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# Trypsin-Sensitive and -Resistant Components in Human T-Cell Membranes Required for Syncytium Formation by Human T-Cell Lymphotropic Virus Type 1-Bearing Cells

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**Human T-cell lymphotropic virus type 1 (HTLV-1) envelope proteins play an important role in viral entry into target cells. In a syncytium formation assay consisting of a coculture of HTLV-1-bearing cells and target cells, mature gp46 and gp21 proteins each inhibited syncytium formation induced by HTLV-1-bearing cells. Experiments with $^{125}$I-labeled proteins showed that $^{125}$I-gp46 bound specifically with MOLT-4 target cells even in the presence of large amounts of gp21, whereas $^{125}$I-gp21 binding to target cells was completely blocked in the presence of large amounts of gp46. These observations suggest that HTLV-1 envelope proteins in syncytium formation interact with at least two components, which are located close to each other on the cell membrane. We isolated two components from MOLT-4 cell lysate, using Sepharose 4B columns coupled with peptides corresponding to amino acids 197 to 216 and 400 to 429, respectively, of the envelope protein. One is a trypsin digestion-sensitive component of approximately 34 to 35 kDa, which interacts specifically with gp46. The other is a nonprotein component, which interacts with gp21. This component was destroyed by sodium periodate oxidation and was partitioned into the methanol-chloroform phase. These observations suggest that these two components play an important role in HTLV-1 entry into target cells via membrane fusion.**

Human T-cell lymphotropic virus type 1 (HTLV-1) is closely associated with adult T-cell leukemia/lymphoma (15, 33, 34, 44) and some neurologic disorders (2, 14, 21, 29). In addition, HTLV-1 has been implicated as causing other diseases, including polymyosis (3, 11, 20) and uveitis (27). Although HTLV-1 has been associated with human malignancies which exhibit the T4 T-lymphocyte cell surface phenotype (4, 33), many human and mammalian cells, including sarcoma cell lines (5, 17, 28, 45) and epithelial and endothelial cells (16, 18), can be infected using a cocultivation technique, since cell-to-cell contact is generally required for infection (41).

Several lines of evidence suggest that the HTLV-1 envelope glycoproteins play a role in viral entry into the target cells (6, 23, 42). (i) Anti-envelope antibodies protect against HTLV-1 infection in vivo (37, 40) and linear peptide targets for neutralizing antibodies are found in the envelope protein (19, 30), and (ii) the introduction of a single amino acid substitution at multiple positions in envelope protein by site-directed mutagenesis affects the syncytium formation activity of HTLV-1 (7, 32). In our previous study (35), we found that the peptides corresponding to amino acids 197 to 216 of the gp46 surface glycoprotein (gp46–197) and 400 to 429 of the gp21 transmembrane glycoprotein (gp21–400) inhibited syncytium formation induced by HTLV-1-bearing cells, suggesting that these two regions may contribute to the interaction between the HTLV-1 envelope and the target cell membrane. In the present study, we show the molecular characteristics of two membrane components which are critical for syncytium formation, as purified from MOLT-4 cell lysate using an affinity column coupled with the peptides gp46–197 and gp21–400, respectively.

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## MATERIALS AND METHODS

**Cells and compounds.** The HTLV-1-bearing T-cell line used was human T-cell line $KT_{252}$ established from an adult T-cell leukemia/lymphoma patient in Kyushu University Hospital. The HTLV-1-negative cell line used was the human T-cell line MOLT-4 (39). These cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Peptides were synthesized using a peptide synthesizer (model 431A peptide synthesizer; Applied Biosystems, Foster City, Calif.) by the t-BOC method, as described previously (24). The purity of individual peptides was examined by reverse-phase high-performance liquid chromatography (HPLC) (Waters 626 pump equipped with a 996 photodiode array detector; Millipore, Milford, Mass.) with an octadecyl ($C_{18}$) silicated column. Peptides were isolated as a single peak. Amino acid sequences of these peptides were confirmed by Edman degradation on a gas-phase sequencer (model 470A; Applied Biosystems).

**Syncytium formation assay.** To monitor syncytium formation in suspension, MOLT-4 cells were used as indicator cells, as described previously (35). The assay consisted of a coculture of $2.5 \times 10^4$ HTLV-1-bearing $KT_{252}$ cells together with $10^5$ indicator MOLT-4 cells. MOLT-4 cells were suspended in RPMI 1640 medium supplemented 10% fetal calf serum and 0.5% normal human sera (referred to as RPMI-medium) at $5 \times 10^6$ cells per ml. Aliquots (20 μl per well) were added to 155 μl of RPMI-medium containing test samples or medium alone in each well of a U-bottom 96-well plate (cell wells 25850; Corning Glass Works, Corning, N.Y.). Then 25 μl of $KT_{252}$ cell suspension ($10^6$ cells per ml of RPMI-medium) was added to each well. After incubation at 37°C for 18 h in a 5% $CO_2$ incubator, the coculture medium was gently mixed with a pipette and aliquots (40 μl of 200 μl of coculture medium) were transferred to 35-mm-diameter tissue culture dishes with a 2-mm grid (Nunc Inc., Naperville, Ill.). Syncytia containing more than five nuclei were then counted under an inverted microscope. In this assay system, the HTLV-1-bearing cell line $KT_{252}$ routinely formed 500 to 800 syncytia per $10^6$ indicator MOLT-4 cells. All experiments were performed in triplicate wells.

**Preparation of HTLV-1 envelope proteins.** Mature gp46 and gp21 proteins (a generous gift from H. Miyakoshi, Diagnostics Research Laboratory, Fujirebio, Tokyo) were purified from culture fluids of HTLV-1-producing TCL-Kan cells. Purification of gp46 was achieved by immunoaffinity chromatography and gel chromatography of culture fluids of TCL-Kan cells (26). The supernatant of a culture fluid was concentrated with an Ultrapore membrane filter and disrupted with 1% Nonidet P-40. This culture fluid was applied to an anti-HTLV-1 gp46 (I-2G8) monoclonal antibody-coupled Sepharose 4B immunoaffinity column and washed with 0.05 M phosphate buffer (pH 7.4) supplemented with 0.5 M sodium chloride. The bound fraction was then eluted with 0.1 M glycine-HCl buffer solution, pH 2.5, and immediately neutralized with 0.5 M Tris solution. The eluted gp46-rich fraction was further purified by fast protein liquid chromatography with a Superdex 200 column (Pharmacia AB, Uppsala, Sweden) to eliminate the contaminated fetal calf serum and cellular components. Mature gp21

was prepared from culture fluid by sucrose density gradient ultracentrifugation and by successive disruption with 1% Nonidet P-40 (12). Briefly, disrupted viral antigens (460 μg/ml, 150 ml) were loaded on a 150-ml column of lentil lectin-Sepharose 4B (Pharmacia Fine Chemicals Co. Ltd., Uppsala, Sweden) and washed with 10 mM Tris-HCl buffer solution, pH 8.3, supplemented with 0.5% sodium deoxycholate. Lectin-bound proteins were eluted with 5% α-methyl D-mannoside and dialyzed against 0.1 M bicarbonate buffer solution, pH 9.6. These two proteins were isolated as single band by protein staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Radiolabeling of protein.** Radioiodination of protein was performed with IODO-BEADS (Pierce, Rockford, Ill.), according to the manufacturer's instructions. Protein (1 μg each) was added to 1.5-ml Eppendorf tubes containing two beads which had been preincubated with 500 μl of 10 mM phosphate buffer, pH 7.2, containing 200 μCi of $Na^{125}I$ (Amersham, Arlington Heights, Ill.) at room temperature for 5 min. The bead mixture was shaken for 2 min by hand. The supernatant was dialyzed against 20 mM phosphate-buffered saline (PBS), pH 7.2. The average specific activity was 15 to 20 μCi of protein per μg.

**Binding experiment with $^{125}$I-labeled proteins.** Duplicate samples of $10^6$ MOLT-4 cells (20 μl each) were added to 160 μl of RPMI 1640 containing 20 mM HEPES (Sigma, St. Louis, Mo.), pH 7.2, in the presence or absence of 100-fold amounts of unlabeled gp46 or gp21, and incubated for 30 min at room temperature. To each tube was added 20 μl of $^{125}$I-labeled gp46 (132,000 cpm/pmol of protein) or $^{125}$I-gp21 (63,000 cpm/pmol of protein) at various concentrations. After incubation for 3 h at 37°C, cells were separated from free ligand by centrifugation through 0.2 ml of an 84% silicone oil–16% paraffin oil mixture in a 400-μl tube (Assist Trading, Tokyo, Japan) for 2 min at 5,500 × *g*, 4°C. The tips of the tubes, which contained the cell pellet, were cut off and cell-bound $^{125}$I-labeled ligands were determined in an Aloka Gamma ARC-360 counter (Aloka Industries, Tokyo, Japan).

**Preparation of membrane component.** Sepharose 4B coupled with peptide was prepared by incubation of 3 mg of synthetic peptide and 0.6 g (dry weight) of cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. MOLT-4 cells ($5 \times 10^8$) were disrupted with 10 ml of 20 mM Tris-HCl buffer, pH 7.4, containing 2 μg of protease inhibitors (leupeptin, pepstatin and aprotinin; Wako Pure Chemicals, Osaka, Japan) per ml, 2 mM phenylmethylsulfonyl fluoride, 50 mM sucrose monocaprate (SM-1000; Dojin Kagaku, Kumamoto, Japan), 1 mM dithiothreitol, 1 mM EDTA, and 150 mM KCl, and the supernatant was then collected by centrifugation at 11,000 × *g* for 20 min. After removing insoluble materials through a Sepharose 4B column (3 ml), the run-through fractions were applied to an affinity column (3 ml) coupled with the gp46–197 or the gp21–400 and exhaustively washed with cell disruption buffer. The materials which bound to the column were obtained using 6 M urea solution and then dialyzed against 10 mM PBS containing 10 mM sucrose monocaprate. The protein concentration was estimated using BCA protein assay reagent (Pierce, Rockford, Ill.), according to the manufacturer's instructions.

**Binding experiment with a plastic plate.** The wells of a 96-well microtiter plate (Nunc; Nunc-Immuno Module Maxisorp) were coated with 1 μg of proteins or peptides dissolved in 10 mM $NaHCO_3$ buffer, pH 9.55, and left overnight at 4°C. Unreacted sites on the solid phase were blocked with 3% bovine serum albumin (BSA, Fraction V; Sigma) in PBS for 3 h at room temperature. After six washings with PBS containing 0.05% Tween 20, samples (1 μl per well) were added to 100 μl of RPMI 1640 containing 20 mM HEPES, pH 7.2, in each well and incubated for 3 h at 37°C. The supernatant was used to determine syncytium formation.

**Other methods.** Trypsin digestion (100 μg/ml; Sigma) was performed by incubation for 1 h at 37°C in serum-free RPMI 1640, and the reaction was stopped by the addition of 5 μl of trypsin inhibitor (200 μg/ml; Wako, Osaka, Japan). $NaIO_4$ oxidation was performed with 10 mM $NaIO_4$ in 50 mM acetate buffer, pH 5.5, for 1 h at room temperature. The reaction mixture was dialyzed against PBS containing 10 mM sucrose monocaprate to remove the reagent and then used to determine syncytium formation. In organic solvent extraction, samples (5 μl each) dissolved in 45 μl of PBS were shaken with 250 μl of chloroform-methanol mixture (2:1) for 2 min. The lower and upper layers were then collected into 1.5-ml Eppendorf tubes, and after drying with a centrifugal concentrator (model 101; TOMY, Tokyo, Japan), the residues were dissolved in 50 μl of PBS containing 10 mM sucrose monocaprate. These were used to determine the presence of syncytium formation. To estimate molecular mass, HPLC was performed using a TSK-GEL G3000SWXL, which was useful within the range 5 to 200 kDa. Analysis was performed in 10 mM PBS containing 10 mM sucrose monocaprate at a flow rate of 0.3 ml/min. SDS-PAGE was performed on a 12.5% polyacrylamide gel. After running on polyacrylamide, the gel was cut into 1-mm strips, placed in a 1.5-ml Eppendorf tube, and homogenized in 100 μl of PBS. After incubation overnight at 4°C, the supernatant was used to determine the presence of syncytium formation. Molecular mass calibration curves were prepared using standard proteins consisting of myosin heavy chain (200 kDa), phophorylase B (97 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18 kDa), and lysozyme (14 kDa).

## RESULTS

**Effects of gp46 and gp21 for syncytium formation.** To confirm that our system for assaying syncytium formation reflected HTLV-1 envelope-dependent entry, we examined the inhibitory effect of mature gp46 and gp21 proteins, which were purified from an HTLV-1-infected cell line (12, 26), on syncytium formation. As shown in Fig. 1, each protein inhibited syncytium formation in a linear manner at low concentration, but the inhibition was limited to approximately 50% at 50 to 100 nM of protein. However, syncytium formation was strongly inhibited with a mixture of gp46 and gp21. The inhibitory effect of this mixture was approximately the sum of those of gp46 and gp21 alone. These observations suggest that syncytium formation in our system is dependent on both the HTLV-1 gp46 surface glycoprotein and the gp21 transmembrane glycoprotein.

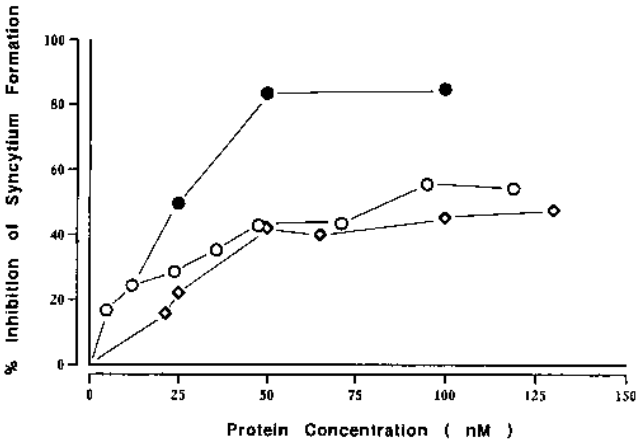


FIG. 1. Inhibitory effect of gp46 and gp21 on syncytium formation. The assay consisted of the coculture of $2.5 \times 10^4$ $KT_{252}$ cells together with $10^5$ MOLT-4 cells. In each case, samples were added to the triplicate wells and syncytia were counted after 18 h of coculture. Each value was estimated in comparison with syncytia in a coculture without sample specimen. ○, gp46; ◇, gp21; and ●, mixture of gp46 and gp21.

**Binding of gp46 and gp21.** To clarify the cell surface component which allows interaction with gp46 and gp21, we tested the binding of $^{125}$I-labeled gp46 and gp21 to MOLT-4 cells in the presence or absence of 100-fold amounts of unlabeled gp46 or gp21. As shown in Fig. 2, $^{125}$I-gp46 clearly bound to the MOLT-4 cells in medium alone (Fig. 2A) and this binding was observed even in the presence of large amounts of unlabeled gp21. As expected, $^{125}$I-gp46 binding was completely blocked in the presence of unlabeled gp46. These observations suggest that gp46 specifically interacts with the component on the MOLT-4 cell surface. On the other hand, $^{125}$I-gp21 easily bound to cells in medium alone. However, in contrast to gp46 binding, while $^{125}$I-gp21 binding to MOLT-4 cells was observed in the presence of unlabeled gp21 (Fig. 2B), such binding was completely blocked in the presence of large amounts of gp46. This result implies the possibility that gp21 binds directly to gp46 without affecting gp46 binding to its own ligand but not vice versa. To analyze this possibility, we performed the binding experiments using a nitrocellulose sheet coated with mature gp46 or gp21. $^{125}$I-gp46 or $^{125}$I-gp21 was added to each nitrocellulose sheet in the presence or absence of 100-fold amounts of unlabeled gp46 or gp21. In these experiments, we have never been able to detect any direct association between gp46 and gp21 on the sheet (data were not shown). These observations suggest that gp21 specifically interacts with a component that is present in large amounts on MOLT-4 cell surface, as shown by complete elimination of gp21 binding in

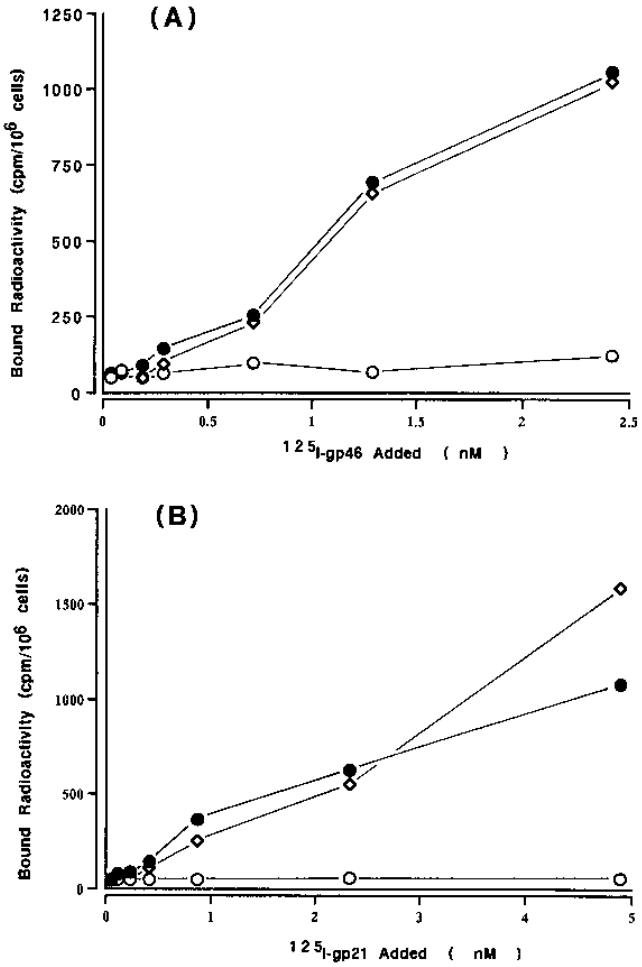


FIG. 2. Binding of $^{125}$I-gp46 and $^{125}$I-gp21 to MOLT-4 cells in the presence of large amounts of unlabeled ligands. Duplicate samples of $10^5$ MOLT-4 cells were incubated with 100-fold amounts of unlabeled gp46 or gp21 for 30 min at room temperature. To each well was added various concentrations of $^{125}$I-gp46 (132,700 cpm/pmol) or $^{125}$I-gp21 (63,300 cpm/pmol). Protein binding was detected as described in Materials and Methods. (A) $^{125}$I-gp46 binding to MOLT-4 cells. ●, medium alone; ○, unlabeled gp46; ◇, unlabeled gp21. (B) $^{125}$I-gp21 binding to MOLT-4 cells. ●, medium alone; ○, unlabeled gp46; ◇, unlabeled gp21.

the presence of gp46. The high amount of gp21 binding may be due to the nature of the gp21-binding component.

Scatchard analysis of the binding of $^{125}$I-gp46 and $^{125}$I-gp21 is depicted in Fig. 3. Analysis of the data reveals the presence of a two-component binding curve for both gp46 and gp21. We estimated that MOLT-4 cells possessed 1,373 high-affinity binding sites per cell and 36,300 low-affinity sites per cell, with a $K_d$ of 108 pM and 6.5 nM, respectively, for gp46, and 1,433 high-affinity binding sites per cell and 19,220 low-affinity sites per cell, with a $K_d$ of 102 pM and 36.3 nM, respectively, for gp21. The values of gp46 obtained on high- and low-affinity binding sites were comparable with those in gp21.

**Isolation of membrane components.** Our previous study showed that the peptides gp46–197 and gp21–400 specifically inhibited syncytium formation induced by HTLV-1-bearing cells (35). This result implies that two peptide regions on the envelope protein may contribute to the specific interaction between the host cell membrane and the HTLV-1 envelope protein, leading to viral entry. Therefore, to confirm the previous result that two peptide regions play important roles in syncytium formation, we examined the effect of materials which bound to gp46–197 and gp21–400 on syncytium formation. In this series of experiments, we prepared Sepharose 4B columns coupled with gp46–197, gp21–400, amino acids 175 to 199 (gp46–175) and 213 to 236 (gp46–213) of gp46, 382 to 403 (gp21–382) and 426 to 446 (gp21–426) of gp21, and 100 to 130 of the p19$^{gag}$ protein (p19–100). MOLT-4 cells were lysed using 50 mM sucrose monocaprate to solubilize proteins and lipids. MOLT-4 cell lysate (1.7 ml) was applied to each Sepharose 4B column (2 ml) coupled with the peptide, and after being washed extensively with PBS containing 10 mM sucrose monocaprate, material that bound to the affinity column was eluted with 6 M urea solution. As shown in Fig. 4, inhibitory activity was seen only in the fractions eluted from the gp46–197 column (gp46–197 eluate) and the gp21–400 column (gp21–

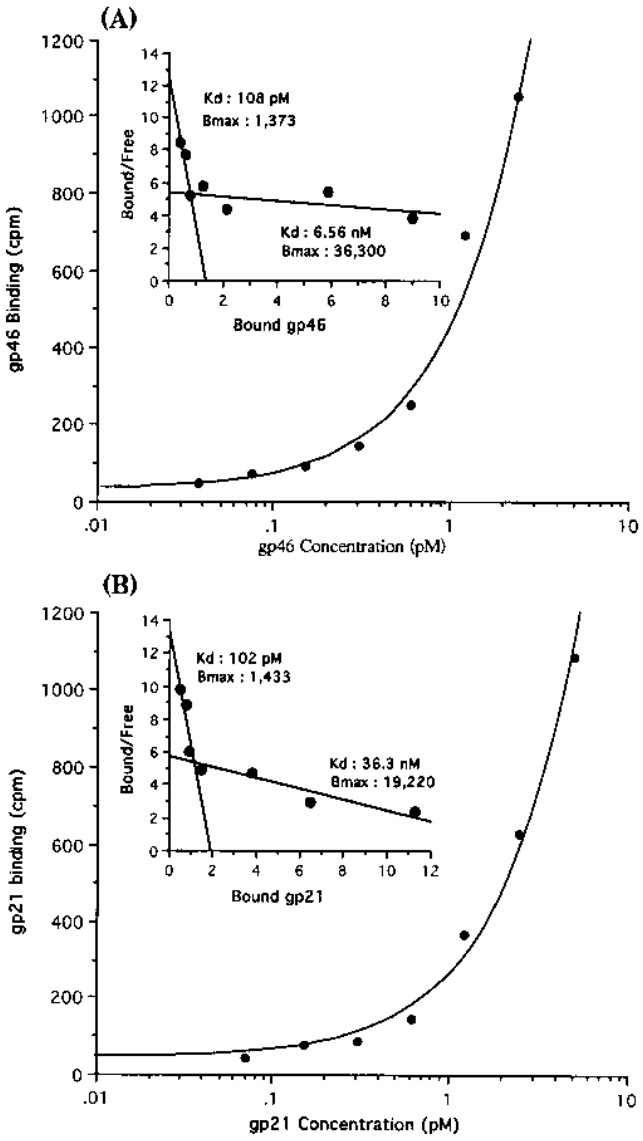


FIG. 3. Binding of gp46 and gp21 to MOLT-4 cells. Specific binding, determined by subtracting the radioactivity bound to cells incubated without ligand, is plotted as a function of protein concentration. Data regarding gp46 and gp21 binding with increasing amounts of proteins are plotted according to the Scatchard equation (insert). (A) gp46 binding. (B) gp21 binding.

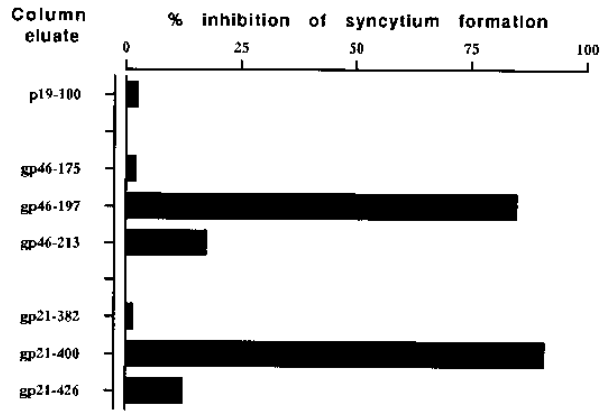


FIG. 4. Inhibitory effect of materials eluted from different columns on syncytium formation. MOLT-4 lysate (1.7 ml) was applied to Sepharose 4B columns coupled with peptide gp46–175, gp46–197, gp46–213, gp21–382, gp21–400, gp21–426, or p19–100. After washing with PBS containing 10 mM sucrose monocaprate, materials that bound to the column were eluted with 6 M urea solution. After dialyzing against PBS containing 10 mM sucrose monocaprate, the dialysates were adjusted to 3 ml with the same buffer. These samples (5 μl each) were used to determine the inhibitory effect on syncytium formation. Each value was estimated in comparison with syncytia in a coculture without any samples.

400 eluate) with 6 M urea solution, whereas no activity was observed in any other fractions. These results indicate that materials which specifically interact with peptides gp46–197 and gp21–400 are involved in syncytium formation induced by HTLV-1-bearing cells.

**Binding specificity.** To clarify the binding specificity of the eluate from the gp46–197 and the gp21–400 columns, we performed adsorption experiments using 96-well microtiter plates coated with gp46, gp21, gp46–197, or gp21–400. The gp46–197 and gp21–400 eluates were added to each well of the microtiter plate. After incubation for 3 h at 37°C, the inhibitory effect of the supernatant on syncytium formation was determined. The results are summarized in Table 1. The inhibitory activity of the gp46–197 eluate was completely eliminated by preincubation with gp46 and gp46–197, whereas no decrease was seen with mature gp21 and gp21–400. This result indicates that the material which allows binding to the gp46–197 column specifically interacts with the HTLV-1 gp46 surface glycoprotein. On the other hand, the inhibitory activity of the gp21–400 eluate was clearly reduced to a residual level by preincubation with gp21 or gp21–400, compared those seen with gp46 and gp46–197. This result indicates that the materials which allows binding the gp21–400 column specifically interacts with the HTLV-1 gp21 transmembrane glycoprotein.

TABLE 1. Binding specificity of the gp46–197 and gp21–400 eluates[a]

| Sample | Preincubation with | No. of syncytia[b] | % Inhibition |
|---|---|---|---|
| None | | 531 | |
| gp46–197 eluate | None | 135 | 74 |
| | gp46 | 515 | 4 |
| | gp21 | 140 | 73 |
| | gp46–197 | 484 | 9 |
| | gp21–400 | 157 | 70 |
| gp21–400 eluate | None | 183 | 66 |
| | gp46 | 245 | 54 |
| | gp21 | 584 | 0 |
| | gp46–197 | 245 | 54 |
| | gp21–400 | 417 | 22 |

[a] Control experiment was performed as follows: RPMI 1640 containing 20 mM HEPES, pH 7.2, was incubated on a plastic plate coated with 3% BSA for 3 h at 37°C and then the supernatant was used to determine the activity for syncytium formation.

[b] Each value was estimated in comparison with syncytia in a coculture without any sample.

TABLE 2. Effects of trypsin digestion, $NaIO_4$ oxidation, and $CH_3OH/CHCl_3$ extraction on the gp46–197 and gp21–400 eluates

| | gp46–197 eluate | | gp21–400 eluate | |
|---|---|---|---|---|
| | No. of syncytia[a] | % Inhibition | No. of syncytia | % Inhibition |
| Control | 736 | | 736 | |
| None | 184 | 75 | 169 | 77 |
| Trypsin-digestion | 691 | 6 | 264 | 64 |
| $NaIO_4$ oxidation | 382 | 48 | 742 | 0 |
| $CH_3OH/CHCl_3$ extraction | | | | |
| $CH_3OH/CHCl_3$ layer | NT[b] | | 309 | 58 |
| Water layer | NT | | 677 | 8 |

[a] Each value was estimated in comparison with syncytia in a coculture of control experiment. All values were expressed as the mean of triplicate experiments.

[b] NT, not tested.

**Characteristics of membrane components.** To assess the biochemical characteristics of the cell surface components, the eluates were treated with trypsin, sodium periodate, and chloroform-methanol (2:1), as described in Materials and Methods. The results are summarized in Table 2. With the gp46–197 eluate, trypsin-digestion markedly reduced the inhibitory activity of syncytium formation, whereas sodium periodate treatment did not produce a clear reduction. These results suggest that this protein is involved in the component which allows binding with gp46. On the other hand, the inhibitory activity of the gp21–400 eluate was completely eliminated with sodium periodate treatment, while there was no reduction with trypsin digestion. Furthermore, the inhibitory activity was partitioned in the organic phase of the methanol-chloroform treatment. These observations imply that a carbohydrate containing lipids is involved in the component which allows binding with gp21.

The gp46–197 eluate was analyzed by HPLC using a TSK-GEL G3000SWXL column (7.7 mm by 300 mm) equilibrated with PBS containing 10 mM sodium monocaprate. The elution profiles of protein and inhibitory activity for syncytium formation in gp46–197 eluate are shown in Fig. 5. Most of the inhibitory activity for syncytium formation was observed at fraction 31 to 33, with an estimated molecular mass of approximately 34 kDa. The eluate was further analyzed by SDS-PAGE. After concentrating the eluate by mixing it with Sepharose 4B-coupled gp46–197, the bound materials was run on a 12.5% polyacrylamide gel. As shown in Fig. 6, a dominant peak of the inhibitory activity for syncytium formation was seen at the position corresponding to a molecular mass of approximately 35 kDa. We also observed an inhibitory activity at the position of approximately 43 kDa. On the other hand, the methanol-chloroform layer of the gp21–400 eluate was analyzed by high-performance thin-layer chromatography (HPTLC; Alufolien Kieselgel 60; Merck, Darmstadt, Germany) using developing solvent $CH_3OH$-$CHCl_3$-$H_2O$ (35:60:8). After developing, bands were stained by anisaldehyde reagent (22), cut into 2- to 3-mm strips, placed in a 1.5 ml Eppendorf tube, and extracted with mixture of chloroform-

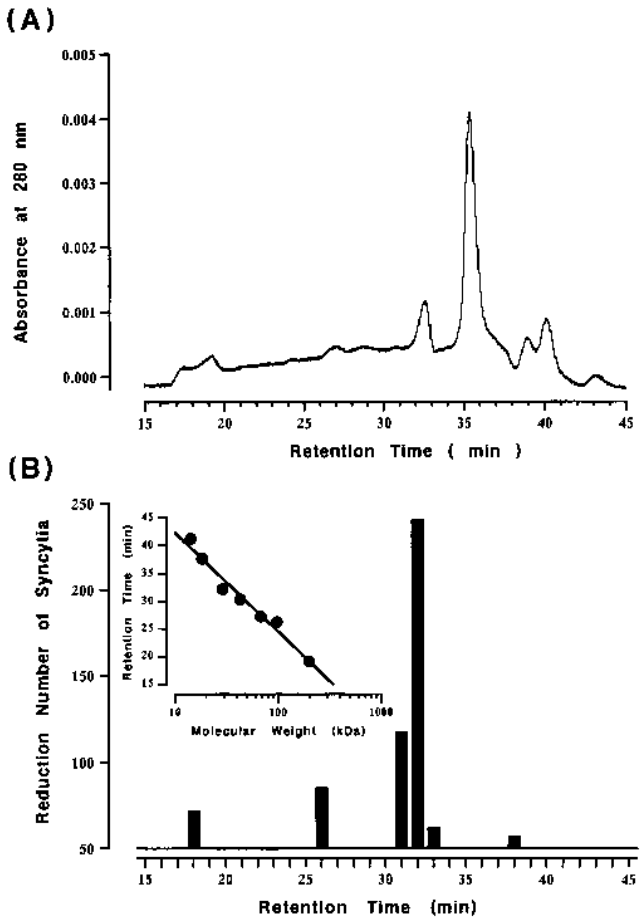


FIG. 5. Representative HPLC analysis of the gp46–197 eluate. The gp46–197 eluate (50 μl) was applied to a TSK-GEL G3000SWXL column (7.8 mm by 300 mm) equilibrated with PBS containing 10 mM sucrose monocaprate and eluted with the same buffer at a flow rate of 0.3 ml/min. After collecting fractions at 1-min intervals, an aliquot (5 μl each) was used to determine its effects on syncytium formation. (A) Elution profile on HPLC. (B) Inhibitory effect of each fraction on syncytium formation. In this assay, the mean number of syncytia in a coculture of $KT_{252}$ and MOLT-4 cells was 780. Each value is expressed as the reduction in the number of syncytia. Molecular mass markers for HPLC (insert) are 14, 18, 29, 43, 68, 97, and 200 kDa.

methanol (2:1). As shown in Fig. 7, inhibitory activity for syncytium formation in the gp21–400 eluate was observed in a weak band at the center of the HPTLC plate.

## DISCUSSION

Syncytium formation induced by HTLV-1-bearing cells is a complex process which includes correct binding to the virus receptor, folding of the envelope, which leads to the exposure of the fusion peptide, and finally membrane fusion. Perturbation of any of these steps is likely to disturb syncytium formation. Our previous studies (35) showed that the peptides gp46–197 and gp21–400 specifically inhibited syncytium formation induced by HTLV-1-bearing cells, suggesting that these two regions may contribute to the interaction between the HTLV-1 envelope and the target cell membrane, leading to membrane fusion. In the present study, using an affinity column coupled with gp46–197 or gp21–400, we identified two components that are critical for syncytium formation induced by HTLV-1-bearing cells. One is a trypsin-digestion-sensitive component that specifically interacts with the gp46 surface protein, and the other is a $NaIO_4$-sensitive, organic solvent-soluble component that interact with the gp21 transmembrane protein. These results suggest that a combination of four components on the opposing cell membranes may contribute to the HTLV-1 envelope-mediated syncytium formation.

In the present study, we used the mature gp46 and gp21 which were purified from the culture fluid of HTLV-1-producing cell lines in the presence of detergent. In HTLV-1, the gp21 transmembrane glycoprotein is usually associated with the gp46 surface glycoprotein, presumably in an ordered configuration that may protects the putative hydrophobic functional domain. Although it is not clear whether purified envelope products would take the same configuration as seen in HTLV-1, we observed that each purified protein had the inhibitory activity on syncytium formation induced by HTLV-1-bearing cells. This result suggests that at least partly purified gp46 and gp21 may have the same configuration as the putative functional domains, as seen in HTLV-1-infected cells.

Although the identity of the HTLV-1 receptor is still uncertain, it has been shown that the gene for the cell surface receptor for HTLV-1 is located in the distal region of human chromosome 17q (38). Recently, Gavalchin et al. (13), using a panel of somatic cell hybrids containing human 17q, showed that the gene for the HTLV-1 receptor was located on the distal region of human chromosome 17q23.2-17q25.3 and that the gene products (approximately 30 to 31 kDa) bound to

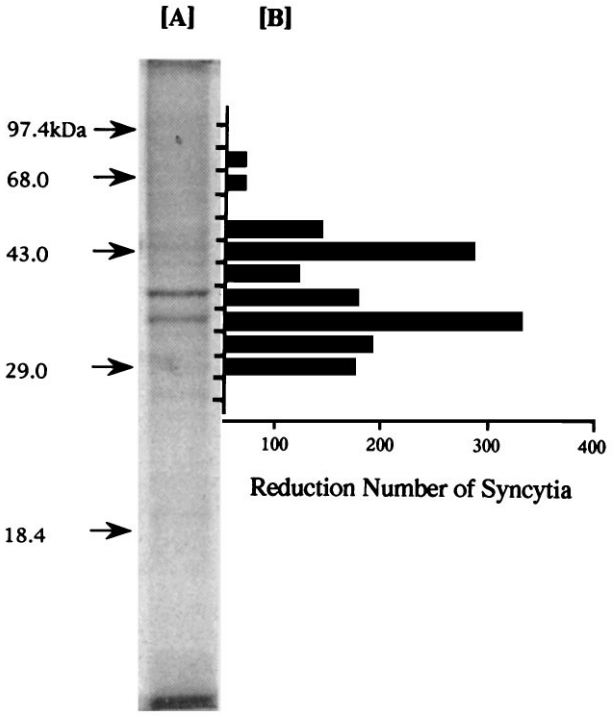


FIG. 6. Representative SDS-PAGE analysis of the gp46–197 eluate. The gp46–197 eluate (300 μl) was mixed with 50 μl of a 50% suspension of Sepharose 4B coupled with gp46–197 in PBS and incubated for 3 h at 37°C. After centrifugation, the pellet was washed three times with 300 μl of PBS, suspended in 30 μl of 250 mM Tris-HCl buffer, pH 6.7, containing 4% SDS, 0.05% bromophenol blue, 50% glycerol, and 10% 2-mercaptoethanol, and incubated for 2 min at 100°C. The reaction mixture was applied to a 12.5% polyacrylamide gel. After running, the gel was cut into 1-mm strips and extracted with 100 μl of PBS as described in Materials and Methods. Protein staining was performed with 0.1% Coomassie brilliant blue. (A) Protein staining. (B) Inhibitory effect of each fraction on syncytium formation. In this assay, the mean number of syncytia in a coculture was 585. Each value is expressed as the reduction in the number of syncytia.

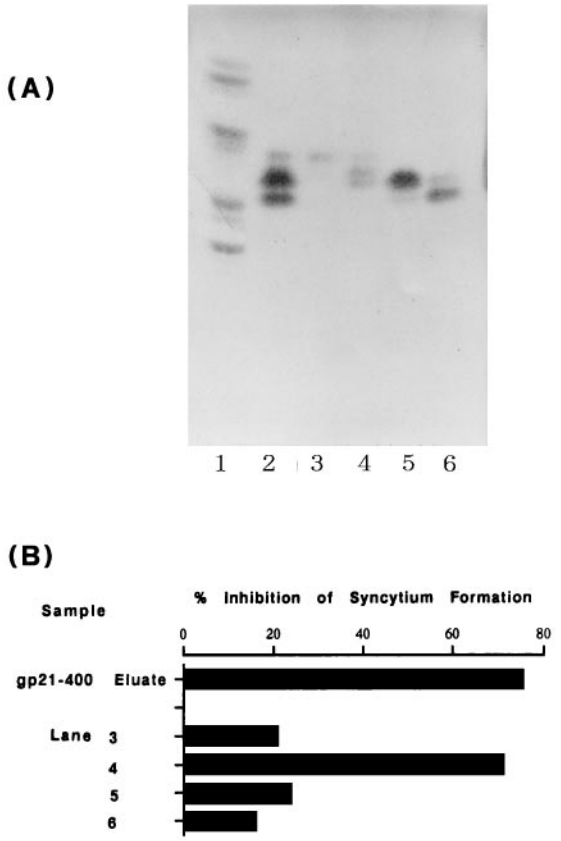


FIG. 7. HPTLC analysis of the gp21–400 eluate. The gp21–400 eluate was extracted with chloroform-methanol (2:1), and the organic layer was dried with a centrifugal concentrator. After dissolving the residue in 50 μl of chloroform-methanol mixture (2:1), an aliquot was applied to a silica plate. The plate was developed with chloroform-methanol-water mixture (60:35:6). (A) The plate was stained with anisaldehyde reagent, and the bands were extracted with chloroform-methanol mixture (2:1). Lanes: 1, standard samples of neutral glycolipids (from the top: ceramide mono-, di-, tri-, and tetrahexoside and its epimers); 2, the gp21–400 eluate; 3 to 6, eluates from the position of the bands. (B) Inhibitory activity of the eluates. After drying up the organic layer, the residues were dissolved in 50 μl of PBS containing 10 mM sucrose monocaprate. Aliquots (2 μl each) were then used to determine the inhibitory effect on syncytium formation. Each value was estimated in comparison with syncytia in a coculture without any samples.

recombinant HTLV-1 envelope gp46. Our experimental results showed that a protein-like component which allowed binding with gp46 had a molecular mass of approximately 34 kDa by HPLC. To further understand the molecular nature of this component, additional studies are underway to determine its fine structure. We showed that the gp21 transmembrane protein including region amino acids 400 to 429 interacts with a nonprotein component, possibly carbohydrate-containing lipids, on the cell surface and this interaction is critical for HTLV-1 envelope-mediated syncytium formation. Denesvre et al. (9) showed recently that chimeric HTLV-1 envelope, into which had been introduced the external domain (amino acids 403 to 447) of Friend murine leukemia virus transmembrane protein, was nonfusogenic. This observation is in good agreement with the data presented here. Combined data from these two studies suggest that the region corresponding to amino acids 400 to 429 of gp21 may be the fusion peptide.

It is considered that the molecular events leading to membrane fusion occur within a short time (8, 25). This short time has led to models in which components for membrane fusion are preassembled in the membrane and exhibit rapid conformational changes leading to the opening of a fusion pore (1). With regard to a model of HTLV-1 infection, it is believed that the fusion pore develops in three steps, in analogy to the model for exocytosis suggested by Almers and Tse (1): (i) insertion of the envelope protein expressed at the surface of HTLV-1-bearing cells into the opposing plasma membrane of the target cell; (ii) a conformational change which produces an opening in the center of the multimer; and (iii) dilation of the pore by dispersal of the multimeric subunits and concomitant incorporation of lipid molecule at the former subunit interfaces. Key features of this model are multimeric association, which resembles spot welding between the membranes, and the dissociation of two fusion components on one membrane, leading to an opening in the center of the multimer (43). HTLV-1 envelope protein is synthesized as a gp61 precursor product cleaved into two mature proteins: a gp46 exterior protein and a gp21, which anchors the envelope at the cell membrane. The envelope mature products gp46 and gp21 are not covalently associated (31). In the HTLV-1 envelope protein, it is feasible that conformational changes lead to the dissociation of gp46 and gp21 through binding between the fusion components in the opposing membrane. In this regard, the HTLV-1 envelope is different from the avian retrovirus envelope mature products, which are linked by disulfide bonds (10). With regard to fusion components of target cells, on the other hand, our experimental results showed that the two components were located close to each other and that the binding of gp46 to one component induced a conformational change in another component, which allowed binding to gp21. gp21 binding was completely blocked in the presence of gp46. Thus, specific interaction of the HTLV-1 envelope proteins with components exposed on the opposing cell membrane is likely to produce an opening in the center of the multimer, allowing formation of a fusion pore.

We showed that $^{125}$I-gp21 binding to the surface of MOLT-4 cells was detected even in the presence of large amounts of unlabeled gp21. This result might be due to the nature of the gp21-binding component. It is believed that membrane lipids can diffuse over molecularly significant distances in a short time. Furthermore, this ability of lipids is undoubtedly enhanced in the presence of protein (36). These characteristics of membrane lipids should be kept in mind when analyzing HTLV-1 envelope-mediated membrane fusion.

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