Attenuation of VLA-5 mediated adhesion of bone marrow-derived mast cells to fibronectin by peptides with inverted hydropathy to EF-hands

René Houtman, J. Edwin Blalock†, Matteo Villain†, Andries S. Koster and Frans P. Nijkamp

†Department of Physiology and Biophysics, Cancer Center, School of Medicine and Dentistry, University of Alabama, Birmingham, USA

(Submitted for publication)
Abstract

Release of allergic mediators from mast cells is enhanced by very late antigen-5 (VLA-5)-mediated interaction of these cells with fibronectin. The extracellular part of the VLA-5 α-subunit was previously shown to contain a cation-binding domain, homologous to the EF-hand of calmodulin, which is important for its function. In this report we show that VLA-5-mediated adhesion of bone marrow-derived mast cells can be induced by two different pathways, first by FcεRI-clustering, which dependents on calmodulin activation and extracellular Ca^{2+}, and second by Mn^{2+} stimulation, independent of calmodulin activation and antagonized by Ca^{2+}. To show a role for EF-hands from different proteins in VLA-5-mediated adhesion we used calcium-like peptides (CALP)1 and CALP2, designed to bind to EF-hands based on inverted hydropathy. CALP1 and more potently CALP2 inhibited FcεRI-induced calmodulin-dependent adhesion to fibronectin, in correlation with their affinity for the calmodulin-EF-hand. Interestingly only CALP2 was able to inhibit Mn^{2+}-induced calmodulin-independent adhesion by interfering with an extracellular target, probably VLA-5. We conclude that CALP1 and 2 can inhibit VLA-5-mediated adhesion of mast cells to fibronectin through binding to EF-hands of multiple proteins, and that these peptides can be used as lead compounds for the development of future therapy against allergy.
Introduction

Integrins are a family of heterodimeric cell surface receptors that bind extracellular matrix and cell surface ligands and play a major role in various processes such as inflammation, cell adhesion, migration, proliferation and differentiation. One example is the modulation of mast cell sensitivity for antigenic stimulation through α5β1 integrin very late antigen-5 (VLA-5)-mediated interaction with the extracellular matrix-component fibronectin. Antigenic stimulation of mast cells by crosslinking of the high-affinity IgE receptor (FcεRI) leads to release of various mediators which cause clinical phenomena associated with an allergic response. The observation that VLA-5 can modulate mast cell degranulation makes this receptor a possible target for future therapeutic strategies against allergy and is the topic of the current study.

Integrin ligand-affinity is modulated by a poorly understood mechanism, referred to as inside-out signaling, whereby these receptors are converted from a low- to high-affinity state. It has been reported that the affinity of VLA-5 for fibronectin can be modulated by cations. Mn2+ induces high-affinity of VLA-5 for the Arginine-Glycine-Aspartic acid (RGD) sequence in fibronectin through binding to the cation-binding motif in the extracellular domain of the VLA-5 α-subunit. This effect can be antagonized by Ca2+. The cation-binding motif of VLA-5 is highly homologous to the Ca2+ binding EF-hand motif of calmodulin (CaM). An EF-hand loop is formed by thirteen amino acids of which five play a role in coordinating the binding of Ca2+ by means of their negatively charged side chain. In the EF-hand-like motif of VLA-5, one of the coordinating amino acids is replaced by an uncharged, nonfunctional, residue and it has been postulated that its coordinating function is taken over by the negatively charged side chain of aspartic acid of the RGD ligand sequence. In this view, high-affinity binding is the result of cooperative coordination of the cation by the integrin and the ligand and stabilization of this complex by the cation itself.

In addition to cations, it was recently shown that clustering of FcεRI triggers VLA-5-mediated adhesion of murine bone marrow-derived mast cells (BMMC) to fibronectin. Although it has been established that influx of Ca2+ and CaM activation play a role in the FcεRI signaling cascade leading to degranulation, it is unclear whether they play a role in the FcεRI-induced VLA-5 affinity modulation.

Accumulating evidence suggests that the gross architecture of a peptide or protein is, at least in part, determined by its pattern of hydrophathy and that exact inversion of this pattern will result in a second peptide with a complementary surface contour to the first because the hydrophobic effect is involved in reversed orientation (reviewed by Blalock). Supporting this theory, CALP1 (for calcium-like peptide 1) designed as an amino acid sequence complementary to that encoding a primordial EF-hand, was shown to mimic Ca2+ inducing CaM activity, binding EDTA, inducing smooth muscle contraction and blocking Ca2+ influx through binding to the EF-hands of Ca2+ channels or indirectly via CALP/CaM interaction with such channels (Manion et al. submitted for publication).
Another complementary peptide, CALP2, was designed by computer-assisted optimal inversion of the hydropathy pattern of the EF-hand 4 amino acid sequence of CaM\textsuperscript{43,102,165}. Due to increased length and optimal hydropathy inversion, the affinity of CALP2 for the EF-hand compared to CALP1 was increased 11-fold as determined by surface plasmon resonance detection. Interestingly, increased affinity by increased reciprocity of the pattern of hydropathy and increased length of the peptide resulted in a change of functional activity. Whereas CALP1 shows biological effects similar to Ca\textsuperscript{2+}, CALP2 acts as an antagonist for CaM\textsuperscript{165}.

Influx of Ca\textsuperscript{2+} and subsequent CaM activation play a possible role in Fc\textsubscript{ε}RI-induced affinity modulation of VLA-5 for fibronectin. In combination with the assumption that based on the pattern of hydropathy the EF-hand-like domain of VLA-5 is a potential binding site for CALP1 and CALP2, this suggests several possible targets for these peptides to interfere with mast cell adhesion. In this report we investigate the ability of CALP1 and CALP2 to inhibit VLA-5-mediated adhesion of BMMC to fibronectin induced either by cationic stimulation or Fc\textsubscript{ε}RI clustering.

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**Materials & Methods**

**Cells**

BMMC were obtained as described previously\textsuperscript{84}. Briefly, bone marrow from femurs of Balb/c mice was flushed and cells were cultured at a density of 2 x 10\textsuperscript{5}/ml in complete RPMI (RPMI1640 medium containing 4 mM L-glutamine, 5 x 10\textsuperscript{-5} M β-mercaptoethanol, 1mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1 mM nonessential amino acids) supplemented with 20% (vol/vol) supernatant from Balb/c splenocytes which were stimulated for 7-days with pokeweed mitogen (Sigma Aldrich NV/SA, Axel, The Netherlands) in complete RPMI. Medium was refreshed once per week. In all graphs mean \( \pm \) s.e.m. of quadruplicates of a representative experiment are shown.

**Design and synthesis of the hydropathically complementary peptides**

The design of the eight residue complementary peptide CALP1 (VAITVLVK) was based on the primordial CaM EF hand 4 motif. Selection of the complementary peptide CALP2 (VKFGVGFKVMVF) was carried out using the computer program AMINOMAT\textsuperscript{®} (Tecnogen ScpA, Italy), with an averaging window r=9, a range of inverted hydropathy of 0.8 and considering also eight amino acids of the flanking regions. The program generated 1,417,176 possible sequences, and chose the one with the lowest Q value (0.0068). This value is defined by the formula: \( Q = [(a_i+b_i)/(n-2s)]^{1/2} \), where \( a_i \) represents the moving averaged hydropathy assigned to every amino acid of the target peptide, \( b_i \) represents the moving averaged hydropathy assigned to every amino acid of each of the complementary peptides generated by the program, \( s \) is \((r-1)/2\) (where \( r \) is the number of amino acids considered in the moving window) and \( n \) is the number of residues in the target peptide.

The peptides were synthesized using continuous flow solid phase peptide synthesis with Fmoc chemistry, on a PerSeptive Biosystems 9050 Peptide synthesizer. Pre-
activated Opfp amino acids with HoAt and preloaded PEG-PS resin were used. The peptides were purified by RP-HPLC on a Waters Delta Pack C18 300 A (300 x 39 mm I.D.). The purity of the product was checked by RP-HPLC on a Dynamax C18 (300 x 4.8 mm I.D.) column equilibrated at a flow rate of 1 ml/min and eluted with a linear gradient from 5 to 80% CH3CN containing 0.1% TFA, in 40 min. MilliQ water previously treated with Chelex 100 to remove any Ca2+ was used in the purification. The identity of the peptides was confirmed by TOF-MALDI MS (U.A.B. Core Facility).

Adhesion assay
For Mn2+-induced adhesion, 4-6 weeks old BMMC were washed with PBS, 1 mM EDTA in PBS and Tris-buffered saline (TBS, 2.9 g/L Tris-Cl, 4 g/L NaCl, 0.2 g/L KCl, 0.4 g/L glucose, 0.1 % BSA) and resuspended at a density of 5 x 10^5 cells/ml TBS. For FceRI-mediated adhesion, 4-6 week old BMMC were incubated 1 hr at 37°C in complete RPMI supplemented with supernatant of the anti-DNP IgE producing hybridoma, 26.82^{98}, washed as described above, and cells were resuspended in TBS with or without 1.8 mM CaCl2 as indicated, at a density of 5 x 10^5 cells/ml. NUNC maxisorp 96 wells plates were coated for 3 hrs at 37°C with 200 µl/well 2.5 mg/ml human fibronectin (CLB, Amsterdam) in PBS, followed by 3 times washing with 200 µl/well PBS. 100 µl cells per well were incubated with 50µl of Mn2+ as indicated or 50 µl of DNP-HSA (final concentration: 3 µg/ml, or as indicated, Sigma Aldrich NV/SA, Axel, The Netherlands), and 50 µl of CALP or W7 (Sanvertech B.V., Heerhugowaard, The Netherlands) (final concentrations as indicated in graphs). CALP2-biotin (2 x 10^{-4} M) was preincubated with or without streptavidin (SA, 1 x 10^{-4} M, Boehringer Mannheim B.V.) in TBS for 1 hr at room temperature under constant rotation, before application to the cells. Cells were incubated on a fibronectin-coated plate at 37°C and after 60 minutes non-adhered cells were removed by three times washing with 200 µl/well PBS. For functional blocking of VLA-5, cells were incubated with blocking antibody BMA5 (a generous gift from Dr. B.M.C. Chan, Univ. Western Ontario, Canada)\textsuperscript{45}, one hour before at room temperature, or various concentrations of RGD peptide (Sigma Aldrich NV/SA, Axel, The Netherlands) during incubation on fibronectin. Adhesion was quantified using Cyquant proliferation assay kit (Molecular Probes Europe B.V., Leiden, The Netherlands) according to the manufacturer’s protocol. In short, plates were frozen overnight at –20°C, thawed and incubated for 60 minutes under continuous agitation at room temperature with a DNA-specific fluorescent dye and lysing reagent. Fluorescence was measured at \lambda_{ex}: 485 nm / \lambda_{em}: 530 nm using a Millipore Cytofluor 2350 microplate reader and adhesion was calculated as the residual fluorescence as a percentage of input fluorescence. In all graphs mean ± s.e.m. of quadruplicates of a representative experiment are shown.

Degranulation assay
4-6 Weeks old BMMC were sensitized with anti-DNP IgE as described above, washed and resuspended at 5 x 10^5 cells/ml in Tyrode’s buffer (Life Technologies B.V., Breda, The Netherlands) supplemented with 10 mM HEPES, 1 g/L NaHCO3, 0.1 % BSA, pH 7.2). 100 µl of cells per well of a Costar 96 well plate were incubated with 50 µl of DNP-HSA (final concentration: 30 ng/ml) and 50 µl of CALP or with CALP alone as indicated, for 30 minutes at 37°C. Supernatants were analyzed for β-hexosaminidase content as parameter for mast cell degranulation as described previously\textsuperscript{84}. In short, 50 µl of supernatant was incubated for 60 minutes at 37°C with 50 µl 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma Aldrich NV/SA, Axel, The Netherlands) in citrate buffer (0.1 M, pH 4.5), the reaction was stopped by addition of 100 µl of glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and fluorescence was measured at â: \lambda_{ex}: 485 nm / \lambda_{em}: 530 nm using a Millipore Cytofluor 2350 microplate reader and adhesion was calculated as the residual fluorescence as a percentage of input fluorescence. In all graphs mean ± s.e.m. of quadruplicates of a representative experiment are shown.
360nm/\text{em.}:460nm using a Millipore Cytofluor 2350 microplate reader. Degranulation was calculated as the amount of \( \beta \)-hexosaminidase activity present in the supernatant as a percentage of the total \( \beta \)-hexosaminidase activity present in the cells, determined in lysates of 5 x 10^4 cells. In all graphs mean \( \pm \) s.e.m. of quadruplicates of a representative experiment are shown.

## Results

### Cationic modulation of BMMC adhesion to fibronectin

To investigate if the previously reported cation-mediated modulation of VLA-5 affinity for fibronectin\textsuperscript{16} can be extrapolated to BMMC, extracellular Ca\textsuperscript{2+} was removed by EDTA treatment and cells were incubated with various concentrations of MnCl\textsubscript{2} on immobilized fibronectin. As is shown in figure 1A,

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\caption{Adhesion of BMMC to fibronectin. BMMC were treated with EDTA to remove extracellular Ca\textsuperscript{2+} and incubated with \textbf{(A)} various concentrations of MnCl\textsubscript{2} or \textbf{(B)} 0.2 mM MnCl\textsubscript{2} and various concentrations of CaCl\textsubscript{2} at 37°C on a fibronectin coated 96 wells plate. \textbf{(C)} Cells, prepared for Mn\textsuperscript{2+}-induced adhesion, were incubated with 15 \( \mu \)g/ml BMA5 one our at room temperature before or various concentrations of RGD peptide during the incubation on fibronectin. After 60 minutes non-adhered cells were removed by washing and adhesion was quantified.}
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MnCl\textsubscript{2} dose-dependently induces the adhesion of BMMC to fibronectin. Optimal adhesion of approximately 75% of cells is reached at 0.2 mM MnCl\textsubscript{2}, and this concentration was used in all subsequent experiments. To investigate if Ca\textsuperscript{2+} can antagonize Mn\textsuperscript{2+}-induced adhesion, cells were treated with EDTA and incubated on immobilized fibronectin with MnCl\textsubscript{2} and various concentrations of
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CaCl₂. Figure 1B shows that CaCl₂ dose-dependently reduces MnCl₂-induced adhesion. Previous data showing that adhesion of BMMC to fibronectin is VLA-5-mediated were confirmed by almost complete inhibition of Mn²⁺-induced adhesion of our cells to fibronectin by soluble ligand, RGD peptide, and blocking antibody BMA5 as is shown in figure 1C. From these data we conclude that in BMMC, the regulation of adhesion to fibronectin by cations parallels earlier published data reporting that Mn²⁺ induces a conformational change of VLA-5 to high-affinity for fibronectin, via a mechanism that is antagonized by Ca²⁺.

A role for extracellular Ca²⁺ in FcεRI-induced adhesion of BMMC to fibronectin

To investigate if the presence of extracellular Ca²⁺ is necessary for the induction of adhesion by FcεRI clustering, anti-DNP IgE sensitized BMMC were treated with EDTA and stimulated with DNP-HSA on immobilized fibronectin in CaCl₂-supplemented buffer (final concentration: 1.8 mM) or calcium-free buffer. As is shown in figure 2, stimulation of BMMC via FcεRI dose-dependently induces adhesion to fibronectin in buffer supplemented with Ca²⁺, however the absence of this divalent cation completely abrogates FcεRI-induced adhesion of these cells. From these results we conclude that in addition to FcεRI-induced mast cell degranulation, the presence of extracellular Ca²⁺ is critical for the FcεRI signaling cascade that induces adhesion of BMMC to fibronectin.

A role for CaM in FcεRI- or Mn²⁺-induced adhesion

To study whether CaM plays a role in antigenic or cationic modulation of VLA-5 affinity for fibronectin, we investigated the effect of the CaM inhibitor, W7, on the adhesion induced by either FcεRI clustering or Mn²⁺ stimulation. Anti-DNP IgE sensitized cells were stimulated with DNP-HSA and EDTA-treated cells were stimulated with Mn²⁺ on immobilized fibronectin with various concentrations of W7. As is shown in figure 3, W7 inhibits adhesion of FcεRI-stimulated BMMC, however it has no effect on adhesion of cells stimulated with MnCl₂. From these data we conclude that adhesion of BMMC to fibronectin due to modulation of VLA-5 affinity by either FcεRI clustering or Mn²⁺ stimulation is mediated via distinct signaling pathways that are CaM-dependent and –independent, respectively.
The effect of CALP1 and CALP2 on FcεRI and Mn²⁺-induced adhesion of BMMC to fibronectin

Our data indicate that FcεRI signaling leading to adhesion is Ca²⁺ and CaM dependent and therefore an effect of both CALP1 and 2 on FcεRI-induced adhesion can be expected. Anti-DNP IgE sensitized BMMC were stimulated with DNP-HSA in the presence of various concentrations of either CALP1 or CALP2 on immobilized fibronectin. Indeed, as is shown in figure 4A, we find that both CALP1 and CALP2 are able to dose-dependently inhibit adhesion induced by FcεRI clustering. We observed that CALP2 is a more potent inhibitor than CALP1 (EC₅₀= 3.5 x 10⁻⁵ and 1 x 10⁻⁴ M respectively) and that both peptides fail to induce adhesion of our cells to fibronectin (data not shown).

The observation that Mn²⁺-induced adhesion of BMMC to fibronectin is mediated via a Ca²⁺- and CaM–independent pathway provides us with the opportunity to investigate if CALP1 and CALP2 are able to interfere with adhesion through binding to a target other than the CaM EF-hand. EDTA-treated BMMC were stimulated with Mn²⁺ and various concentrations of either CALP1 or CALP2 on immobilized fibronectin. As is shown in figure 4B, CALP1 does not have an effect on Mn²⁺-induced adhesion, however CALP2 dose-
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dependently inhibits Mn$^{2+}$-induced adhesion of our cells to fibronectin, suggesting a target for CALP2 other than CaM because this pathway is CaM-independent.

Effect of prevention of CALP2 cell entry on its ability to inhibit adhesion

Our data show that CALP 2, as well as CALP1, are able to inhibit the Ca$^{2+}$- and CaM-dependent adhesion, suggesting an intracellular target for these peptides. Only CALP2 is able to attenuate Ca$^{2+}$- and CaM-independent adhesion, and we suggest that the target for CALP2 is the EF-hand-like domain of VLA-5 which is located extracellularly.

To test for an extra- versus intracellular “non-CaM” target, CALP2 was labeled with N-terminal biotin. This peptide was incubated and complexed with SA to prevent it from entering the cells, and to specifically target extracellular sites. BMMC were stimulated on immobilized fibronectin either via FcεRI or with Mn$^{2+}$ together with free or complexed CALP2-biotin. Figure 5 shows that prevention of cell entry largely abrogates the inhibiting effect of CALP2 on FcεRI-induced adhesion, while the inhibiting effect of this peptide on Mn$^{2+}$-induced adhesion of BMMC to fibronectin is largely retained. These data show that besides the ability of CALP2 to inhibit adhesion through binding to intracellular targets such as CaM, this peptide can also attenuate adhesion of BMMC to fibronectin by way of binding to extracellular targets.

**Figure 5. Effect of prevention of cell entry of CALP2 on its adhesion inhibiting potency.** BMMC were stimulated via clustering of FcεRI or with Mn$^{2+}$ and incubated with free CALP2-biotin (no SA) or with CALP2-biotin complexed with streptavidin (SA) on immobilized fibronectin. After 60 minutes non-adhered cells were removed by washing and adhesion was quantified.

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<tr>
<th>% inhibition</th>
<th>IgE</th>
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<tr>
<td>no SA</td>
<td>75</td>
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<td>+ SA</td>
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Effect of CALP1 and CALP2 on mast cell degranulation

Our data show that both CALP1 and 2 inhibit FcεRI-induced adhesion of BMMC to fibronectin, which suggest that these peptides interfere with Ca$^{2+}$-influx and/or CaM activation. To investigate this, we tested the effects of these peptides on FcεRI-degranulation, a mast cell parameter which has been shown to be dependent on Ca$^{2+}$-influx and CaM activation, which should be comparable to those on FcεRI-induced adhesion. Anti-DNP IgE sensitized BMMC were stimulated with DNP-HSA in the presence of various concentrations of CALP1 or CALP2 and the release of granular β-hexosaminidase, as a measure for degranulation, was determined. As is shown in figure 6A, CALP1 and more potently CALP2, parallel to adhesion, dose-dependently inhibited β-hexosaminidase release from BMMC (EC$_{50}$: 2 x 10$^{-5}$
and 6 x 10^{-6} M respectively). Both peptides lacked the intrinsic ability to induce mast cell degranulation, as is shown in figure 6B. From these data we conclude that CALP1 and 2 both attenuate Fc\(_{\varepsilon}\)RI-induced degranulation by interfering with the same targets as those in Fc\(_{\varepsilon}\)RI-induced adhesion, probably Ca\(^{2+}\)-influx and CaM activation.

**Discussion**

The observation that VLA-5 can modulate mast cell degranulation makes this receptor a possible target for future therapeutic strategies against allergy. Our data show that high-affinity of VLA-5 and subsequent adhesion of BMMC to fibronectin can be induced via two separate pathways, first by clustering of Fc\(_{\varepsilon}\)RI, which is dependent on activation of CaM and extracellular Ca\(^{2+}\), and second by Mn\(^{2+}\)-stimulation which is independent of CaM and even antagonized by Ca\(^{2+}\).

Because Fc\(_{\varepsilon}\)RI-induced adhesion as well as degranulation are both dependent on extracellular Ca\(^{2+}\) and activation of CaM, it is logical to assume that both are mediated by the same signaling cascade and therefore affected similarly by CALP1 and 2. Inhibition by CALP2 seemed predictable as this peptide was shown to be an antagonist for CaM activation\(^{165}\) and should therefore have the same affect as the CaM inhibitor, W7. Inhibition of adhesion and degranulation by CALP1 can not be explained by inhibition of CaM, since it was shown that this peptide mimics Ca\(^{2+}\) by activating CaM\(^{38,165}\). Importantly, it was shown...
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recently that CALP1 not only mimics Ca\(^{2+}\) in its binding and activating characteristics towards CaM, but is also able to directly, as well as indirectly, through CaM, block the N-methyl-D-aspartate type calcium channel of rat neuronal cells and the nonselective cation channel (NSCC) of Jurkatt cells which provide negative feedback for Ca\(^{2+}\) influx (Manion et al. Submitted for publication) These channels were shown to be present in mast cells and play a role in mast cell degranulation\(^{72,73,129,130}\) and these receptors could serve as targets for CALP1 in our cells. The closure of cation channels also explains the lack of intrinsic ability of CALP1 to induce degranulation, which is characteristic of Ca\(^{2+}\) ionophores\(^{15}\). Of CALP1 and CALP2 the latter was the most potent inhibitor of both adhesion and degranulation which can be explained by the fact that this peptide has the higher affinity for the CaM EF-hand.

The EC\(_{50}\) of both peptides for inhibition of adhesion was approximately fivefold higher than that for inhibition of degranulation. In our experiments we find that the amount of allergen needed for optimal degranulation is approximately tenfold higher than that for optimal adhesion (unpublished observations), this suggest that a stronger Fc\(\varepsilon\)RI signal is needed for degranulation and is therefore more sensitive to the inhibiting effects of CALP1 and 2.

Mn\(^{2+}\)-induced adhesion is independent of CaM activation and an inhibiting effects of any CALP on adhesion is, therefore, mediated by different targets than those described for the Fc\(\varepsilon\)RI pathway. We find that CALP2 but not CALP1 is able to inhibit Mn\(^{2+}\)-induced adhesion of BMMC to fibronectin, which could be explained by the higher affinity of this peptide for EF-hands. The EC\(_{50}\) of CALP2 for inhibition of Mn\(^{2+}\)-induced adhesion is approximately threefold higher than that for inhibition of Fc\(\varepsilon\)RI-induced adhesion. This suggests either lower affinity of CALP2 for this EF-hand than for CaM, or a higher quantity of EF-hands that need to be bound for a comparable effect. We claim that the target for CALP2 in the inhibition of Mn\(^{2+}\)-induced adhesion is the EF-hand-like domain of VLA-5, and because this motif has a hydropathy profile identical to the CaM EF-hand, it should therefore bind CALP2 with the same affinity. Therefore we propose that the difference in target quantity is the explanation for this observation.

In our experiments using SA-complexed CALP2, a partial inhibition of Fc\(\varepsilon\)RI-induced adhesion can be observed. There are several explanations for this phenomenon. First, the presence of some unbound CALP-2-biotin could attenuate CaM activation, we show that an effect of CALP2 on IgE-mediated degranulation could already be observed at low concentrations. Second, there could be a direct effect of CALP2 on the EF-hand like domain of VLA-5 in Fc\(\varepsilon\)RI-induced adhesion. In the same experiment we also observed a partial attenuation of the inhibiting capacity of complexed CALP2 compared to free CALP2. This could be explained by the fact that the binding of CALP2 to the EF-hand like domain of VLA-5 is partially interfered spherically by the bulky SA-molecule to which it is bound.

Although we have no data showing direct interaction between VLA-5 and CALP2, several arguments support our conclusion that this integrin is targeted during inhibition of CaM independent adhesion. First, based on hydropathy and sequence homology to CaM EF-hands the EF-hand-like domain of VLA-5 forms a potential binding site for CALP2. Second, the affinity of CALP2 and Ca\(^{2+}\) for CaM was shown to be comparable\(^{34,165}\). Accordingly, we find that the EC\(_{50}\) of these agents for inhibition of Mn\(^{2+}\)-induced adhesion is also comparable. Third, although EF-hand motifs are present in numerous proteins that play a role in
various cellular processes\textsuperscript{163}, most of these are localized intracellularly. The observation that our target is located extracellularly, narrows the list of possible candidates drastically and mainly leaves us with the integrins. We and others have shown that in 4-6 weeks old BMMC, adhesion to fibronectin is entirely VLA-5-mediated\textsuperscript{45,87}, which strongly suggests this integrin as the most obvious candidate.

There are several mechanisms by which CALP2 could inhibit VLA-5 binding to fibronectin. Binding of the peptide to the VLA-5 EF-hand-like domain could induce a conformational change of the integrin to a low-affinity state, a mechanism which was also postulated for the antagonizing effect of Ca\textsuperscript{2+} in cation modulation of VLA-5 affinity\textsuperscript{116,118}. Although it was shown that EF-hand-binding to troponin C of CALP2 induces a conformational change of this protein, this conformational change was different from the one induced by Ca\textsuperscript{2+}\textsuperscript{165}. Another possible mechanism is that due to binding to the EF-hand-like domain, which is localized in the ligand-binding site of the integrin, binding of the ligand by the integrin is physically blocked and thereby adhesion is prevented. Previous studies have shown that hydrophobic patterning can be used to tailor make peptide ligands targeted to the EF-hand of CaM and that by increasing affinity the functional activity of the peptide can be modulated from agonistic to antagonistic\textsuperscript{38,165}. In this report we show that these peptides can be applied to block cell adhesion by interfering with CaM and other targets, presumably the EF-hand-like domain of VLA-5. Their ability to block adhesion and thereby modulate the activity of mast cells indicates that these peptides could serve as possible lead compounds for the development of future therapies against allergy and other pathologies in which proteins with EF-hands are involved.