenerative tropical spastic paraparesis/HTLV-associated myelopathy (TSP/HAM). Complementary to the classical models of disease progression, we propose new schemes that emphasize the potential metabolic alterations caused in different cellular compartments. Finally, we review the potential use of HTLV Env-derived constructs as tools for labeling GLUT1 *in vivo* and inhibiting GLUT1 transport in tumor cells.

2. INTRODUCTION

2.1. Foreword

Here, we will not pretend to offer a review of the last twenty years of research on human T-cell leukemia/lymphoma virus (HTLV) and HTLV-related retrovirus envelopes and their cell membrane-associated interacting components. Neither will we try to offer a review of the literature on GLUT1, the ubiquitous glucose transporter that we identified as a receptor for HTLV envelope-mediated viral entry. Rather, we will attempt to provide some insights into the context in which we identified GLUT1 as a receptor for HTLV envelope-mediated infection. We will also highlight some of the questions related to this identification and explore a few issues with regards to HTLV infection and physiopathology that may be advanced by this work.

2.2. Retroviral entry, the viral envelope, and Env, the retroviral envelope glycoprotein

The primary events that condition infection by exogenous retroviruses rely on interactions between the viral

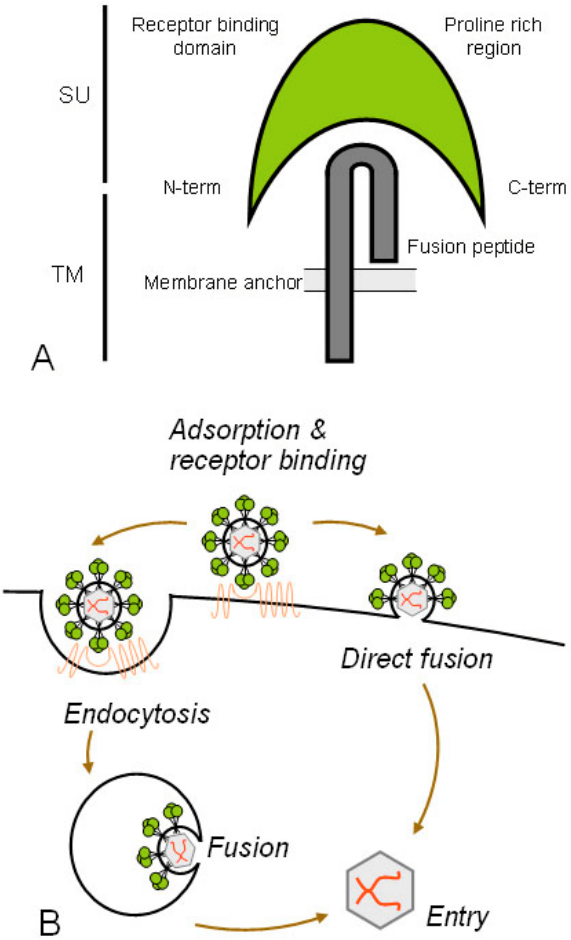

Figure 1. Schematic representation of the envelope glycoprotein of Gammaretroviruses. (A) The envelope glycoprotein comprises a surface subunit (SU) and a transmembrane subunit (TM). The former contains receptor-binding properties and stabilizes the fusion peptide present in the amino terminus of the TM which is anchored in the viral envelope or at the cell surface of infected cells. (B) Upon receptor binding, the envelope SU is shed, triggering the release of the fusion peptide required for fusion of the viral and cellular membranes. This fusion process takes place either at the cell surface plasma membrane or after endocytosis, depending on the retrovirus and the target cell. After the formation of a fusion pore, the viral nucleoprotein complex is released into the cytoplasm.

envelope and outer cell surface components. While most interactions between exogenous virions and neighboring cells are likely to lead to the sheer engulfment and catabolism of viral particles via cellular endocytosis (see for instance (1, 2), viral entry leading to productive infection appears to require interactions between the retrovirus envelope and particular cellular receptors. Successful envelope-receptor interactions lead to membrane fusion and the release of viral capsids into the cytoplasm. Depending on the retrovirus but also on the cell type, fusion is either pH-independent and occurs directly at the cell plasma membrane, or pH-dependent and takes place after endocytosis in intracellular vesicles (figure 1B).

The retroviral envelope is dragged out of the cell plasma membrane of virion-producing cells and the viral-encoded envelope glycoprotein (Env) is inserted within. The very first steps of infectious retroviral entry are governed by Env. Functional Env molecules of vertebrate retroviruses are synthesized as glycoprotein precursors that are cleaved in the Golgi into two main components: an entirely extracellular and glycosylated surface component (SU) and a transmembrane component (TM) to which SU is associated (figure 1A) (see (3). All retroviral SU have been shown to harbor the receptor binding determinants, whereas the TM, which harbors a typical fusion peptide at its amino terminus, is directly responsible for the initiation of fusion pores. As worked out in different retroviral models, Env-mediated infectious viral entry appears to include at least the following steps: adsorption of the particles to the cell surface; SU-Env receptor interaction; Env conformational changes that release the TM fusion peptide; formation of TM-mediated fusion pores; and membrane fusion. In the case of oncoretroviruses that belong to the Gammaretrovirus genus, such as murine leukemia viruses (MLV), conformational changes that follow the primary SU-receptor interaction seem to be conditioned by a subsequent interaction between amino and carboxy terminal SU sequences (see for instance (4-6).

2.3. Cell surface receptors for retroviral envelopes

The human immunodeficiency virus (HIV) pandemic has triggered a great deal of effort toward the identification of receptors used by retroviruses to enter and infect cells. Since the identification of CD4 as the HIV Env receptor (7, 8), the list of receptors continues to grow (see table 1). Interestingly, CD4 is one retroviral receptor for which coreceptors, multimembrane-spanning chemokine receptors, have been formally identified. The generally accepted scheme is that an initial interaction of HIV Env with CD4 is followed by the recruitment of chemokine receptors. It is also noteworthy that all receptors identified for mammalian oncoviruses belonging to the Gammaretrovirus group are multimembrane-spanning molecules whose function is to transport different nutrients, such as amino acids, inorganic phosphate and myo-inositol (9-11). In contrast, avian Alpharetroviruses and retroviruses other than mammalian Gammaretroviruses use single membrane spanning or lipid-anchored proteins (10). Despite the fact that HTLV-1 was the first human retrovirus isolated, identification of its Env receptor awaited another twenty years.

2.4. HTLV, HTLV Env-mediated tropism, fusion and viral entry: facts and questions

2.4.1. HTLV and STLV, endemic primate retroviruses of the Deltaretrovirus PTLV species

Since the first description of adult T cell leukemias and the isolation of HTLV-1 in endemically infected population in Southern Japan (12) and in a patient with cutaneous T-cell lymphoma (13), the two known types of HTLV, HTLV-1 and the related HTLV-2 type, have been found to be more widespread than initially thought. HTLV is endemic to all populated continents, with the

Table 1. Identified retroviral receptors

Retrovirus envelope	Host [1]	Receptor	Topology	Cell function	References
Ecotropic-MLV	Mouse	CAT1	14 transmembrane domains	Basic amino acid transporter	175
Amphotropic-MLV 10A1-MLV[2]	Mouse	PiT2	12 transmembrane domains	Inorganic phosphate transporter	176, 177
Xenotropic Polytropic-MLV	Mouse	XPR	8 transmembrane domains [3]	ND [4]	178-180
M813	Mouse	SMIT1	14 transmembrane domains [3]	Myo-inositol transporter	9
10A1-MLV[2] FeLV-B FeLV-T GaLV	Mouse Cat Cat Monkey	PiT1	12 transmembrane domains	Inorganic phosphate transporter	181
SRV ; BaEV[2] RD114 HERV-W [2, 5] SNV ; REV-A	Monkey Cat Human Birds	ASCT1; ATB[0]	10 transmembrane domains	Neutral amino acid transporter	182, 183
FeLV-C	Cat	FLVCR	12 transmembrane domains	Facilitative transporter	183, 184
PERV-A[2]	Pig	PAR1; PAR2	10-11 transmembrane domains [3]	ND	185
HTLV-1; HTLV-2 STLV	Human Monkey	GLUT1	12 transmembrane domains	Facilitative glucose transporter	45
HIV-1 ; HIV-2 SIV-1 ; SIV-2	Human Monkey	CXCR4[6] ; CCR5[6]	7 transmembrane domains	G-protein coupled chemokine receptor	186-189
HIV ; SIV	Primate	CD4		TCR signaling	7, 8
MMTV	Mouse	TrfR	Single	Transferrin receptor	190
ALV-A	Birds	TVA	Transmembrane	LDL receptor-like protein	191
ALV-B ; ALV-D ; ALV-E	Birds	TVB		Fas/NFR-like receptor	192, 193
JSRV ENTV	Sheep Goat	HYAL2	GPI anchor	ND	194
FeLV-T	Cat	FeLIX	Soluble	FeLV-B Env origin; Interacts with PiT1	195

[1] Refers to the natural host in which the retrovirus has been isolated, and does not refer to the virus tropism which usually covers a wider species spectrum; [2] Retroviruses that use two different receptors; [3] Debated number of transmembrane domains; [4] ND, not determined; [5] HERV-W is an endogenous Env sequence which is not part of an infectious retrovirus; [6] Only the two main coreceptors identified are indicated. Abbrevations: MLV, murine leukemia virus; FeLV, feline leukemia virus; GaLV, gibbon ape leukemia virus; SRV, simian retrovirus; BaEV, baboon endogenous virus; HERV, human endogenous retrovirus; SNV, spleen necrosis virus; REV, reticuloendotheliosis virus; PERV, porcine endogenous retrovirus; HTLV, human T-cell leukemia virus; STLV, simian T cell leukemia virus; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; MMTV, mouse mammary tumor virus; ALV, avian leukosis virus; JSRV, Jaagsiekte sheep retrovirus; ENTV, enzootic nasal tumour virus.

apparent exception of North Africa and Europe (see (14, 15) and references therein for a review). However, HTLV infections have also been established in these latter areas as sporadic infectious agents, concomitant with the pandemic spread of HIV. A high HTLV incidence has been reported in several indigenous populations of sub-saharan Africa, Middle-East, southwestern Japan, Melanesia, and Americas (15). The endemic presence of HTLV in humans parallels the endemic infection of numerous non-human primate species with closely related simian viruses of several types. Simian T-cell leukemia virus type 1 and 2 (STLV-1 and -2), isolated from several species of monkeys around the world, including new-world spider monkeys, exhibit a close relationship with their human counterparts. On the other hand, a human equivalent for a third STLV type, STLV-3, isolated from different species of monkeys in Eastern and Western Africa (16-18), has yet to be identified. Human HTLV and simian STLV belong to the PTLV (for primate T-cell leukemia virus) retroviral species, which are the main primate members of the Deltaretrovirus genus. Other Deltaretroviruses include the baboon T cell leukemia virus (BTLV) and bovine leukemia virus. The BTLV Env sequence (accession number U56855) is hardly distinguishable from PTLV-1 (19), whereas BLV Env appears to represent a distinct *bona fide* protein.

2.4.2. Restricted *in vivo* tissue distribution versus extended *in vitro* tropism

It has been extensively documented that HTLV-1 infection *in vivo* takes place preferentially in CD4+ T lymphocytes (20), whereas HTLV-2 seems to be more restricted to CD8+ T cells (21). This cellular confinement of HTLV infection *in vivo* is in sharp contrast with the remarkably extended tropism observed for both HTLV types *in vitro*. Thus, HTLV infections *in vitro* have been shown to rapidly propagate in numerous cell types, among which are non-T lymphoid cells, osteosarcoma cells, microglial cells, endothelial cells, placental villous cells and many other cell types (22-27). Moreover, the restricted *in vivo* cell type distribution of HTLV contrasts with the fact that HTLV-1 and HTLV-2 Env-mediated infection,

with replication-defective recombinant viruses, can be established in all tested vertebrate cell lines (28-30). Accordingly, the HTLV-1 Env receptor appears to be present on all tested vertebrate cell lines as assayed by the competitive binding of an isolated HTLV-1 SU (31). In light of these observations, the possibility of a broader *in vivo* tropism for both HTLV types has emerged. Indeed, several reports have shown that in addition to CD4+ and CD8+ T cells, monocytes and B cells can also be infected by HTLV-1 and -2 *in vivo* (32-34).

Because of the intrinsic difficulty in examining patients at the very early stages of infection, the exhaustive investigation of HTLV infection in non-hematopoietic tissues cannot be performed. To circumvent this obstacle, Kazanji *et al*. have used *de novo* HTLV-1 infected squirrel monkeys as a model for the early installment of HTLV-1 infection (35). Although these authors found a marked preferential lymphoid distribution of HTLV-1 proteins, they also reported that numerous non-hematopoietic tissues, including muscle, secretory glands, pancreas and intestine, appear to support HTLV-1 replication (35). The ability of HTLV to infect non-lymphoid tissues in humans is supported by a report describing the infection of breast epithelial cells in a rare case of combined adult T-cell leukemia (ATL) and gynecomasty in a man (36). Thus, while preferential infection of hematopoietic cells, with a marked predilection for T lymphocytes, appears to be the rule for HTLV infections, targeting of HTLV to non-hematopoietic tissues early after infection cannot be excluded and deserves closer examination.

The vast research on HTLV-infected individuals leaves little doubt that T lymphocytes are the major, although not exclusive, virus reservoir *in vivo*. The origin for this T cell-preferred distribution *in vivo* can be due to differences in the availability of Env receptors and co-factors for viral entry (37), as well as to differences at post-entry levels (for a review see for instance (38). Env-independent cell specific restrictions, taking place at pre and post-integration levels, have been identified for other retroviruses. For example, the murine *Fv-1* and human *Ref-1* genes exert a post-entry restriction on MLV capsids before integration (see (39) and references therein) as well as simian TRIM5a on HIV; also the CEM15/APOBEC-3G cytidine deaminase inhibits infection by targeting the neosynthesized proviral DNA but this effect is neutralized by the HIV Vif protein (see (39) and references therein); and a lack of HIV Tat transcription-enhancing activity in the absence of cyclin T1 results in a cell type-specific restriction (40). Although similar mechanisms have not been documented for HTLV, it is interesting to note that HTLV-1 capsid-containing retroviral cores have been shown to play a role in the low infectivity of HTLV-1 (41). It remains to be determined whether this is due to capsid-directed restrictive cellular factors as described above. Finally, in addition to direct virus-cell interactions, antiviral activities of the host organism, such as the immune response (42) and cell-specific Env-mediated cytotoxic effects (43-45) certainly have a major impact on the *in vivo* retrovirus tissue distribution. In conclusion, the *in vivo* cell type distribution of HTLV infection, as generally evaluated late after the initial infection, results from a full array of interactions between the virus and the host, and is not likely to precisely match the *in vitro* cellular tropism, *stricto sensu*, of the virus.

2.4.3. Cell-free and cell-to-cell infection

Although achievable, *in vitro* infection with cell-free HTLV-1 virions remains remarkably inefficient when compared to infection by Gammaretroviruses or lentiviruses. Following the earliest attempts to spread HTLV infection *in vitro*, it became clear that infection is remarkably dependent on cell-to-cell contacts (see (46) and references therein). This property has been beautifully illustrated by Igakura *et al.* who used confocal microscopy to visualize the transfer of different virion components from lymphocytes of infected patients to non-infected recipient lymphocytes (46).

The low infectious ability of HTLV-1 virions was also reproduced with replication-defective recombinant vector systems and seems to be due to both the HTLV core (41) and Env proteins (28-30, 47). However, from our own experience, while MLV or HIV cores pseudotyped with HTLV-1 envelope are indeed poorly infectious in cell-free preparations, with infectious titers of approximately 10^2 FFU/ml, pseudotyping with HTLV-2 Env leads to titers of 10^5 FFU/ml (45). Whether this higher titrating property of the HTLV-2 Env is common to all HTLV-2 strains or is particular to the prototypic Env of the HTLV-2 NRA isolate (48) used in these studies is under investigation. It will also be important to determine whether the low infectious titers of HTLV-1- pseudotyped virions is specific to the Env in the HTLV-1 MT-2 isolate (49). Nevertheless, in the absence of viremia, cell-to-cell infection appears to be the exclusive mode of *in vivo* HTLV transmission (see for instance (50) for a review).

It is important to note that cell-to-cell transmission is also the most efficient mode of transmission for all retroviruses, including HIV and MLV which are generally presented as paradigms of cell-free transmitting retroviruses (see for instance (51). In the presence of a strong immune response and the absence of viremia, it is likely that cell-to-cell spreading represents the sole mode of *in vivo* transmission for most retroviruses, despite the fact that *in vitro* models of retrovirus replication have favored efficient high-titering viral production at the expense of cell-to-cell transmission. Indeed, when seeding high-titer replication-competent MLV on fully permissive adherent cell monolayers, no rampant viral spreading is observed as monitored by viral antigen expression. Rather, *de novo* infected cells remain confined within restricted foci of infection, even in the absence of any movement-restricting gel which is generally required for plaque assays with cell-free spreading lytic viruses (52) (figure 2). Therefore, as previously put forth by D. Derse, we believe that retroviral spreading arising from cell-to-cell contact between infected and non-infected cells is probably not specific for HTLV but is likely to be « *the rule rather than the exception* » (53). Accordingly, natural transmission of retroviruses requires fluid exchanges that include transfers of significant numbers of infected cells (54).

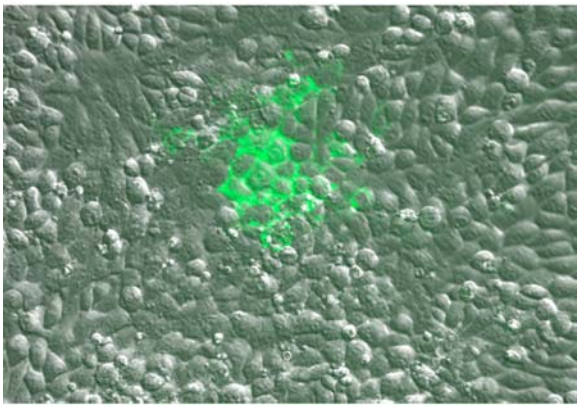

Figure 2. Visualization of a Friend murine leukemia virus (F-MLV) infection focus. As it is the case for HTLV, propagation of replication-competent F-MLV occurs mainly through cell-to-cell contact, resulting in confined foci of infected cells. NIH 3T3 cells were infected at a low multiplicity of infection with F-MLV. A focal immunofluorescence assay [52] was performed 3 days later by staining with an anti F-MLV Env SU monoclonal antibody (H48) followed by a FITC-conjugated secondary antibody.

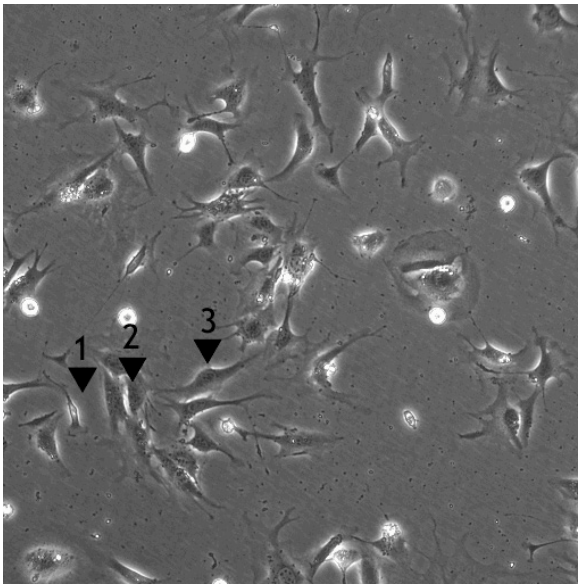

Figure 3. Time-lapse video microscopy of NIH 3T3 cells transfected with a syncytial Env expression vector. Following transfection, images were collected every minute for 16 hours using a wide field microscope. Adherent cells are constantly in motion and establish contacts through filopodiae and lamellipodiae. However, syncytia formation occurs only when cells are expressing fusiogenic Env glycoproteins. Three cells that seem to initiate a syncytium are numbered. Cell #1 fuses originally with one daughter of parental cell #3. Immediately after, cell #2 joins the syncytium. The syncytium then expands rapidly, leading to the recruitment, at the center of the syncytium, of more than 20 nuclei that show planar circular movements. DNA-liposome complexes used for transfection are visible as subcellular structures which are rapidly captured and dissolved by cells.

2.4.4. Env-mediated receptor binding and syncytia formation

The presence of cellular receptors for Env-mediated viral entry conditions the first steps of retroviral infection and virus cell tropism. The most commonly used assays to study HTLV receptor expression are based on cell-to-cell fusion that take advantage of the highly fusiogenic property of HTLV Env, leading to the rapid formation of multinucleated giant syncytia (figure 3). Using adequate target cell types, syncytial properties can be observed for most retrovirus Env (55). In the case of HTLV Env, syncytia formation is spectacularly rampant and develops in almost all vertebrate cell lines tested. However, Env-receptor interactions and HTLV Env-mediated viral entry in the absence of syncytia formation has been observed in a few cell lines (see for instance (29). Therefore, although syncytia formation appears to be a useful means of monitoring HTLV Env-cell membrane interactions (37, 40), it is not strictly indicative of receptor recognition by HTLV Env. Rather, it is likely to reflect post-binding events required for membrane fusion processes (see (56, 57).

2.4.5. pH dependence and receptor requirements

Retrovirus entry requires the fusion of viral and cell membranes to allow the release of viral cores in the cytoplasm through a fusion pore. This fusion event can take place either at the plasma membrane in a pH-independent manner or after endocytosis when the low pH environment in the endosome triggers Env conformational changes leading to membrane fusion (57). Based on cell-to-cell fusion and vesicular stomatitis virus (VSV) pseudotype infection assays, HTLV-1 was first classified, like most retroviruses, as pH-independent (58-60), consistent with the ability of HTLV to form rampant syncytia. Since we described cells that are specifically resistant to HTLV Env-mediated syncytia formation but remain fully susceptible to HTLV-1 and -2 Env-mediated infection (29), it will be interesting to monitor pH dependence for viral entry in these cells. Indeed, the observation of HTLV particles within endosomes is also consistent with the existence of a pH-sensitive endocytic pathway (61). Interestingly, Trejo *et al.* have reported that HTLV-1 Env-pseudotyped HIV particles infection exhibits intermediate pH-sensitivity on HeLa cells (30), suggesting the use of different routes of infection by HTLV according to the cell type used. Alternatively, acidification may be required at a post-binding level, similar to what has been described for the Env of avian retroviruses (62). Accordingly, it has been suggested that poor HTLV-1 transmission may be due to differences in post-binding events that favor the evolution of a fusion-competent envelope TM protein conformation (37). Thus, low or high abundance of secondary molecules involved in fusion events and different from the primary binding receptor, may influence the tropism of HTLV particles and their mode of entry, as documented for HIV (56, 59). The close evaluation of pH dependence in different physiological settings such as described previously (46) will help bringing new clues to these questions.

2.4.6. Cellular factors involved in HTLV Env-mediated membrane fusion and viral entry

The initial search for cell surface components

that are involved in interactions with HTLV Env, leading to viral entry, relied on the use of monoclonal antibodies directed against HTLV Env (63). Thus, cellular components interacting with HLA molecules were suspected to bind HTLV Env at the cell surface because of the cross reactivity reported between HTLV Env and a monoclonal antibody that reacts against the region of an HLA-B locus gene which codes for the extracellular portion of a class I histocompatibility antigen (64). Also, an antibody raised against hIL-2 was found to cross react with Env, suggesting that HTLV receptor could be the hIL-2 receptor (65). On the basis of a relative resistance of murine fibroblasts to HTLV Env-mediated infection and syncytium formation, it was reported that the HTLV-1 and -2 receptor maps to a single locus on human chromosome 17, in 17q21-23, near the thymidine kinase 1 (TK-1) locus (66, 67). However, this hypothesis lost ground before being completely abandoned when mouse fibroblasts were shown to be susceptible to HTLV Env-mediated fusion (68) and infection (28, 30) and the presence of human chromosome 17 in mouse cell hybrids increased neither infection (28) nor HTLV Env binding (31). Interestingly, it was later shown that HTLV could infect numerous cell types in an *in vivo* mouse model (69). Accordingly, mouse cells have been found to bind soluble HTLV-1 Env SU immunoadhesins (31).

Syncytia formation has also widely been used as a read-out for the screening of antibodies directed against Env-receptor interactions. Thus, several monoclonal antibodies found to recognize tetraspanin antigens have been shown to block HTLV Env-mediated syncytia formation and tetraspanins, which are involved in cell adhesion, have been proposed as potential HTLV receptors (70, 71). However, it was later shown that the interaction of CD82 tetraspanin with HTLV envelope inhibits HTLV fusion and cell-to-cell transmission, thus discarding its potential role as a receptor (72).

Several unidentified proteins, ranging from 31 to 70 kDa, have also been suspected as potential receptors based on their recognition by the 34-23 monoclonal antibody which inhibits HTLV-1 virion binding to activated lymphocytes (73). At the time, this was thought to be concordant with the fact that the antigen recognized by 34-23 was found to be expressed from human chromosome 17, at the 17q23.2-17q25.3 position, in the TK-1 locus. It is noteworthy that NIH3T3(TK$^-$) cells do not form syncytia upon expression of the HTLV envelope, although these cells possess a functional HTLV receptor (29). Therefore, the syntenic mouse and human locus containing the TK-1 gene may contain a gene coding for a protein involved in a post-receptor binding step.

Peptides derived from the HTLV Env SU were also used to screen for inhibition of syncytia formation in attempts to identify the receptor. Such a peptide (position Asp197-Leu216 of HTLV-1 Env (74) was found to bind the 71-kDa heat shock cognate protein (HSC70) within a beta actin-containing complex (75). Interestingly, HSC70 appeared to be a cell-fusion enhancing factor (76) which interacts with the capsid protein of many enveloped and non-enveloped viruses (see (77). Against a role for HSC70 as a component of the primary receptor is also the fact that the peptide that binds HSC70 maps to the central proline rich region (PRR) homologue of the HTLV Env SU, while we mapped the receptor binding determinants of the HTLV Env upstream of the PRR homologue (see below) (78).

The finding that HTLV infection is tightly dependent on cell-to-cell contacts has encouraged the evaluation of diverse adhesion molecules in HTLV Env-mediated processes. E-selectin, LFA-I, VLA-4, L-selectin, and CD44 have been shown to be involved in HTLV transmission (79). Moreover, ICAM-1, ICAM-3 and VCAM-1 were also found to modulate syncytia formation, while antibodies directed against integrin beta 2 and beta 7 where shown to inhibit syncytia formation (37, 80). The intervention of non-protein molecules in HTLV receptor functions has also been evaluated. Palmitoyl(16:0)-oleoyl(18:1)-phosphatidylglycerol and lipid rafts were found to be involved in syncytia formation (81, 82). Recently, an implication for heparan sulfate proteoglycans in HTLV Env-mediated fusion and infection has been demonstrated (83).

As discussed above, syncytia formation is a multi-step process that depends first on Env-receptor binding but also on post-binding events which bring the fusion pore into syncytial dynamics. The study of HTLV Env-mediated syncytia formation has favored the identification of cellular components involved in post-binding events rather than those implicated in primary receptor binding.

3. HTLV AND STLV ENVELOPE GLYCOPROTEINS: A SIMPLE ENV IN A COMPLEX RETROVIRUS

PTLV belong to the Deltaretrovirus genus which is part of the complex retrovirus group that includes the Lentivirus and Spumavirus genera. Complex retroviruses are characterized by the production of several multiple-spliced mRNAs and the production of a whole array of regulatory proteins. Retroviruses which, like MLV, are characterized by single-intron splicing belong to the simple retrovirus group. Phylogenetic analyses based on the conservation of the *pol* gene place HTLV and MLV at the most distant branches of the Retroviridae family phylogenetic tree (84). Therefore, the striking conservation of Env SU motifs between HTLV-1 and the Friend strain of ecotropic MLV (F-MLV) was surprising (44). Those motifs are located upstream of a conserved CxxC motif (85, 86), where x represents hydrophobic residues, and downstream of the MLV SU amino terminal domain that harbors the variable regions sufficient to bind MLV Env receptors (87-89). Based on this SU motif conservation between viruses with phylogenetically distant *pol* sequences and very different overall genetic organizations, we postulated that the MLV and HTLV SU are derived from closely related ancestor genes that were “captured” by ancestral HTLV and MLV retrotransposons (44, 90).

The alignment of the F-MLV SU with the SU of three prototypic PTLV types underscores the presence of a

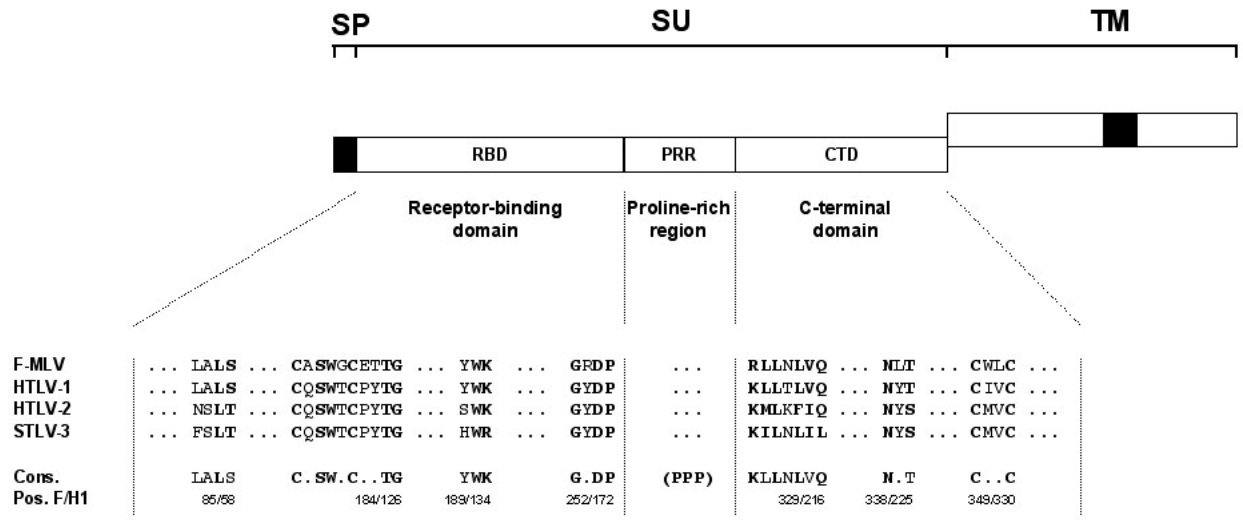

Figure 4. Conservation of motifs in the Env SU of MLV and PTLV. Amino acid sequences of F-MLV (accession number X02794), HTLV-1 (accession number M37747), HTLV-2 (accession number L20734) and STLV-3 (accession number CAA61321) SU were aligned using the ClustalW algorithm, and conserved motifs are shown (4 in the receptor binding domain (RBD), and 3 in the carboxy terminal domain). There is no sequence homology in the proline-rich region (PRR) which is very heterogeneous in length (52 residues for F-MLV and 26 for HTLV-1 and –2), as it is the case among Gammaretroviruses. Residues which are either identical between the 4 sequences or belong to the same polarity group are indicated in bold. Other indicated residues in the consensus sequence correspond to shared residues between F-MLV and at least 1 PTLV. The positions of the last residue of each motif are shown for F-MLV and HTLV-1. SP: signal peptide.

PRR homologue as well as several short conserved motifs in the intervariable regions of the MLV receptor binding domain (RBD) and in the carboxy-terminal domain (figure 4). We determined that these conservations reflect a similar functional organization of the HTLV and MLV SU modules. Indeed, a chimeric Env in which the mouse-restricted ecotropic F-MLV RBD is replaced by the HTLV SU amino terminal domain is fusiogenic for both mouse and human cells, thus indicating the recognition of an HTLV Env receptor (44).

The fusion triggered by the binding of ecotropic MLV Env to its receptor depends on the interaction of the SU carboxy terminus with a conserved His residue, or any other aromatic residue, at position 8 of the MLV SU (4-6). However, no conserved aromatic residue around this position was found in PTLV SU. It will therefore be interesting to determine whether the HTLV RBD (HRBD) exerts properties similar to those associated with the MLV SU His at position 8. Alternatively, the absence of an equivalent mechanism for triggering fusion may provide additional clues concerning the molecular bases for the low cell-free infectivity of HTLV virions.

4. HTLV ENV RECEPTOR EXPRESSION AND T CELL ACTIVATION

As T cells appear to be the main target for HTLV transformation as well as the major *in vivo* reservoir, we tracked expression of the HTLV receptor on different T cell populations, including CD4 helper T cells, CD8 cytotoxic T cells as well as naïve and memory CD4 subsets, using tagged forms of HRBD. As opposed to all cell lines, including transformed T cell lines which express HTLV receptors, we were surprised that none of these primary T cell populations bound our tagged HRBD. Unlike transformed T cells such as Jurkat, circulating primary T cells are almost entirely in the G_0 phase of the cell cycle and do not express activation markers. The stimulation of primary T cells by antigen occurs *via* the T cell receptor (TCR) and, under normal conditions, promotes an efficient immune response. Upon activation, T cells can undergo 6-8 divisions and secrete various cytokines. This transient phase is associated with the modulation of numerous cell surface markers. Indeed, we and others determined that HTLV receptor expression is transiently induced by TCR activation (45, 91). We also found that receptor expression preceded DNA synthesis and was not strictly dependent on cell division.

Our results indicate that transient expression of the HTLV receptor on T cells following TCR stimulation is likely to be crucial for the constitution of these cells as HTLV reservoirs *in vivo*. In this respect, it is notable that human T cells, including HTLV-infected T cells, can probably survive for more than 20 years in the peripheral circulation. Our data also leads us to speculate that infection of T lymphocytes by the virus preferentially occurs in sites where T cells become activated, such as in inflammatory sites and secondary lymphoid organs. The illustration by Igakura *et al.* of an accumulation of HTLV Gag proteins and genomes at lymphocyte cell-cell junctions and their rapid transfer to uninfected lymphocytes, favors a scenario whereby virus transmission occurs more readily upon extensive contact between cells of the immune system, such as in lymph nodes or inflammatory sites. In a symmetrical scenario, CD4+ T cells directed against HTLV-1 antigens have been shown to be preferentially infected in

HTLV-1 seropositive individuals (92). This may reflect an additional route of transmission from passively infected cells to reactive T lymphocytes. Moreover, when infected with HTLV or transduced with HTLV accessory proteins, T cells express different factors, such as integrins (93, 94), that are likely to enhance the probability of cell contacts.

Breast feeding and accompanying cell transfers that occur between mother and infant have been proposed to be a major route of HTLV transmission (see (54, 95) for a review). The particular "immaturity" of the newborn's immune system may account for this preferential route. Indeed, in contrast to adult peripheral T cells which present a 1:1 ratio of naïve and memory T cells, neonatal T cells are almost exclusively naïve cells, which proliferate and change to a memory phenotype upon encountering with a cognate antigen. Furthermore, the peripheral lymphocytes in newborns have only recently differentiated in the thymus, and as such are designated as recent thymic emigrants (RTE). RTE respond to antigenic and cytokine stimulation differently from adult memory T cells (96-98). RTE enter more rapidly into cycle when activated through the TCR, with 24% of newborn RTE going into G_1 versus 1% for adult T cells, following 15 hours of TCR stimulation (representative data from S. Jaleco and N. Taylor, unpublished results). Moreover, neonatal, and not adult, T cells undergo multiple divisions in response to the IL-7 cytokine (97, 98). Indeed, IL-7 triggered surface expression of the HTLV receptor in CD4+ lymphocytes isolated from umbilical cord blood, albeit at significantly lower levels than that observed in TCR-activated lymphocytes (99). This expression of the HTLV receptor is not a constitutive characteristic of neonatal T cells but is directly dependent on IL-7 treatment, since HTLV receptor was not observed in freshly isolated quiescent neonatal T cells. Interestingly young HTLV-1 seropositive patients have a decreased proportion of naïve T lymphocytes and display higher levels of plasma IL-7 than non infected individuals (100). Thus, expression of the HTLV receptor by IL-7-stimulated neonatal T cells suggests a possible mechanism *via* which HTLV can be transmitted from an infected mother to the infant, even in the absence of antigenic stimulation.

5. GLUT1 AS A HTLV ENV RECEPTOR FOR VIRAL ENTRY

As described above, the HTLV SU RBD appears to share the same organization as the SU of MLV-related retroviruses, and all identified MLV receptors are multimembrane-spanning nutrient transporters (table 1). As such, we focused our search for an HTLV Env receptor on multimembrane-spanning molecules potentially involved in the transport of nutrients.

Transfection of cells with HTLV Env or HRBD constructs specifically blocked milieu acidification. As most of the H+ proton accumulation in *in vitro* cultured cell lines is due to the activity of H+/lactate symporters (101), we first evaluated proteins involved in lactate transport, such as monocarboxylate transporter 1 (MCT1) and its chaperone protein CD147 (102, 103), as potential HTLV Env receptors (45). Because the HRBD-mediated block of medium acidification was not associated with these candidate receptors and most of the lactate produced in cell cultures is derived mainly from glucose degradation, we next evaluated glucose transporters as HTLV Env receptors.

Vertebrate glucose transporters belong to the GLUT family which comprises 14 members divided into 3 classes that include other hexose transporters, and members of unknown functions (104, 105). Upon treatment with cytochalasin B, an inhibitor of GLUT glucose transporters (106, 107), HRBD binding as well as Env-mediated infection are impaired (figure 5B), in agreement with the involvement of GLUT molecules in HTLV Env-mediated entry process (45). Within this family, we hypothesized that GLUT1 best fit all the known properties of the HTLV receptor. However, because all classical cell culture conditions force cell adaptation to fermentation which tightly relies on glycolysis, all established vertebrate cell lines express GLUT1. As such, they are not suitable for potential receptor screening by classical cDNA transfection strategies. Also, upon transient transfection of GLUT1, or the related GLUT3 isoform cells acquire a round shape and eventually detach from the support, a typical consequence of cell toxicity that can tentatively be attributed to an excess in intracellular glucose levels. Indeed, the selection of stable clones over-expressing GLUT1 was unsuccessful in our hands. For this reason, we based our screening strategy on a transient gain-of-function assay based on glucose transport, HRBD binding and HTLV Env-mediated retroviral infection.

We developed several approaches in order to vary GLUT1 availability at the cell surface. Upon transient transfection of GLUT1 cDNA in several mammalian cell lines, we observed a striking elevation of HTLV Env binding and HTLV Env-mediated infection (45). Based on this observation, we further confirmed the role of GLUT1 as an HTLV Env receptor using two additional assays. First, we evaluated the ability of GLUT1 to specifically restore HTLV Env-mediated infection after blocking the HTLV Env receptor with Env itself (figure 5C). In this interference assay, the HTLV RBD is expressed in cells concomitantly with receptor candidates. Upon expression of the HRBD, the availability of the receptor decreases leading to a drop in viral titers of at least 2 logs. This drop was totally abolished upon cotransfection of GLUT1 but not the GLUT3 isoform, demonstrating a direct effect of GLUT1, independent of its glucose transport activity, in restoring HTLV RBD binding and HTLV Env-mediated infection (45). Furthermore, in a second assay, we used small interfering RNAs to down-modulate endogenous levels of GLUT1 RNA (figure 5D). Following this down-modulation, HTLV Env binding and Env-mediated infection were significantly reduced, while binding and infection mediated by the amphotropic MLV Env were not reduced. In this system as well, introduction of GLUT1, but not the GLUT3 isoform, fully restored HTLV Env binding and infection (45). In theses systems, GLUT1 was found to serve as cell surface receptor for both HTLV-1 and -2 infection (45) and unpublished observations).

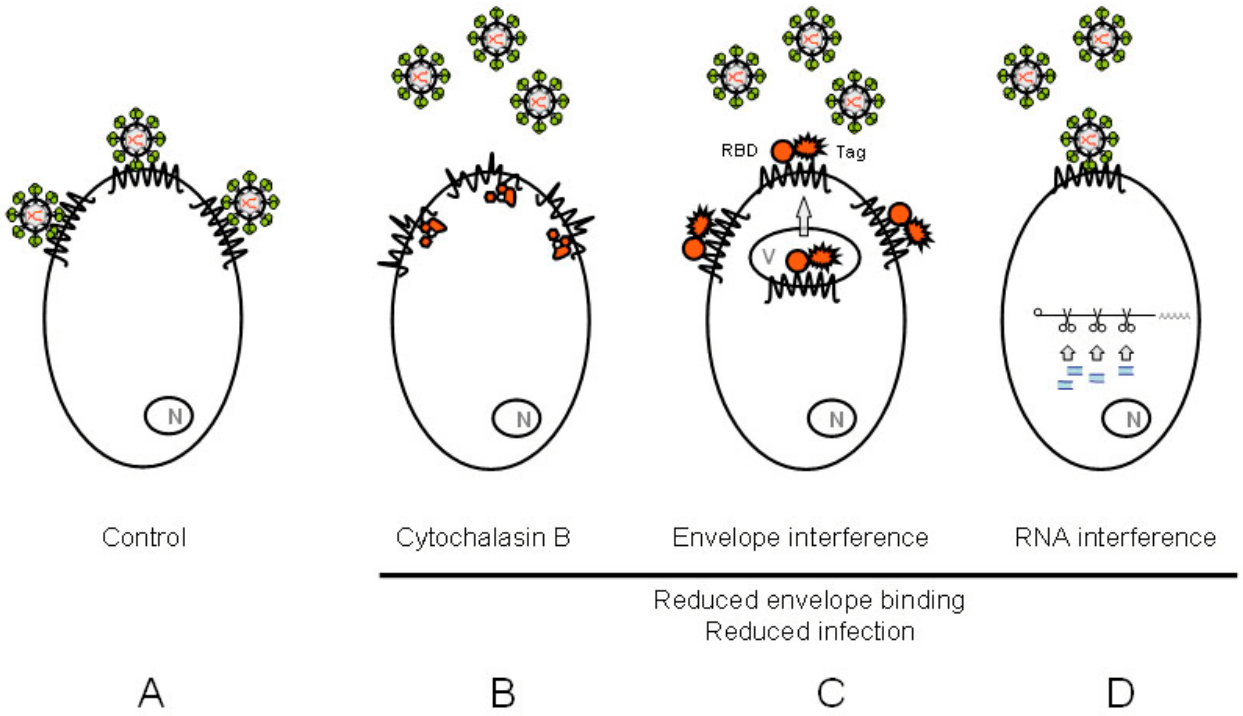

Figure 5. Schematic representation of assays designed to downmodulate GLUT1 transport and expression. Three techniques were used to inhibit GLUT-1 availability or expression in 293T cells. Cells were (A) not treated; or treated with (B) cytochalasin B which inhibits glucose transport activity by interacting with the cytoplasmic face of GLUT1; (C) an interfering HTLV RBD that saturates vesicular or plasma membrane GLUT1 via direct binding to the transporter; and (D) interfering RNAs directed against the 3' untranslated region of the GLUT1 mRNA. In all three of the latter conditions, HTLV Env-mediated binding and infection were significantly reduced as compared to the non treated cells. Moreover, binding and infection could be reestablished by overexpressing GLUT-1, but not the related glucose transporter GLUT-3, in conditions C and D. N: Nucleus; V: Vesicle.

Immunoprecipitation of HTLV RBD with an HTLV Env antibody was accompanied by an efficient pull-down of GLUT1, confirming the close physical interaction between the HTLV RBD and GLUT1 (45). Further analyses of GLUT1-HRBD complexes may lead to the identification of additional partners, such as potential coreceptors. In this regard, it will be interesting to compare pull-down experiments performed with entire SU immunoadhesins (31, 91), and different forms of truncated HTLV SU or chimeric HTLV-MLV Env (44, 45, 78).

GLUT1 physically interacts with an amino terminal RBD fragment of approximately 160 residues that we identified as the minimal receptor-binding domain (78). Furthermore, we identified a conserved tyrosine residue (Y114 for HTLV-1 and Y110 for HTLV-2) as required for receptor binding (45, 78), and our preliminary results suggest that HTLV-1 and -2 RBD interact with the same surface epitope of GLUT1. Thus, the same motifs and probably the same conformations govern the interaction between GLUT1 and the HTLV-1 and HTLV-2 SU. However, this does not preclude differences in their respective affinities for GLUT1 or differences in the recruitment of other membrane-associated cellular factors, including potential coreceptors. In this regard, it is interesting to note that upon transfection of GLUT1, the increased binding of an HTLV-1 HRBD appeared relatively homogenous whereas HTLV-2 HRBD binding reached higher levels but was more heterogeneous (N. Manel, unpublished observations). Thus, it is possible that other molecules differentially modulate the binding of HTLV-1 and -2 envelopes.

6. LYMPHOCYTE ACTIVATION AND GLUT1-DEPENDENT METABOLISM

Using the HRBD, we were not able to detect surface GLUT1 expression on the vast majority of freshly isolated CD4 or CD8 T lymphocytes (99). This is either due to a true absence of surface GLUT1 or to the level of sensitivity of our tagged-HRBD that does not allow detection of low amounts of this protein. Our conclusion that the vast majority of primary human T lymphocytes express low or absent levels of GLUT1 is supported by data wherein GLUT1 expression was not detected in freshly isolated human T cells using a polyclonal rabbit anti-GLUT1 antibody, directed against the intracellular carboxy-terminal domain (108). Nath *et al.* did detect HTLV receptor expression, i.e. GLUT1, on primary human T lymphocytes using an SU immunoadhesin on lymphocytes isolated by counter elutriation, wherein the cells were subjected to a high speed centrifugation for an extended time period, followed by positive selection with CD4 or CD8 antibodies (91). Moreover, when these authors

assessed GLUT1 expression in lymphocytes isolated via negative selection and in the absence of elutriation, only a very low percentage of T cells bound the HTLV SU immunoadhesin (91). In this regard, it is important to note that several recent studies, including work performed in our own laboratory, have shown that the choice of blood coagulant as well as the method used to isolate T lymphocytes can modulate surface expression of receptors pertinent to T lymphocyte function (109).

The observation that glucose metabolism increases upon T lymphocyte activation was reported over 30 years ago (110-113). Soon thereafter, it was also shown that mitogen stimulation increases glucose transport in thymocytes (114). Indeed, in 1978 Whitesell and Regen reported that the thymus contains two populations of cells ; quiescent cells where the glucose transport equilibrates with a half-time of 30-50 minutes and "active" cells where the half-time is approximately 1 minute (115). While glucose transport is crucial to the survival, differentiation and proliferation of thymocytes and mature T lymphocytes, the vast majority of studies concerning glucose metabolism in these cells were performed in the late seventies and early eighties, thus precluding studies of GLUT members.

The first glucose transporter (GLUT1) was identified by Mueckler *et al.* in 1985 (116), and in 1994, Mookerjee and colleagues assessed the expression and subcellular distribution of different GLUT isoforms in primary human T lymphocytes (108). They found that GLUT1 was not expressed on quiescent human T lymphocytes but was induced, predominantly at the plasma membrane, by 24 hours post mitogen stimulation. While other GLUT isoforms (GLUT-2 and GLUT-3) were found to be expressed on quiescent T cells, the importance of GLUT1 in T cell activation was strongly suggested by the finding that only its expression was correlated directly with glucose transport activity (108). Later was shown that the serine/threonine kinase Akt induces glucose uptake and cellular glycolysis in 3T3-L1 fibroblasts. This occurs via a translocation of the insulin-responsive GLUT-4 transporter to the plasma membrane and an increase in the synthesis of GLUT1 (117). Studies such as these, performed in several cell systems, were then transposed to T lymphocytes. Indeed, very recently, increased glycolysis due to expression of an activated Akt in murine T cells was shown to result in increased size, resistance to death-by-neglect, and T-cell malignancy (118). Under normal conditions, GLUT1 protein does not appear to be expressed in quiescent murine T cells (119, 120), as is the case for their human counterparts. After the original description that thymocytes can be divided into two groups with regards to glucose transport (115), A. Singer's group explored the molecular basis of this process and showed that there is a large heterogeneity in GLUT1 expression in murine thymocyte subsets (121). Moreover, stimulation of thymocytes with the IL-7 cytokine results in a significant increase in GLUT1 expression and an associated upregulation of the anti-apoptotic Bcl-2 protein (121). Thus, it appears that GLUT1 expression plays a role in the survival, proliferation and transformation of T lymphocytes.

It is important to note that in the vast majority of these studies, total cellular GLUT1 expression, and not surface GLUT1, was assessed because of a lack of reagents directed against the extracellular portion of GLUT1. While several commercially-available antibodies have been advertised as recognizing extracellular GLUT1, none of these antibodies were found to be reliable in our hands (unpublished data). Many of our studies, assessing the surface expression of GLUT1 on human T lymphocytes, were performed using tagged HRBD even before our identification of the HTLV receptor as GLUT1. Our data, demonstrating that expression of the HTLV receptor is associated with T cell survival and proliferation, is in complete agreement with that reported for GLUT1. The identification of GLUT1 as the HTLV receptor and the HRBD tools that we derived will help to further explore HTLV infection and the physiological consequences of GLUT1 expression in different human lymphocyte subsets.

7. IMPACT OF HTLV ENV-GLUT1 INTERACTIONS ON METABOLISM

Glucose provides a key supply of energy and carbon and glucose transport is a universally conserved property of living organisms. In vertebrates, GLUT1 appears to be the main glucose transporter and its presence at the cell surface as a functional glucose transporter is influenced at many levels. Each GLUT1 molecule harbors two glucose binding sites. The current model of GLUT1 topology (reviewed in (122) proposes that the two binding sites face opposite sides of the cell membrane and that they are in reverse conformation with regards to glucose binding. In the "in" state of the GLUT1 molecule, it is the cytoplasmic site that binds glucose, whereas in the "out" state glucose binding occurs at the exofacial glucose binding site. A functional transporter is thought to be a GLUT1 dimer, with each monomer in opposite "in" and "out" states, and their opposite states maintained by simultaneous flipping. GLUT1 expression is induced by various treatments (reviewed in (123), including glucose starvation (124, 125), hypoxia (126), inhibition of oxydative phosphorylation (127) and osmotic stress (128). Under stress, GLUT1 is induced at several levels: increased rate of transcription (129), stabilization of the mRNA (130), and unmasking of glucose binding sites on the GLUT1 molecule (131).

The first level of regulation of GLUT1 depends on its availability at the plasma membrane (132, 133). In *in vitro* models of insulin-induced increase in glucose uptake, GLUT4 massively translocates to the plasma membrane, and although to a lesser extent, translocation of GLUT1 from intracellular pools is also triggered (134, 135). GLUT1 translocation is also accompanied by increased transcription (136) and translation (137). GLUT1 induction by insulin varies with the cell line used and seems to be inversely proportional to the amount of GLUT1 already present at the membrane (138). Another level of modulation is exerted directly on cell surface-associated GLUT1 molecules. GLUT1 binds intracellular ATP (139) in the absence of hydrolysis, which results in inhibition of GLUT1-mediated glucose uptake (140), due to alterations in susbtrate binding affinity (141).

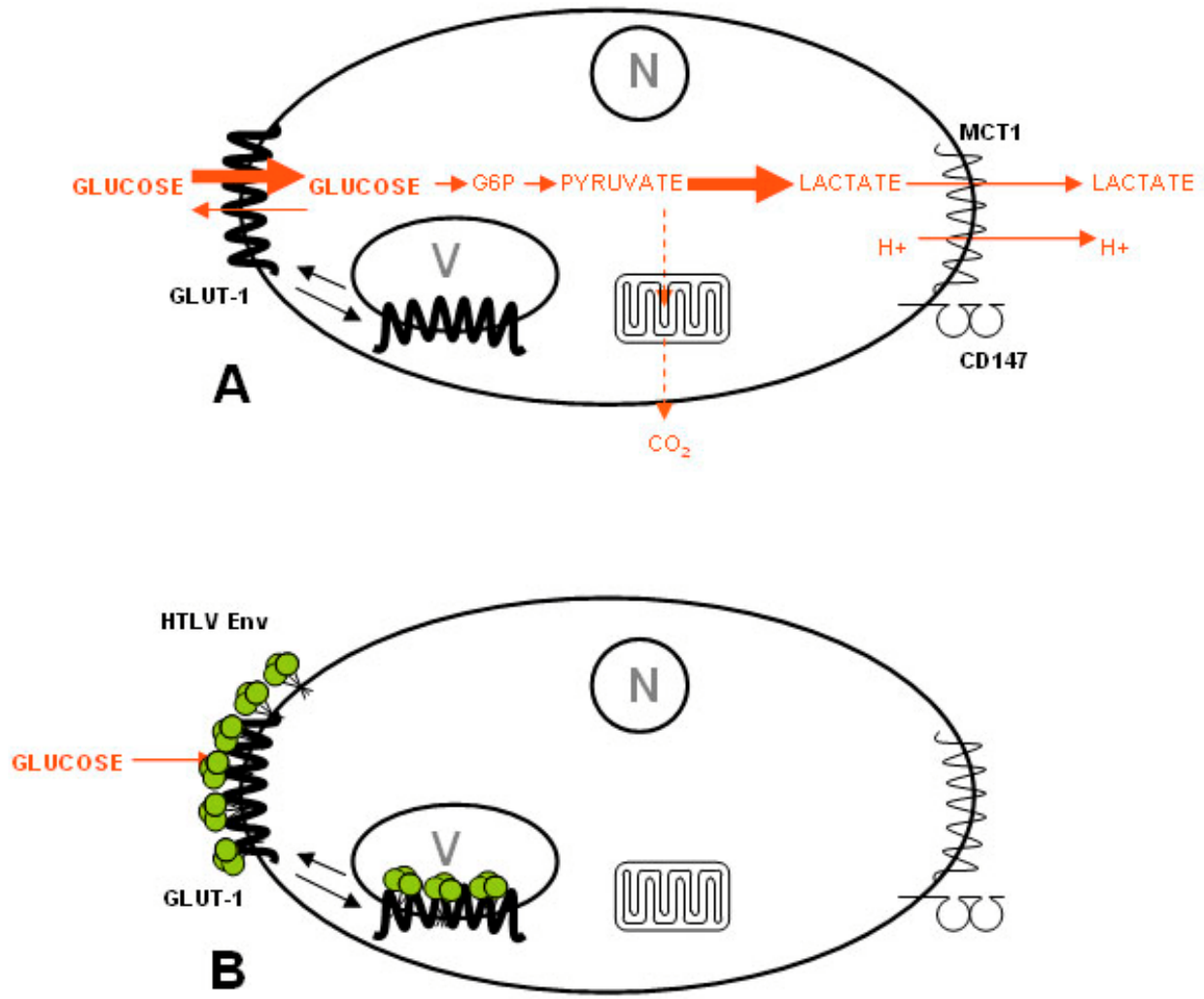

Figure 6. Impact of HTLV Env on glucose metabolism. In control cells grown in *in vitro* culture (A), glucose is taken up by cells and is then converted to pyruvate through glycolysis. Because cells are grown under limited O2 availability, 95% of the pyruvate is degraded into lactate, which is then secreted together with protons via the monocarboxylate transporter (MCT) leading to acidification of the culture medium. CD147, tightly associated with the main transporter MCT1, is shown. In the presence of HTLV Env (B), glucose transport into cells is strongly decreased, and glycolysis is hampered as observed by the significantly decreased production of lactate and a lack of acidification of the culture medium. Note that although several HTLV Env are depicted as being bound to GLUT1 in this figure, the precise stoichiometry of HTLV Env binding per GLUT1 molecule is not known. N: Nucleus; V: Vesicle.

As indicated earlier, in classical mammalian cell culture systems, cells have adapted from *in vivo* respiration to *in vitro* fermentation, thus relying primarily on glycolysis to maintain their ATP pool (142). Although the selective pressure that drives this switch remains to be fully elucidated, the limited availability of O_2 in culture medium provides a reasonable explanation. This fermentative adaptation to reduced oxygenation has been proposed to be a key step in the *in vivo* development of tumors (143). To fuel fermentation, glucose is the preferential metabolite, and under these conditions, GLUT1 plays a key role as the major glucose transporter (116). Following HTLV Env expression, glucose uptake is reduced due to the binding of HTLV envelope to GLUT1. Reduction of intracellular glucose in cultured cells reduces the glycolysis and thus production of its end-product, lactate. In culture, most of the lactate is released with protons via the MCT1/CD147 symporter system (102, 103) and figure 6). Therefore, a decrease in lactate production is accompanied by a drop in the acidification of the culture medium (figure 6). Protons co-secreted with lactate from fermentation are thought to account for 95% of the evolution of extracellular medium pH in *ex vivo* cultures (J. Pouyssegur, personal communication). As such, the inhibition of GLUT1 activity by HTLV envelope expression or by GLUT1 siRNA (our unpublished observations) prevents the color change of the pH indicator, phenol red, commonly added to the culture medium.

Interestingly, a switch to galactose as a source of

carbon tends to bias cell metabolism toward respiration (see for instance (144). When substituting galactose for glucose, we observed that HTLV Env binding increases, thus mimicking glucose starvation (our unpublished observations). Under these culture conditions, the induction of GLUT1 at the surface of 293T or HeLa cells is maintained during several weeks of culture and multiple passages (our unpublished observations).

The exact mechanism by which HRBD inhibits GLUT1 is unclear. To date we have not been able to inhibit glucose transport by adding exogenous HRBD to the cell culture medium. This might be due to insufficient amounts of soluble HRBD molecules produced by HRBD-transfected 293T cells (our main source of soluble HRBD), or because inhibition occurs only upon intracellular production of HRBD. Therefore, it remains to be determined whether binding of HTLV Env and HRBD to GLUT1 inhibits its transport activity, directly by competitive binding with glucose or by inducing conformational changes in GLUT1. Alternatively, GLUT1 inhibition by intracellular HRBD may alter GLUT1 trafficking, resulting in decreased cell surface-associated GLUT1 molecules. The first model is currently supported by our observation that bound tagged HRBD protein can be readily detected at the cell surface of HRBD-producing cells (our unpublished observations) arguing in favor of the presence of significant amounts of HRBD-GLUT1 complex at the cell surface.

Since GLUT1 and glucose transport occupy a central place in cell metabolism, HRBD-GLUT1 interactions also influence the balance of other essential metabolites upstream of lactate production, at all levels of glycolysis. Interestingly, GLUT1 can transport ascorbic acid under certain conditions (see (145) for a review). Potential alteration of this transport by HRBD-harboring HTLV derivatives could exert an influence on *in vivo* cell survival and integrity of the host organism, due to the role of vitamin C (ascorbate) in counteracting oxidative damage of proteins. Also, type 2 adenosine receptor agonists were found to inhibit the cell binding of HTLV virions (146). Although the effect of these compounds on Env-receptor interactions remains to be assessed, the fact that GLUT1 is an adenine nucleotide binding protein under an ATP feedback control regulation (147) may open new alleys for exploring the role of these nucleoside receptors in the early steps of retroviral infection.

In the experimental systems we used, HTLV Env and HRBD proteins were produced by transient transfection. We were unable to obtain stable HTLV Env expression, even when using cell lines that were resistant to syncytia formation, such as the NIH3T3(TK$^-$) described above. Attempts to derive such HTLV-expressing cells failed even when the HTLV SU-derived truncated HRBD was used in conjunction with other hexoses than glucose, confirming the essential function of GLUT1 in cell culture. Therefore, the question of the adaptive processes that allow HTLV-producing cell lines like MT-2, to maintain glucose uptake activity and GLUT1 expression remains unanswered. While it is clear that the HTLV envelope is expressed at the surface of such cell lines, as demonstrated by their ability to form syncytia with other cells, they might be expressed at a sufficiently low level as not to block GLUT1 activity. Alternatively, HTLV-producing cell lines might rely on another glucose transporter.

In vivo, only a few cells, among which are muscle cells, rely on anaerobic glycolysis for energy production. Interestingly, when thymocytes undergo proliferation, a transition from aerobic to anaerobic metabolism occurs, despite the presence of O_2. This phenomenon has been referred to as the *Crabtree effect* (148). At this stage of lymphocyte differentiation, glucose utilization increases 18-fold (148). Furthermore, proliferating thymocytes are strictly dependent on glucose, in contrast to hepatoma cells or human primary fibroblasts, for which uridine can be substituted (149). It is thus tempting to propose that in the course of an HTLV infection, an HTLV Env block of GLUT1 activity in infected thymocytes would provide a strict selective pressure, and potentially promote the emergence of tumorigenic T lymphocyte clones.

8. CONCLUSIONS AND PERSPECTIVES

8.1. Determinants of HTLV Env-GLUT1 interactions

The identity of the HTLV Env receptor remained elusive for approximately two decades and the search was hampered by the fact that HTLV entry can take place in all established vertebrate cell lines. This long search has been the source of numerous speculations as to the nature of the receptor, including the possibility that a dedicated cellular receptor may not be required for HTLV infection or that many different receptors can be used by HTLV. Based on the elucidation of the modular organization of the receptor binding domain (HRBD) and the generation of new tools which comprised only this region of the HTLV Env (44, 78), we obtained new clues that were key to our finding that GLUT1 is a receptor for HTLV Env. The first clue was based on the MLV-related organization of the HTLV SU (44, 78, 90) and figures 1 and 3). Since all identified Env receptors of MLV and related mammalian retroviruses are transporter-like multimembrane spanning molecules, or their chaperone molecules, we hypothesized that the HTLV receptor would belong to this class of cell surface molecules (table 1). The second clue was that expression of the HTLV Env receptor corresponded to an early marker of T cell activation (99). The third major clue was that transfection of HRBD blocks extracellular acidification and lactate accumulation which, *in vitro*, are both closely dependent on the integrity of the glycolytic pathway. Although GLUT1 appears to a primary HTLV receptor, it is not excluded that other cell surface molecules can be used by HTLV for infection, or alternatively, can potentiate infection.

Experiments of Env cross-interference to superinfection showed that HTLV-1 and -2 and STLV-1 use a common receptor (66, 150). We also observed this phenomenon when using HTLV-1 and -2 HRBD in cross-interference assays based on their cell surface binding (45, 78). Accordingly, the Tyr residue at position 114 of the

HTLV-1 SU, that we identified as a major determinant for receptor-binding (45, 78), is also conserved among all known strains of PTLV-1 and 2. To our knowledge, no Env interference assays have been performed with STLV-2 and STLV-3 strains. However, the high level of conservation of this residue and its surrounding residues in all reported PTLV strains (unpublished observations) augurs for the same receptor usage for all PTLV.

In addition to PTLV, the Deltaretrovirus genus comprises BLV for which the nature of the Env receptor remains uncertain. Given the close relationship between these two viral species (151, 152), the search for a BLV Env receptor that belongs to the multimembrane spanning molecules seems reasonable. It is interesting to note that despite a strikingly different organization of the HIV SU, whose RBD is constituted of a complex array of non contiguous determinants (153), multimembrane spanning molecules (chemokine receptors) serve as HIV Env coreceptors (154). Furthermore, cellular partners of GLUT1 appear to be recruited during HTLV entry as coreceptors (D. Ghez, Y. Lepelletier, C. Pique and O. Hermine, personal communication). It is tempting to speculate that many retroviruses have coreceptors but under conditions where the coreceptor is ubiquitously expressed, they have not been recognized to play this role. In the case of HTLV, further documentation of the interactions between Env, GLUT1 and additional partners will likely benefit from finer mapping of the respective interacting determinants. To this aim, we are currently testing HTLV Env-derived peptides as well as GLUT chimeras, constructed between GLUT1 and other GLUT isoforms, for their abilities to physically interact and modulate infection.

8.2. HTLV Env infection and GLUT1 expression: How to conciliate ubiquity and restricted distribution?

One of the recurrent paradoxes in the HTLV field is the restricted tissue distribution of the virus as opposed to the wide range of Env-mediated tropism in cell culture. Indeed, the HTLV Env-mediated tropism *in vitro* is one of the widest of all known retroviruses. Accordingly, GLUT1 is a molecule that is expressed on almost all dividing or metabolically active cells, hence its general expression on established cell lines. Although no definitive explanation can ultimately conciliate this apparent discrepancy, a whole array of observations hint to adequate explanations.

Retroviral tropism depends on viral entry, and for that purpose on Env-receptor interactions. However, a whole sequence of post-entry events leading to particle assembly and release are modulated by the cellular context and can thereby restrict *in vivo* tropism. Whether such restriction(s) play a role in the constitution of the *in vivo* reservoir of HTLV remains to be assessed and may benefit from the use of animal models (35). Another level of explanation comes from a more relative perception of the *in vivo* GLUT1 "ubiquity". Indeed, there are considerable differences in the levels of cell surface GLUT1 expression in different tissues. These differences constitute the basis for the *in vivo* detection of highly metabolically active and glycolysis-dependent cancer cells, by tracking GLUT1 activity with positron emission tomography after injection of 2-(18F)fluoro-2-deoxy-D-glucose, a non-metabolized glucose analogue (for a review see (155, 156). Precise analyses concerning the number of cell-surface GLUT1 molecules and viral particles that are required to initiate a successful viral entry, as well as the availability of potential coreceptor molecules in different cellular contexts, is likely to provide further information on the *in vivo* distribution of HTLV. It will be of interest to monitor, in experiments such as those described by Igakura *et al*. (46), the respective localizations of cell surface-expressed GLUT1 and Env molecules, as compared to other viral components, during cell-to-cell contacts between different cell types of infected and non-infected cells. Additional explanations for the *in vivo* tropism include the potential elimination of productively infected cells by the immune system (157, 158) and the fact that, like most retroviruses, HTLV infection of T cells requires cell division for integration, replication and further spreading. The ensemble of these restrictions will most likely efficiently limit the *in vivo* expansion of an HTLV infection.

We believe that the interaction of HTLV Env with GLUT1 is likely to be detrimental in certain cells with respect to metabolic function and membrane structures (see for instance (29, 45). This hypothesis is supported by the observations that virion components are extremely difficult to detect in the blood of infected patients and that virion production by T cells from asymptomatic as well as ATL patients is not readily detectable (157, 158) and O. Hermine, personal communication). Thus, productively infected cells may be rapidly eliminated *in vivo*. This would result in an *in vivo* distribution that would be confined to those cells which have by-passed these expected restrictions. We may therefore ask what makes lymphocytes are able to sustain HTLV infection for long periods rather than question why non-lymphoid cells are not able to be stably infected with the virus.

8.3. New insights into HTLV-mediated pathogenesis

The relatively low incidence of ATL among HTLV-infected patients (<5%) and the long latency observed before disease onset (20-40 years) mirror the multivariable nature of the leukemogenic process (159). As mentioned above, selection pressures are likely to include Env-GLUT1 interactions. In this context, it is conceivable that lymphoid cells are more likely to survive such pressures because of their ability to switch from a GLUT1-dependent status, when activated, to a GLUT1-independent survival state under conditions of quiescence (108) and figure 7). The ultimate emergence of leukemogenic clones may then result from additional effects such as those produced by Tax and other HTLV components (50, 160), and from potential mutations compensating for GLUT1 impairments brought on by recurrent HTLV Env expression. Closer assessment of GLUT1-associated parameters in the lymphocytes of asymptomatic patients and patients at different stages of ATL will allow us and others to determine the validity of this hypothesis.

The identification of GLUT1 as a receptor for HTLV Env and the ability of HTLV Env to block glucose transport may have considerable consequences on neurological

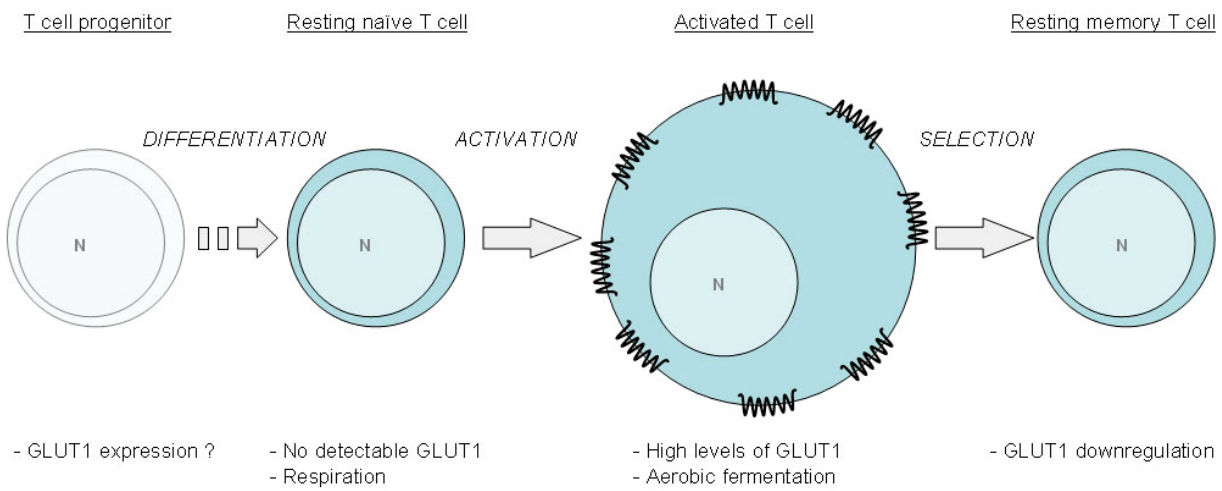

Figure 7. Model of GLUT1 expression during *in vivo* T cell activation. The vast majority of peripheral T-cells (>95%) are in the G0 phase of the cell cycle. Resting naïve T cells do not express GLUT1 at their plasma membrane and transport glucose at low basal levels. Upon activation through the T cell receptor (TCR), GLUT1 expression is strongly increased, concomitant with a significantly augmented glucose transport. Activated T cells acquire the phenotype of memory T cells and a small percentage survives and returns to a resting state. These quiescent memory T cells, like their naïve counterparts, do not express significant levels of GLUT1. GLUT1 expression during early stages of T lymphocyte differentiation remains to be determined.

function as exemplified by the neurodegenerescence associated with HTLV infection (157, 161). In this context, it is particularly relevant that the brain is strictly dependent on glucose consumption and moreover, that its usage relies on glycolysis. This process hinges on GLUT1 as shown by the fact that mutations in the GLUT1 gene lead to an impairment of glucose transport that is the cause of a severe cerebral deficiency (162). Moreover, the major clinical symptoms in patients with heterozygous GLUT1 mutations are neurological, with infantile seizures, developmental delay, microcephaly and ataxia (163). Astrocytes are the natural intermediate between brain vessels and neurons (see (164) and a generally accepted scenario of astrocyte-neuron carbohydrate exchanges is that most of the circulating glucose in the brain blood is consumed by glial cells via GLUT1. The glucose is converted into lactate by fermentation, in an aerobic environment, and the secreted lactate constitutes the major energy source for neurons (165-167). Interestingly, HTLV-1 infected lymphocytes have been shown to alter astrocytic functions (for instance see (168). HTLV Env-mediated impairment of glucose transport may therefore impact on neurons through a dysfunction of glucose metabolism in glial cells, such as astrocytes and oligodendrocytes. Finally, since ascorbic acid transport appears to prevent oxidative damage leading to cerebral degenerative processes and GLUT1 relays this transport under certain conditions (169), the presence of HTLV Env in the brain capillary and endothelial cells may favor the triggering of neurodegenerative processes such as those associated with HTLV infection (157). Evaluation of the impact of HTLV Env on ascorbic acid transport in these different cell types should bring additional clues.

Since contact between neuronal cellular components and HTLV-infected cells is likely to occur through the blood flow, the observation that GLUT1 is particularly abundantly expressed on human erythrocytes may bear some significance to HTLV-mediated neurodegenerescence. Thus, approximatvely 15,000 GLUT1 molecules can be detected at the surface of a single mature human erythrocyte, corresponding to 5% of the entire cell membrane (123), while only 700 molecules are present on a rat erythrocyte (138). Whether HTLV particles can be titered out from the circulation by red blood cells opens to new horizons. For instance, it becomes important to determine whether there is a potential link between the relatively rapid development of TSP/HAM after blood contamination (170) and the ability of human erythrocytes to bind HTLV virions. This could potentially occur via viral transfer from lymphoid and red blood cells to endothelial cells of blood vessels. This infection would in turn decrease local glucose uptake by endothelial cells and glial cells, leading to a disturbance of metabolic exchanges such as those described in the "astrocyte-neuron lactate shuttle" (165). In this perspective, to determine whether bound particles enter red cells or remain at their surface and can be passed on to new cells may become of a major significance. To this regard it is important to note that erythrocyte-associated GLUT1 has a different electrophoretic migration pattern than GLUT1 from other cells, due to different glycosylation, as well as different kinetic properties (122, 171). Whether such differences prevent viral entry in erythrocytes and allow red cells to circulate virions to other organs could be addressed ex vivo. Consequently, it will be important to assess whether GLUT1 glycosylation modulates HTLV entry.

The abundance of GLUT1 on erythrocytes is a property that humans share with bats (172) and sea mammals (173). This property correlates with, respectively, high glucose metabolism of human in the central nervous system, the ability of bats to undertake sustained flight, and maximization of glucose delivery under hypoxic stress, respectively. In contrast, GLUT1 appears to be expressed at

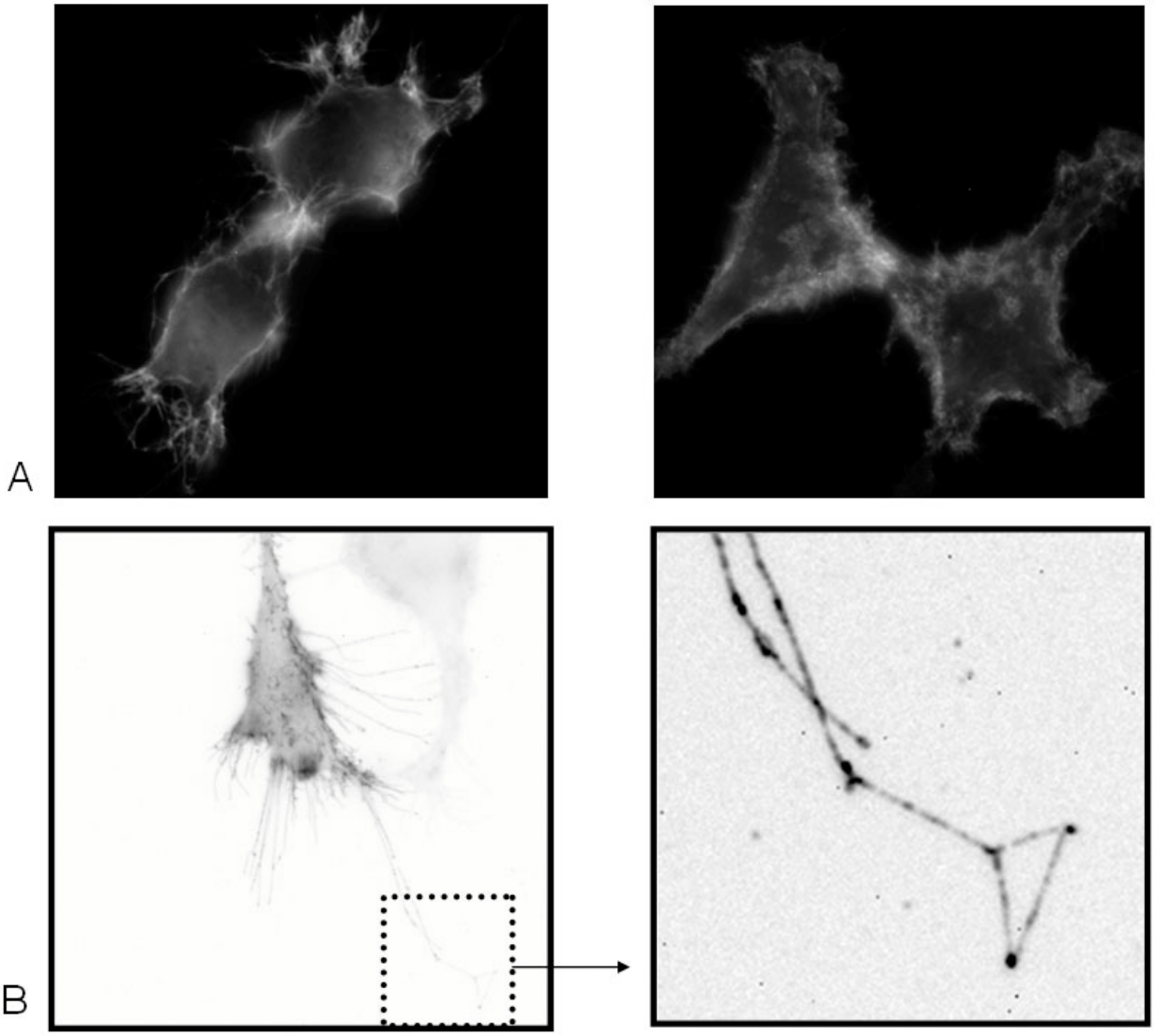

Figure 8. Distribution of GLUT1 on human HeLa cells. The receptor binding domain of HTLV Env was fused to the green fluorescent protein (EGFP) in order to track the distribution of GLUT1. HeLa cells were transfected with this fusion protein. The pattern of fluorescence indicates that GLUT1 is enriched at cell contacts (A). In isolated cells, GLUT1 is enriched at the extremity of membrane extensions (B).

significantly lower levels on non-human primate erythrocytes, as shown by immunoelectronmicroscopy of brain erythrocytes (174). As such, while STLV-1 most likely also recognizes GLUT1 as a receptor (150) and our unpublished observations), viral dynamics and viral dissemination in relation with erythrocyte binding are likely to be fundamentally different between human and monkeys.

8.4. HRBD as a marker for GLUT1 surface expression

EGFP-tagged HRBD binds diffusely to fibroblasts with a preferential accumulation at cell-cell contact areas (figure 8A), and filopodiae-like structures (figure 8B). Labeling of cells with transfected or externally added tagged-HRBD-containing conditioned media allowed the detailed visualization of filopodiae extending tens of microns from the main cell plasma membrane (figure 8B). These observations illustrate the close association of a major metabolic transporter, such as GLUT1, in the construction of membrane structures involved in cell-cell contact and "explorative" extensions. This likely reflects the adaptive plasticity conferred to the cell by these two synergic architectural and metabolic functions under local changes of resources. The exploration of the association between resource availability and the construction of different types of membrane extensions may provide a new angle at studying cell adaptation and differentiation at a metabolic and an architectural level. The tools derived from HRBD will undoubtedly be useful for these investigations.

8.5. HRBD-GLUT1interactions as diagnostic and therapeutical tools

As mentioned above, glucose uptake via GLUT1

activity is the basis for the *in vivo* detection of tissues with high metabolic activities and tumors (155, 156). Specific detection of cell surface-expressed GLUT1 molecules has been hampered by its high level of conservation, and the lack of reliable reagents recognizing exofacial GLUT1 determinants (M. Mueckler, personal communication). Therefore, conditioned medium containing HRBD-derived molecules constitutes such a convenient source of non-toxic reagents that can be used to easily detect cell surface GLUT1. Thus, these tools can be used to monitor GLUT1 expression in relation to various physiopathological conditions. Finally, as endogenous expression of HRBD will generally exert a toxic effect due to the inhibition of GLUT1, its ectopic expression may help eliminating GLUT1-overexpressing tumor cells (see for instance (155, 156).

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