Binding and Internalization of an LFA-1-Derived Cyclic Peptide by ICAM Receptors on Activated Lymphocyte: A Potential Ligand for Drug Targeting to ICAM-1-Expressing Cells

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Purpose. The interaction of cell-adhesion molecules LFA-1/ICAM-1 is critical for many inflammatory and immune responses. Blockades of this interaction using antibodies or peptide analogs are being developed as therapeutic approaches for inflammatory and autoimmune diseases. The aim of this study is to examine the binding and internalization mechanisms of LFA-1 peptide [cLAB.L or cyclo-(1,12)-PenITDGEATDSGC] mediated by ICAM receptors on the surface of lymphocytes.
Methods. The binding and internalization of cLAB.L were evaluated using fluorescence-labeled cLAB.L on activated Molt-3 cells, measured by flow cytometry. Confocal fluorescence microscopy was also used to image the distribution of peptide binding and internalization.
Results. The binding of FITC-cLAB.L exhibited bimodal cell distribution and was enhanced by Ca^{2+} and Mg^{2+}. Marked differences in peptide binding were found between 37 and 4°C, as well as between activated and non-activated cells. Unlabeled peptide, low temperature, and the absence of cell activation suppress the peptide binding. The presence of peptide in the cytoplasm was detected in 37 but not 4°C binding. Peptide cLAB.L inhibited the binding of monoclonal antibodies to domain D1 of ICAM-1 and domain D1 of ICAM-3.
Conclusions. Peptide cLAB.L can bind to the D1-domain of ICAM-1 and, to a lesser extent, to ICAM-3 on activated T-cells. Peptide binding indicates responses to the multiple and dynamic states of activated receptor ICAMs; this peptide may also be internalized by ICAM receptors on T-cells. This work suggests that cLAB.L has a therapeutic potential to target drugs to ICAM-1 expressing cells including autoreactive lymphocytes and inflamed tissues.

KEY WORDS: adhesion-molecule peptide; lymphocyte function-associated antigen-1; intercellular adhesion molecule-1; Molt-3 T-cells.

INTRODUCTION

One major complementary signal for sustaining T-cell activation is the interaction of lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18) and intercellular adhesion molecule-1 (ICAM-1; CD54) and B7/CD28. These complexes induce the movement of actin molecules in the cytoskeleton, which produces an accumulation of surface receptors such as LFA-1 and ICAM-1 at the interface between T-cells and antigen-presenting cells (APC) (1). LFA-1 also interacts with ICAM-2 and ICAM-3, in addition to ICAM-1, for different functions. ICAM-2 predominantly is expressed on resting endothelium and interacts more with LFA-1 on non-activated than activated circulating-T-cells while ICAM-3 is expressed by monocytes and resting lymphocytes with a major role in initiating the immune response (2). Activation of LFA-1/ICAM-1 interaction can be triggered by CD2, CD3, phorbol esters, and MHC class II molecules through stimulation of protein kinase (3,4). Divalent cations are essential for LFA-1/ICAM-1 interactions in enhancing the functional activity of the adhesion molecules (4), and the ion type requirement relates to the specific domains of LFA-1 and ICAM-1 (5).

Immune response can be modulated using inhibitors of LFA-1/ICAM-1 interaction. Monoclonal antibodies (mAbs) against these cell adhesion-molecules have been successfully investigated as potential immunosuppressants (6–8). However, the relative efficacy/safety ratio of mAbs application often faces challenges in its systemic administration and immunogenic potential. Adhesion-molecule peptides offer better physicochemical stability than do antibodies and may not have any immunogenic properties. Small peptide fragments derived from ICAM-1 sequences have been shown to inhibit LFA-1/ICAM-1 interaction (9–11). The conformation of an adhesion-molecule peptide may play an important role in its selectivity for the receptor (12). We have shown that a cyclic peptide (cIBR) derived from the sequence of ICAM-1 can inhibit homotypic T-cell adhesion (13–15); it can bind and be internalized by surface receptors of T-cells (11). Several studies have also observed the internalization of adhesion molecules and other surface receptors (16–19); receptor internalization can be a complementary mechanism of the inhibition of cell-cell adhesion. The internalization of ligand to cell adhesion receptor can be used to target drugs to the cells expressing these receptors. In this case, the drugs can be conjugated to the ligand of the cell adhesion receptors for selectively delivering drug to the targeted cells.

Here, we investigate the binding and internalization characteristics of cLAB.L [cyclo-(1,12)-PenITDGEATDSGC], a cyclic LFA-1 peptide containing 10 amino acids. This peptide is derived from the I-domain of the α-subunit of LFA-1. The understanding of binding and internalization characteristics of cyclic peptide cLAB.L combined with the strategic conjugation of this peptide to anti-cancer or anti-inflammatory drugs may provide the basis of future therapeutics for T-cell mediated immune disorders or inflammatory diseases that are characterized by elevated ICAM-1 expression. Penicillamine and cystein residues were added to the N- and C-termini (14) to form the cyclic peptide cLAB.L via a disulfide bond between the Pen1 and Cys12 residues. The formation of a cyclic peptide restricts the peptide conformation to produce conformational stability; thus, it has a better selectivity for cell surface receptors than its linear counterpart (13). We have shown that cLAB.L can inhibit homotypic T-cell adhesion in a concentration-dependent manner (13,14), presumably via inhibition of LFA-1/ICAMs interactions. The mechanisms may involve binding of the cLAB.L to the LFA-1-binding region of ICAM-1 and ICAM-3, thus blocking the LFA-1/ICAMs interactions. Alternatively, the cLAB.L may bind to the β-subunit of LFA-1, disrupting the heterodimeric formation of the α- and β-subunits of LFA-1 necessary for ICAM

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binding. An *in vivo* study of a collagen-induced rheumatoid arthritis (CIA) mice model showed that cLAB.L effectively lowered the arthritis score in a reproducible manner (unpublished work). In this study, fluorescence-labeled and unlabeled cLAB.L peptides were used to evaluate the mechanisms of action of the peptide on the surface receptors of activated and non-activated Molt-3 T-cells. The binding sites to ICAMs and the influence of divalent cations (i.e., Ca^{2+} and Mg^{2+}) were also determined.

MATERIALS AND METHODS

Cell Culture and Cell Activation

Molt-3 cells obtained from the American Type Culture Collection (Rockville, MD) were maintained and grown as previously described (11). For the purpose of cell activation, phorbol 12-myristate-13-acetate (PMA) from Sigma (St. Louis, MO) or anti-CD3 antibody from Chemicon (Temecula, CA) were used in concentrations of 0.2 μM and 10 μg/ml, respectively. Incubation with PMA was varied (1, 2, 4, 16, 24, 40, and 48 hours) to study the effect of time on receptor activation.

Peptide Labeling

Cyclic peptide cLAB.L was purchased from Multiple Peptide Systems (San Diego, CA). The pure product was analyzed by NMR and fast atom bombardment mass spectrometry (FABMS). Peptide cLAB.L (50 mg) was dissolved in a minimal amount of Milli-Q water and added with fluorescein-5-isothiocyanate (FITC) at two times the molarity of the peptide. The FITC reacted with the N-terminus of cLAB.L. The pH mixture was adjusted to 9 with 1 N NaOH and the reaction was allowed to proceed for 1 h; the pH was then brought to 7 by adding 10% acetic acid. The mixture was lyophilized and purified using preparative reversed-phase high-performance liquid chromatography (RP-HPLC). The molecular weight of the fraction containing FITC-cLAB.L was confirmed by FABMS to give M+1 = 1586.

Peptide Binding Experiment

Cells were centrifuged at 3000 g for 5 min and resuspended in serum-free medium to reach a concentration of 3.5 × 10^6/ml. Peptide stock solution was prepared in phosphate-buffered saline (PBS). Binding experiments were carried out in a 48-well cell culture cluster (Corning) and incubated at either 4 or 37°C. In experiments to block FITC-cLAB.L binding, the cell suspension was pre-incubated with unlabeled cLAB.L for 1 hour at 4°C. The FITC-cLAB.L was then added, followed by incubation for another hour. At the end of incubation, cell suspensions were centrifuged at 3000 g for 3 minutes, decanted, and rinsed with 10 mM Hepes/PBS. The cell pellet was fixed using 4% paraformaldehyde/PBS for 20 minutes at room temperature, washed twice with 10 mM Hepes/PBS, and resuspended in PBS. Samples were analyzed with a flow cytometer (Becton-Dickinson) and median values of fluorescence intensity were taken as the binding intensities. As many as 10,000 cells were counted for every sample during acquisition and each experiment was done at least in triplicate. Cells without peptide addition were used as controls, by which measurements samples histograms were subjected to automatic subtraction. The control histogram was placed within 10^0 to 10^1 on the log scale of FL1-Height by adjusting the FL1 detector. The binding intensities were represented as their relative values to the reference conditions.

Antibody Binding Experiment

Peptide cLAB.L was used to block the binding of saturated concentrations of FITC-labeled mAbs against LFA-1, ICAM-1, or ICAM-3 on Molt-3 cells in a one-step direct analysis. MAbs against LFA-1 were anti-human CD11a (LFA-1 integrin α_L) clone 38 and anti-human CD18 (β_2 integrin) clone IB4 from Ancell (Bayport, MN). MAbs against ICAMs were clone 15.2 and clone 8.4A6 (Ancell), which recognize the D1 and D2 domains of ICAM-1, respectively, and clone 186-2G9 of the D1 domain of ICAM-3. Molt-3 cells were treated with peptide for 1 hour before the addition of the antibody. Flow cytometric analysis was as described for peptide binding experiments. Irrelevant murine IgG (Sigma, St. Louis, MO) was used as a control for non-specific binding. A cyclic 11-amino acid peptide containing an RGD sequence (20) was used as a control peptide. This peptide was chosen because ICAMs and LFA-1 (unlike other integrins i.e., β_3 integrins, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$ and β_1 integrins) do not recognize the tripeptide sequence RGD (21).

Calculation of K_d Like Values

The total number of cell ICAM-1 receptors was estimated from the fluorescence intensity of FITC-labeled mAb against ICAM-1 at saturated concentration. It was specified in the product information that the ratio of antibody/FITC conjugate was 0.55 mg/ml and the FITC/IgG molar ratio was 10.4. The molar ratio of FITC: peptide was measured as 1.0. By correlating the peptide binding signals with the above parameters, the number of moles of cell-bound peptide was determined. The ratio of bound peptide to the total number of receptors (υ) was then plotted against the number of free peptides (A) and fitted to the following Eq. (1):

$$\upsilon = [A]/\{K_d + [A]\} \quad (1)$$

K_d-like values were obtained from the analysis of the hyperbolic equation of the plot in Figure 3A using Sigma Plot software. Alternatively, K_d-like values were calculated using the equation adapted from previously published work on the binding of myasthenogenic peptides on human T-cells (22), in which reciprocal conversion of flow-cytometric readings of peptide binding described as median fluorescence intensities (MFI) was plotted versus the reciprocal of the peptide concentrations [D]. The double reciprocal conversions of two parameters were fitted to Eq. (2). Linear regressions of the plots were then calculated using Sigma Plot software. K_d-like values from Eqs. (1) and (2) were very similar.

$$1/\mathrm{MFI} = K_d/(\mathrm{MaxSignal}) \times 1/[D] + 1/(\mathrm{MaxSignal}) \quad (2)$$

Fluorescence and Confocal Microscopy Studies

Samples from peptide-binding experiments at 4 and 37°C were photographed using an epi-fluorescence microscope Nikon Eclipse TE300 and a confocal microscope (Biorad

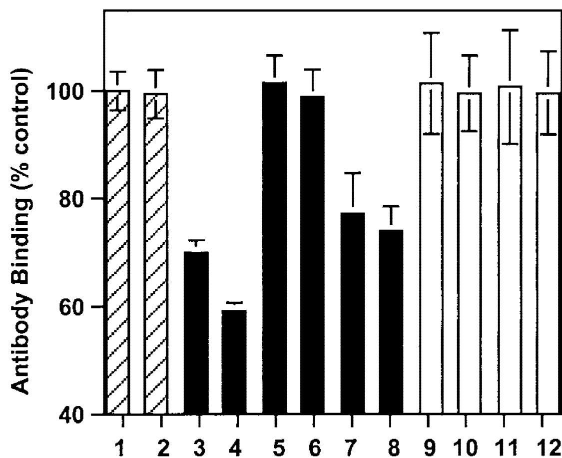

Fig. 1. Blocking on the binding of FITC-conjugate antibodies against ICAM-1, ICAM-3 and LFA-1 by control peptide and cLAB.L. Bars 1 and 2 represent the average binding of all the tested adhesion-molecule antibodies upon blocking by the control peptide at 80 and 160 μM, respectively. Bars 3 to 12 are the results of blocking by cLAB.L peptide. Bars 3 and 4 are the binding of anti-ICAM-1 (D1 domain) upon blocking by cLAB.L at 80 and 160 μM, respectively. Bars 5 and 6, ICAM-1 (D2 domain); bars 7 and 8, ICAM-3 (D1 domain); bars 9 and 10, LFA-1 (CD11a, α_L); bars 11 and 12, LFA-1 (CD18, β_2). Data represent mean of six determinations ± SE.

MRC100 Laser Scanning Confocal Imaging System connected to a Nikon Diaphot 2000 microscope).

RESULTS

Inhibition of Antibody Binding to LFA-1, ICAM-1, and ICAM-3

To determine whether the peptide binds to LFA-1, ICAM-1, or ICAM-3, the ability of cLAB.L peptide to inhibit binding of saturated concentrations of FITC-labeled mAbs against LFA-1, ICAM-1, and ICAM-3 was evaluated (Fig. 1). Binding of mAb against the D1-domain of ICAM-1 was blocked by cLAB.L peptide in a concentration-dependent manner. However, the cLAB.L peptide did not inhibit binding of mAb against the D2-domain of ICAM-1 nor the binding of all the mAbs against LFA-1. This result suggests that the cLAB.L peptide binds to the D1-domain of ICAM-1, but not to the D2-domain of ICAM-1 nor to the LFA-1. Interestingly, the cLAB.L peptide can also bind to the D1-domain of ICAM-3 because it can block the binding of anti-ICAM-3 binding to the D1-domain. At a concentration of 160 μM, the peptide blocks binding of anti-ICAM-1 (D1) and anti-ICAM-3 (D1) to 59% and 74%, respectively. Thus, cLAB.L prefers binding to ICAM-1 rather than to ICAM-3, suggesting that the major mechanism of activity of the peptide is inhibition of LFA-1/ICAM-1.

Binding Specificity of FITC-cLAB.L

Flow cytometry analyses showed that the binding of FITC-cLAB.L to Molt-3 cells exhibited two-population distribution (Fig. 2, A and B); in contrast, binding of FITC-labeled mAbs against ICAM-1 or LFA-1 show only one cell population (Fig. 2C). The first population (population-1) in Figs. 2A and B has a high number of cells with low fluorescence intensity while the second population (population-2) has low cell numbers but high fluorescence intensity. This suggests that the receptors in population-2 facilitate more peptide binding than do those in population-1.

To characterize receptor-mediated binding of the peptide, two different experiments were performed—binding saturation of FITC-cLAB.L and blocking of FITC-cLAB.L binding by the unlabeled cLAB.L. FITC-cLAB.L binding over a wide range of peptide concentrations (0–1000 μM) exhibited multiple-equilibrium characteristics (data not shown). The saturation curve was found to be within 0–25 μM as shown in Figure 3A; the K_d-like-values were calculated to be 1.8 and 6.0 μM for population-1 and -2, respectively. The higher K_d-like-value of population-2 may reflect the recycle mode of binding and receptor internalization on population-2. The calculated K_d-like value in this work is intended only to describe the relative binding efficiencies of population-1 and population-2 and not in any way to quantify the potency

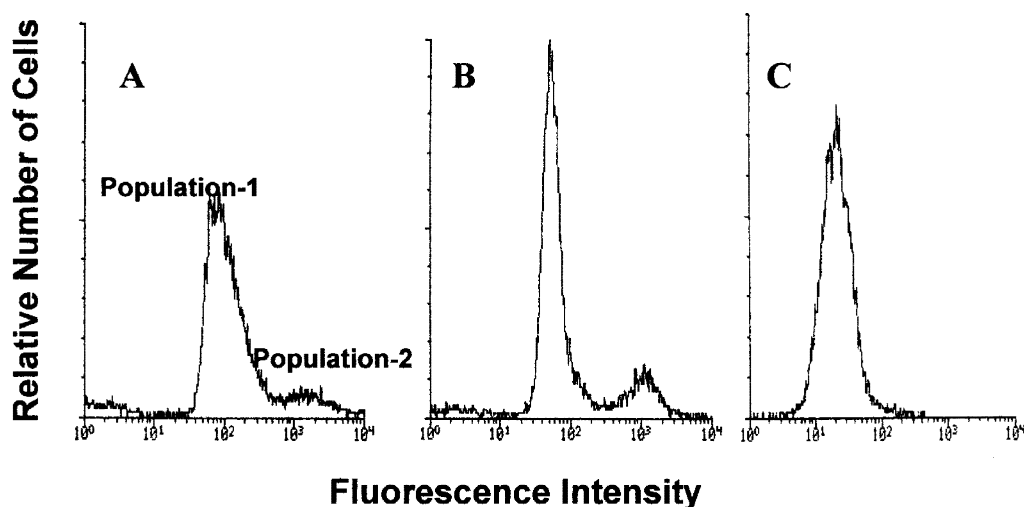

Fig. 2. Distribution of Molt-3 cell-population in response to the binding to FITC-cLAB.L whereby cells were activated with PMA for 4 hours (A) or 48 hours (B); distribution of Molt-3 cell population in response to the binding of FITC-conjugate antibody against the D1 domain of ICAM-1 (C). Peptide concentration was 25 μM and antibody was used at the dilution suggested by the manufacturer.

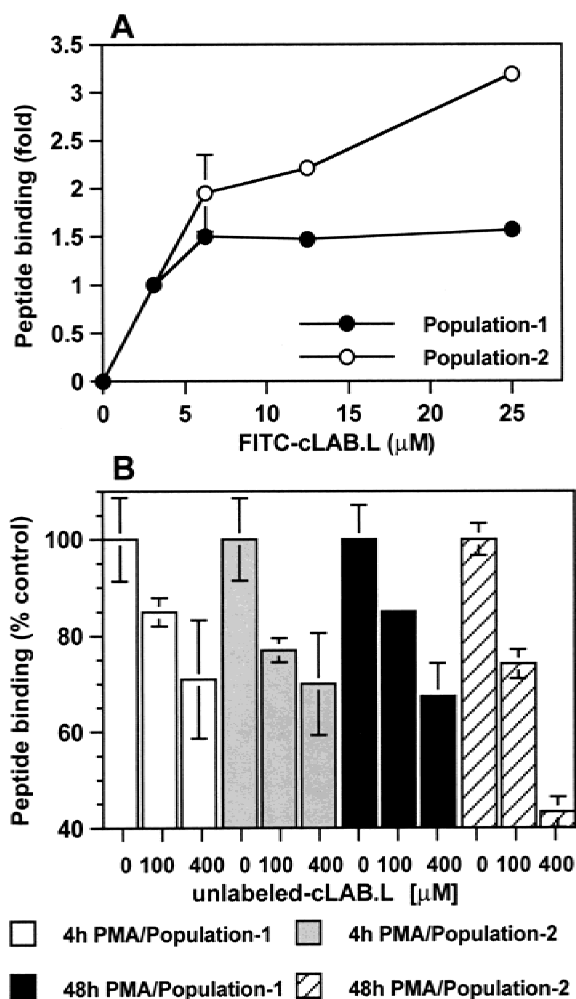

Fig. 3. Concentration-dependent profile of FITC-cLAB.L on population-1 and -2 of Molt-3 cells (A), and the binding of FITC-cLAB.L following cell incubation with unlabeled cLAB.L (B). Peptide binding in Fig. 3A was relative to the binding intensity of 3.12 μM FITC-cLAB.L. In Fig. 3B, comparison was made between cells activated with PMA for 4 and 48 hours. Percentage of peptide binding was taken in reference to the binding intensity of 25 μM FITC-cLAB.L without the addition of unlabeled cLAB.L. Four determinations were carried out for both Figs. 3A and 3B. Error bars are eliminated when the values are very small or less than the size of the symbol.

of the peptide in impairing the interaction of purified proteins. Blocking of the FITC-cLAB.L binding by the unlabeled peptide was conducted using cells that had been activated for 4 and 48 hours (Fig. 3B). The results in Figure 3B showed that the unlabeled cLAB.L inhibits the binding of FITC-cLAB.L on cell population-1 and -2 in a concentration-dependent fashion. Cell activation times of 4 and 48 hours indicated comparable inhibitory profiles on population-1 but a slightly different trend on population-2; 48 hour-activated cells were more prone to blocking by unlabeled peptide than were those of 4-hour activation. When FITC-cLAB.L bindings on cells of both 4- and 48-hour activation were further studied, it was apparent that binding on 4-hour activation was always higher than those on 48h-activation (Fig. 4, A and B). These data suggest that 4-hour activation produces a more active state of the receptors.

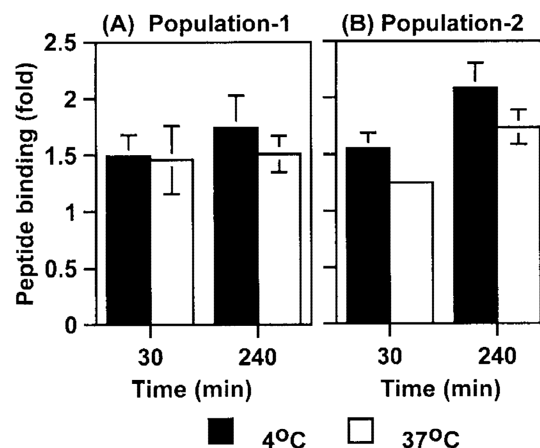

Fig. 4. The ratio of binding intensities between 4- and 48-h activation on cell-population 1 (A) and cell-population 2 (B). Binding of 25 μM FITC-cLAB.L on Molt-3 cells at 4 and 37°C was followed every 30 minutes for up to 240 minutes. Peptide binding in both Figs. 4A and 4B was relative to the binding intensity of 25 μM FITC-cLAB.L on 48-hour activated-cells. Each value represents the mean of four determinations ± SE. Error bars are eliminated when the values are very small.

Effect of Cell Activation, Temperature, and Cations

The proportion and fluorescence intensities between the two cell populations as indicated in Fig. 2(A and B) changed at various time of PMA activation. The cell numbers of population-2 increased with increasing activation time while the cell numbers in population-1 decreased proportionately (Fig. 5A). At the same time, the fluorescence intensity in population-1 increased sharply in the first four hours, then it reached a steady state at 40 hours of PMA activation (data not shown).

The effect of PMA as an activator was further examined along with that of anti-CD3 and a mixture of cations Ca^{2+} and Mg^{2+}. The peptide binding was evaluated at 4 and 37°C. Results for population-1 are shown in Fig. 5B; similar profiles were observed for population-2 (data not shown). It is clear that at either 4 or 37°C, the peptide binds significantly on PMA- or CD3-activated Molt-3 cells. The fluorescence intensities of peptide binding at 37°C increased over 4 hours of experiments wheras a nearly constant pattern was found at 4°C. This suggests that at 37°C both binding and uptake of the peptide by the receptors occurred on activated cells. Fig. 5B also showed that little or no binding was observed on non-activated cells and that Ca^{2+} and Mg^{2+} were not sufficient to promote peptide binding on non-activated cells. However, when a mixture of Mg^{2+} and Ca^{2+} was added to the PMA-activated cells, binding of FITC-cLAB.L to population-1 and -2 improved 3.4- and 1.8-fold, respectively (Fig. 5C). It is also apparent that divalent cations have a greater influence on FITC-cLAB.L binding to the receptors in cell population-1 than to the receptors in population-2. Addition of Ca^{2+} cations caused a 1.5-fold binding enhancement of FITC-cLAB.L on population-1, but no effect was detected on population-2. No peptide binding was observed with the addition of Mg^{2+} alone. These data suggest that binding of cLAB.L to surface receptors is influenced by synergistic effects of Ca^{2+} and Mg^{2+}.

The difference in binding at 4 and 37°C was further

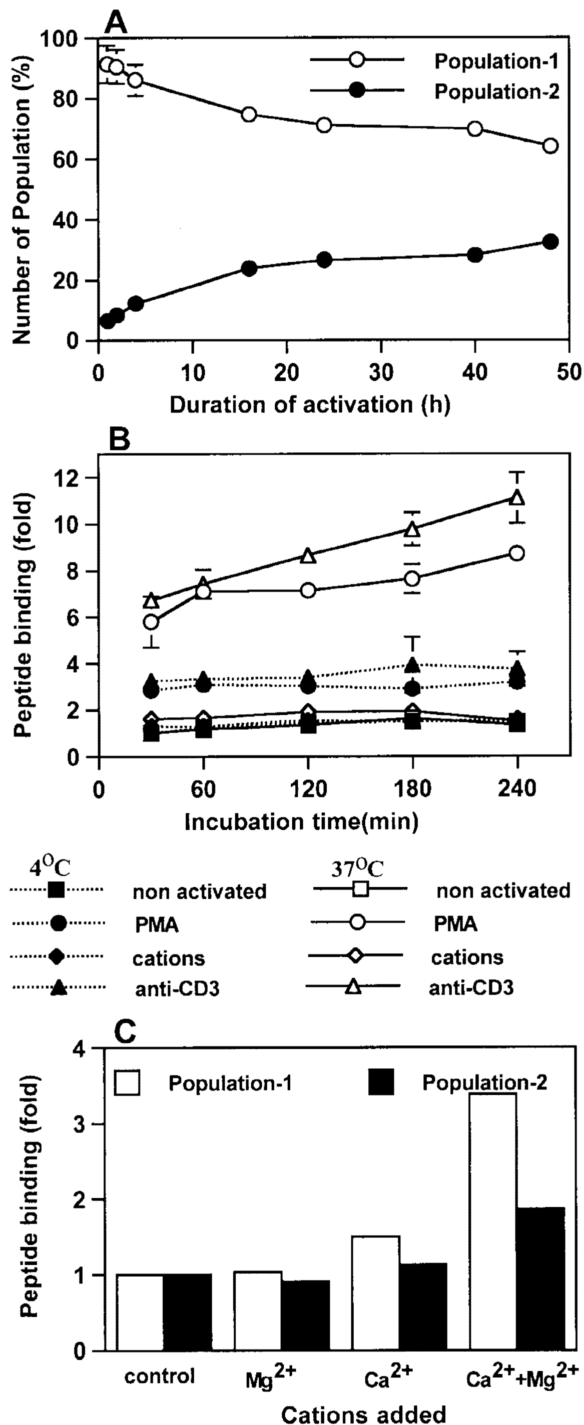

Fig. 5. Proportions of cell population-1 and -2 in response to the duration of PMA activation (A). Effect of activators on the binding of 25 μM FITC-cLAB.L to cell population-1 (B). Binding experiments were carried out in the presence of activators anti-CD3, PMA, or divalent cations (mixture of Ca^{2+} and Mg^{2+} at a final concentration of 1.5 mM) at 4 or 37°C. Peptide binding on non-activated cells at 4°C was used as a reference. Data in Figs. 5A and B represent the mean of four determinations ± SE. Error bars are eliminated when less than the size of symbol. Effect of cation addition on the binding intensity of FITC-cLAB.L to PMA-activated Molt-3 cells (C). Final concentrations of Ca^{2+}, Mg^{2+}, and Ca^{2+} Mg^{2+} were 1.5 mM. Control was the binding intensity of FITC-cLAB.L to PMA-activated cells without added cations. Data represent the mean of three determinations ± SE. Error bars are eliminated when the values are very small.

evaluated by visualization using an epi-fluorescence microscope (Fig. 6, A–C) and a confocal-fluorescence microscope (Fig. 6, D–F). Surface binding of FITC-cLAB.L on an aggregate of Molt-3 is indicated by the green fluorescence seen with the epi-fluorescence microscope (Fig. 6A), and by the white-to-gray color observed with the confocal-fluorescence microscope (Fig. 6D). Observation of single cells clearly showed a higher binding intensity at 37°C (Fig. 6B) than at 4°C (Fig. 6C). Sectional images of cells from the two different temperatures suggested the presence of peptide in the cytoplasm after binding at 37°C (Fig. 6E) but not at 4°C (Fig. 6F).

DISCUSSION

This study was designed to evaluate the characteristics and mechanisms of the action of cLAB.L peptide, derived from the I-domain of LFA-1 α-subunit, in inhibiting LFA-1/ICAM-1-mediated homotypic T-cell adhesion. As mentioned above, there are two possible mechanisms of action of this peptide in inhibiting homotypic T-cell adhesion. One is that the cLAB.L binds to ICAMs (ICAM-1 and ICAM-3) and inhibits LFA-1/ICAMs interactions. Alternatively, the cLAB.L peptide can bind to the β-subunit (CD18) of LFA-1 and disrupt the integrity of LFA-1, thus inhibiting the LFA-1/ICAMs interactions. The use of intact cells rather than purified substrates in this work is based on two important native characteristics of ICAM-1 on the cell surface. First, the native structure of ICAM-1 is a dimer. Soluble ICAM-1 may experience an alteration of the oligomeric native biochemical necessary for its activity to bind to integrin and to inhibit cell adhesion events; this form is conserved when using membrane-bound ICAM-1 (23). Secondly, certain adhesion molecules (including ICAM-1) interact with the cytoskeleton through their cytoplasmic tails. This interaction supports cellular functions that include receptor-ligand interactions and receptor internalization (17).

We found that cLAB.L peptide binds primarily to the D1 domain of ICAM-1 and to a lesser extent to the D1 domain of ICAM-3 (Fig. 1); this is supported by the ability of this peptide to inhibit the binding of mAbs against the D1 domain of both ICAM-1 and ICAM-3. A weaker blocking effect of cLAB.L on the binding of anti-ICAM-3 compared to anti-ICAM-1 may be due to the higher sequence and/or conformational selectivity of cLAB.L for ICAM-1 than for ICAM-3. Peptide cLAB.L contains at least three important residues claimed to be involved in LFA-1/ICAM-1 interaction, Thr^{243} and Ser^{245} (24) and Glu^{241} (25). Although the binding sites in the I-domain of LFA-1 for ICAM-1 and ICAM-3 have been suggested as distinct but partially overlapping (26), none of the reported residues is part of the cLAB.L sequence. ICAM-1 and ICAM-3 have 52% homology between them that is largely contributed by domain D2 (77%), but the important residues for LFA-1 binding to ICAM-1 as well as ICAM-3 are in domain D1 (27,28). Thus, the non-conserved residues in the D1 domain contribute to integrin binding. Furthermore a weaker blocking of cLAB.L on the binding of anti-ICAM-3 may also be attributed by the fact that LFA-1/ICAM-1 and LFA-1/ICAM-3 interactions function at different stages during immune response (the predominant role of LFA-1/ICAM-3 occurs during the initiation of immune response), and that the LFA-1/ICAM-3 interaction has a much lower affinity than LFA-1/ICAM-1 (26,29). We carried out

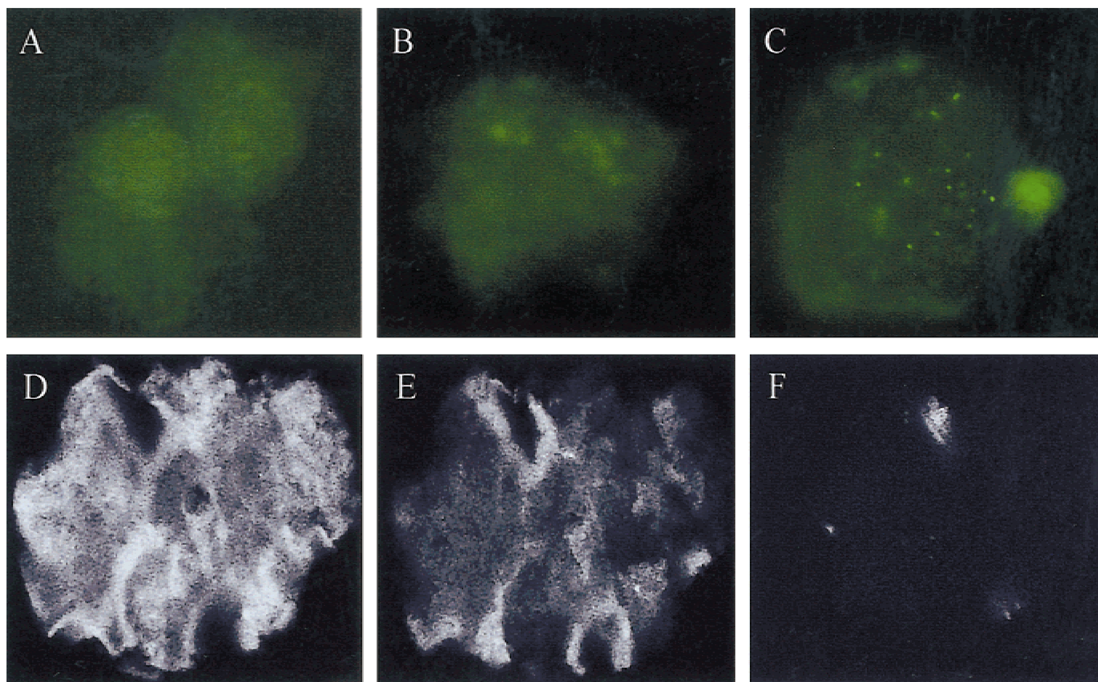

Fig. 6. Microscopy of FITC-cLAB.L binding and internalization on Molt-3 cells from two different temperatures of binding. Epi-fluorescence and confocal-fluorescence microscopes were used to observe surface binding and internalization, respectively. Epi-fluorescence microscopy of Molt-3 cell-clump after incubation at 37°C (A), single Molt-3 cell at 37°C (B), and single cell at 4°C (C). Confocal microscopy of surface projection of Molt-3 cell following 37°C incubation (D), single confocal section showing the distribution of the FITC-cLAB.L in the cytoplasm following 37°C incubation (E), and single confocal section showing a minimal distribution of the FITC-cLAB.L on the cell peripheral following 4°C incubation (F).

additional experiments using CD, NMR and molecular dynamics simulation (MD) and a computational docking using the program AUTODOCK. The combined results of CD, NMR and MD show that cLAB.L can bind to Ca^{2+} and Mg^{2+}. The results of computational docking show that the peptide can bind to the D1 domain of ICAM-1 via peptide residues of Pen1, Ile2, Glu6, and Ser10 (unpublished work). Our results support the work by other groups on the inhibitory activity of a linear peptide containing nine amino acids similar to cLAB.L (30) in which 2 mM of this linear peptide was able to reduce 30% of T-cells binding to ICAM-1, thus making it the most potent of the tested I-domain peptides. Although a direct comparison cannot be made, it is worth noting that we used a much lower concentration (160 μM) of cLAB.L to reduce anti-ICAM-1 binding to T-cells by 41% (Fig. 1). This may suggest that the conformational rigidity of cyclic peptide cLAB.L contributes to its binding selectivity to ICAM-1 (14).

The bimodal distribution characteristics of FITC-cLAB.L binding (Figs. 2 and 5A) suggest the occurrence of multiple and dynamic states of receptors, i.e., ICAM-1 and/or ICAM-3. This is in agreement with earlier reports on the other adhesion-molecule receptors LFA-1 (31) and $\alpha_5\beta_1$ integrin (32). The latter indicated that activation led to two or more different conformational states and reflects distinct stages in adhesion signaling. Thus, a similar explanation may be used for FITC-cLAB.L binding to ICAMs whereby short (4 hours) and long (48 hours) activation times produced two different activated states of ICAMs and, hence, different binding intensities of FITC-cLAB.L. It is plausible that a longer activation time may provide membrane receptor and cytoskeleton rearrangements, thereby increasing the proportion of popoulation-2. Population-2 may have cluster forms of active ICAMs, whereas population-1 has active ICAMs that are spread out through the cell surface

The ability of PMA and anti-CD3 to promote binding is an indication that this LFA-1-derived peptide binds selectively to the activated T-cells in which the pathway of the protein kinase is directly or indirectly regulated. The use of divalent cations was found to be beneficial only in enhancing peptide binding to already activated T-cells (Fig. 5C); this supports the earlier finding that a section of the I-domain binds to the ICAM-1 via divalent cations (5).

A higher binding intensity on activated cells at 37°C than at 4°C suggested an ATP-dependent process (Fig. 5B). Combining the latter with images from the confocal microscope (Fig. 6, D–F) and the dynamic responses of peptide binding to cell activation provides indirect evidence of receptor-mediated internalization of the peptide. Internalization in terms of receptor recycling to control the cell-receptor activity is common in the case of adhesion molecules; receptor-ligand binding is often followed by endocytosis as one of the mechanisms for the termination of receptor activity (17,18). The plausible characteristic of receptor-mediated internalization, combined with other methods such as drug conjugation, may lead to a useful application of peptide cLAB.L in targeting leukocyte-related diseases. To date, we have successfully conjugated an anti-inflammatory drug with an ICAM-1 cyclic-peptide through its N-terminal. Work is currently in progress to determine the efficacy of the conjugate.

In conclusion, direct binding between cLAB.L on the surface of activated Molt-3 T-cells is via binding to ICAM-1 and to a lesser extent to ICAM-3. The bimodal cell-

population distribution of peptide binding suggests the responses to the occurrence of multiple and dynamic state of activated receptor ICAMs. The peptide binding is influenced by a mixture of Ca^{2+} and Mg^{2+}, and the peptide may also be internalized by ICAMs. This work suggests that cLAB.L peptide is a potent inhibitor of LFA-1/ICAM-1 interaction and its selective binding mechanism may be used to designate a drug conjugate to target cells having elevated ICAM-1 expression.

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