The I Domain of Integrin Leukocyte Function-associated Antigen-1 Is Involved in a Conformational Change Leading to High Affinity Binding to Ligand Intercellular Adhesion Molecule 1 (ICAM-1)*

(Received for publication, July 6, 1998, and in revised form, July 30, 1998)

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On T cells the leukocyte integrin leukocyte function-associated antigen-1 (LFA-1) (CD11a/CD18) can be induced to bind its ligand intercellular adhesion molecule 1 (ICAM-1) (CD54) either by increasing the affinity of the receptor with Mg²⁺ and EGTA or by receptor clustering following activation with phorbol ester. The existence of these two adhesion-inducing pathways implies that alternative mechanisms might exist by which LFA-1 engages ICAM-1. The LFA-1 α subunit I domain contains a major binding site for ICAM-1. In this study we show that soluble LFA-1 I domain blocks ICAM-1 binding of the high affinity Mg²⁺-induced form of LFA-1 but not the phorbol ester-induced form. Under conditions of Mg²⁺-activation, the soluble I domain also prevents expression of an activation dependent epitope on LFA-1, implying that it inhibits a conformational change necessary for conversion to the high affinity form of this integrin. In addition, the binding of Mg²⁺-activated LFA-1 to ICAM-1 is blocked by peptides covering the α4-β3 loop, the β3-α5 loop, and the α5 helix of the I domain, whereas none of the peptides tested blocks phorbol ester-mediated adhesion. The blocking peptides localize to the same face of the crystal structure of the LFA-1 I domain and define an area that, during activation, may be involved in association of the I domain with another region of LFA-1, potentially the β-propeller domain. This is the first evidence linking a structural domain of an integrin, in this case the I domain, with a particular activation mechanism.

Adhesion mediated by the interaction of the integrin LFA-1 (CD11a/CD18) with one of its ligands, ICAM-1, ICAM-2, or ICAM-3, is crucial to the inflammatory process (reviewed in Refs. 1–3). LFA-1 is not constitutively able to bind ligand, but requires activation, with the characteristics of the active LFA-1 depending on the method of stimulation. In vitro, triggering of T cells with phorbol ester or by increasing intracellular Ca²⁺ concentration does not induce a detectable change in the affinity of the individual LFA-1 molecules. These stimuli, however, induce clustering of LFA-1, thereby increasing the overall strength of binding (4, 5), which is described as an increase in avidity. In contrast, activation from the outside of the cell with Mg²⁺ and EGTA results in the formation of a higher affinity form of LFA-1, as assessed by an increased ability to bind soluble ICAM-1, and in expression of an activation reporter epitope recognized by mAb 24 (1, 6, 7). In this situation there is no evidence for LFA-1 clustering (5). These findings and those of others (8, 9) suggest that physiological stimuli regulate adhesion by two major mechanisms involving alterations either in the affinity of the individual integrin molecules or in the overall avidity of binding. Such different forms of integrin adhesion might dictate the nature of signals transmitted into the cell and have functional consequences. It is not completely understood how these two forms of adhesion relate to the in vivo activation of LFA-1; however, the idea of a two-stage model of activation has been proposed in which integrin clustering is followed by a ligand-induced affinity increase (1).

Whether LFA-1 adhesion activated by different stimuli involves distinct regions within the integrin is unknown. Several ligand binding sites have been identified that colocalize with sites where divalent cation is also thought to bind (reviewed in Refs. 10 and 11). Most attention has focused on the ligand binding activity of the “T” domain, a ~200 amino acid region inserted into the amino-terminal region of 7 of the 16 integrin α subunits (reviewed in Refs. 1 and 10). Isolated I domains can bind directly to ligand (12–17). The crystal structures of the LFA-1 (18, 19), Mac-1 (CD11b/CD18) (20, 21), and αβ2I (CD49b/CD29) (22) I domains show homology to the classical dinucleotide binding fold (the Rossmann fold), composed of a central sheet of 6 β strands surrounded by 7 helices. A Mg²⁺/Mn²⁺ binding site, termed the metal ion-dependent adhesion site (MIDAS), is located at the “top” of the domain (20) and is conserved between I domains. This motif is critical for ligand binding in LFA-1 (23, 24), Mac-1 (13, 23, 25, 26), and αβ2I (15, 27). The seven N-terminal repeat sequences of a typical integrin α subunit have been modeled as a β-propeller fold (28), and the I domain is predicted to bind to the upper surface of this model, resembling the relationship between the α and β subunit of a heterotrimeric G protein (29).

In this study, we use recombinant I domain and constituent peptides in order to understand how this domain participates in LFA-1 binding to ICAM-1 when different integrin activators are used. We demonstrate that soluble I domain inhibits Mg²⁺-induced LFA-1-mediated adhesion to ICAM-1 but not phorbol...
ester-induced binding. We provide evidence that such a conformational change in LFA-1, which leads to the high affinity form, is blocked by the soluble I domain. Using peptides, the inhibiting activity is pinpointed to two loops associated with the α4 and α5 helices that lie on one face of the I domain.

EXPERIMENTAL PROCEDURES
Preparation of LFA-1 I Domain and ICAM-1Fc Proteins
The LFA-1 I domain resides Leu111–Ser327, termed (Leu111–Ser327)I dom, includes the predicted I domain sequence and an amino-terminal extension of ~17 amino acids (30). It was made by a polymerase chain reaction from a full-length LFA-1 α subunit cDNA using a standard protocol. The expressed glutathione S-transferase fusion protein was purified as described previously (17), and was directly cleaved from the glutathione S-transferase moiety using thrombin (Amersham Pharmacia Biotech) while still attached to the glutathione-Sepharose (Amersham Pharmacia Biotech). The electrophoretic mobility of the isolated protein, as determined by SDS-polyacrylamide gel electrophoresis, corresponded to the predicted size of ~25 kDa.

Preparation of Synthetic Peptides
Peptides spanning the LFA-1 I domain were synthesized on a model 4360 Applied Biosystems solid phase synthesizer using 9-fluorophenylamino-

RESULTS
LFA-1 I Domain Blocks Mg2+ but Not Phorbol Ester-induced Adhesion—The role of the I domain in different forms of LFA-1-mediated adhesion to ICAM-1 was investigated by testing the ability of soluble recombinant (Leu111–Ser327)I dom to interfere with Mg2+/EGTA or PdBu-stimulated T cell binding to immobilized ICAM-1. Experimental conditions were chosen such that similar percentages of T cells added to the reaction adhered with both stimuli. The I domain inhibited Mg2+/EGTA-induced adhesion in a dose-dependent manner with half-maximal blocking at 2 μM, but did not interfere with PdBu-induced LFA-1 adhesion (Fig. 1A). Both forms of adhesion were completely blocked by mAbs against ICAM-1 or LFA-1 (Fig. 1B and data not shown). Inactive I domain (control protein, see “Experimental Procedures”) did not interfere with Mg2+-induced adhesion (Fig. 1B) indicating that the blocking effect of the active I domain was not due to nonspecific protein interactions.

One possible reason for the failure of the (Leu111–Ser327)I dom to block PdBu-mediated adhesion is that PdBu might induce a higher overall binding strength than is achieved with Mg2+/EGTA and therefore the binding of Mg2+/EGTA-treated cells would be more easily inhibited than the binding of cells treated with phorbol ester. To investigate this we titrated the amount of anti-ICAM-1 mAb 15.2 required to prevent adhesion (Fig. 2A). The titration curves overlap, showing that both forms of adhesion are inhibited equally by a blocking antibody. To
Role of LFA-1 I Domain in High Affinity Binding to ICAM-1

One of the characteristics of Epitope 24 on T Cell LFA-1—

A. binding of T cells to immobilized ICAM-1Fc following activation with Mg$^{2+}$/EGTA or PdBu in the presence of 1–4 μM ([Leu$^{111}$-Ser$^{327}$]I dom. B. binding of T cells activated with Mg$^{2+}$/EGTA in the presence of mAbs at 10 μg/ml, or 4 μM ([Leu$^{111}$-Ser$^{327}$]I dom or control protein (see “Experimental Procedures”). Data from one representative experiment (n = 4) are shown (mean of triplicates ± S.D.).

Substantiate these data we compared the overall adhesive strength of Mg$^{2+}$/EGTA- and of PdBu-activated cells by a second method. T cells were allowed to adhere to ICAM-1 and then the strength of adhesion was evaluated by a quantitative centrifugal removal assay (35), in which the cells were subjected to increasing relative centrifugal forces (Fig. 2B). The number of cells that remained bound to ICAM-1 at each relative centrifugal force was the same for both forms of adhesion indicating that treatment of cells with either 1 mM Mg$^{2+}$ and 1 mM EGTA or with 50 μM PdBu results in a very similar overall strength of adhesion.

We have previously shown that ([Leu$^{111}$-Ser$^{327}$]I dom binds to ICAM-1 under conditions similar to the Mg$^{2+}$/EGTA activation procedure (17) raising the possibility that the lack of inhibition of PdBu-mediated T cell adhesion is due to the inability of soluble I domain to bind ICAM-1 under the PdBu assay conditions. When investigated by solid phase enzyme-linked immunoasorbent assay, however, the soluble I domain was found to bind ICAM-1 to a similar extent when either PdBu or Mg$^{2+}$/EGTA assay buffers were used (data not shown).

One explanation for the blocking effect of ([Leu$^{111}$-Ser$^{327}$]I dom might be that it competes for ICAM-1 binding with Mg$^{2+}$/EGTA activated LFA-1 but not with PdBu activated LFA-1. Another explanation is that soluble I domain inhibits by interacting with LFA-1 on T cells thus preventing a conformational change essential for high affinity binding induced by Mg$^{2+}$/EGTA. We attempted to discriminate between these two possibilities by preincubating either T cells or immobilized ICAM-1 with ([Leu$^{111}$-Ser$^{327}$]I dom, then removing unbound protein prior to the adhesion assay (Fig. 3). Neither preincubation of T cells nor of immobilized ICAM-1 with soluble I domain caused inhibition of T cell adhesion, therefore preventing assessment, using this approach, of which of the two mechanisms of inhibition applies. The need for the constant presence of the I domain in the assay, however, implies that the interaction of the I domain with ICAM-1 or T cells is of low affinity.

Soluble I Domain Inhibits the Expression of the Activation Epitope 24 on T Cell LFA-1—One of the characteristics of Mg$^{2+}$-induced LFA-1 adhesion is the expression of an activation reporter epitope recognized by mAb 24 (1, 6, 7). Exposure of this epitope is thought to reflect a conformational change in LFA-1 that leads to a high affinity receptor. PdBu-stimulated adhesion, in contrast, is not associated with any significant increase in mAb 24 expression (4). In order to assess whether soluble I domain interacts with T cells under the condition of Mg$^{2+}$-activation, we investigated whether it affects the expression of the mAb 24 epitope. T cells were incubated with ([Leu$^{111}$-Ser$^{327}$]I dom and Mg$^{2+}$/EGTA in the same manner as for an adhesion assay (except that they were not exposed to immobilized ICAM-1) and the expression of mAb 24 was measured by flow cytometry. Soluble I domain inhibited mAb 24 expression in a dose-dependent manner (Fig. 4, A and B). However, soluble I domain did not affect the expression of three mAbs, S6F1, G25.2, and TS2/4, which, like mAb 24, are not expressed on the I domain (12), but are directed against epitopes on the β-propeller domain of LFA-1 (36) (Fig. 4, C and D, and data not shown). These data show that the soluble I domain interferes specifically with expression of a conforma-

B. Leitinger, unpublished results.
tion-sensitive epitope on LFA-1, indicating that there is a direct interaction of the soluble I domain with T cells. This implies that the I domain inhibits T cell binding to ICAM-1 by interacting with LFA-1 and preventing a conformational change necessary for conversion to the high affinity form of this integrin.

The Effect of I Domain Peptides on Mg\(^{2+}\)/EGTA and PdBu-induced T Cell Binding to ICAM-1—To characterize the regions within the I domain required specifically for Mg\(^{2+}\)-stimulation, constituent peptides (Table I) were tested for their ability to interfere with ICAM-1 binding of T cells following the two protocols already mentioned. When T cell LFA-1 was activated by treatment with Mg\(^{2+}\)/EGTA, only peptides I(217–233) and I(238–254) consistently blocked LFA-1 binding to ICAM-1 (Fig. 5A). The average level of inhibition for I(238–254) was 72% and for I(217–233) was 50% at 2 \(\mu M\) (Fig. 5B). It is of interest that I(130–159), which covers the major part of the MIDAS motif (D\(^{137}\)X\(^{138}\)S\(^{139}\)X\(^{140}\)S), was not inhibitory at 2 \(\mu M\) (data not shown). As anticipated, when LFA-1-mediated adhesion was induced by PdBu, I(217–233), I(238–254), and the other peptides tested had no blocking activity (Fig. 5B). This result indicates that I domain regions I(217–233) and I(238–254) are specifically involved in Mg\(^{2+}\)-induced recognition of ICAM-1.

In order to further pinpoint the critical residues, we made a set of overlapping peptides concentrating on the sequence Thr\(^{217}\)-Ile\(^{254}\) (Table I). Peptides corresponding to the N-terminal, mid, and C-terminal regions of the most inhibitory peptide, I(217–233) and I(238–254), i.e. I(238–246), I(242–254), and I(247–258), respectively, all blocked T cell binding to ICAM-1 in an equivalent, dose-dependent manner and were as effective as the parent
peptide I(238–254), whereas control peptide I(256–275) did not interfere at the same concentrations (Fig. 6A). We considered peptide I(242–254) to represent the minimally active sequence. Similarly, peptide I(223–233) corresponded in blocking activity to the parent peptide I(217–233) (data not shown). Unfortunately, peptide I(210–223) had limited solubility and could not be tested. The dose-dependent inhibition of T cell binding to ICAM-1 by I(223–233) and I(238–254) was compared with scrambled versions of both these peptides (see Table I), which did not significantly affect binding (Fig. 6B).

To test whether the peptide inhibition occurs by the same mechanism as soluble I domain inhibition of T cell binding to ICAM-1, we analyzed the effect of peptide I(238–254) on mAb 24 expression. Fig. 7 shows that I(238–254) at 2 mM, but not its scrambled control, inhibited mAb 24 expression. The average level of inhibition of 24 epitope expression was 55% (n = 4). These results indicate that I(238–254) and soluble I domain interact with T cells in a similar manner.

Mapping of Blocking Peptides on the LFA-1 I Domain Structure—When the two function blocking peptides are superimposed on the crystal structure of the LFA-1 I domain (18), they span two adjacent areas. The sequence Glu223–Val233 corresponds on the loop following the 5 helix which terminates in the β strand and the sequence Ala242–Ile254 consists of the β3–α5 loop and follows the loop to the end of the small α5 helix (see Fig. 8A).

![Fig. 5. Effect of I domain peptides on T cell adhesion to ICAM-1.](image)

The main findings of this study are: (i) that soluble LFA-1 α subunit I domain interferes with Mg2+-activated but not phorbol ester-induced LFA-1 adhesion to ICAM-1, indicating that it has a distinct role in adhesion of high affinity LFA-1; (ii) that a key region required for Mg2+-activation is localized to one face of the I domain and includes the α4–β3 loop, the β3–α5 loop, and the α5 helix; and (iii) that this region in the I domain is involved in a conformational change necessary for conversion to the high affinity form of LFA-1. This is the first evidence linking a conformational alteration involving a structural element, in this case the I domain, with a particular activation mechanism for an integrin.

There are several possible explanations for why the soluble I domain blocks binding to immobilized ICAM-1 of the high affinity Mg2+-induced form but not of the low affinity PdBu form of LFA-1. Although both of these methods of activation of LFA-1 have distinct characteristics, they result in a similar overall strength of cell adhesion, thus discounting this as a possible reason for the difference in behavior of the soluble I domain. Another possibility is that the two forms of LFA-1 bind ICAM-1 in distinctive ways; however, there is no evidence so far to suggest more than one type of binding site on ICAM-1. Both forms of adhesion are blocked in a similar manner by anti-ICAM-1 mAb 15.2, as seen in this study, and by mAbs to

**Table I**

| Peptides and amino acid sequences | Amino acid sequence |
|----------------------------------|--------------------|
| First series                     |                    |
| I(130–159)                       | VDLVFLEDGS. MSLQDFEQK. I LDKNKVMK |
| I(193–202)                       | DALLKHVKHM         |
| I(217–233)                       | TVFREELGA. RPDATKV |
| I(238–254)                       | TDGEATDSGN. IDAOKI |
| I(246–275)                       | RYYGIGKRF. QTKEOETNH |
| I(276–295)                       | KFASKPASEF. VKILDIFKL |
| I(296–312)                       | KDLFTELQKK. IYIEGT |
| Second series                    |                    |
| I(223–233)                       | ELGARPAETK. V     |
| I(258–265)                       | TDGEATDSGN        |
| I(242–254)                       | ATDSGNIDAA. KDI   |
| I(247–258)                       | NIAAKOIRIR. YI    |
| Scrambled controls               |                    |
| I(223–233)                       | VKLETGADR. P      |
| I(238–254)                       | DIDKAEAGAD. TAINSOG |
other epitopes on ICAM-1 (32). In addition, both PdBu- and Mg\(^{2+}\)-induced LFA-1 binding are equally sensitive to mutation of Glu\(^{34}\) or Gln\(^{73}\) in ICAM-1. These mutations have been shown to severely disrupt LFA-1 binding (17, 38). Together, these results indicate that the two forms of LFA-1 recognize the same critical features of the ligand-binding motif on ICAM-1.

The I domain contains the major binding site for ICAM-1 (12, 39), with the region implicated in ligand binding surrounding the metal binding MIDAS motif (18–20, 22). It is speculated that this motif may directly contact ligand and that a metal ion may provide a bridge between the ligand and I domain (17, 40–42). Mutagenesis and switching a M for a L segments or mouse for human sequences around the MIDAS motif have further confirmed the ligand binding status of this area (36, 43, 44). The distinctive role for the I domain in Mg\(^{2+}\)-induced adhesion could be explained if the I domain operates as a ligand binding site only for this form of adhesion. However, the same anti-I domain mAbs interfere with both PdBu-induced as well as Mg\(^{2+}\)-induced LFA-1 adhesion (4, 39, 45), which is consistent with the idea that the I domain serves as the ligand binding site for both forms of LFA-1.

One feature that distinguishes the Mg\(^{2+}\)-induced form of LFA-1 from the PdBu-induced form is the expression of an activation reporter epitope recognized by mAb 24 (6, 7), the exposure of which is thought to result from a conformational change. The inhibition of expression of this epitope by soluble I domain suggests that the soluble I domain prevents a conformational change within LFA-1 required for transition to the high affinity form of this integrin. Furthermore, the reduction in 24 epitope expression implies that the inhibitory effect of the soluble I domain occurs via interaction with T cell LFA-1 rather than immobilized ICAM-1. Although the 24 epitope has not been precisely mapped on the LFA-1 \(\alpha\) subunit (6), it is certain that it does not reside within the I domain (12). This suggests that an interdomain movement involving the I domain is associated with exposure of the cryptic 24 epitope located elsewhere in LFA-1 (46) and that we are not measuring a conformational change in the I domain itself.

The inhibitory I domain peptides used in this study mimicked the behavior of the soluble I domain in preventing Mg\(^{2+}\)-induced adhesion, and for the I(238–254) peptide in preventing mAb 24 epitope expression. The LFA-1 I domain crystal structure shows that these inhibitory sequences cover the \(\alpha_4\)-\(\beta_3\) loop (Glu\(^{223}\)–Val\(^{238}\)) and the \(\beta_3\)-\(\alpha_5\) loop to the \(\alpha_5\) helix (Ala\(^{242}\)–Ile\(^{254}\)) (18). These regions are spatially close, lying on the same face of the domain. For other I domain-containing integrins, there is evidence that adjacent loops distal to the MIDAS motif can influence the ability of the integrin to function. For example, the mutation of the \(\alpha_2\)-\(\alpha_3\) loop and \(\alpha_1\)-\(\beta_3\) loop in Mac-1 I domain resulted in an active integrin able to spontaneously bind fibronogen (47). Moreover, mutation of Pro\(^{192}\) or Pro\(^{195}\) within the \(\alpha_2\)-\(\alpha_3\) loop of LFA-1 and Mac-1, respectively, interfered with adhesion to their ligands ICAM-1 (24) and I3B (25).

Recently the seven N-terminal repeat sequences of a typical integrin \(\alpha\) subunit have been modeled as a \(\beta\)-propeller fold (28) homologous to the heterotrimeric G protein \(\beta\) subunit (37). The I domain, which is structurally homologous to the G protein \(\alpha\) subunit, can be positioned on the upper surface of the \(\beta\)-pro-
peller, to one side of the central axis, mimicking the relationship between G protein α and β subunits (28, 29). Looking down on the central axis of the β-propeller (see Fig. 8B), the Ala242–Ile254 sequence protrudes out over the central cavity, and the Glu223–Val233 sequence potentially makes contact with the β-propeller domain. The positioning of these α4 and α5 loop regions suggests that they represent possible contact points between the I domain and the β-propeller. The speculation is that the conformational change in LFA-1 that occurs upon binding to the Glu223–Val233 sequence potentially makes contact with the α4 and α5 subunits of transducin (29). From our data we conclude that the addition of the soluble I domain or peptides interferes with the formation of the high affinity form of LFA-1 in ligand binding following PdBu stimulation. This does not preclude the involvement of I domain of intact LFA-1 activation by PdBu implies that PdBu stimulation does not involve conformational changes involving the I domain, in keeping with the fact that PdBu does not cause an affinity alteration or a detectable conformational change in LFA-1. This does not preclude the involvement of I domain of intact LFA-1 in ligand binding following PdBu stimulation.

Although crystallographic studies show that changes in the tertiary structure of the I domain are possible (21) these would not have been detected in our study. From our data we conclude that the addition of the soluble I domain or peptides interferes with alterations in interdomain or intersubunit associations and thereby prevents changes in the quaternary structure of LFA-1. Our results give rise to the idea that activation of LFA-1 to a high affinity form involves interdomain movement, which alters the association of the I domain with the β-propeller domain, and in this way leads to structural changes that increase the affinity for ligand.

Acknowledgments—We are extremely grateful to Nicola O’Reilly and Dhira Gadhia (Imperial Cancer Research Fund) for the synthesis of the peptides used in this study, to Dan Leathy (Baltimore) for the LFA-1 I domain coordinates, and to Suhail Islam (Imperial Cancer Research Fund) for the PREPI program. We thank our colleagues in the Leukocyte Adhesion Laboratory and M. J. E. Sternberg for valuable discussion in connection with this manuscript.

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