An antibody that blocks human T-cell leukemia virus type 1 six-helix-bundle formation in vitro identified by a novel assay for inhibitors of envelope function

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INTRODUCTION

Entry of retrovirus capsids into the host-cell cytoplasm requires fusion of the lipid membranes that surround both the virus and the target cell. Membrane fusion is catalysed by the viral envelope glycoproteins (Env), which are presented on the surface of the virus or infected cell as a trimer of surface glycoprotein (SU) subunits anchored to a trimer of transmembrane glycoproteins (TM). Binding of SU to a cell-surface receptor is thought to induce dramatic changes in Env conformation that convert Env from a metastable, non-fusogenic state to a fusion-active form (reviewed by Eckert & Kim, 2001; Sodroski, 1999). The receptor-stimulated changes in Env conformation promote insertion of the fusion peptide of TM into the target-cell membrane and, through a cascade of molecular rearrangements, promote membrane fusion (Eckert & Kim, 2001; Sodroski, 1999). For both human immunodeficiency virus (HIV) and human T-cell leukemia virus (HTLV), this model of virus entry is supported by the observations that mutations within Env (Delamarre et al., 1994, 1997, 1999; Pique et al., 1990, 1992, 1993; Poon & Chen, 1998; Rosenberg et al., 1997), anti-Env antibodies (Baba et al., 1993; Blanchard et al., 1999; Burton et al., 2004, 2005; Desgranges et al., 1994; Kuroki et al., 1992; Palker et al., 1992; Shida et al., 1987; Tanaka et al., 1991), recombinant competitive ligands (Deen et al., 1988; Fisher et al., 1988; Jassal et al., 2001), soluble polyanions (Moulard et al., 2000; Piñón et al., 2003b) and Env-derived synthetic peptides (Brighty & Jassal, 2001; Jiang et al., 1993; Jinno et al., 1999; Piñón et al., 2003a; Sagara et al., 1996; Wild et al., 1992) all interfere with envelope-mediated membrane fusion and potently block retrovirus infection of cells. The ability to interfere with envelope activity has generated considerable hope that small-molecule antagonists of envelope function or immunological agents targeting envelope can be developed as clinically relevant therapies to combat retrovirus infections.

Recently, the crystal structure for one conformation of the extracellular region of HTLV-1 TM has been solved and reveals that, in common with other viral fusion proteins (Baker et al., 1999; Bullough et al., 1994; Chan et al., 1997;
Fass et al., 1996; Kobe et al., 1999; Malashkevich et al., 1998, 1999; Rosenthal et al., 1998; Weissenhorn et al., 1998; Wilson et al., 1981), HTLV-1 TM adopts a trimer-of-hairpins motif (Fig. 1). For HTLV-1 TM (gp21), the N-terminal α-helices of three gp21 monomers assemble to form a central, triple-stranded coiled coil. At the base of the coiled coil, each monomer forms a 180° loop stabilized by formation of a disulphide bond, thereby reversing the polypeptide-chain direction; the C-terminal sequences adopt an extended structure, including a short helical region that lies in an antiparallel manner within the grooves formed by the core coiled coil to yield the characteristic six-helix-bundle or trimer-of-hairpins conformation of retroviral TM (Fig. 1) (Kobe et al., 1999).

Evidence suggests that the trimer of hairpins represents a post-fusion conformation of TM, and a model for membrane fusion proposes that insertion of the N-terminal fusion peptide into the target-cell membrane results in the formation of a transient pre-hairpin intermediate. In the pre-hairpin conformation, the C-terminal end of TM is anchored in the viral membrane, whilst the N-terminal

fusion peptide is embedded in the target-cell membrane. Ultimately, the pre-hairpin intermediate resolves to the trimer-of-hairpins structure, which draws the viral and cellular membranes together, facilitates their destabilization and induces membrane fusion (Eckert & Kim, 2001).

Previously, our group and others have characterized synthetic peptides that potently inhibit HTLV-1 Env-mediated membrane fusion and virus entry into cells (Brighty & Jassal, 2001; Jinno et al., 1999; Piñón et al., 2003a; Sagara et al., 1996). One of the inhibitory peptides, P-400, mimics the extended C-helical region (aa 400–429) of HTLV-1 TM. By using pull-down and electrophoretic mobility-shift assays, Brighty and colleagues (Piñón et al., 2003a) demonstrated that C-helix-mimetic peptides interact directly with the core coiled-coil domain of HTLV-1 TM, and that the inhibitory properties of the peptides are correlated with their ability to bind to the core coiled coil. Whilst it is clear that the inhibitory peptides interact directly with the core coiled-coil motif and that this interaction is crucial to the peptides’ inhibitory properties, it is currently unclear whether this is the only site of interaction with

![Fig. 1. TM structure and expressed TM-fusion proteins. (a) Structure of the trimer-of-hairpins motif of HTLV-1 TM. The central triple-stranded coiled coil is shown in space-filling form, with the extended peptide and C-helical region shown in green. (b) Model of the core coiled-coil structure of HTLV-1 TM used in this study represented in ribbon format fused to maltose-binding protein (MBP; white space-filling model). All structures were plotted by using MacPymol software. (c) Representation of the functional regions of HTLV-1 TM. The N-helical and C-helical regions are indicated as boxes; amino acid coordinates based on the envelope protein precursor are shown. Boxed regions below TM highlight the motifs that are fused to MBP. Black bars above TM highlight the regions that are mimicked by synthetic peptides.](http://vir.sgmjournals.org)
envelope. Nevertheless, the demonstration that P-400-related peptides inhibit membrane fusion by targeting the core coiled coil of TM raises the possibility that additional antagonists of membrane fusion targeting the core coiled coil can be identified.

To date, the assays available to monitor association of C-helix-mimetic peptides or the C-helical region of TM with the core coiled coil are laborious, qualitative rather than quantitative and not suited to the high-throughput methodologies required for screening panels of reagents for inhibitory activity. To overcome these obstacles, an in vitro assay has been developed to examine in a quantitative manner the association of C-helix peptides with recombinant HTLV-1 TM. The assay has been used to examine the specificity of binding of C-helix mimetics to derivatives of HTLV-1 TM and to investigate the inhibitory activity of a variety of TM-derived synthetic peptides. Finally, we have used the assay to identify a novel monoclonal antibody (mAb) that disrupts formation of the HTLV-1 six-helix bundle in vitro. The assay developed in this study is of utility in the biochemical analysis of peptide association with the core coiled coil and will facilitate rapid screening of reagents for the ability to disrupt formation of the six-helix bundle of HTLV-1 TM and, ultimately, to identify novel inhibitors of envelope-mediated membrane fusion.

**METHODS**

**Cells.** HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (DMEM/10% FBS).

**Plasmids.** The plasmids pHTE-1 (Dokhelar et al., 1989), pMAL-gp21hairpin and pMAL-gp21fishhook, and the control maltose-binding protein (MBP) vector pMAL-stop, have been described previously (Piñón et al., 2003a). The vectors pMAL-N-helix (MBP–N-helix), encoding aa M338–W387, and pMAL-C-helix, encoding aa P-41 to W387 of the TM region of HTLV-1 envelope were generated by PCR amplification of the respective coding regions using primers that incorporate a 5′ EcoRI and a 3′ HindIII site. The PCR products were digested and the fragments were cloned into the EcoRI and HindIII sites of pMalC2.

**Peptide synthesis.** Peptides (Table 1) were synthesized by using standard solid-phase Fmoc chemistry and, unless stated otherwise, have acetylated N termini and amidated C termini. The peptides were purified by reverse-phase HPLC and verified for purity by MALDI-TOF (matrix-assisted laser desorption/ionization-time-of-flight) mass spectrometry. All peptides were dissolved in DMSO, the concentration of peptide stock solutions was confirmed by measurement of A280 in 6 M guanidine hydrochloride and peptides were used at the final concentrations indicated.

**Expression and purification of MBP–TM proteins.** Escherichia coli JM109 cells, transformed with pMAL-stop or the MBP–TM expression plasmids (Piñón et al., 2003a), were grown at 37°C in the presence of ampicillin (100 μg ml−1) until the OD600 reached 0.6. Cells were induced with IPTG at a final concentration of 0.5 mM for 4 h at 37°C. Cells were harvested, resuspended in column buffer [20 mM Tris/HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA] supplemented with PMSF (1 mM) and aprotinin (1 μg ml−1) and lysed by sonication. Clarified cell lysates were diluted 1:5 in column buffer and loaded onto an amylose column pre-equilibrated with column buffer. The column was washed with 12 column volumes of column buffer and the MBP and MBP–TM fusion proteins were eluted with column buffer containing 10 mM maltose. The concentration of the fusion proteins was estimated by Bradford assay. The oligomerization states of our MBP–TM chimera were assessed by Superdex 200 gel-filtration chromatography in PBS (Piñón et al., 2003a; data not shown).

**ELISA.** Microtitre 96-well plates (MaxiSorb; Nunc) were coated overnight at 4°C with the chimeric MBP–TM fusion proteins (MBP–Fishhook, MBP–Hairpin, MBP–N-helix, MBP–C-helix or control MBP; all at 10 μg ml−1) in PBS (pH 7.2). Plates were blocked (5% Marvel in PBS/0.2% Tween 20) for 1 h at room temperature. After washing (five times), immobilized antigen was incubated with antibodies at the concentrations indicated for 2 h at room temperature. After washing (five times), horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:10 000 dilution) (Sigma) was added and the bound antibody was detected by using fresh ABTS substrate at 15 mg ml−1 [in 0.1 M citric acid, 60% H2O2 (30% w/v)]. After 10–20 min, colour development was stopped and A415 was read.

**Peptide-binding and competition-binding assays.** Microtitre 96-well plates (MaxiSorb; Nunc) were coated overnight at 4°C with MBP–Fishhook or the test MBP-fusion protein (10 μg ml−1) in PBS (pH 7.2). Plates were blocked (5% Marvel/PBS/0.2% Tween 20) for 1 h at room temperature. Washed (five times) and immobilized antigen was incubated with antibodies at the concentrations indicated for 2 h at room temperature. After washing (five times), horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:10 000 dilution) (Sigma) was added and the bound antibody was detected by using fresh ABTS substrate at 15 mg ml−1 (in 0.1 M citric acid, 60% H2O2 (30% w/v)). After 10-20 min, colour development was stopped and A415 was read.

**Table 1.** Amino acid sequences of synthetic peptides used in this study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid position</th>
<th>Sequence</th>
<th>Mass (Da)</th>
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<tbody>
<tr>
<td>P-400</td>
<td>gp21 400–429</td>
<td>COOH-CCFLNITNSHVLSQERPPLENVRTGLWGL-NH2</td>
<td>3411</td>
</tr>
<tr>
<td>Bio-P-400</td>
<td>gp21 400–429</td>
<td>Bio-CCFLNITNSHVLSQERPPLENVRTGLWGL-NH2</td>
<td>3750</td>
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<td>P-NS407YF</td>
<td>gp21 400–429</td>
<td>COOH-CCFLNITNSHVLSQERPPLENVRTGLWGL-NH2</td>
<td>3520</td>
</tr>
<tr>
<td>P-400</td>
<td>gp21 400–429</td>
<td>COOH-CRFPIITNSHVPSQERPPLENVRTGLWGL-NH2</td>
<td>3458</td>
</tr>
<tr>
<td>P-197</td>
<td>gp46 197-216</td>
<td>COOH-DHILEPSPWKSQILTVLQ-NH2</td>
<td>2331</td>
</tr>
<tr>
<td>Bio-P-197</td>
<td>gp46 197-216</td>
<td>Bio-DHILEPSPWKSQILTVLQ-NH2</td>
<td>2773</td>
</tr>
<tr>
<td>P-80</td>
<td>gp46 80–96</td>
<td>COOH-SLYLPFWHTKPPRRNGG-NH2</td>
<td>2118</td>
</tr>
<tr>
<td>P-338</td>
<td>gp21 338–368</td>
<td>COOH-MSLASGKSLHHKDKDQLTQAVKRNKHL-NH2</td>
<td>3459</td>
</tr>
<tr>
<td>P-360</td>
<td>gp21 360–389</td>
<td>COOH-AIFVKNHKLKIAQYAAQNRRCGDLFWEQ-NH2</td>
<td>3593</td>
</tr>
<tr>
<td>C-34</td>
<td>gp41 628–661</td>
<td>COOH-WMEWDREINNYSILHSLIESQNPQEEKNEQELL-NH2</td>
<td>4351</td>
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in the presence or absence of peptide competitors at the concentrations specified, at room temperature for 1 h. When examining the ability of mAbs to block binding of Bio-Pcr-400 to the coiled coil, the peptide was added at 2.5 μg ml⁻¹ in wash buffer without DTT. Subsequently, plates were washed (five times) to remove unbound peptide and incubated for 1 h with 100 μl streptavidin–HRP (Sigma) (1 : 10000 dilution) at room temperature. Plates were washed five times to remove unbound streptavidin–HRP and twice with PBS to remove residual detergent. Finally, bound streptavidin–HRP and therefore bound Bio-Pcr-400 was detected by using ABTS substrate and A415 was read (Bio-Rad plate reader).

**Syncytium-interference assay.** Syncytium-interference assays were performed as described previously (Jassal et al., 2001). Briefly, 3 × 10⁵ HeLa cells, transfected with the envelope expression vector pHTE-1, were added to 7.0 × 10⁶ untransfected HeLa target cells. The effector and target cells were co-cultured in the absence or presence of the P-400-related peptides at the concentrations specified. The cells were incubated for 12–15 h at 37 °C, washed twice with PBS and fixed in PBS/3 % formaldehyde. Assays were performed in triplicate and the number of syncytia from five low-power fields per replicate was scored by light microscopy.

## RESULTS

We have expressed fragments of the extracellular domain of HTLV-1 TM (Fig. 1) fused to MBP (Piñón et al., 2003a). One of these fusion proteins, MBP–Fishhook, faithfully mimics the central core coiled coil and disulphide-bonded loop of TM (Fig. 1b, c); the fusion protein forms stable trimers, it binds to a peptide inhibitor of envelope-mediated membrane fusion in *in vitro* pull-down assays and, in syncytium-interference assays, it competes with viral envelope as a target for the inhibitory peptide Pcr-400 (Piñón et al., 2003a). Having demonstrated that the core coiled-coil domain interacts directly with the inhibitory C-helix mimetic P⁴⁰⁰, we wished to examine this interaction in more detail with the specific objective of developing a facile assay that can be used to examine the binding reaction between the C-helix peptides and the core coiled coil and to identify inhibitors of the interaction between the core coiled coil and the C-helical region of HTLV-1 TM.

To examine the core coiled co-binding properties of P⁴⁰⁰, a derivative peptide was synthesized that is modified by addition of a biotin group fused to the terminal amine of the peptide by a six-carbon spacer arm. The biotin group allows rapid capture and detection of the peptide by using immobilized streptavidin or streptavidin–HRP conjugates, respectively. The spacer arm was included to avoid steric interference between the biotin group of the modified C-helix peptide and the peptide-binding site on the core coiled coil. As covalent modification of the peptide may affect its biological activity adversely, syncytium-interference assays were used to compare the inhibitory properties of the biotinylated peptide with those of the parental peptide, P⁴⁰⁰. Briefly, HeLa cells were transfected with the HTLV-1 Env expression vector pHTE-1 and the transfectants were used as effector cells in cell-fusion assays. Co-culture of untransfected HeLa cells with Env-expressing HeLa cells induced rampant syncytium formation in untreated cultures, but syncytium formation was inhibited dramatically in the presence of the peptide P⁴⁰⁰ (Fig. 2a). This inhibition of syncytium formation was specific to P⁴⁰⁰, as an irrelevant control peptide derived from the SU region of envelope had no inhibitory effect on cell fusion (Fig. 2a). Moreover, a derivative of P⁴⁰⁰ in which aa N407 and S408 were substituted with the bulky aromatic amino acids tyrosine and phenylalanine, respectively, also failed to inhibit syncytium formation. Importantly, the biotinylated peptide Bio-P⁴⁰⁰ blocked syncytium formation just as effectively as the parental peptide, demonstrating that the biotin spacer-arm modification had no adverse effect on the biological activity of the inhibitory peptide.

Next, the ability of Bio-P⁴⁰⁰ to bind to the recombinant core coiled-coil domain of HTLV-1 TM was examined by using a novel enzyme-linked binding assay. Recombinant core coiled coil fused to MBP (MBP–Fishhook) was captured onto 96-well ELISA plates; subsequently, the plates were blocked, washed and incubated with increasing concentrations of the indicated peptides. (a) Syncytium-interference assay using HeLa cells transfected with the envelope expression vector HTE-1 and co-cultured with untransfected HeLa target cells in the presence or absence of the indicated peptides. (b) Binding of Bio-P⁴⁰⁰ and the control peptide Bio-P-197 to immobilized core coiled coil was examined by an enzyme-linked binding assay.

![Fig. 2. An N-terminally biotinylated P⁴⁰⁰ peptide inhibits envelope-mediated syncytium formation and binds to a TM-derived recombinant core coiled-coil fusion protein.](http://vir.sgmjournals.org)
concentrations of Bio-Pcr-400 or a control peptide, Bio-P-197, derived from the SU region of envelope. Unbound peptide was washed away and bound peptides were detected. As predicted, Bio-Pcr-400 bound efficiently to the core coiled coil in a dose-dependent manner. In contrast, the biotinylated SU-derived control peptide, Bio-P-197, did not bind to the core coiled coil at any of the peptide concentrations tested (Fig. 2b), indicating that binding to the core coiled coil was a specific property of the C-helix-mimetic peptide.

To examine the target specificity of C-helix peptides and the conformational requirements of TM for peptide binding, the association of Bio-Pcr-400 to a panel of MBP–TM and Env-derived fusion proteins was explored. Published size-exclusion chromatography data and non-denaturing PAGE analysis indicate that two of these MBP-fusion proteins, MBP–Hairpin and MBP–Fishhook, are trimeric and faithfully recapitulate the trimer-of-hairpins structure and the core coiled coil, respectively, of HTLV-1 TM (Píñón et al., 2003a). In addition, two truncated TM derivatives that are incapable of forming trimers (Fig. 3a, b; our unpublished results), but represent the monomeric N-helix and the monomeric C-helix, were also tested for the ability to bind Bio-Pcr-400. In these assays, Bio-Pcr-400 bound specifically only to the trimeric recombinant core coiled coil (Fig. 3c). Bio-Pcr-400 did not bind to the control MBP-carrier protein and, most significantly, we observed little or no binding to the recombinant fusion protein that mimics the trimer-of-hairpins motif of HTLV-1 TM. In addition, Bio-Pcr-400 failed to bind to truncated TM derivatives that represent monomeric forms of the N-helical or C-helical regions of TM. Moreover, as inhibitory peptides may interact with more than one region of envelope (Liu et al., 2005), binding of the biotinylated peptide to intact SU (gp46–Fc; Jassal et al., 2001) was explored by pull-down and enzyme-linked binding assays; however, to date, no additional interactions have been detected (data not shown).

**Disruption of Bio-Pcr-400–core coiled coil interaction**

Having demonstrated that Bio-Pcr-400 binds specifically only to the core coiled coil of HTLV-1 TM, the ability of envelope-derived peptides to competitively inhibit Bio-Pcr-400 binding to the core coiled coil was tested. A constant amount (2.5 μg ml⁻¹) of Bio-Pcr-400 was incubated with the core coiled coil in the presence of increasing concentrations of peptide competitor, unbound peptide was washed away and the bound Bio-Pcr-400 was detected.

As expected, the unmodified C-helix mimetic Pcr-400 was found to be a highly effective competitive inhibitor of Bio-Pcr-400 binding to the core coiled coil (Fig. 4a). This competitive inhibition was specific, because a control peptide, C-34, which mimics the C-helical region of HIV-1 envelope, or P-80, an irrelevant peptide derived from the SU region of HTLV-1 Env, failed to inhibit binding of Bio-Pcr-400 (Fig. 4a). In contrast, a C-helix peptide based on the variant HTLV-1 strain ATK (Pcr-400), which is a relatively weak inhibitor of membrane fusion catalysed by envelope from the HTLV-1 strain CR, blocked binding of Bio-Pcr-400 to the core coil, but was not as effective as the homologous peptide inhibitor Pcr-400 (Fig. 4b). Moreover, the biologically inactive mutant peptide Pcr-NS407YF also failed to block binding of Bio-Pcr-400 to the core coiled coil (Fig. 4b). The ability of peptides derived from the N-helical regions to competitively block binding of Bio-Pcr-400 to the core coil was also examined. Synthetic peptides P-338 and P-360, which mimic overlapping elements of the N-helical region of HTLV-1 TM, also failed to inhibit binding of Bio-Pcr-400 to the core coiled coil, suggesting that the C-helical peptides...
have little or no affinity for truncated forms of the N-helical region of TM (Fig. 4b). These data also suggest that the N-helix-mimetic peptides are unlikely to intercalate into, or disrupt the conformation of, the preformed core coiled coil. In keeping with these data, the N-helical-mimetic peptides do not inhibit syncytium formation or membrane fusion catalysed by HTLV-1 envelope (data not shown).

Identification of antibodies that prevent assembly of the six-helix bundle

The demonstration that C-helix peptides and their derivatives can competitively inhibit binding of Bio-P\textsuperscript{P\textgamma}-400 to the core coiled coil in direct binding assays indicated that this assay may be of utility in identifying novel inhibitors of the core coiled coil–C-helix interaction. In particular, this assay may be of use in the search for antibodies targeting the fusion-active structures of HTLV TM; it is anticipated that such antibodies may exhibit neutralizing activity. To test this view, a panel of murine mAbs raised against HTLV-1 envelope were surveyed for the ability to block the interaction of Bio-P\textsuperscript{P\textgamma}-400 with the core coiled coil. Each of these mAbs is reactive with viral envelope expressed in HTLV-1-infected cells by one or more assays that include Western blotting, ELISA against partially purified envelope, and positive flow-cytometry signals against infected but not uninfected cells; however, the epitopes recognized by these mAbs have yet to be defined.

Seven mAbs reactive with HTLV-1 envelope protein and two control mAbs, one reactive with the V3 loop of HIV-1 envelope and the other reactive with the MBP-carrier protein, were examined for the ability to inhibit binding of Bio-P\textsuperscript{P\textgamma}-400 to the core coiled coil. The vast majority of these mAbs, even at high concentration (5 \textmu g ml\textsuperscript{-1}), were unable to block binding of the bioactive peptide to the recombinant core-coiled-coil region of HTLV-1 TM (Fig. 5). However, mAb 18-8 antagonized binding of Bio-P\textsuperscript{P\textgamma}-400 to the core coiled coil in a concentration-dependent manner (Fig. 5). Thus, in vitro, mAb 18-8 is able to interfere with formation of the six-helix-bundle structure of HTLV-1 TM.

Analysis of mAb reactivity

Several mechanisms for mAb-mediated inhibition of C-peptide binding to the core coiled coil can be proposed. The mAbs may interact directly with the groove on the surface of
the coiled coil, thereby blocking access of C-peptides to their target binding site. Alternatively, mAbs may interact directly with the C-helix-mimetic peptides, resulting in steric occlusion of the peptides from the channel formed on the core coiled coil. Finally, the mAbs may bind to the core of TM in a manner that disrupts the trimeric structure of the coiled coil, resulting in loss of the TM conformation required for C-peptide binding. As mAb 18-8 is a potent inhibitor of C-peptide binding to the core coiled coil, each of these possibilities was explored. The inhibition of Bio-P$^{CT}$-400 binding to the core coiled coil implies that mAb 18-8 recognizes an epitope within TM and that recognition of this epitope by the mAb is incompatible with C-peptide binding. The reactivity of mAb 18-8 with recombinant derivatives of HTLV-1 TM protein was therefore examined by ELISA.

Derivatives of TM fused to MBP were examined for reactivity with mAb 18-8 (Fig. 6). The mAb did not bind to the control fusion partner MBP, and little or no binding of mAb 18-8 was observed with the intact trimer-of-hairpins form of TM. Moreover, mAb 18-8 failed to bind to the N-helix derivative (MBP-N-helix) or the trimeric core coiled coil. These data indicate that mAb 18-8 does not recognize an epitope within the N-helical and disulphide-bonded loop regions of TM. Instead, strong reactivity of mAb 18-8 was observed with the MBP–C-helix fusion protein, indicating that the mAb probably recognizes an epitope contained within, or at least partially overlapping, the C-helical region of HTLV-1 TM. Moreover, recognition of this epitope appears to be conformation-dependent, as mAb 18-8 failed to bind to the trimer-of-hairpins form of recombinant TM.

Given that mAb 18-8 blocks binding of Bio-P$^{CT}$-400 to the core coiled coil and demonstrates reactivity with the C-helical region of TM, it was anticipated that mAb 18-8 would neutralize HTLV-1 entry by interfering with the conformational changes in envelope that are required for envelope-mediated membrane fusion. However, despite exhaustive analysis, no neutralizing activity could be detected for mAb 18-8 in either syncytium-interference assays or infection assays using HTLV envelope-pseudo-typed virus particles (data not shown).

**DISCUSSION**

The demonstration that a synthetic peptide that mimics the C-helical region of HTLV-1 TM is a potent inhibitor of envelope-mediated membrane fusion suggests that inhibition of virus entry is a viable objective of antiretroviral therapy for HTLV-1-associated disease. Recent evidence and the data presented here indicate that the major target for the inhibitory peptide is the core coiled-coil region of fusion-active TM. Importantly, the ability to modify the inhibitory peptide without loss of biological activity has provided an opportunity to examine the binding specificity of the inhibitory peptides in a more quantitative and systematic manner than was possible previously.

Association of P$^{CT}$-400 with the core coiled coil has been demonstrated in pull-down assays and electrophoretic mobility-shift assays (Piñón et al., 2003a), but the interaction of the inhibitory peptide with other regions of envelope had not been explored. Here, by using a quantitative binding assay, it is demonstrated that binding of the inhibitory peptide to HTLV-1 TM is highly selective, and efficient binding is observed only for the trimeric coiled-coil motif of TM. No binding is observed to the trimer-of-hairpins form of TM, suggesting that, once formed, the six-helix-bundle structure is extremely stable and that synthetic peptides are unable to displace the C-helical region of TM from the grooves in the core coiled coil. Therefore, the peptide inhibitors must bind to fusion-active envelope prior to formation of the six-helix-bundle structure. Importantly, amino acid side chains of each N-helix monomer make direct contact with the C-helical region of TM and, in the trimeric state, these contacts are critical for C-helix binding (Piñón et al., 2003a; Kobe et al., 1999); despite the importance of these side-chain interactions, monomeric forms of the N-helical region are unable to bind the inhibitory peptides. Thus, trimerization is a critical structural requirement for association of the inhibitory peptides with the fusion-active intermediates of HTLV-1 TM. From the data presented here, the coiled coil appears to be the sole target for binding of the C-helix mimetic P$^{CT}$-400, as binding to other regions of HTLV TM is not observed. In contrast, for HIV, the well-characterized peptide inhibitor T20 appears to interact with multiple regions of HIV envelope, including the core coiled coil, the membrane-proximal region of gp41 and a CD4-induced co-receptor-binding region on gp120 (Liu et al., 2005). It is worth noting that, for HTLV-1 and the closely related Bovine leukemia virus, accumulating evidence indicates that an interaction occurs between the chain-reversal region of TM and SU, and that this involves formation of a disulphide bond between a CX$_4$CC motif in TM and a reactive CXXC motif in SU (Johnston & Radke, 2000; Wallin et al., 2004). Although P$^{CT}$-400 encodes both Cys400 and Cys401, which are required

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**Fig. 6.** mAb 18-8 is reactive with the C-helical region of HTLV-1 envelope. The reactivity of mAb 18-8 with recombinant fragments of TM fused to MBP was examined by ELISA.
for TM–TM and TM–SU disulphide interactions, we have been unable to detect any interaction of P\textsuperscript{Ct}-400 with SU in our binding and pull-down assays. Thus, for the HTLV-inhibitory peptide P\textsuperscript{Ct}-400, the potential spectrum of binding sites appears to be restricted to the trimeric core coiled coil of the pre-hairpin intermediate of TM.

A notable aspect of the assay described here is that association of the biotinylated peptide with the core coiled coil to yield a six-helix bundle is exquisitely sensitive to competitive inhibition. Control or mutant peptides that fail to inhibit HTLV-1 syncytium formation also fail to compete with Bio-P\textsuperscript{Ct}-400 for association with the coiled coil. Conversely, and as expected, the untagged P-400 peptides based on sequences of HTLV-1 strains ATK and CR are efficient competitors of Bio-P\textsuperscript{Ct}-400 binding to the core coiled coil. Moreover, the homologous CR-derived peptide is the most active inhibitor of syncytium formation and is by far the most efficient competitive inhibitor in the plate-binding assays. Thus, the assay exhibits properties that make it ideal for high-throughput screening of reagents to identify inhibitors of the six-helix-bundle conformation of TM.

Interestingly, synthetic peptides that mimic fragments of the N-helical region of HTLV-1 TM failed to compete with the core coiled coil for binding to Bio-P\textsuperscript{Ct}-400. Moreover, none of the N-helix-mimetic peptides described so far inhibits envelope-mediated membrane fusion (this study; Sagara et al., 1996). In contrast, synthetic peptides that correspond to overlapping fragments of the HIV-1 N-helix are effective inhibitors of HIV-induced membrane fusion (Eckert & Kim, 2001; Wild et al., 1992). A distinguishing feature of the HIV-derived N-helical peptides is that truncated forms of the HIV-1 N-helical region have a tendency to form trimeric structures (Louis et al., 2003; Lu & Kim, 1997; Lu et al., 1995). From the accumulating data, it is tempting to speculate that the HTLV-1 N-helical peptides are unable to inhibit membrane fusion due to an inability to form stable trimeric coiled coils. In keeping with this view, the data presented here demonstrate that monomeric forms of the HTLV-1 N-helices fail to bind C-helical peptides in direct binding assays. It is not clear why truncated forms of HTLV-1 TM that encode the entire leucine/isoleucine heptad-repeat region, or fragments thereof, lack the propensity to form stable trimers, but this aspect of TM function is currently being investigated.

The inhibition of HTLV-1 Env-induced membrane fusion by C-helix peptides suggests that strategies designed to elicit neutralizing antibodies reactive with the core coiled coil or C-helix of TM would be of value in the development of an HTLV-1 vaccine. The development of a plate-based assay for association of C-helix peptides with the core coiled coil has permitted a survey of mAbs for the ability to prevent six-helix-bundle formation. This strategy successfully identified a mAb that, in a dose-dependent manner, prevents association of the C-helix peptide P\textsuperscript{Ct}-400 with the core coiled coil. Epitope mapping confirmed that the mAb recognizes a motif that is contained within, or overlaps with, the C-helical region of TM. Given the reactivity of mAb 18-8, it is likely that the antibody binds directly to the C-helical peptides and prevents their association with the coiled coil sterically. It is therefore surprising that, whilst the antibody is able to prevent six-helix-bundle formation in vitro, it is unable, even at high concentrations, to block envelope-mediated membrane fusion in syncytium-interference assays or virus entry in pseudotyping assays. Whilst several plausible explanations could account for the lack of neutralizing activity displayed by mAb 18-8, by far the simplest explanation is that the epitope recognized by this antibody is inaccessible during the fusion process.

Precedents for lack of neutralizing activity of antibodies targeting the fusion-dependent structures of retroviral TM have been documented. In the case of HIV, a mAb (NC-1) reactive with the gp41 six-helix-bundle structure failed to neutralize virus infection (de Rosny et al., 2004), whilst polyclonal antibodies directed to the trimeric N-helix peptides demonstrated inhibitory activity only under very selective conditions (Golding et al., 2002). Taken together, the published studies and the data presented here imply that it will be technically challenging to generate antibodies that possess the required target specificity and neutralizing activity against retrovirus TM proteins. Nevertheless, a notable success has been reported. By using phage-display technology, Miller et al. (2005) isolated a single-chain antibody, denoted D5, that recognizes trimeric N-helical peptides that mimic the HIV core coiled coil. When converted to a full IgG molecule, N-helix-binding activity was retained, D5 IgG blocked HIV envelope-mediated membrane fusion and the antibody neutralized a range of HIV-1 clinical isolates (Miller et al., 2005), confirming elegantly that neutralization of virus infection by antibodies targeted to the pre-hairpin intermediate of TM is achievable. It remains to be determined whether neutralizing antibodies reactive with the pre-hairpin intermediate of HTLV-1 TM can be identified, but the identification of an antibody that recognizes the C-helical region of TM and antagonizes six-helix-bundle formation in vitro indicates that an extensive search for such antibodies is warranted.

Retrovirus entry into cells is emerging as a validated target for antiretroviral therapy (Kilby & Eron, 2003; Kilby et al., 1998; Lalezari et al., 2003) and inhibition of HTLV-1 entry by neutralizing antibodies or small-molecule antagonists of envelope function appears to be a viable objective for therapeutic intervention in HTLV-1-associated disease. From this study, it is clear that antibodies targeting the relevant pre-hairpin structures of HTLV-1 TM can be identified, but it remains to be determined whether antibodies with the appropriate neutralizing activity can be generated. Nevertheless, the development of a quantitative assay for binding of C-helix peptides to the core coiled coil will facilitate the characterization of pre-hairpin reactive antibodies, permit further biochemical analysis of inhibitory peptides and, ultimately, be of utility in the identification of small-molecule antagonists of envelope function.
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REFERENCES


