Neuropilin-1 Is Involved in Human T-Cell Lymphotropic Virus Type 1 Entry

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Human T-cell lymphotropic virus type 1 (HTLV-1) is transmitted through a viral synapse and enters target cells via interaction with the glucose transporter GLUT1. Here, we show that Neuropilin-1 (NRP1), the receptor for semaphorin-3A and VEGF-A165 and a member of the immune synapse, is also a physical and functional partner of HTLV-1 envelope (Env) proteins. HTLV-1 Env and NRP1 complexes are formed in cotransfected cells, and endogenous NRP1 contributes to the binding of HTLV-1 Env to target cells. NRP1 overexpression increases HTLV-1 Env-dependent syncytium formation. Moreover, overexpression of NRP1 increases both HTLV-1 and HTLV-2 Env-dependent infection, whereas down-regulation of endogenous NRP1 has the opposite effect. Finally, overexpressed GLUT1, NRP1, and Env form ternary complexes in transfected cells, and endogenous NRP1 and GLUT1 colocalize in membrane junctions formed between uninfected and HTLV-1-infected T cells. These data show that NRP1 is involved in HTLV-1 and HTLV-2 entry, suggesting that the HTLV receptor has a multicomponent nature.

Human T-cell lymphotropic virus type 1 (HTLV-1) causes adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis (49). Unlike other retroviruses, free HTLV-1 virions are poorly infectious, with cell-to-cell contact being the major route of viral transfer in vivo (14). The importance of intercellular contacts for efficient HTLV-1 transmission was highlighted by Bangham and collaborators, who showed that an essential determinant of HTLV-1 cell-cell spreading is the establishment of a viral synapse (21).

On the viral side, HTLV-1 entry depends on the 46-kDa surface glycoprotein (SU), which is responsible for receptor recognition, and the 21-kDa transmembrane glycoprotein (TM), which triggers the fusion between viral and cellular membranes (32). Both proteins are produced by cleavage of the 61-kDa envelope (Env) precursor (42, 46). Regions in the 313-amino-acid-long SU encompassing residues 100 and 200 were shown to be the targets of neutralizing antibodies (2, 43, 57). Consistent with these observations, we and others showed that mutations introduced in these regions reduce the ability of HTLV-1 Env to trigger syncytium formation and/or virus infection (11, 12, 48, 52, 59).

Originally detected in CD4⁺ T cells (50), HTLV-1 infects other cell types in vivo, including CD8⁺ T cells, monocytes,

endothelial cells, and dendritic cells (18, 20, 30, 33). In contrast to this limited tropism in vivo, the HTLV receptor appears to be expressed in almost all cell lines. Moreover, the HTLV receptor is highly conserved in vertebrate species (41, 56). As a result of Env/receptor interactions, the HTLV-1 receptor is down-regulated or nonfunctional at the surface of chronically infected T cells (17, 47). Cell fusion induced by HTLV-2, a closely related nonpathogenic retrovirus, is also prevented in chronically HTLV-1-infected T cells, demonstrating that HTLV-1 and HTLV-2 share the same receptor (55).

Heparan sulfate proteoglycans have been reported to play a role in the binding of HTLV-1 to target cells (44). Recently, heparan sulfate proteoglycans were also found to contribute to HTLV-1 infection of primary CD4⁺ T cells (26). Other cell surface proteins may also be involved in HTLV-1 Env-induced cell fusion (15, 53), although their roles as entry receptors have not been clearly established. The lack of nonpermissive vertebrate cells has considerably hindered research into the HTLV entry receptor, and its identity remained unknown for more than 20 years. A major advance came with the demonstration that the ubiquitous glucose transporter GLUT1 plays a major role in HTLV entry. GLUT1 binds to HTLV-1 and HTLV-2 envelope proteins, and GLUT1 depletion in target cells reduces infection by HTLV-2-enveloped pseudotypes (36). The overexpression of GLUT1 increases the susceptibility of resistant cells to HTLV-1 Env-mediated cell fusion and infection (9), and an antibody directed to GLUT1 blocks HTLV-1 Env-mediated cell fusion and infection of primary CD4⁺ T lymphocytes (23). Mutation of residue 106 or 114 of Env reduces or abolishes the interaction of SU with GLUT1 (36), account-

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ing for the importance in HTLV-1 Env functions of the amino acid 100 region of the SU.

Various viruses use several molecules to interact with target cells. Therefore, it is possible that more than one molecule contributes to HTLV-1 entry. Given the particular mode of transmission of HTLV-1, such molecules are expected to be recruited within the HTLV-1 viral synapse or to be proteins found in T-lymphocyte junctions. We have previously shown that Neuropilin-1 (NRP1), a highly conserved 130-kDa single-spanning transmembrane protein, is a constituent of the immune synapse (58). NRP1 was first described as a receptor of semaphorin-3A, which plays a critical role during central nervous system embryogenesis (19, 29), and is also a coreceptor for vascular endothelium growth factor F-A165 (VEGF-A165) (54). NRP1 is expressed in T cells, dendritic cells (58), and endothelial cells (54), which are the main targets of HTLV-1 infection. Interestingly, activation of the CD4⁺ CD45RO⁺ T-cell subset, which represents most of the HTLV-1-infected cells in vivo (50), up-regulates NRP1 production (Y. Lepelletier, unpublished data), a feature reminiscent of the finding observed with the HTLV receptor (37, 39). The HTLV-1 SU inhibits T-cell proliferation in a mixed lymphocyte reaction (MLR) (39), which is similar to the inhibition of MLR by anti-NRP1 antibodies and soluble Fc-NRP1 (58). NRP1 is overexpressed in vitro in many tumor-derived cells and thus is overexpressed in most established laboratory cell lines (38, 54). As these properties are similar to those associated to the HTLV receptor, we investigated the role of NRP1 in HTLV entry.

MATERIALS AND METHODS

Cells and transfections. Cos cells stably transfected with an HIVLTR-LacZ construct (Cos-HIVLTR-LacZ), HeLa cells stably expressing the HIV-1 Tat protein (HeLa-Tat) (12), and 293T cells were maintained in Dulbecco's modified Eagle medium, and the HTLV-1-infected T-cell line C91/PL, the uninfected CD4⁺ T-cell line CEM, and the breast cancer cell line MDA-MB-453 (a kind gift from J. P. Peyrat, Lille, France) were maintained in RPMI 1640 medium, each medium supplemented with 10% fetal calf serum (FCS) (Invitrogen, France). The HTLV-1-infected T-cell line CIB, which has been described previously (3) and which was established from a patient with HTLV-1-associated myelopathy, was cultivated in complete RPMI 1640 medium supplemented with 100 U/ml of interleukin 2 (IL-2) (Roche, France). Peripheral blood mononuclear cells from healthy donors were separated by Ficoll Hypaque density gradient centrifugation followed by isolation of primary T cells using the pan T-cell negative-selection kit (Miltenyi Biotec, France). T cells were stimulated with 1 µg/ml of phytohemagglutinin (PHA-M; Sigma, France) in the presence of 20 U/ml IL-2 (Roche, France). Dendritic cells were prepared and used for primary T-cell stimulation as previously described (58). Cos-HIVLTR-LacZ and HeLa-Tat cells were transfected by the calcium phosphate precipitation method, and MDA-MB-453 cells were transfected using DMR1E-C (Invitrogen, France).

Plasmids and siRNA. The CMV-Env-LTR construct encoding the full-length HTLV-1 envelope proteins and the CMV-Env2 construct encoding the full-length HTLV-2 envelope proteins, both under the control of the cytomegalovirus (CMV) promoter, have been described elsewhere (12, 51). We used a construct containing a stop codon at the beginning of the *env* gene (CMV-Env-Stop) (12) as a negative control of Env expression. The HTLV-1 SU-rFc plasmid (residues 1 to 313 of the HTLV-1 surface Env protein fused to the rabbit immunoglobulin G [IgG] Fc constant region) described previously (25) was kindly provided by K. S. Jones (NCI, Frederick, Md.). The HIV-1 env pMA243 plasmid was from M. Alizon (Institut Cochin, Paris, France). The pMT21-HA-NRP1 (HA-NRP1) and pMT21-HA-NRP1ΔTM-myc (HA-NRP1-ΔTM-myc) plasmids encoding full-length and soluble forms of NRP1, respectively, and the pCHIX-GLUT1-HA plasmid (kindly provided by M. Sitbon, Institut de Genetique Moleculaire de Montpellier, Montpellier, France) have been described previously (19, 34, 36). We used the empty vector pSG5M (8) as a control plasmid. Sequences of the control (4) and anti-NRP1 small interfering RNAs (siRNAs) (QIAGEN,

France) were TTCTGGACCTATCACTTCA and AGCCAGAGGAGTACG ATCA (nucleotides 2232 to 2249 of human *nrip1* gene), respectively.

Cell fusion assays. In all assays, β-galactosidase (β-Gal) production was measured using the chemiluminescence β-Gal reporter gene assay kit (Roche, France) as previously described (46).

Cos-HIVLTR-LacZ cells were transfected with the CMV-Env-LTR or CMV-Env-Stop plasmid, and HeLa-Tat cells were transfected with the HA-NRP1 or pSG5M control plasmid. Two days after transfection, the cells were mixed at equal ratios and cocultivated for 24 h, and β-Gal produced as a result of Tat-dependent transactivation of the human immunodeficiency virus type 1 (HIV-1) promoter was measured. MDA-MB-453 cells were transfected with either the pSG5M or HA-NRP1 plasmid and with the CR-LacZ plasmid in which the *lacZ* gene is under the control of the HTLV-1 promoter. 293T cells were transfected with either the CMV-Env-LTR or the CMV-Env-Stop construct, which also encodes HTLV-1 Tax. Two days after transfection, transfected target cells and Env/293T cells were mixed at equal ratios and cocultivated for 24 h, and β-Gal produced as a result of Tax-dependent transactivation of the HTLV-1 promoter was measured.

Infection assay. Replication-defective LacZ retroviral vectors pseudotyped with either the HTLV-2 (H2), HTLV-1 (H1), or amphotropic murine leukemia virus (A-MLV) envelope proteins were produced by transfection of 293T cells with Gag/Pol, Env, and LacZ plasmids (a kind gift from M. Sitbon, Montpellier, France) as described elsewhere (36). Target cells were plated on 24-well plates (5×10^4) after 24 h for plasmid transfection or after 48 h for siRNA transfection. After 24 h, the supernatants from H1-MLV (1/4), H2-MLV (1/5 to 1/10), or A-MLV (1/50 to 1/100) pseudotype-producing cells were added, and the infectivity was assessed 48 h later by measuring the level of β-Gal activity with the β-Gal reporter gene assay kit (Roche, France).

Coimmunoprecipitation assays. Cells were lysed in a solution containing 20 mM Tris, pH 8, 120 mM NaCl, 0.2 mM EGTA, 0.2 mM NaF, 0.2% sodium deoxycholate, and 0.5% NP-40 supplemented with mixed protease inhibitors (Roche, France). For Env precipitation, cell lysates were incubated overnight at 4°C with 3 µl of pooled serum samples from HTLV-1-infected or healthy donors or of a serum sample from an HIV-1-infected individual. Reciprocal immunoprecipitation of HA-NRP1 was carried out using 3 µg of the anti-hemagglutinin (HA) 12CA5 monoclonal antibody (MAb). For NRP1/GLUT1 coimmunoprecipitation experiments, HA-NRP1-ΔTM-myc was immunoprecipitated overnight at 4°C with 5 µg of the anti-myc 9E10 MAb. Precipitated or total cell proteins were mixed with 2× Laemmli buffer (Sigma, France) (without boiling for GLUT1 detection), resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed by Western blotting using either the anti-HA 12CA5 antibody or sera from HTLV-1-infected patients and the corresponding peroxidase-conjugated secondary antibodies (Promega, France).

Cell surface NRP1 detection and Env binding assays. Cell surface expression of NRP1 was detected by cytometry using the phycoerythrin-coupled BDCA4 MAb (Miltenyi Biotec) or a phycoerythrin-coupled control antibody. Env binding assays were carried out with proteins produced in 293T cells. Cells were transfected either with the plasmid encoding the soluble HTLV-1 SU-rFc or with the pSG5M control plasmid. Intracellular proteins were recovered after 48 h by cell sonication as previously described (24). The levels of fusion proteins were quantified using a rabbit IgG enzyme-linked immunosorbent assay quantification kit (Bethyl). Target 293T cells were transfected with the control pSG5M plasmid or with plasmids encoding HA-NRP1 or GLUT1-HA or were preincubated with 50 ng/ml of VEGF-A165 (R&D, France) for 30 min at 4°C. For Env binding, the procedure described previously (24) was used. Cells were fixed in 1% paraformaldehyde for 15 min, washed twice in phosphate-buffered saline (PBS), and then incubated with 200 ng of sonicates for 60 min at room temperature. After two washes in PBS containing 2% FCS, the cells were incubated with FITC-conjugated goat anti-rabbit antibody (Chemicon, Euromedex, France) for 30 min at 4°C and washed twice in PBS buffer containing 2% FCS. SU-rFc binding was analyzed by flow cytometry.

Intracellular staining and T-cell conjugate formation. For NRP1 binding to target cells, recombinant NRP1 fused to the Fc fragment of human IgG (NRP1-Fc, R&D, France) was added to HTLV-1-infected CIB primary T cells for 30 min at 4°C (10 µg in 50 µl of medium). After the cells were washed, they were labeled with a fluorescein isothiocyanate (FITC)-conjugated anti-human antibody (Sigma, France) and further stained with the anti-Env 68/4.11.21 MAb (Interchim, France) followed by a cyanin-3 anti-mouse secondary antibody (Jackson ImmunoResearch, Interbiotech, France).

Cell conjugates were formed by mixing HTLV-1-infected C91/PL cells (2×10^5) in 400 µl of complete RPMI 1640 medium with an equal number of uninfected CEM T cells or peripheral blood mononuclear cells (PBMC) pre-stimulated for 4 days with PHA-M (1 µg/ml) and IL-2 (20 U/ml). Cells were

FIG. 1. NRP1 is up-regulated in activated T cells. Flow cytometry analysis of NRP1 surface expression in activated T cells. Purified primary T cells were stimulated with phytohemagglutinin in the presence of IL-2 (PHA/IL-2) or were incubated with activated dendritic cells (DC-activated). Cell surface NRP1 was detected by cytometry after labeling with phycoerythrin-conjugated control or anti-NRP1 (BDCA4) MAbs.

centrifuged for 10 min at 1,000 rpm, incubated for 30 min at 37°C, and then plated onto poly-L-lysine-coated glass slides. The cells were fixed by 20 min of incubation in PBS containing 4% paraformaldehyde, quenched by 5 min of incubation in PBS containing 100 mM glycine, and incubated in permeabilization buffer (PBS, 0.2% bovine serum albumin, and 0.05% saponin) for 15 min at 4°C. All subsequent steps were carried out at 4°C in permeabilization buffer. Non-specific sites were saturated by incubation with normal human serum (1/10) for 20 min, and HTLV-1 envelope proteins were stained using sera from HTLV-1-infected donors (1/3,000) and FITC-conjugated anti-human IgG (Sigma, France). NRP1 was detected using a rabbit anti-NRP1 antibody (1/100) and a cyanin-3-conjugated anti-rabbit IgG (Jackson ImmunoResearch, Interbiotech, France). GLUT1 was detected with MAb 1418 (1/100; R&D, France) and a cyanin-5-conjugated anti-mouse IgG (Jackson ImmunoResearch, Interbiotech, France). Slides were mounted in Mowiol (Calbiochem, Merck Eurolab, France) and examined by using a confocal microscope (LSM Meta 510; Zeiss, France).

RESULTS

NRP1 is up-regulated upon T-cell activation. Binding assays carried out with soluble HTLV-1 or HTLV-2 SU proteins have demonstrated that the HTLV receptor is absent from resting T cells and is up-regulated upon T-cell activation (37, 39). To determine whether NRP1 expression was consistent with this profile, we examined the distribution of NRP1 on T cells during activation. The cell surface level of NRP1 was analyzed by cytometry on primary T lymphocytes obtained from uninfected healthy donors, just after isolation (time zero) or at various times after stimulation (Fig. 1). We found that there were almost no detectable NRP1 in resting T cells. By contrast, endogenous NRP1 was up-regulated upon either polyclonal stimulation or dendritic cell-induced activation. These expression profiles are consistent with the relationship between NRP1 and the molecules involved in HTLV entry.

NRP1 is involved in HTLV-1 Env-mediated cell fusion. To test whether NRP1 is a functional partner of HTLV-1 Env, we examined the effect of NRP1 overexpression on HTLV-1 Env-dependent cell fusion using quantitative assays based on HTLV-1 or HIV promoter-dependent β -galactosidase production (12). For target cells, we used HeLa-Tat cells, which ex-

A. NRP1 cell surface expression

B. Fusion with HeLa-Tat

C. Fusion with MDA-MB-453

FIG. 2. Overexpression of NRP1-enhanced HTLV-1 Env-mediated cell fusion. (A) Cell surface expression of endogenous NRP1 by HeLa-Tat and MDA-MB-453 cells. Cells were labeled with phycoerythrin-coupled control (white peaks) or anti-NRP1 (gray peaks) antibodies. The mean fluorescence intensities (MFI) of the NRP1-positive populations after subtracting the MFI of the control antibody (ΔMFI) is given on each plot. (B) Syncytium formation assay using HeLa-Tat as target cells. HeLa-Tat cells transfected with a control plasmid (white bar) or various amounts of the HA-NRP1 plasmid (black bars) were cocultivated with Cos-HIVLTR-LacZ cells transfected with the CMV-Env-Stop (Env-) or the CMV-Env-LTR (Env+) construct. β -Galactosidase activity was determined after subtracting the background from a coculture of Cos-HIVLTR-LacZ transfected with the CMV-Env-stop plasmid and total protein normalization. Results are expressed as percentages of β -Gal produced by HeLa-Tat cells transfected with the control plasmid and are the means $\pm$ standard deviations (error bars) for a representative experiment (out of two experiments). (C) Syncytium formation assay using MDA-MB-453 as target cells. MDA-MB-453 cells transfected with the control (Cont.) plasmid (white bar) or the NRP1 plasmid (black bar) and with the indicator HTLV-1LTR-LacZ plasmid (CR-LacZ) were cocultivated with 293T cells transfected with the CMV-Env-Stop (Env-) or the CMV-Env-LTR (Env+) plasmid. β -Galactosidase activity was determined after subtracting the background from a coculture of 293T cells transfected with the CMV-Env-stop plasmid and total protein normalization. Results are expressed as percentages of β -Gal produced by MDA-MB-453 cells transfected with the control plasmid and are the means $\pm$ standard deviations (error bars) for a representative experiment (out of three experiments).

press NRP1 at the cell surface, and the breast cancer cell line MDA-MB-453, which produces a very low level of NRP1, as previously described (54) (Fig. 2A).

First, we used a previously described quantitative fusion assay with HeLa cells stably expressing the HIV-1 Tat protein and Cos cells stably transfected with a HIVLTR-LacZ construct (12). HeLa-Tat cells were transfected with increasing doses of a plasmid encoding the rat form of NRP1 tagged with an HA epitope (HA-NRP1). We observed a dose-dependent increase in the level of fusion with Env-transfected Cos-HIVLTR-LacZ cells (Fig. 2B). We then investigated the effect of

NRP1 expression in NRP1^{low} cells. Transfection of MDA-MB-453 cells with a green fluorescent protein plasmid consistently led to green fluorescent protein expression in less than 15% of the cells (not shown), indicating a low transfection efficiency. However, in our assay, MDA-MB-453 cells were cotransfected with HA-NRP1 and the indicator gene construct, restricting the quantification of the fusion events within the population of cells overexpressing NRP1. HA-NRP1 expression in MDA-MB-453 cells resulted in a fourfold increase in β -Gal production (Fig. 2C).

These data show that the amount of NRP1 on target cells influences their capacity to fuse with HTLV-1 Env-producing cells.

NRP1 contributes to both HTLV-1 and HTLV-2 Env-mediated virus infection. We next performed infection assays based on *lacZ* gene-carrying murine leukemia virus particles pseudotyped with the HTLV-1 (H1), HTLV-2 (H2), or amphotropic MLV (A-MLV) envelope proteins as described elsewhere (36). We first compared the susceptibility of NRP1-positive 293T cells and NRP1^{low} MDA-MB-453 cells to infection (Fig. 3A). Because infection by A-MLV particles of MDA-MB-453 cells was more than 15 times lower than that of 293T cells (see the legend to Fig. 3A), we divided the values obtained with 293T cells by 15 for a comparison of the cells (giving 109.037 for MDA-MB-453 cells versus 103.120 arbitrary units for 293T cells; MDA-MB-453/293T infection ratio of 1). Comparison of pseudotype infections further showed that MDA-MB-453 cells were infected at lower levels than 293T cells by both H1-MLV and H2-MLV pseudotypes (MDA-MB-453/293T infection ratios of 0.44 and 0.25, respectively). We then investigated the effect of NRP1 expression on MDA-MB-453 cell infection. As already mentioned, a transfection efficiency of only 15 to 20% can be achieved in this cell line. Moreover, because the indicator gene is provided by the pseudotyped viruses and not by the target cells, we were unable to measure cell infection only in NRP1-transfected cells. Nevertheless, MDA-MB-453 cells transfected with the HA-NRP1 construct showed a higher level of infection by both H1-MLV and H2-MLV pseudotypes than cells transfected with the empty vector (considered 100% infection). By contrast, no increase was found for A-MLV infection (Fig. 3B). We also analyzed the effect of NRP1 overexpression in 293T cells. 293T cells transfected with the HA-NRP1 construct showed higher levels of infection by H2-MLV and, to a lesser extent, by H1-MLV, than by A-MLV pseudotypes (Fig. 3C). We then investigated the importance of endogenous NRP1 on HTLV Env-dependent infection by siRNA-mediated down-modulation. In 293T cells transfected with the anti-NRP1 siRNA, the cell surface level of NRP1 decreased significantly (Fig. 3D, left panel; siNRP1/siCont. Δ MFI ratio of 0.4 where Δ MFI is the mean fluorescence intensity [MFI] of NRP1-positive cells after subtracting the MFI of the control antibody), which was associated with specific reductions in the levels of both H1-MLV and H2-MLV pseudotype infections (Fig. 3D, right panel, 35% and 51% inhibition, respectively).

Together, these data indicate that NRP1 also contributes to the viral infection process mediated by both the HTLV-1 and HTLV-2 envelope glycoproteins.

NRP1 and HTLV-1 Env interact at the cell surface. Molecules involved in HTLV-1 entry presumably bind Env at the

cell surface. Therefore, we tested whether cell surface NRP1 binds to the HTLV-1 SU protein using a construct encoding the HTLV-1 surface glycoprotein fused to the rabbit Fc fragment (SU-rFc) (25) (Fig. 4). Due to the presence of endogenous receptor(s), 293T cells transfected with the empty vector were able to bind to the SU-Fc protein (Fig. 4A). The binding of the SU-rFc protein increased in HA-NRP1-transfected 293T cells and, to a lesser extent, in GLUT1-HA-transfected cells. The increased binding upon NRP1 overexpression was moderate but reproducible ($140\% \pm 16\%$ in four experiments). To evaluate the contribution of endogenous NRP1 on Env binding, 293T cells were treated with the anti-NRP1 siRNA (Fig. 4B). In a representative experiment, anti-NRP1 siRNA treatment reduced cell surface NRP1 by 50% versus cells treated with the control siRNA (Fig. 4B, left pair of panels; siNRP1/si-Cont. Δ MFI ratio of 0.5), which was associated with a 20% reduction in the level of SU-rFc binding (right pair of panels; siNRP1/si-Cont. Δ MFI ratio of 0.81). The results of three independent experiments showed that the SU-rFc binding decreased by $20\% \pm 0.2\%$ in cells in which NRP1 was reduced by $55\% \pm 5\%$. We also pretreated 293T cells with VEGF-A165, one of the NRP1 ligands, and found that this also reduced SU-rFc binding (Fig. 4B, bottom pair of panels; $49\% \pm 10\%$ in three experiments).

We also investigated whether soluble NRP1 can bind cell surface Env (Fig. 4C). The chronically HTLV-1-infected CIB primary T cells were incubated with recombinant NRP1 fused to the Fc domain of human Ig (NRP1-Fc) and the corresponding secondary antibody, and the cells were further stained for cell surface expression of Env proteins. As previously described, we found that envelope proteins were strongly polarized in CIB cells, which retained the morphology of activated T cells (3). NRP1-Fc proteins also decorated a polarized area of the plasma membrane, within which they were completely colocalized with Env (Fig. 4C).

These findings indicate that NRP1 and HTLV-1 Env proteins can associate at the cell surface. Moreover, they show that NRP1 partially accounts for the ability of target cells to bind to the HTLV-1 surface protein.

Overexpressed NRP1 forms intracellular complexes with HTLV-1 or HTLV-2 Env. Because they are all synthesized in compartments of the secretory pathway, envelope proteins can form intracellular complexes with their cellular membrane-interacting partners (10, 28, 45). We then investigated whether NRP1/Env complexes are formed in cells by coprecipitation experiments in 293T cells transfected with plasmids encoding Env and HA-NRP1 (Fig. 5). HA-NRP1 was detected after precipitation of HTLV-1 Env with sera from HTLV-1-infected patients and blotting with the anti-HA antibody (Fig. 5A, lane 4). No signal was detected in the absence of HA-NRP1 (lane 2) or HTLV-1 Env (lane 3) or with a serum sample from an uninfected donor (lane 1). Moreover, HA-NRP1 did not coprecipitate with the envelope glycoproteins of human immunodeficiency virus (top blot, lane 5) in 293T cells transfected with a plasmid encoding HIV-1 Env, despite the fact that HIV Env was efficiently immunoprecipitated by the anti-HIV-1 serum (bottom blot). The reciprocal immunoprecipitation of HA-NRP1 with an anti-HA antibody allowed both the HTLV-1 gp61 precursor and SU proteins to be recovered (Fig. 5B, lane 3). Intracellular complexes with NRP1 were also de-

A. Cell comparison **B. MDA-MB-453/plasmid** **C. 293T/plasmid** **D. 293T/siRNA**

FIG. 3. NRP1 is involved in HTLV-1 and HTLV-2 Env-mediated virus infection. Infection assays using *lacZ*-carrying MLV particles pseudotyped with the HTLV-1 (H1-MLV) or HTLV-2 (H2-MLV) Env protein or amphotropic (A-MLV) envelope protein. β -Gal production was measured using a chemiluminescence assay after subtracting the background of noninfected cells and total protein normalization. (A) Infection assay using NRP1-positive 293T cells or NRP1^{low} MDA-MB-453 as target cells. Results are expressed as β -Gal activity units and are the means $\pm$ standard deviations (SD) (error bars) for a representative experiment (out of two experiments). To facilitate comparison, the β -Gal activity of 293T cells was divided by 15 (indicated by an asterisk). (B) Effect of the transient expression of HA-NRP1 on MDA-MB-453 cell infection. MDA-MB-453 cells were transfected with the pSG5M or HA-NRP1 plasmid. Results are expressed as percentages of β -Gal produced by MDA-MB-453 cells transfected with the control (Cont.) plasmid and are the means $\pm$ SD of two experiments. (C) Effect of the transient overexpression of HA-NRP1 on 293T cell infection. Results are expressed as percentages of β -Gal produced by 293T cells transfected with the control (Cont.) plasmid and are the means $\pm$ SD of two experiments. (D) Effect of siRNA-mediated down-regulation of endogenous NRP1 on 293T cell infection. Cells were transfected with a control siRNA (siCont.) or a siRNA directed to NRP1 (siNRP1). (Left) Endogenous cell surface NRP1 as described in the legend to Fig. 2A, showing the MFI of NRP1-positive cells after subtracting the MFI of the control antibody (Δ MFI). (Right) Infection of siRNA-treated cells. Results are expressed as percentages of β -Gal produced by 293T cells transfected with the control siRNA and are the means $\pm$ SD of three experiments.

tected in cells producing the HTLV-2 envelope proteins (Fig. 5C, lane 2) and in cells producing the HTLV-1 envelope glycoproteins and a soluble version of HA-tagged NRP1 (HA-NRP1- Δ TM-myc) (Fig. 5D, lane 4).

All together, these results show that envelope proteins from both HTLV-1 and HTLV-2 associate with NRP1 within cells and that only the ectodomain of NRP1 is needed for these interactions.

Overexpressed NRP1 and GLUT1 form intracellular complexes that are increased in the presence of Env. As both GLUT1 (27, 36) and NRP1 bind Env, we investigated whether these two proteins could form intracellular complexes in the absence or presence of Env (Fig. 6). We used the HA-NRP1- Δ TM-myc and GLUT1-HA constructs to precipitate NRP1 with the anti-myc antibody and reveal GLUT1 with the anti-HA Mab. No cellular proteins were detected in the absence

FIG. 4. NRP1 and HTLV-1 Env interact at the cell surface. (A) Effect of HA-NRP1 or GLUT1-HA overexpression on SU-rFc binding. 293T cells transfected with the control (Cont.) plasmid or plasmid encoding HA-NRP1 or GLUT1-HA were incubated with a control cell sonicate (white peaks) or a cell sonicate containing SU-rFc proteins (gray peaks), then incubated with an FITC-conjugated goat anti-rabbit antibody, and analyzed by flow cytometry. One representative experiment out of three is given, showing the MFI of the positive populations after subtracting the MFI of the control sonicate (ΔMFI). (B) Effect of endogenous NRP1 down-regulation on SU-rFc binding. 293T cells were transfected with a control siRNA (si-Cont.) or an siRNA directed to NRP1 (si-NRP1) or were pretreated with VEGF-A165 (50 ng/ml). Env binding was determined as described above. One representative experiment out of three is given, showing the mean fluorescence intensities of the Env-positive populations after subtracting the MFI of the control sonicate (ΔMFI). (C) Soluble NRP1 colocalized with HTLV-1 Env at the cell surface. The HTLV-1-infected CIB primary T cells were incubated with 10 μg of recombinant NRP1-hFc (human Fc) proteins for 30 min at 4°C. Cells were then labeled with an FITC-conjugated anti-human Fc antibody and then stained with the anti-Env 68/4.11.21 MAb followed by cyanin-3 anti-mouse secondary antibody, and analyzed by confocal microscopy.

of HA-NRP1- ΔTM -myc (lanes 1 to 4), showing that the precipitation process was specific. In cells producing HA-NRP1- ΔTM -myc and GLUT1-HA but not Env (lane 6), we observed a specific band migrating around 50 kDa (indicated by an asterisk), corresponding to fully glycosylated forms of GLUT1 (1). In the presence of Env and HA-NRP1-myc (lane 5), the level of recovered GLUT1 increased, and an additional intense band corresponding to a lower-molecular-weight GLUT1 species appeared. The Env proteins were also found in the anti-NRP1 precipitate (middle blot,

lane 5). Since GLUT1-HA/Env complexes cannot be precipitated by the anti-myc antibody (lane 1), this shows that a NRP1/Env/GLUT1 ternary complex was formed within the cells. Env proteins were also detected in the NRP1 precipitate obtained from cells overexpressing HA-NRP1- ΔTM -myc but not GLUT1-HA (middle blot, lane 7), confirming the NRP1/Env association.

These results indicate that GLUT1 and NRP1 can spontaneously interact at low levels and that they are strongly recruited by the HTLV-1 Env to form a ternary complex.

FIG. 5. NRP1 forms intracellular complexes with HTLV-1 or HTLV-2 envelope glycoproteins. (A) NRP1 coprecipitates with HTLV-1 but not with HIV-1 Env glycoproteins. 293T cells were transfected with a control plasmid (lane 2) or with the HA-NRP1 plasmid in the presence of the CMV-Env-LTR construct (lanes 1 and 4) or the CMV-Env-Stop construct (lane 3). Cellular proteins were precipitated using a serum sample from an uninfected donor (lane 1) or a pool of sera from HTLV-1-infected individuals (HTLV-1 serum, lanes 2 to 4). Recovered proteins were blotted with the anti-HA MAb (top blot) or the HTLV-1 serum (bottom blot) MAb. A similar experiment was carried out with 293T cells transfected with a construct encoding the HIV envelope proteins after precipitation with a serum sample from an HIV-1-positive individual (HIV-1 serum). IP, immunoprecipitation. (B) HTLV-1 Env proteins coprecipitated with HA-NRP1. 293T cells were transfected with the HA-NRP1 plasmid and with the CMV-Env-Stop construct (lane 2) or the CMV-Env-CMV construct (lane 3). Cellular proteins were immunoprecipitated with the anti-HA antibody (IP HA), and recovered proteins were blotted with HTLV-1 serum (Blot HTLV1). (C) NRP1 interacts with the HTLV-2 envelope proteins. 293T cells were transfected with a construct encoding HTLV-1 Env (lane 1), HTLV-2 Env (lane 2), or HIV-1 Env (lane 3) in the presence of the HA-NRP1 vector. Total proteins were immunoprecipitated using a pool of sera from HTLV-1-infected individuals (IP HTLV) (which cross-reacted with the HTLV-2 Env proteins). Recovered proteins were blotted with the anti-HA MAb (top blot). Blotting of total cellular proteins with HTLV-1 sera was also performed to assess Env protein expression (bottom blot). (D) The ectodomain of NRP1 also coprecipitated with HTLV-1 Env. 293T cells were transfected with only a control plasmid (lane 1) or HA-NRP1 plasmid (lane 2) or with the Env plasmid in the presence of the construct encoding the full-length NRP1 (HA-NRP1, lane 4) or the soluble form of NRP1 (HA-NRP1-ΔTM-myc, lane 3). Cellular proteins were immunoprecipitated using the HTLV-1 sera (IP HTLV). Recovered proteins were blotted with the anti-HA MAb (top blot). Blotting of total cellular proteins with the anti-HA MAb (middle blot) or HTLV-1 sera (bottom blot) were also performed to assess protein expression.

Endogenous NRP1 and GLUT1 colocalized in membrane junctions formed between infected and uninfected T cells. Finally, we investigated the relationship between endogenously expressed NRP1 and GLUT1 by examining their subcellular localizations in T cells. To this end, cell conjugates were formed between uninfected T cells or between uninfected T cells and T cells chronically infected by HTLV-1. Env proteins

FIG. 6. NRP1 and GLUT1 form intracellular complexes which increased in the presence of Env. Coprecipitation of NRP1 and GLUT1 in the presence (+) or absence (–) of HTLV-1 Env. 293T cells were transfected (+) with HA-NRP1-ΔTM-myc plasmid and with plasmids encoding Env or GLUT1-HA as indicated. The CMV-Env-Stop and pSG5M plasmids were used as negative controls. Cellular proteins were immunoprecipitated with the anti-myc antibody (IP Myc), and recovered proteins were blotted with either the anti-HA MAb (top blot) or HTLV-1 serum (middle blot). Blotting of total cellular proteins with the anti-HA antibody (bottom blot) were also performed to assess GLUT1 expression. Since both overexpressed NRP1 and GLUT1 possess the HA epitope, the top of the membranes containing NRP1 was removed to avoid antibody recognition interference.

were also labeled to identify areas of the cell membranes in which Env/receptor interactions occur. We found that GLUT1, but not NRP1, was present within cell contact sites in conjugates formed between two uninfected CEM T cells (Fig. 7A). By contrast, when CEM T cells were clustered with infected C91/PL cells, NRP1 was polarized towards, and concentrated within, the cell contact area (Fig. 7B). GLUT1 proteins were also found in this area, and spots of NRP1/GLUT1 colocalization were observed. Env proteins were strongly polarized on the opposite side of the contact, corresponding to the plasma membrane of the infected T cell, showing that it did indeed correspond to a viral transmission area (Fig. 7B). Similarly, concentration of NRP1 and colocalization with GLUT1 at the level of the cell junctions were observed in conjugates formed between C91/PL cells and activated PBMC (Fig. 7C). We also observed partial cell-cell fusion (Fig. 7D), in which there was an intense polarization and colocalization of Env, GLUT1, and NRP1 in the areas of membrane mixing.

The results of these analyses document the relationship between HTLV-1 Env, NRP1, and GLUT1 in an endogenous context and strongly suggest that NRP1 plays a role in HTLV-1 transmission between T cells.

DISCUSSION

The molecular and cellular mechanisms of HTLV-1 transmission have remained unidentified for more than 20 years. However, two recent findings have improved our knowledge of HTLV-1 infection. First, HTLV-1 was found to be transmitted through a viral synapse (21). Second, it was shown that GLUT1 is a receptor for HTLV-1 and the related HTLV-2 virus (36). Here, we show that HTLV-1 and HTLV-2 envelope proteins

FIG. 7. Endogenous NRP1 is localized in the cell junctions formed between HTLV-1-infected and uninfected T cells. Cell conjugates were formed between (A) uninfected CEM T cells, (B) uninfected CEM T cells and chronically HTLV-1-infected C91/PL T cells, or (C and D) uninfected activated PBMC and infected C91/PL T cells. NRP1, GLUT1, and Env were stained in permeabilized cells as described in Materials and Methods. Each row shows a single optical plane recorded independently in the red (NRP1), green (Env), or blue (GLUT1) channel by confocal microscopy. Phase-contrast microscopy, NRP1 staining, and costaining of NRP1 and Env or of NRP1 and GLUT1 (right) are shown as indicated. Areas of colocalization between NRP1 and Env appeared as yellow pixels, and areas of colocalization between GLUT1 and NRP1 signals appeared as white pixels. Enlargements of the cell contact areas are also shown.

also interact specifically with Neuropilin-1 and that this interaction modulates HTLV Env functions.

The cell surface molecules involved in HTLV-1 Env binding are up-regulated upon T-cell activation (37, 39). In agreement with these observations, we found that the cell surface expression of NRP1 increased in activated primary T cells. Functional assays provided direct evidence that NRP1 is involved in HTLV-1 Env-mediated cell fusion and viral infection. The introduction of the rat NRP1 gene into NRP1^{low} MDA-MB-453 cells increased their fusion with HTLV-1 Env-expressing cells. NRP1 overexpression also increased fusion in HeLa cells, although to a lower extent than in MDA-MB-453 cells, probably due to the presence of endogenous NRP1 in HeLa cells. In addition, NRP1 overexpression enhanced the infection of MDA-MB-453 cells by MLV particles enveloped by either

HTLV-1 or HTLV-2 envelope proteins, but not by A-MLV. Increased infection by HTLV-1 and HTLV-2-enveloped pseudotypes was also demonstrated in 293T cells, and conversely, siRNA-mediated depletion of endogenous NRP1 in these cells reduced infection. These data reveal that NRP1 is a previously unknown functional partner for both HTLV-1 and HTLV-2 envelope proteins.

We also used biochemical and cellular approaches to demonstrate that NRP1 and HTLV-1 envelope proteins interact both within cells and at the cell surface. We found that intracellular complexes with NRP1 do not form with HIV-1 envelope glycoproteins, showing that NRP1 specifically interacts with HTLV envelope proteins. Recombinant NRP1-Fc proteins colocalized with HTLV-1 Env proteins in plasma membrane clusters. Conversely, the level of cell surface NRP1 in-

fluenced the binding of the HTLV-1 SU protein on target cells. Although this was reproducible, the effect of NRP1 modulation on SU-rFc binding was only moderate. This suggests that most of the Env binding to target cells is mediated by cell surface molecules other than NRP1. These may be heparan sulfate proteoglycans (26, 44) and/or GLUT1 (36).

An important issue now to be addressed is the precise function of NRP1 and GLUT1 during HTLV-1 entry. We tried to transfect 293T cells with both anti-NRP1 and anti-GLUT1 siRNAs. However, this cotreatment was highly toxic for cells, making investigation of the fusion capacity or infectivity of NRP1/GLUT1 double-negative cells difficult. We could not use antibodies directed against GLUT1 or NRP1 in functional assays, since we were unable to find antibodies that can recognize surface GLUT1 on live cells and since we found that commercial anti-NRP1 antibodies affect cell viability in long-term assays. However, although we cannot yet define the precise role of NRP1 in the HTLV-1 entry process, we showed that NRP1 and GLUT1 form intracellular complexes that were regulated by Env. In the absence of Env, small amounts of GLUT1 can be precipitated with NRP1, implying that the two proteins are constitutively associated, even in a nonviral context. In the presence of Env, the amount of NRP1/GLUT1 complexes massively increased. This shows that NRP1 and GLUT1 concomitantly bind to HTLV-1 Env to form a ternary complex, suggesting that they interact with distinct regions. In the presence of Env, NRP1 precipitated with low-molecular-weight forms of GLUT1, corresponding to immature N-glycosylated products (1). This suggests that the NRP1/Env/GLUT1 ternary complexes are formed in early compartments of the secretory pathway.

NRP1 and GLUT1 have been reported to bind to the PDZ-domain protein GLUT1CBP (GLUT1 C-terminal binding protein) via a consensus C-terminus PDZ-binding motif (6, 7). GLUT1CBP, also named GAIP-interacting protein, C terminus (GIPC) (13), is a scaffold protein that promotes the assembly of multimolecular complexes containing membrane receptors and cytoskeleton-associated molecules or cell signaling molecules (5, 6, 22). We found that the soluble form of NRP1 could precipitate GLUT1, suggesting that NRP1/GLUT1 complex formation does not involve the recruitment of GLUT1CBP by the cytoplasmic domain of NRP1. However, since NRP1 can form homodimers (62), we cannot exclude the possibility that the association of the truncated protein with endogenous NRP1, which contains the PDZ-binding motif, may facilitate NRP1/GLUT1 association. The HTLV-1 and HTLV-2 envelope proteins also possess a consensus C-terminal PDZ-binding motif that could potentially recruit GLUT1CBP. Indeed, we have previously shown that hDIg, another PDZ-domain scaffold protein, binds to the cytoplasmic domain of HTLV-1 Env and assists the cell-to-cell virus transmission (3). Further investigations are therefore needed to determine whether GLUT1CBP/GIPC recruitment, via its association to the cell signaling pathway and/or cytoskeleton, also plays a role in HTLV-1 transmission.

As well as the intracellular association of overexpressed NRP1 and GLUT1, we showed that endogenous NRP1 and GLUT1 are in close contact in areas of HTLV-1 transmission. GLUT1 is found in cell contact sites independently of HTLV-1 infection (35). Consistent with this, we observed GLUT1 in the

cell junctions formed between uninfected CEM T cells. By contrast, NRP1 was not found in these cell contact sites, despite the fact that it was partially colocalized with GLUT1 in small intracellular regions, which probably correspond to synthesis and maturation compartments. Unlike in uninfected T-cell conjugates, we found that NRP1 was concentrated and partially colocalized with GLUT1 within the membrane junctions formed between HTLV-1-infected and uninfected T cells. This suggests that NRP1 was recruited in the cell contact areas via an Env-dependent mechanism, supporting its role in HTLV-1 transmission. NRP1 is specifically expressed in the main target cells of HTLV-1 infection *in vivo* and is overexpressed in the transformed cell lines used *in vitro*. The selective localization of NRP1 in viral transmission areas and the contrasting NRP1 expression profile between primary T cells and cell lines may partly explain the paradox of HTLV-1 tropism *in vitro* and *in vivo*.

Partners of retroviral envelope proteins may be the bona fide entry receptors or may be molecules involved in virus transport and transmission, such as DC-sign for HIV (16). In contrast to the enhancing effect of NRP1 on HTLV-1 Env-dependent fusion, the interaction between DC-sign and HIV inhibits Env-dependent syncytium formation (40). Alternatively, NRP1 may be a receptor for HTLV-1 entry. NRP1 has properties similar to those of the HTLV receptor, such as wide expression among cell lines from various origins, localization in T-cell junctions, up-regulation in activated T cells, and the ability to inhibit MLR (38, 54, 58). This suggests that HTLV-1 entry may require more than one cellular molecule. The observation that NRP1 forms an Env-regulated ternary complex with GLUT1 is reminiscent of the situation found in the HIV-1 model, in which complexes are formed between HIV-1 gp120, its binding receptor CD4, and one of the fusion receptors, CXCR4 or CCR5 (31, 61). NRP1 may therefore be either the binding or fusion receptor for HTLV-1. Alternatively, NRP1 and GLUT1 may function in concert to constitute a high-affinity binding site for Env. In line with this hypothesis, it has been shown that NRP1 acts as a coreceptor for VEGF receptor 2 by increasing its binding to VEGF-A165 (54).

NRP1 triggers repulsive or attractive cues that govern axonal guidance. The semaphorin/NRP1 interaction induces cytoskeleton rearrangements, which in turn regulate the formation of cell adhesion sites (60). This suggests that the HTLV-1 SU/NRP1 interaction at the interface between infected and uninfected T cells may also play an active role in the formation and/or stability of the HTLV-1 viral synapse. Whatever the exact role NRP1 plays in HTLV entry, its large contribution to the homeostasis of the nervous, vascular, and immune systems allows new perspectives to explain the multifaceted physiopathology of HTLV-related diseases.

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