Pharmaceutical Research, Vol. 20, No. 10, October 2003 (© 2003) *Research Paper*

Mechanism of Binding and Internalization of ICAM-1-Derived Cyclic Peptides by LFA-1 on the Surface of T Cells: A Potential Method for Targeted Drug Delivery

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Received February 20, 2003; accepted June 9, 2003

Purpose. Peptides derived from the Domain 1 of the adhesion molecule ICAM-1_{1-21} are being developed as targeting ligands for LFA-1 receptors expressed on activated T cells. This work aims to elucidate the binding and internalization of ICAM-1-derived cyclic peptides (cIBL, cIBC, and cIBR) to LFA-1.
Methods. Ninety-six-well plates coated with soluble LFA-1 (sLFA-1) were used to characterize the binding of FITC-labeled peptide. An anti-CD11a antibody to the I-domain of LFA-1 was used to inhibit the binding of these peptides, which was quantified using a fluorescence plate reader. An unrelated FITC-labeled cyclic peptide was used as a negative control, and PE-labeled anti-CD11a antibodies (PE-R3.2 and PE-R7.1) were used as positive controls. Peptide binding to cell surface LFA-1 was visualized using colocalization of FITC-cIBR peptide and PE-labeled anti-CD18 antibody (LFA-1 β-subunit) on SKW-3 T cells by fluorescent microscopy. Inhibition of ICAM-1 binding to LFA-1 by peptides was evaluated using a Biacore assay. Binding and internalization of FITC-labeled peptides were evaluated by flow cytometry and confocal microscopy at 4°C and 37°C.
Results. These FITC-labeled cyclic peptides bind to sLFA-1 and can be blocked by an anti-CD11a antibody to the I-domain, suggesting that their binding site is on the I-domain of LFA-1. The FITC-cIBR peptide was localized with an anti-CD18 antibody on the surface of T cells, indicating that the FITC-cIBR peptide binds to LFA-1 on the cell surface. Flow cytometry and confocal microscopy demonstrated that FITC-labeled peptides were internalized in a temperature-dependent manner. Biacore analysis demonstrated that these peptides did not inhibit sICAM-1 from binding to immobilized sLFA-1. However, the binding properties of the soluble forms of LFA-1 and ICAM-1 may not correlate to their interaction at the cell surface.
Conclusions. Cyclic ICAM-1–derived peptides (cIBL, cIBC, and cIBR) bind to the I-domain of LFA-1 and are internalized by LFA-1 receptors on the surface of T cells. Therefore, these peptides could be used to target and deliver drugs to the cytoplasmic domain of T cells.

KEY WORDS: ICAM-1; LFA-1; binding; internalization; T cells; targeted drug delivery.

INTRODUCTION

Autoimmune diseases involve the activation and proliferation of T cells that react to "self" antigens. Activated T cells secrete cytokines to recruit other cells to engage in the immune response. Activation of T cells requires two different signals. The first signal (signal-1) is mediated by the interaction between the T-cell receptor (TCR) on T cells and an MHC:peptide complex on the surface of antigen-presenting cells (APC) such as B cells or dendritic cells (1). The second or costimulatory signal (signal-2) is delivered by several different pairs of molecules at the interface between T cells and APC, including the interaction between leukocyte function-associated antigen-1 (LFA-1) on T cells and intercellular adhesion molecule-1 (ICAM-1) on the APC (1). At the interface of T cells and APC, these receptor–ligand pairs can form a supramolecular complex within a specialized cell–cell junction called the "immunologic synapse" (2,3). The formation of this synapse has been followed by observing the dynamic movements of fluorescently labeled MHC-II–peptide complex and ICAM-1 on artificial planar membranes after the addition of T cells (2). Initially, TCR/MHC-peptide complexes (signal-1) are visible as an outer ring region while ICAM-1/LFA-1 complexes (signal-2) form an inner region of the synapse. During T-cell activation, the TCR/MHC-peptide complexes (signal-1) move to the inner region to form the central zone supramolecular activation complex (cSMAC), and the ICAM-1/LFA-1 clusters form an outer ring called the peripheral zone supramolecular activation complex (pSMAC) (3,4). The formation of the "immunologic synapse" takes several minutes, and the duration of this engagement is key to the signaling process (2).

The type of costimulatory signal (signal-2) can influence the activation and commitment of T cells for Th1 or Th2 differentiation. Inhibition of the interaction between CD28 and B7 blocks IL-4 and IL-5 (Th2 cytokines) production, and blocking the interaction between ICAM-1 and LFA-1 increases Th2 cytokine production (5). Furthermore, costimulation by ICAM-1 strongly inhibits IL-10 production, which may favor the development of Th1 rather than Th2 cells (6). In addition, inhibition of ICAM-1/LFA-1 interaction has been shown to suppress T-cell activation to prevent allograft rejection (7,8) and insulin-dependent diabetes mellitus (IDDM) (9,10). Monoclonal antibodies (mAbs) to ICAM-1 and LFA-1 have been developed for treating autoimmune diseases such as rheumatoid arthritis (11,12) and psoriasis (13,14).

Previously, we have discovered that linear and cyclic peptides derived from the α- and β-subunits of LFA-1 can inhibit homotypic and heterotypic T-cell adhesion as well as mixed lymphocyte reaction (MLR) (15,16). The α-subunit peptides were derived from the I-domain (17,18) and the divalent cation-binding region of LFA-1 (18,19). A cyclic peptide (cLABL) from the I-domain was identified that bound to the D1 domain of ICAM-1 and ICAM-3 and was internalized by ICAM-1 into the cytoplasmic domain (17). Peptides derived from the divalent cation-binding region of the α-subunit were found to inhibit homotypic T-cell adhesion and could complex with calcium and magnesium ions as in the LFA-1 receptor (19). Finally, β_2-subunit-derived peptides can bind to ICAM-1 and inhibit homotypic T-cell adhesion and suppress MLR (15,16). Interestingly, a β-subunit peptide, LBE, was found to enhance the binding of cLABL peptide to ICAM-1 (20). This result suggested that the LBE peptide may change the conformation of ICAM-1 so that its affinity for cLABL peptide is higher.

We also discovered linear and cyclic peptides derived from ICAM-1 that can inhibit ICAM-1/LFA-1–mediated T-

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0724-8741/03/1000-1523/0 © 2003 Plenum Publishing Corporation

cell adhesion (15,16,18). Linear peptides ($ICAM_{1-21}$ and $ICAM_{25-50}$) derived from the D1-domain of ICAM-1 were found to inhibit homotypic T-cell adhesion and mixed lymphocyte reaction (MLR) (15). $ICAM_{1-21}$ peptide (called IB peptide) inhibits binding of anti-CD11a antibody to LFA-1, suggesting that this peptide can bind the α-subunit of LFA-1 (18). In contrast, anti-CD11a antibody binding was enhanced by $ICAM_{25-50}$ peptide (called IE peptide), suggesting that this peptide binds to a different site on LFA-1, which may induce a conformational change within LFA-1 to a more favorable one for antibody binding (18). The $ICAM_{1-21}$ was reduced to three overlapping sequence cyclic peptides: cIBL ($ICAM_{1-10}$), cIBC ($ICAM_{6-15}$), and cIBR ($ICAM_{12-21}$). The cyclic peptides were also able to inhibit homotypic and heterotypic T-cell adhesion, presumably by binding to the α-subunit of LFA-1 (16). However, the detailed mechanisms of binding of these peptides to LFA-1 on the surface of T cells are not well characterized.

In this report, we have studied the mechanism of binding of ICAM-1_{1-21} peptide and cyclic peptides cIBL, cIBC, and cIBR Table 1 to LFA-1. Our hypothesis is that the activity of these peptides derives from their ability to mimic the ICAM-1 interaction with LFA-1. The site of ICAM-1 binding to LFA-1 is the I-domain of the α-subunit (21); this suggests that the binding site of these peptides may also be within the I-domain. Furthermore, we hypothesize that the activity of ICAM-1 peptides in inhibiting T-cell adhesion is related to their ability to induce LFA-1 receptor internalization (22). The internalization of an ICAM-1 peptide ligand by LFA-1 presents an attractive and novel drug delivery system to target reactive T cells involved in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. If a therapeutic drug is conjugated to this small peptide, the drug would be targeted into the cells that express LFA-1 receptor, reducing indiscriminant activity of the drug and its potential side effects.

METHODS AND MATERIALS

Materials

Phorbol ester (phorbol 12-myristate-13-acetate, PMA) was used to activate the cells and was purchased from Sigma (St. Louis, MO). All peptides were purchased from Multiple Peptide System (San Diego, CA). Membrane probe DiD was purchased from Molecular Probes (Eugene, OR). Soluble ICAM-1 (sICAM-1), full-length soluble LFA-1 (sLFA-1) (23), and monoclonal antibodies (R3.1, R7.1, R15.7, and TS2/4) were from Boehringer-Ingelheim (Ridgefield, CT).

Cell Culture

MOLT-3 cells, a leukemia-derived human T-cell line, were purchased from ATCC (Rockville, MD), and SKW-3 cells were obtained from Boehringer-Ingelheim. The cells were propagated in RPMI-1640 medium containing 10% v/v fetal bovine serum and penicillin/streptomycin (100 mg/L medium) and incubated at 37°C with 95% humidity and 5% CO_2. As necessary, cells were activated with 10% v/v PMA-containing medium. The final PMA concentration was 2 μM, and optimum activation was achieved after 16 h of incubation.

FITC-Labeled Peptides

FITC-labeled peptides were synthesized using procedures previously developed in our laboratory (22). The labeled peptides were purified by semipreparative HPLC. The pure products were identified by analytic HPLC and FAB-MS.

For FITC-peptide–binding experiments, 1% (w/v) BSA was used to block the nonspecific binding. The effect of BSA on the fluorescence intensity of FITC-labeled peptides was evaluated. FITC-peptides were dissolved in PBS, and their fluorescence was measured as a function of increasing concentrations of BSA added. The change in fluorescence intensity leveled at approximately 1% BSA/PBS.

ELISA Assays to Determine ICAM-1 Peptide/LFA-1 Binding

The binding of ICAM-1 cyclic peptides (cIBL, cIBC, and cIBR) to sLFA-1 was measured using three different ELISA assays: (a) direct binding of FITC-labeled peptide to sLFA-1-coated wells, (b) inhibition of FITC-labeled peptide binding to LFA-1 by its corresponding unlabeled peptide, and (c) inhibition of FITC-labeled peptide binding to LFA-1 by anti–LFA-1 antibody. For each experiment, the ELISA plates were prepared using the following method. Diluting buffer (DB), which consisted of Dulbecco's PBS with calcium and magnesium, 2.0 mM $MgCl_2$, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), was first used to dissolve both peptides and sLFA-1. Plates were coated with 50 μL of sLFA-1 at a concentration of 10–20 μg/mL on 96-well MaxiSorp ImmunoPlates from NUNC (Roskilde, Denmark) and incubated

Table I. The Sequences of ICAM-1 Peptides That Are Used in This Study

Peptide name	Sequence number	Amino acid sequence	MW (g/mol)
IB	ICAM-$1_{1\text{-}21}$	QTSVSPSKVILPRGGSVLVTG	2082.2
cIBL*	ICAM-$1_{1\text{-}10}$	Cyclo(1,12)-<u>Pen</u>-QTSVSPSKVI<u>C</u>-OH	1667.6
cIBC*	ICAM-$1_{6\text{-}15}$	Cyclo(1,12)-<u>Pen</u>-PSKVILPRGG<u>C</u>-OH	1227.6
cIBR*	ICAM-$1_{12\text{-}21}$	Cyclo(1,12)-<u>Pen</u>-PRGGSVLVTG<u>C</u>-OH	1174.5
cLABL*	LFA-$1_{237\text{-}247}$	Cyclo(1,12)-<u>Pen</u>-ITDGEATDSG<u>C</u>-OH	1197.5
FITC-cIBL†	ICAM-$1_{1\text{-}10}$	FITC-Cyclo(1,12)-<u>Pen</u>-QTSVSPSKVI<u>C</u>-OH	2057.5
FITC-cIBC†	ICAM-$1_{6\text{-}15}$	FITC-Cyclo(1,12)-<u>Pen</u>-PSKVILPRGG<u>C</u>-OH	1729.0
FITC-cIBR†	ICAM-$1_{12\text{-}21}$	FITC-Cyclo(1,12)-<u>Pen</u>-PRGGSVLVTG<u>C</u>-OH	1563.4
FITC-cLABL†	LFA-$1_{237\text{-}247}$	FITC-Cyclo(1,12)-<u>Pen</u>-ITDGEATDSG<u>C</u>-OH	1587.4

* The formation of cyclic peptide was done by forming a disulfide bond between the penicillamine-1 (Pen^1) and cysteine-12 (Cys^{12}) residues. Residues 2 through 11 were derived from the ICAM-1 sequence.

† The FITC-label was conjugated to the N-terminal of the peptide.

for 30 min at room temperature (RT). The plates were washed twice with 200 μL of DB supplemented with 2% BSA and then incubated with 2% BSA/DB for 30 min at 37°C.

For direct binding studies, 50 μL of FITC-labeled peptides at 40 μM or 50 μL of anti-CD11a mAbs (R3.1 and R7.1) at 5 μg/mL were added to the plate and incubated for 1 h at 37°C. The plates were then washed twice with 200 μL of DB. To detect CD11a mAb binding, 50 μL of PE-conjugated goat antimouse secondary antibody was added at a 1:1000 dilution and incubated for 30 min at 37°C. These wells were then washed twice with 200 μL of DB. The fluorescence of bound peptide and antibody was measured using a PerSeptive Biosystems CytoFluor (Series 4000) fluorescence plate reader.

The second ELISA was performed to determine the effect, if any, of the FITC moiety and the specificity of peptide binding to LFA-1 by inhibiting their binding using the respective unlabeled peptide. In this experiment, plates were coated with sLFA-1 and blocked with 2% BSA, as above; 50 μL of unlabeled peptide (cIBL, cIBC, or cIBR) at different concentrations (133 μM, 400 μM, and 533 μM) was added to LFA-1 coated wells and incubated for 30 min at 37°C. The plate was then washed twice with DB. The corresponding FITC-labeled peptide was added to these wells, followed by a 30-min incubation at 37°C. The plate was washed with DB, and the fluorescence was measured.

A third ELISA was performed to determine the binding site of peptides cIBL, cIBC, and cIBR on LFA-1. In this experiment, LFA-1–coated plates were prepared as previously described (see above). Next, 50 μL of an anti-CD11a antibody (R3.1 at 10 μg/mL) that binds to the I-domain of LFA-1 was added to sLFA-1–coated wells. Incubation for 30 min at 37°C was followed by washing with 200 μL of DB. FITC-labeled peptides (50 μL) at a concentration of 40 μM were then added to the plate and allowed to incubate for 30 min at 37°C. The plate was washed with DB, and the fluorescence intensity measured using the ELISA plate reader. FITC-labeled cLABL peptide that does not bind to LFA-1 was used as a negative control (24). As a reference and positive control, the results from anti-CD11a mAb (R3.1) and FITC-peptide direct binding ELISA assay were plotted next to results from this ELISA to demonstrate relative reduction in binding to LFA-1.

Confocal Microscopy of FTIC-cIBR Colocalized with Anti–LFA-1 (CD18) Antibody

SKW-3 T cells (150 μL at 1 × 10^6 cells/mL) were treated with 50 μL of FITC-cIBR (200 μM) for 1 h at 4°C and then spun and washed in the following manner to remove unbound peptide. T cells were centrifuged for 3 min at 1800 rpm, and the supernatant was decanted. Cells were resuspended in 500 μL of PBS, and they were centrifuged followed by removal of the supernatant. The cells were then resuspended in 150 μL of PBS/1% BSA followed by addition of 50 μL of R15.7 (10 μg/L), an anti-LFA-1 antibody to the β-chain of LFA-1. The cells were incubated with the antibody for 45 min at 4°C, and then washed as before. Anti–LFA-1 antibody was detected with R-phycoerythrin (PE)-labeled goat antimouse antibodies in the following manner. PE-labeled secondary goat antimouse mAb (50 μL) was added (at a stock dilution of 1:50 in PBS/1% BSA/0.1% sodium azide) and allowed to incubate for 30 min at 4°C. The cells were washed as described previously, resuspended in 2% paraformaldhyde in PBS, and kept at 4°C until mounted. The cells were mounted onto microscope slides by spinning at 400 rpm for 4 min in a Shandon Cytospin 3; a coverslip was then applied to protect the cells. A Zeiss LSM510 confocal microscope with an argon ion laser and 2 HeNe lasers was used to visualize cells, and the PE and FITC fluorophores were excited at λ = 488 nm and read at λ = 505–530 nm using a BP filter.

Confocal Microscopy of FTIC-peptide Internalization

SKW-3 cells (150 μL at 1 × 10^6 cells/mL) were treated with 50 μL of FITC-cIBR (200 μM) for 1.5 h at either 37°C or 4°C. Cells were then washed as previously described and resuspended in 2% paraformaldehyde in PBS. Membrane probe DiD from Molecular Probes (Eugene, OR) was then added to a final concentration of 10 μM to label the cell membranes, and cells were kept at 4°C until mounted on slides. FITC-cIBR was excited as described previously; however, the DiD membrane probe was excited at λ = 644 nm with emission at 665 nm. Software used for multitrack two-color specimens was provided by Zeiss LSM V2.8. Z-sections through cell clusters were made at 0.4-μM intervals.

BIACORE and TIRF Assays

All surface plasmon resonance (SPR) experiments were conducted on a Biacore-1000 instrument from Biacore (Piscataway, NJ). The LFA-1/ICAM-1 interaction was evaluated by immobilizing soluble LFA-1 with TS2/4 mAb. The TS2/4 mAb was immobilized to the chip by the amine-coupling procedure. Briefly, a carboxymethylcellulose surface (MC5 chip purchased from Biacore) was activated with EDC/NHS for 7 min according to the manufacturer's protocol. TS2/4 mAb at 50 μg/mL in 10 mM sodium acetate at pH 4.5 was then injected over the activated surface for 8 min at a rate of 5 μL/min. The surface was blocked with 1.0 M ethanolamine. This protocol resulted in approximately 3000 to 4000 resonance units (RU) of immobilized mAb. sLFA-1 at 50 μg/mL in 10 mM Tris and 1.0 to 2.0 mM $MgCl_2$ at pH 7.5 was adsorbed on to the TS2/4 mAb surface at a flow rate of 2.0 μL/min until saturation was achieved (~1000 RU).

The inhibition experiments were conducted in buffer consisting of the following reagents: 10 mM Tris, 150 mM NaCl, and 1.0 to 2.0 mM $MgCl_2$ at pH 7.5. Peptides were dissolved in running buffer at concentrations of 100 μM and 1.0 mM. As a positive control, 15 μL of sICAM-1 (1.0 μM) was injected at 15 μL/min. For inhibition experiments, 15 μL of peptide (100 μM and 1.0 mM) was injected simultaneously with sICAM-1 (1.0 μM) at a rate of 15 μL/min over the surface of LFA-1. Evidence of LFA-1/ICAM-1 inhibition is derived from the differences between the dissociation slopes (k_d) of the sensograms after the injection of sICAM-1 with peptide compared to sICAM-1 alone. An increase in the dissociation slope of peptide/sICAM-1 compared to sICAM-1 alone would be evidence that LFA-1/ICAM-1 binding is blocked. Sensograms were collected and evaluated using the BIAevaluation software from Biacore (Piscataway, NJ).

RESULTS

Binding of ICAM-1 Cyclic Peptides (cIBL, cIBC, and cIBR) to LFA-1

A series of ELISA assays was used to investigate the binding of ICAM-1–derived peptides to sLFA-1. Direct bind-

ing of FITC-labeled peptides (FITC-cIBL, FITC-cIBC, and FITC-cIBR) to ELISA plates coated with sLFA-1 is shown in Fig 1A (open bars). The binding affinity of FITC-labeled peptides to sLFA-1 is in the following order: FITC-cIBR > FITC-cIBC > FITC-cIBL. FITC-labeled cLABL was used as a negative control. This peptide, which was derived from the I-domain of LFA-1, binds to domain-1 (D1) of ICAM-1. This negative control peptide has a very weak binding to the LFA-1-coated plate, suggesting nonspecific interaction with the LFA-1 receptor. As a positive control, anti–LFA-1 mAbs R3.1 and R7.1 (10 μg/mL) were used (Fig. 1A, gray bars). Peptide cIBC, which has an overlapping sequence with cIBR, has slightly less affinity for LFA-1 than cIBR. cIBL has no sequence overlap with cIBR and shows the weakest affinity for LFA-1. These results suggest that the overlapping sequence between cIBR and cIBC contributes to the binding of these peptides.

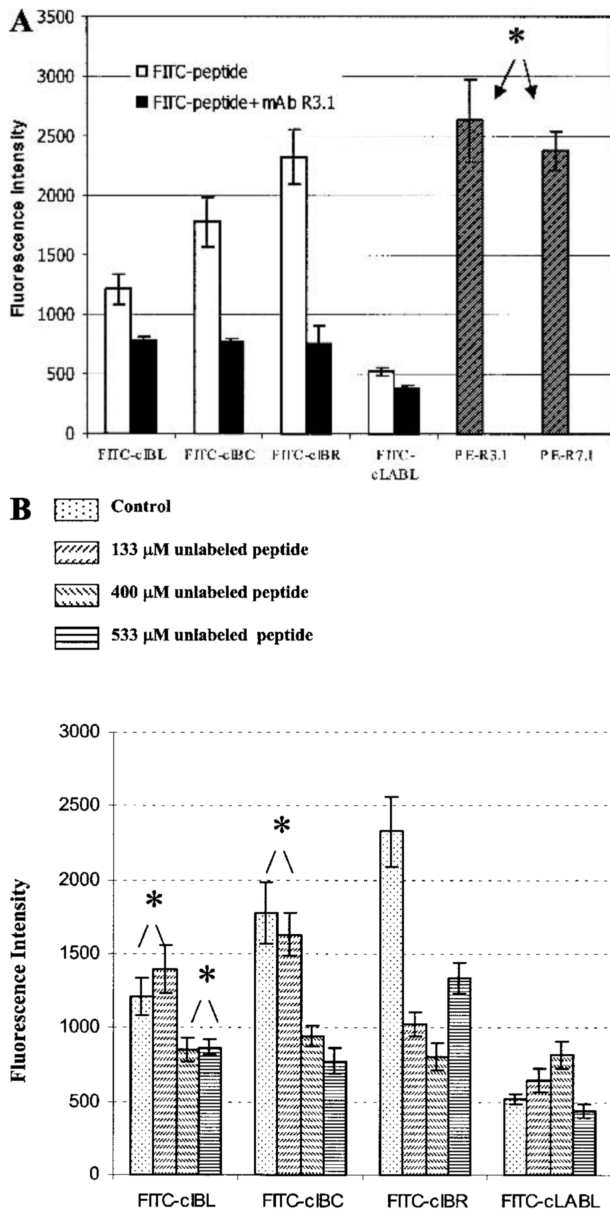

Fig. 1. (A) Binding properties of FITC-labeled cyclic peptides (FITC-cIBL, FITC-cIBC, and FITC-cIBR) to sLFA-1–coated wells relative to FITC-cLABL (open bars). Anti–LFA-1 antibodies (R3.1 and R7.1; hashed bars) were used as positive controls. Inhibition of FITC-labeled cyclic peptides binding to sLFA-1 by anti–LFA-1 antibodies (R3.1; filled bars). (B) Binding specificity of FITC-labeled cyclic peptides to sLFA-1 demonstrated by their inhibition using increasing concentrations of the respective unlabeled peptides (cIBL, cIBC, and cIBR). *Denotes these values are not significantly different from each other.

The binding site of ICAM-1 peptides on sLFA-1 was evaluated by blocking FITC-labeled peptide binding with an anti-CD11a mAb (R3.1), which binds to the I-domain of LFA-1. Figure 1A (black bars) shows that R3.1 mAb can block the binding of FITC-cIBL, FITC-cIBC, and FITC-cIBR to LFA-1. As a negative control, the antibody did not block the nonspecific binding of FITC-cLABL. The binding of anti–LFA-1 antibody (R3.1) to sLFA-1 is shown as a reference to demonstrate the affinity of the antibody for sLFA-1. These results suggest that all three ICAM-1 peptides bind to the I-domain of LFA-1, but cIBR peptide demonstrates more binding site overlap with the R3.1 antibody than do the cIBC or cIBL peptides.

To evaluate the binding specificity of the FITC-labeled peptide for sLFA-1, the respective unlabeled peptide was used to block binding of FITC-labeled peptide to LFA-1 (Fig. 1B). Different concentrations (133, 400, and 533 μM) of unlabeled peptides were incubated in sLFA-1–coated wells, and the plates were washed to remove unbound peptides. Then, the corresponding FITC-labeled peptide was added to the well. Figure 1B shows that the unlabeled ICAM-1 peptide can block binding of the FITC-labeled peptide to LFA-1 in a dose-dependent manner. However, the decrease in fluorescence does not correspond linearly to the concentration of unlabeled peptide added. This may suggest that secondary peptide–peptide interaction may occur at higher doses affecting the binding of the FITC-labeled peptide to LFA-1. As a negative control, the unlabeled cLABL peptide did not block the binding of FITC-cLABL, suggesting that its binding was nonspecific. Taken together, these results suggest that FITC-labeled peptides bind to the same site as the unlabeled peptide and that the FITC group did not interfere with peptide binding.

Peptide Binding and Internalization Properties on T cells

Although we have demonstrated that these peptides can bind sLFA-1 and inhibit binding of mAb to the I-domain of LFA-1, their ability to bind LFA-1 on the surface of T cells was still in question. Therefore, colocalization studies can demonstrate the direct binding between the peptide and LFA-1 on the cell surface. FITC-cIBR peptide was colocalized with anti-CD18 mAb (R15.7; anti–β-subunit) of LFA-1 on SKW-3 cells. Previously, the cIBR peptide had been shown not to inhibit anti-CD18 mAb binding to LFA-1, suggesting that this peptide did not bind to the β-subunit. The peptide–antibody colocalization was followed by confocal microscopy. Fig 2 shows that FITC-cIBR (green) and R15.7 mAb (red) are bound in close proximity at the cell surface in clusters that result in the apparent yellow color at the cell edge because of the overlap of both red and green fluorescence. An especially bright yellow spot can be seen at the contact point between T cells, where a cluster of LFA-1 receptors interacts with the peptide. Furthermore, because this image was taken at some depth within the cell, small spots of

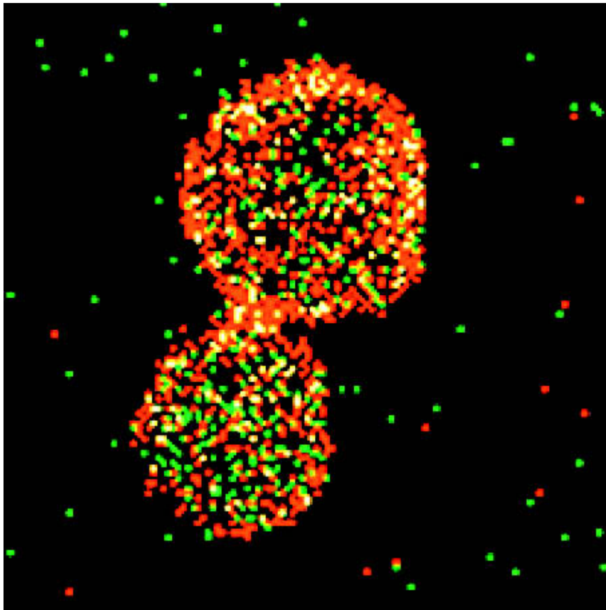

Fig. 2. Confocal microscopy image of the colocalization of FITC-cIBR (green) with an anti-CD18 mAb (R15.7; red) on LFA-1 on the surface of SKW-3 T cells. The colocalization of FITC-cIBR and anti-CD18 on LFA-1 receptors gives yellow image.

green fluorescence can be identified throughout the T-cell cytoplasm; these were later identified as internalized FITC-cIBR by scanning through the entire cell cytoplasm in 0.4-μm increments using confocal microscopy.

To evaluate the potential internalization of the peptides by LFA-1 receptors, temperature-dependence binding studies were performed using FITC-cIBR, FITC-cIBC, and FITC-cIBL and were evaluated by flow cytometry Fig. 3. The binding of FTIC-labeled peptides to activated T cells depends on the incubation time. The binding of FITC-cIBR plateaus at 60 min (Fig. 3C), and FITC-cIBL at 120 min (Fig. 3A). In contrast, FITC-cIBC binding does not appear to reach a plateau after 240 min of incubation time (Fig. 3B). On the basis of the maximum fluorescence, the binding affinity of FITC-labeled peptides to LFA-1 on T cells at 37°C is in the following order: FITC-cIBR > FITC-cIBC > FITC-cIBL. This order is similar to that observed for the peptide binding to sLFA-1 using ELISA. T cells treated with FITC-labeled peptides at 37°C have fluorescence intensities five- to eightfold greater than those treated at 4°C (Fig. 3). The results suggest that the fluorescence intensity at 37°C was caused by the surface binding and internalization of the FITC-labeled peptide. On the other hand, the fluorescence intensity at 4°C was caused only by surface binding because, at this temperature, the receptor-mediated, energy-dependent process of internalization is restricted.

To test the hypothesis that the FITC-labeled peptide can be internalized by LFA-1, confocal microscopy was used to evaluate the effect of temperature on FITC-cIBR peptide binding and internalization. In this study, SKW-3 T cells were incubated with FTIC-cIBR peptide at either 37°C or 4°C (Fig. 4). The cell membranes were stained with DiD (red color), which quenched the fluorescence of membrane-bound FITC-cIBR. A series of confocal microscope images of 0.4 μM Z-sections through a cluster of SKW-3 T cells were recorded at

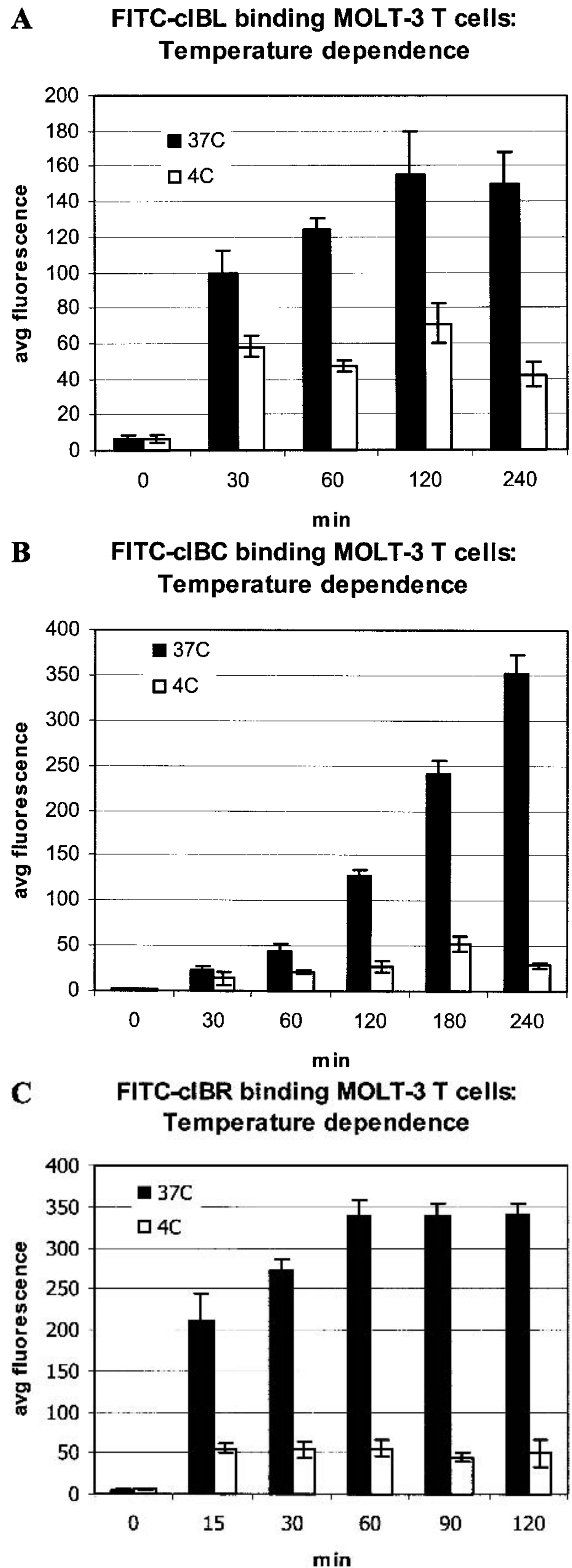

Fig. 3. Binding of FITC-labeled cyclic peptides to T cells at 37°C (open bars) and 4°C (filled bars) as a function of time; the binding property was determined by flow cytometry: (A) FITC-cIBL; (B) FITC-cIBC; (C) FITC-cIBR.

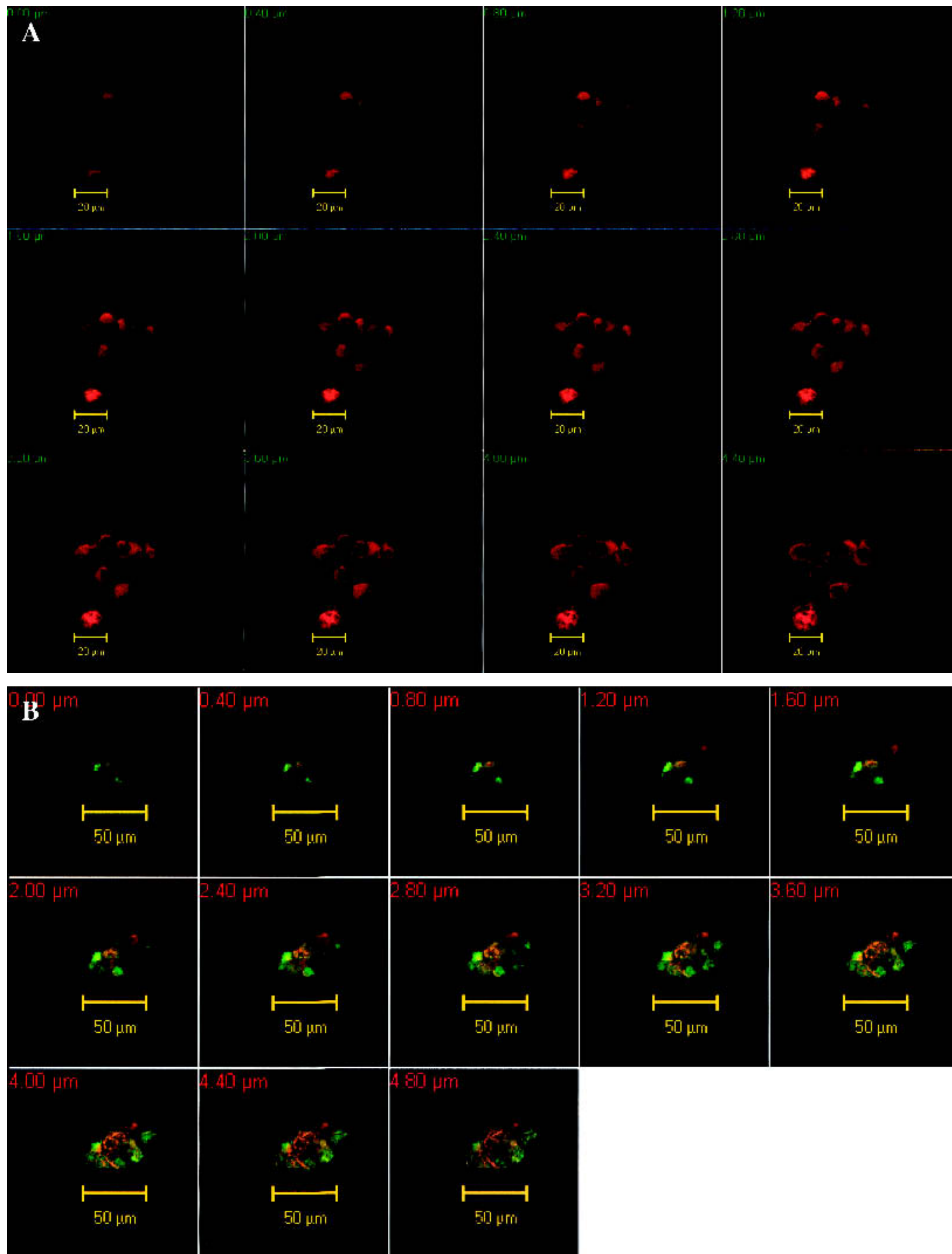

Fig. 4. Confocal microscopy images of SKW-3 T cells after incubation of FITC-cIBR and membrane probe DiD at 37°C (A) and 4°C (B). In both A and B, each frame represents an image taken at a depth 0.4 µm greater than the previous one within a cluster of SKW-3 T cells.

both 37°C and 4°C (Fig. 4). The results show that FITC-cIBR (green fluorescence) could be identified throughout the cytoplasm of cells that were treated at 37°C (Fig. 4B). In contrast, there was no green fluorescence found inside the cells that were treated at 4°C (Fig. 4A). These results strongly indicate that FITC-cIBR peptide binds to and is internalized by the LFA-1 receptors on T cells at 37°C.

Biacore and TIRF Assays

The ability of ICAM-1 peptides to inhibit binding of soluble ICAM-1 (sICAM-1) to sLFA-1 immobilized onto SPR chips was evaluated by both Biacore and TIRF assays. As a standard and control, sICAM-1 was first injected onto the chip for a period of time to reach equilibrium binding with

sLFA-1. After injection of sICAM-1, the Biacore or TIRF signal decreases, indicating the dissociation of LFA-1–bound sICAM-1 from the chip surface. The off rate (slope) of bound ICAM-1 was used as a reference to determine whether ICAM-1–derived peptides were able to inhibit sICAM-1 binding. We expected to observe a faster off rate for sICAM-1 than for controls if the ICAM-1–derived peptides bound to the sLFA-1 chip and blocked sICAM-1 from binding. However, we found that these peptides did not alter the off rate of sICAM-1 in either the Biacore or the TIFR assay. These results suggest that the peptide cannot inhibit the binding of sICAM-1 to immobilized LFA-1; however, several factors may influence their activity in these assays. First, because purified LFA-1 constitutively exists in its high-affinity form, this may affect the ability of these peptides to bind. Also, because sLFA-1 is immobilized to the chip via the TS2/4 anti-LFA-1 antibody, this may limit or restrain conformational changes of LFA-1 that may occur on peptide binding. Second, it is unclear whether the high-avidity form of LFA-1, characterized by receptor clustering on the cell surface, is represented on the Biacore chip surface. In this *in vitro* environment, sICAM-1 may therefore bind to sLFA-1 in a slightly different manner than it would on the cell surface. Therefore, the inability of these peptides to block sLFA-1/sICAM-1 interaction may not be representative of their activity at the cellular level to inhibit LFA-1/ICAM-1 interaction.

DISCUSSION

This study was designed to characterize the binding of cyclic ICAM-1 peptides (cIBL, cIBC, and cIBR) and LFA-1 on the surface of T cells (see Table I). These cyclic peptides have overlapping sequences that are derived from ICAM-1_{1-21} peptide (IB peptide). Cyclization of cIBL, cIBC, and cIBR peptides was accomplished by the formation of a disulfide bond from the Pen^1 to the Cys^{12} residue. Peptide cyclization stabilizes its conformation to a form that may enhance the specificity of the peptide for binding to LFA-1. These cyclic peptides (cIBL, cIBC, and cIBR) were found to inhibit homotypic T-cell adhesion as well as heterotypic T-cell adhesion to Caco-2 cell monolayers better than the parent IB peptide. However, previous studies presented no direct evidence to show that these cyclic peptides bind LFA-1.

In the work presented here, the binding of cyclic peptides (cIBL, cIBC, and cIBR) to the LFA-1 receptor was confirmed using (a) direct binding of FITC-labeled peptides to sLFA-1 receptors, (b) inhibition of FITC-labeled peptide-binding to LFA-1 by an anti-CD11a mAb, (c) colocalization of FITC-cIBR peptide and anti-CD18 mAb to LFA-1 on T cells, and (d) temperature-dependent binding of FITC-labeled peptide to T cells. The order of binding affinity of the cyclic peptides to LFA-1 is as follows: FITC-cIBR > FITC-cIBC > FITC-cIBL (Fig. 1A, open bars). The binding of these peptides to LFA-1 can be inhibited by the binding of anti-CD11a mAb (R3.1) to the I-domain, suggesting that these peptides bind to the I-domain of LFA-1 (Fig. 1A, filled bars). Fluorescent microscopy images demonstrated that FITC-cIBR peptide (green) colocalized with a PE–anti-CD18 mAb (red) to give a yellow image on the surface of SKW-3 T cells (Fig. 2). These results suggest that the FITC-cIBR peptide binds to the α-subunit of LFA-1 at the I-domain.

The cyclic peptides (cIBL, cIBC, and cIBR) were derived from the A, A′, and B strands of the ABED face of the D1 of ICAM-1 Fig. 5 (25,26). Previously, mutation studies suggested that ICAM-1 binds to the I-domain of LFA-1 via its GFC face through the Glu^{34} residue. Furthermore, it has also been suggested that ICAM-1 binding to LFA-1 utilizes nonlinear epitopes (27). However, others have shown that ICAM-3 utilizes both the ABED and the GFC face to bind LFA-1 (28). Our results support this type of binding scenario because these peptides, whose sequences are found on this ABED face, also bound to the I-domain. Although ICAM-1 and ICAM-3 have distinct binding sites, there is much similarity between the binding sites of these two ligands to LFA-1. Furthermore, LFA-1 interaction with ICAM-3 is essential for LFA-1/ICAM-1–dependent adhesion and signal transduction during the initiation of an immune response (29). Therefore, the unique interaction of ICAM-3 with LFA-1 through the ABED and GFC faces may induce receptor signaling different from that by ICAM-1 and may facilitate the specific function of ICAM-3.

The binding affinity of cIBR and cIBC peptides to LFA-1 is higher than that of the cIBL peptide; the cIBR and cIBC peptides have an overlapping Pro-Arg-Gly-Gly (PRGG) sequence with a β-turn structure (30,31). This epitope has a similar β-turn conformation in the X-ray structure of D1 of ICAM-1 (Fig. 5). Alanine scanning also showed that this PRGG sequence is important for peptide activity (data not shown). The presence of this β-turn structure could stabilize the peptide interaction with the I-domain of LFA-1. Other studies have demonstrated the importance of proline residues flanking the RGD sequence. It is thought that proline may contribute to maintaining a favorable conformation for the solvent-exposed RGD site for its recognition by integrins (32).

In resting T cells, LFA-1 is expressed in a low-affinity state for ICAM-1; on activation, the LFA-1 changes conformation to a high-affinity state. The LFA-1 I-domain has been shown to bind ICAM-1 at the metal-ion–dependent adhesion site (MIDAS) by facilitating the interaction between LFA-1 residues coordinating a divalent cation (Mn^{2+}/Mg^{2+}) and ICAM-1 residues (33,34). When Mg^{2+} is bound within the I-domain, a conformational change results in the C-terminal α7 helix swinging away from the central β-sheet. This form is referred to as the opened (high-affinity) form of LFA-1 (35). However, it has been suggested that when Ca^{2+} is bound to the MIDAS, it inhibits the expression of the high-affinity form of LFA-1 and restrains LFA-1 conformation in the closed form. LFA-1 affinity for ICAM-1 can also be controlled by small molecules, which bind to an allosteric control site called the I-domain allosteric site (IDAS) (35,36). These small molecule antagonists, such as statins, *p*-arylthio cinnamides, and hydantoin derivatives, are thought to prevent the conformational change from the closed to the open form of LFA-1 (36,37). Further studies have demonstrated that the binding of our ICAM-1–derived peptides to T cells is enhanced by the presence of Ca^{2+}, suggesting that peptide binding favors the closed conformation of LFA-1. It may also suggest possible activation-dependent activity (data not shown).

The isolated sLFA-1 receptor used in the Biascore assays is in the high-affinity form, limiting the dynamic conformational changes of LFA-1 from closed to open. Therefore, these peptides' activation-dependent sensitivity to the confor-

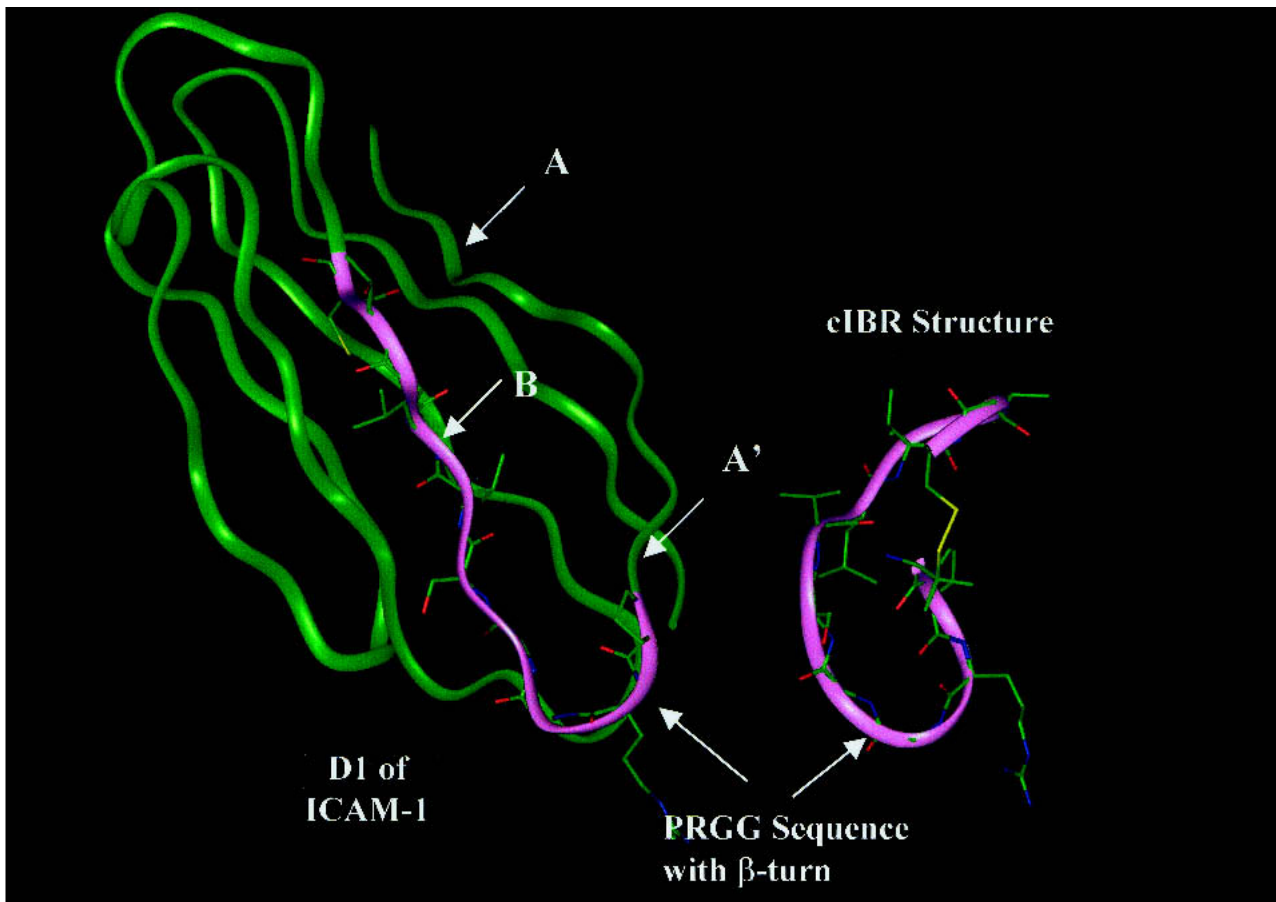

Fig. 5. X-ray crystal structure of ICAM-1 domain-1 (D1) (25,26) and the NMR structure of cIBR peptide (magenta) (30). The cIBL, cIBC, and cIBR peptides are located along the face of the A, A′, and B strands on D1 of ICAM-1. The sequence of cIBR peptide in D1 of ICAM-1 is highlighted in magenta, and the β-turn sequence (PRGG) is identified with arrows.

mation of LFA-1 may explain why these peptides were not able to inhibit sLFA-1/sICAM-1 interaction in Biacore experiments.

Internalization of ligand-bound integrins is a mechanism used to control a number of cellular processes. For example, β_2-integrins may participate in regulating neutrophil adhesiveness by removing activated receptors or permitting receptor recycling (38). The kinetics and morphology of endocytosis of mammalian cell integrin receptors have been studied using flow cytometry, and, similarly to what we observed of the internalization of FITC-cIBR peptide, the internalization of integrin–ligand complexes was also dependent on temperature (39). Many viruses are known to "highjack" integrins to mediate their entry into cells, and RGD-peptide ligands of integrins have been used to mediate gene delivery in synthetic and viral vector systems. Antiviral therapies are also being developed based on peptide ligands for integrins. The HIV-1 virus uses the ICAM-1/LFA-1 interaction for entry into T cells (40), and peptides derived from ICAM-1 (D1) were able to inhibit HIV-1 entry (41).

Although temperature-dependence studies alone are not unequivocal evidence of FITC-peptide internalization, further evidence of their internalization was confirmed by flow cytometry and confocal microscopy studies. Incubation of the FITC-labeled cyclic peptides on T cells at 37°C shows a time-dependent increase in fluorescence intensity; incubation at 4°C does not show this increase (Fig. 3). This result indicates that the FITC-labeled peptides are internalized by LFA-1, because cell metabolism and receptor-mediated internalization are negligible at 4°C. To prove this concept, we studied the internalization of FITC-cIBR peptide using confocal microscopy at 37°C and 4°C (Fig. 4). The membranes of T cells were labeled with DiD (red), which quenches the fluorescence from the membrane-bound FITC-cIBR peptide (green). Cross-sections of the confocal microscopy images of T cells incubated with FITC-cIBR peptide at 37°C demonstrate the presence of green fluorescence in the cytoplasm (Fig. 4), whereas cells incubated at 4°C did not show green fluorescence in the cytoplasm. This result strongly indicates that the peptide was internalized by the LFA-1 receptor into the cytoplasmic domain of T cells. The internalization of LFA-1 on binding to peptides raises the possibility of another mechanism by which these peptides may inhibit T-cell adhesion. The inhibition of T-cell adhesion could be through internalization of LFA-1 that is mediated by binding of ICAM-1 peptide. This mechanism could be an alternative to the direct inhibition of ICAM-1 binding to LFA-1 by peptide blocking the binding site of ICAM-1 on LFA-1. In the future, it would be interesting to evaluate this hypothesis by determining if there was a correlation between an increase in T-cell adhesion an LFA-1 reexpression after peptide internalization.

CONCLUSION

We have shown that cIBL, cIBC, and cIBR peptides can bind to the I-domain of LFA-1 in isolated LFA-1 as well as on the surface of T cells. These peptides were internalized by LFA-1 on T cells, suggesting that they can be used to target drugs to the cytoplasmic domain of T cells. In the future, we will show the utility of these peptides in delivering cytotoxic drugs to T cells.

ACKNOWLEDGMENTS

We would like to express thanks to Boehringer Ingelheim for providing M.E.A. a summer internship opportunity and to the following individuals for their helpful contributions to this work: Deborah D. Jeanfavre, Carol Stearns, Mark E. Labadia, Joseph R. Woska, Jr., and Patricia L. Reilly. We also thank Dr. Mehran Yazdanian who supervised M.E.A. while at Boehringer Ingelheim. We would like to thank the PhRMA Foundation for the predoctoral fellowship to M.E.A. This work was also supported by Self-Faculty Scholar to T.J.S. from The University of Kansas and the National Institutes of Health (GM-08359). We thank Nancy Harmony for proofreading the manuscript.

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