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Identification and Mapping of Functional Domains on Human T-Cell Lymphotropic Virus Type 1 Envelope Proteins by Using Synthetic Peptides

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To identify the regions that are important in human T-cell leukemia virus type 1 (HTLV-1) envelope function, we synthesized 23 kinds of peptides covering the envelope proteins and examined the inhibitory effect of each peptide on syncytium formation induced by HTLV-1-bearing cells. Of the 23 synthetic peptides, 2, corresponding to amino acids 197 to 216 on gp46 and 400 to 429 on gp21, inhibited syncytium formation induced by HTLV-1-bearing cells but did not affect syncytium formation induced by human immunodeficiency virus type 1-producing cells. The peptide concentrations giving 50% inhibition of syncytium formation for gp46 197 to 216 and gp21 400 to 429 were 14.9 and 6.0 μM, respectively. A syncytium formation assay with overlapping synthetic peptides containing amino acids 175 to 236 and 391 to 448 of the envelope proteins showed that syncytium formation was inhibited by peptides that contained the amino acid sequences 197 to 205 (Asp-His-Ile-Leu-Glu-Pro-Ser-Ile-Pro) and 397 to 406 (Gln-Glu-Gln-Cys-Arg-Phe-Pro-Asn-Ile-Thr). These observations suggest that the two regions corresponding to amino acids 197 to 216 and 400 to 429 are involved in HTLV-1 envelope function.

Human T-cell lymphotropic virus type 1 (HTLV-1), a type C retrovirus, is closely associated with adult T-cell leukemia/lymphoma (9, 30) and some neurologic disorders (3, 13, 25). HTLV-1 has been implicated as a causative factor in other diseases, including some cases of polymyositis, polyarthritis, uveitis, and infectious dermatitis (1). This virus is transmitted from mother to infant by breast-feeding (8, 17, 23), between male and female by sexual intercourse (19, 38), and by blood transfusion (24). In these forms of transmission, HTLV-1 genome-carrying lymphocytes appear to be responsible for the infection, since there is no evidence of viral expression in vivo or HTLV-1 infection as a result of blood transfusion in patients who receive blood cell components from seropositive donors (24).

The HTLV-1 envelope glycoprotein is synthesized as a precursor product of 488 amino acids (34). This glycoprotein is then cleaved by cellular protease into two mature products, a surface protein (gp46) of 313 amino acids and a transmembrane protein (gp21) which allows anchoring of the gp46-gp21 complex at the cell surface. These HTLV-1 glycoproteins are suggested to be involved in viral infection by the following experimental observations: (i) sera from HTLV-1-infected individuals neutralized the infectivity of a pseudotype virus expressing HTLV-1 envelope antigen (42); (ii) animals were protected from HTLV-1 infection by immunization with either recombinant HTLV-1 envelope antigen (14, 22) or HTLV-1 *env* gene expressing recombinant vaccinia virus (35); (iii) most sera from HTLV-1-infected individuals contained antibodies directed to peptides from the carboxy terminal of gp46 (15, 28, 40); and (iv) linear peptide targets for neutralizing antibodies were localized around amino acids 88 to 98 (27) and 187 to 198 (12, 16, 39) on gp46. These imply that regions of HTLV-1 envelope glycoproteins play an important role in viral entry into the target cell, including binding to a specific receptor, followed by postbinding events leading to fusion of the viral envelope with the plasma membrane of the target cell.

The purpose of this work is to identify the functional domains on envelope proteins in HTLV-1 infection by using synthetic peptides covering the envelope protein. In the present study, we show that two peptides corresponding to amino acids 197 to 216 on gp46 and amino acids 400 to 429 on gp21 inhibit syncytium formation induced by HTLV-1-bearing cells.

MATERIALS AND METHODS

Cells and compounds. The HTLV-1-bearing T-cell lines used were human T-cell line KT_{252}, established from an adult T-cell leukemia/lymphoma patient in Kyushu University Hospital, and C91/PL (31). The HTLV-1-negative cell lines used were the human T-cell line MOLT-4 (37) and feline kidney fibroblast cell line 8C, transformed by sarcoma virus (5). MOLT-4/HTLV-IIIB cell was the MOLT-4 cell line persistently infected with the HTLV-IIIB strain of human immunodeficiency virus type 1 (HIV-1) (20) and used as an HIV-producing cell. These cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. High-molecular-weight, high-sulfite-containing dextran sulfate (average molecular weight, 500,000; sulfite content, 17%) was purchased from Pharmacia, Uppsala, Sweden.

Peptide synthesis. Peptides were synthesized according to the predicted amino acid sequence based on the nucleotide sequence of the *env* gene of HTLV-1 (34) and HTLV-2 (36). Peptides were synthesized in a peptide synthesizer (model 431A; Applied Biosystems, Foster City, Calif.) with *tert*-butoxycarbonyl (*t*-Boc) aminoacyl-4-(oxymethyl)-Pam resin as solid support. Protected amino acids were obtained from The Protein Institute, Osaka, Japan. The α amino groups were protected with *t*-Boc. The peptides were cleaved from resins with trifluoromethanesulfonic acid as specified by the manufacturer and lyophilized. After being dissolved in 5% acetic acid, the peptide solution was applied to a Sephadex G-25 column equilibrated with 5% acetic acid and eluted with the same solution. The purity of individual peptides was examined by reverse-phase high-performance liquid chromatography with an octadecyl (C_{18}) silicated column. Most of the peptides were isolated as a single peak and used in this study without further purification. Five peptides (HTLV-1 gp46 175 to 199, 197 to 216, and gp21 400 to 429, HTLV-2 gp46 194 to 213 and gp21 396 to 425) were sequenced by Edman degradation on a gas phase sequencer (model 470A; Applied Biosystems). The

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TABLE 1. Synthetic peptides used in this study

Peptide[a]	Peptide code	Amino acid sequence	M_w
env gp46			
1–25	gp46-1	MGKFLATLIL FFQFCPLIFG DTSPS	2,856
20–49	gp46-20	GDYSPSCCTL TIGVSSYHSK PCNPAQPVCS	3,102
46–70	gp46-46	PVCSWTLDLL ALSADQALQP PCPNL	2,666
68–92	gp46-68	PNLVSYSSYH ATYSLYLFPH WTKKP	3,000
89–115	gp46-89	TKKPNRNGGG YYSLSYSDPC SLKCPYL	2,996
111–138	gp46-111	KCPYLGCQSW TCPYTGAVSS PYWKFQHD	3,254
136–161	gp46-136	QHDVNFTQEV SRLNINLHFS KCGFPF	3,078
159–183	gp46-159	FPFSLLVDAP GYDPIWFLNT EPSQL	2,867
175–199	gp46-175	FLNTEPSQLP PTAPPLLPHS NLDHI	2,749
197–216	gp46-197	DHILEPSIPW KSKLLTLVQL	2,331
213–236	gp46-213	LVQLTLQSTN YTCIVCIDRA SLST	2,643
235–254	gp46-235	STWHVLYSPN VSVPSSSSTP	2,132
253–282	gp46-253	TPLLYPSLAL PAPHLTLPFN WTHCFDPQIQ	3,432
277–292	gp46-277	FDPQIQAIVS SPCHNS	1,743
288–317	gp46-288	PCHNSLILPP FSLSPVPTLG SRSRRAVPVA	3,170
env gp21			
313–333	gp21-313	AVPVAVWLVS ALAMGAGVAG G	1,896
332–352	gp21-332	GGITGSMSLA SGKSLLHEVD K	2,087
350–386	gp21-350	VDKDISQLTQ AIVKNHKNLL KIAQYAAQNR RGLDLLF	4,237
382–403	gp21-382	LDLLFWEQGG LCKALQEQCR FP	2,595
400–429	gp21-400	CRFPNITNSH VPILQERPPL ENRVLTGWGL	3,458
426–448	gp21-426	GWGLNWDLGL SQWAREALQT GIT	2,573
445–462	gp21-445	TGITLVALLL LVILAGPC	1,780
458–488	gp21-458	LAGPCILRQL RHLPSRVRYP HYSLIKPESS L	3,601

[a] The numbers represent amino acid positions counting from the Met corresponding to the initiation codon of the envelope.

sequences of these peptides were identical with predicted amino acid sequences based on the nucleotide sequence of the *env* gene.

Syncytium formation assay. To monitor syncytium formation in suspension, MOLT-4 cells were routinely used as indicator cells. The assay consisted of a coculture of 2.5×10^4 KT_{252} or C91/PL cells together with 10^5 MOLT-4 cells. MOLT-4 cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.5% normal human serum (referred to as RPMI medium) at 5×10^6 cells per ml. Aliquots (20 μl per well) were added to 155 μl of RPMI medium containing synthetic peptides 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} or C91/PL 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, coculture medium was gently mixing with the pipette, aliquots (40 μl of the 200-μl coculture medium) were transferred to 35-mm tissue culture dishes with a 2-mm grid (Nunc Inc., Naperville, Ill.). Syncytia containing more than five nuclei were then counted with an inverted microscope. The syncytium formation assay with KT_{252} and 8C was described previously (11). Briefly, 8C cells were seeded into 24-well plastic plates (Nunc Inc., Naperville, Ill.) at 2×10^5 cells per well in Iscove modified Dulbecco medium (Boehringer, Mannheim, Germany) containing 10% fetal calf serum and 2 μg of Polybrene per ml (Sigma, St. Louis, Mo.). After 24 h, IMDM medium containing synthetic peptide or medium alone was added to the wells containing 8C cells. KT_{252} cells were then added to each well at 2×10^5 cells per well. The cells were cocultivated for 3 days. After removal of the culture medium, the cell monolayer was fixed and stained with Diff-Quick solution. Syncytia containing more than five nuclei were then counted under an inverted microscope. The syncytium formation assay for HIV-1 infection was described previously (6). The assay consisted of a coculture of 5×10^3 MOLT-4/HTLV-IIIB cells together with 2×10^4 MOLT-4 cells. After 48 h of coculture, syncytia were counted under a microscope after fixation with 5% formalin. All experiments were performed in triplicate wells.

RESULTS

To determine the regions on the HTLV-1 envelope protein important for envelope functionality, we synthesized 23 kinds of peptides (16 to 37 amino acids long), which overlapped by 2 to 5 amino acids and covered the entire envelope proteins. These peptides were tentatively referred to as protein *X*, with *X* referring to the position of the first amino acid of the peptide (where Met, the initiation codon of the envelope, is residue 1) (Table 1). The effect of each peptide in HTLV-1 envelope function was examined through the inhibition of syncytium formation induced by HTLV-1-bearing cells. The assay consisted of a coculture of HTLV-1-bearing T-cell line KT_{252} together with MOLT-4 indicator cells. In this assay, KT_{252} cells routinely formed 800 syncytia per 10^5 indicator cells. Figure 1 shows the activity of each peptide (10 μM) for inhibiting syncytium formation. Two peptides corresponding to amino acids 197 to 216 of gp46 (gp46-197) and amino acids 400 to 429 of

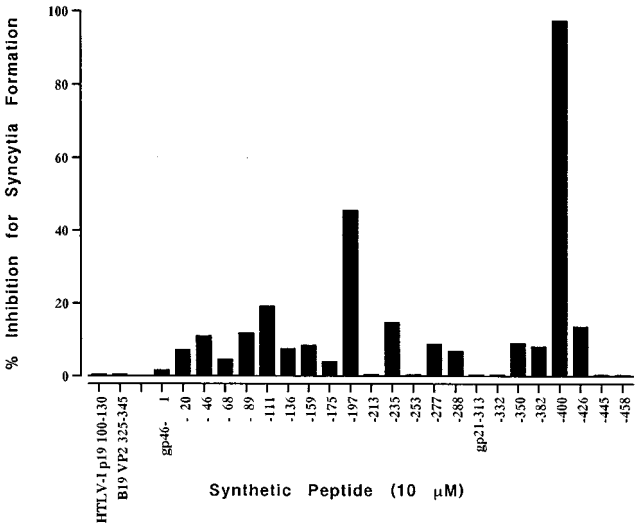

FIG. 1. Inhibitory effect of synthetic peptides on syncytium formation. The assay consisted of a coculture of 2.5×10^4 KT_{252} cells together with 10^5 MOLT-4 cells. In each case, 10 μM synthetic peptide was added to the triplicate wells and syncytia were scored after 18 h of coculture. Each value was estimated in comparison with syncytia in a coculture without synthetic peptide. Amino acid sequences of p19 100–130 and B19 VP2 325–345 are PPPPSSPTHDPDSDPP QIPPPYVEPTAPQVL and RISLRPGPVSQPYHHWDTDKY, respectively.

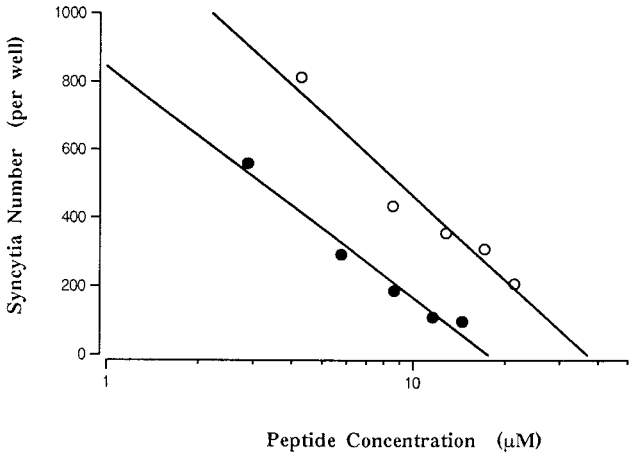

FIG. 2. Inhibitory effect of gp46 197–216 and gp21 400–429 on syncytium formation. The mean number of syncytia was plotted against the peptide concentration. Symbols: ○, gp46 197–216; ●, gp21 400–429.

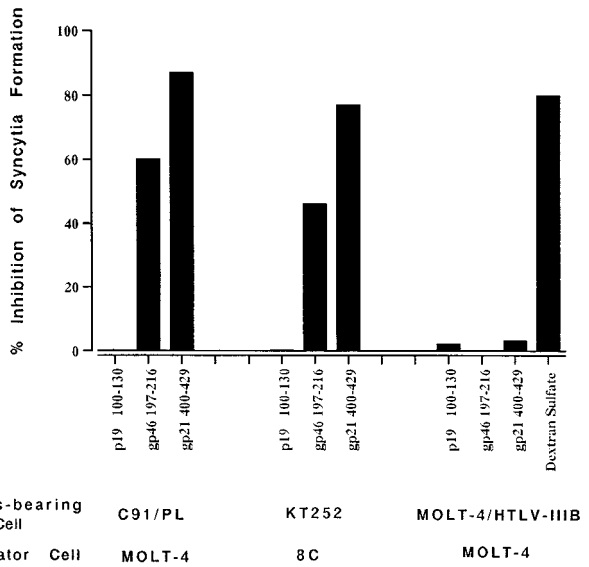

FIG. 3. Peptide effect on syncytium formation induced by C91/PL and MOLT-4/HTLV-IIIB cells. These assays were performed as described in Materials and Methods. In these assays, the mean number of syncytia was 820 in a coculture of C91/PL and MOLT-4, 360 in a coculture of KT_{252} and 8C, and 110 in a coculture of MOLT-4/HTLV-IIIB and MOLT-4. Dextran sulfate was used at 10 μg/ml.

gp21 (gp21-400) inhibited syncytium formation strongly. gp46-197 and gp21-400 (10 μM each) inhibited syncytium formation by 45 and 97%, respectively. When serially diluted peptides were added to the syncytium assay system, the mean number of syncytia decreased linearly depending on the peptide concentration (Fig. 2). The peptide concentrations causing a 50% inhibition of syncytium formation (ID_{50}) for gp46-197 and gp21-400 were 14.9 and 6.0 μM, respectively. We also observed a weak activity with the peptides corresponding to the region from amino acids 46 to 161 on gp46 (gp46-46, gp46-89, and gp46-111). The other synthetic peptides had no apparent activity; nor did control peptides, which correspond to immunodominant regions on HTLV-1 p19 (p19 100 to 130) (15) and human parvovirus B19 VP2 (VP2 325 to 345) (32).

To clarify the specificity of the peptide inhibitory effect for syncytium formation, we examined the effect of peptides on syncytium formation in other fusion assays performed with a coculture of other HTLV-1-bearing cells (C91/PL) or HIV-producing cells (MOLT-4/HTLV-IIIB) together with indicator cells (MOLT-4 or 8C) (Fig. 3). The two peptides (gp46-197 and gp21-400) inhibited syncytium formation induced by HTLV-1-bearing cells (C91/PL and KT_{252}), whereas the peptides had no activity for syncytium formation induced by HIV-1-producing cells (MOLT-4/HTLV-IIIB). The peptide p19 100–130 had no activity for inhibition of syncytium formation in any of the three assays. These results suggest that the inhibitory effect of the peptide for syncytium formation is specific.

To obtain the fine map of the functional domains on these two peptides (gp46 197–216 and gp21 400–429), we synthesized a series of overlapping 10- and 12-mer peptides spanning amino acids 194 to 220 of gp46 (gp46 194–205, 197–208, 200–211, 203–214, 206–217, 209–220, and 213–236) and 391 to 448 of gp21 (gp21 391–400, 397–406, 403–412, 409–418, 415–424, 421–430, and 426–448) (Fig. 4) and examined the inhibitory effect of each peptide on syncytium formation. In a series of peptides derived from gp46 197–216, syncytium formation was inhibited with peptide gp46 194–205 and gp46 197–208 (Fig. 5A). This implies that the amino acid sequence 197 to 205 (Asp-His-Ile-Leu-Glu-Pro-Ser-Ile-Pro) is essential for inhibiting syncytium formation by gp46 197–216. HTLV-2 peptide (amino acids 194 to 216 of the HTLV-2 Env protein) corresponding to HTLV-1 gp46 197–216 also inhibited syncytium formation, comparable to gp46 197–216. In the essential amino acid sequence of HTLV-2 peptide, amino acid substitution occurred at Asp-179 (to Glu), Ile-199 (to Val), Glu-201 (to Thr), Ile-204 to Thr, and Pro-205 to Ser. This shows that these amino acid substitutions do not affect the inhibiting activity of gp46 197–216 on syncytium formation. On the other hand, in a series of fragment peptides of gp21 400–429, syncytium formation was inhibited by peptides gp21 391–400, 397–406, and 403–412 (Fig. 5B). This implies that the amino acid sequence between 397 and 406 (Gln-Glu-Gln-Cys-Arg-Phe-Pro-Asn-Ile-Thr) is essential for inhibition of syncytium formation by gp21 400–429. In recent studies, the variation of amino acid sequence in the near-essential sequence of this peptide (amino acids 397 to 412) has been shown in isolates from different geographical origins (4, 26, 33, 43). These amino acid substitutions occurred at Arg-401 (to Cys or Tyr), Pro-403 (to Leu), Pro-411 (to Ser), and Ile-412 (to Met). The peptides with amino acid substitutions at these positions (gp21 397–406/M-1, 397–406/M-2, and 397–114/M-3) have the same activity as HTLV-1 gp21 397–406. Furthermore, HTLV-2 peptide (amino acids 396 to 425 of the HTLV-2 Env protein) with amino acid substitutions at Arg-401 (to Cys), Pro-403 (to Leu), Thr-406 (to Ser), Ser-408 (to Thr), Pro-411 (to Ser), and Ile-412 (to Val), also inhibited syncytium formation as did HTLV-1 gp21 400–429. Taken together, these results show that these amino acid substitutions do not affect the inhibition of syncytium formation.

DISCUSSION

In the present study, we synthesized 23 kinds of peptides covering the HTLV-1 envelope proteins and examined the effect of each peptide on syncytium formation induced by HTLV-1-bearing cells. We found that two peptides corresponding to amino acids 197 to 216 on the gp46 surface protein and amino acids 400 to 429 on the gp21 transmembrane protein inhibited syncytium formation induced by HTLV-1-bearing cells, whereas three peptides had no effect on syncytium formation induced by HIV-1-producing cells. Furthermore,

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                          175                                                                  235
                            FLNTEPSQLPPTAPPLLPHSNLDHILEPSIPWKSKLLTLVQLTLQSTNYTCIVCIDRASLST
HTLV-I  gp46  175-199       FLNTEPSQLPPTAPPLLPHSNLDHI
              194-205                          SNLDHILEPSIP
              197-208                             DHILEPSIPWKS
              200-211                                LEPSIPWKSKLL
              203-214                                   SIPWKSKLLTLV
              206-217                                      WKSKLLTLVQLT
              209-220                                         KLLTLVQLTLQS
              213-236                                            LVQLTLQSTNYTCIVCIDRASLST

              197-216                             DHILEPSIPWKSKLLTLVQL
                                                   * * **  *  * *   **
HTLV-II gp46  194-213                             EHVLTPSTSWTTKILKFIQL

                          391                                                                  448
                            GLCKALQEQCRFPNITNSHVPILQERPPLENRVLTGWGLNWDLGLSQWAREALQTGIT
HTLV-I  gp21  391-400       GLCKALQEQC
              397-406             QEQCRFPNIT
              403-412                   PNITNSHVPI
              409-418                         HVPILQERPPL
              415-424                               ERPPLENRVL
              421-430                                     NRVLTGWGL
              426-448                                           GWGLNWDLGLSQWAREALQTGIT

              400-429                  CRFPNITNSHVPILQERPPLENRVLTGWGL
                                       * * ** * **  ******** ** *****
              397-406/M-1         QEQCCFLNIT
              397-406/M-2         QEQCYFLNIT
              397-414/M-3         QEQCCFLNITNSHVSMLQ
HTLV-II gp21  396-425                CCFLNISNTHVSVLQERPPLEKRVITGWGL
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FIG. 4. Amino acid sequence of the synthetic peptide for mapping the functional domain. Amino acid sequences of the peptides gp21 397–406/M-1, 397–406/M-2, and 397–406/M-3 were obtained from isolates in Japan (35), the Caribbean (33), and North America (26), respectively. Asterisks (*) indicate identical amino acid residues.

amino acid substitutions around the inhibitory peptide regions did not affect the inhibition of syncytium formation by these two peptides. These results suggest that the peptide inhibitory effect for syncytium formation is specific on HTLV-1 infection and general phenomena in spite of the kinds of HTLV-1 target cell lines.

We show that the peptide corresponding to amino acids 400 to 429 on the gp21 transmembrane protein profoundly affected syncytium formation, indicating that the central region on gp21 is important for envelope function. In HTLV-1, the gp21 transmembrane protein is bound to the gp46 surface protein and would ensure the anchorage of the envelope protein at the membrane. It is believed that gp21 may also be responsible for fusion between the virus and target cell membrane, as well as for syncytium formation between infected and receptor-bearing cells (10, 21). Analysis of the HTLV-1 envelope protein sequence indeed showed that the putative amino-terminal part of gp21 bears a 29-amino-acid hydrophobic stretch which has

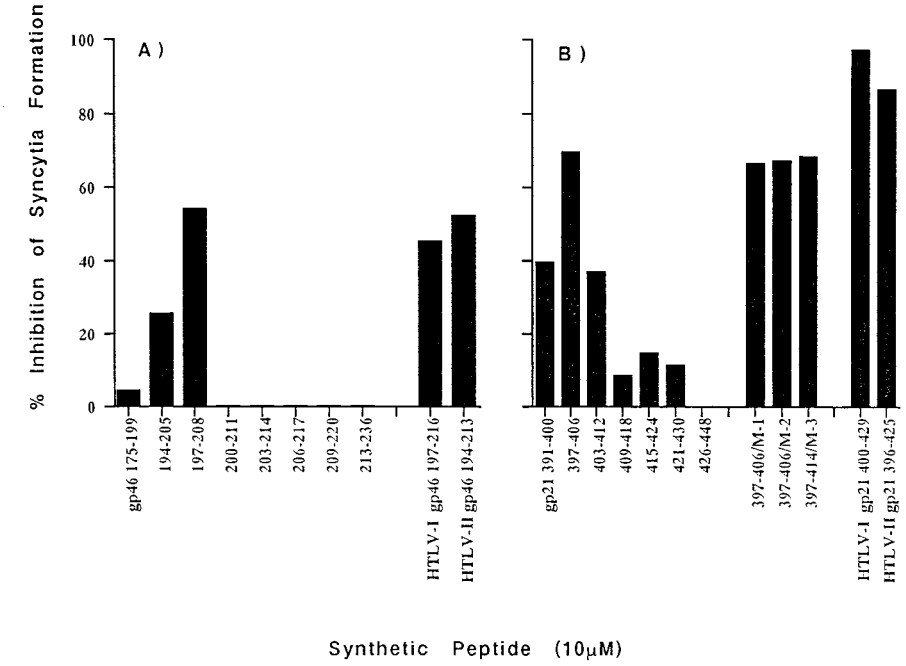

FIG. 5. Mapping of inhibitory domains on gp46 197–216 and gp21 400–429 for syncytium formation. The assay consisted of a coculture of 2.5×10^4 KT_{252} cells together with 10^5 MOLT-4 cells. In each case, 10 μM synthetic peptide was added to the triplicate wells and syncytia were scored after 18 h of coculture. Each value was estimated in comparison with the syncytium count in a coculture without synthetic peptide. (A) gp46 197–216. (B) gp21 400–429.

characteristics of a fusion peptide (41); the anchorage domain would correspond to a hydrophobic sequence (amino acids 446 to 465) located near the carboxyl terminus of gp21. Peptide gp21 400–429, which profoundly affects syncytium formation, corresponds to the site between the anchorage domain and the fusion domain of gp21. Syncytium formation induced by virus-bearing cells requires several steps, including binding to the virus receptor and folding of the envelope, leading to exposure of the fusion domain and membrane fusion. The inhibitory peptide region might directly affect binding or act on a subsequent step of the fusion process required for syncytium formation.

The peptide corresponding to amino acids 197 to 216 of gp46 clearly inhibited syncytium formation. The gp46 surface protein is believed to be responsible for the interaction of the envelope with an undefined target receptor. When HTLV-1 envelope protein is aligned with murine or feline retrovirus envelope protein, analogy to the attachment site for cell recognition is found in a region between amino acids 138 and 202 of the HTLV-1 envelope (7). The study with site-directed mutagenesis showed that the region corresponding to amino acids 195 and 205 on gp46 is important for envelope-dependent syncytium formation. Furthermore, linear peptide targets for neutralizing antibody have been found near amino acids 188 to 197 on gp46 (16, 39). Our results support the view that the central region of gp46 is important for envelope function. Taken together, these results imply that the region around amino acids 197 to 203 on gp46 might be the receptor-binding site.

In the present study, we observed a weak inhibitory activity on syncytium formation by the peptides corresponding to the region of amino acids 46 to 161 on gp46. Parker et al. (27) have shown that a peptide corresponding to amino acids 88 to 98 of gp46 was capable of neutralizing syncytium formation. Delamarre et al. (2) showed that the region between amino acids 75 and 101 of gp46 was important for syncytium formation. Our results support their findings and suggest that the region containing amino acids 46 to 161 on gp46 plays a certain role in envelope-dependent syncytium formation. However, it is unclear how each functional region on the envelope proteins, including the above two functional regions, is involved in the process which disturbs syncytium formation.

Delamarre et al. (2) previously showed that Asp-197 is an essential amino acid for syncytium formation and that a mutant with Asp changed to Glu had remarkably diminished envelope functionality for syncytium formation. We showed here that the HTLV-2 peptide, which Glu-197, has the same activity as HTLV-1 peptide gp46-197. This discrepancy might be due to the cell type dependency of the effect of this domain on envelope function, as observed in the intracytoplasmic domain in envelope intracellular maturation (29).

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