Structural Insights into Calmodulin-regulated L-selectin Ectodomain Shedding

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The L-selectin glycoprotein receptor mediates the initial steps of leukocyte migration into secondary lymphoid organs and sites of inflammation. Following cell activation through the engagement of G-protein-coupled receptors or immunoreceptors, the extracellular domains of L-selectin are rapidly shed, a process negatively controlled via the binding of the ubiquitous eukaryotic calcium-binding protein calmodulin to the cytoplasmic tail of L-selectin. Here we present the solution structure of calcium-calmodulin bound to a peptide encompassing the cytoplasmic tail and part of the transmembrane domain of L-selectin. The structure and accompanying biophysical study highlight the importance of both calcium and the transmembrane segment of L-selectin in the interaction between these two proteins, suggesting that by binding this region, calmodulin regulates in an “inside-out” fashion the ectodomain shedding of the receptor. Our structure provides the first molecular insight into the emerging new role for calmodulin as a transmembrane signaling partner.

Background: Calmodulin inhibits the proteolysis of L-selectin’s extracellular domains through an unknown mechanism.

Results: Calmodulin binds the juxtamembrane and predicted membrane-spanning regions of L-selectin in a calcium-dependent manner.

Conclusion: Binding of calmodulin to the cytoplasmic/transmembrane domain of L-selectin enacts a conformational change in the extracellular domains preventing cleavage.

Significance: Elucidating the mechanisms of L-selectin shedding is critical to understanding leukocyte trafficking.

Cell adhesion molecules expressed on leukocytes and endothelial cells intricately coordinate both the transit of granulocytes and monocytes from the bloodstream to inflamed tissue and the homing of naive lymphocytes to peripheral lymphoid organs (1, 2). Described by the adhesion cascade, several adhesion molecule families act to sequentially recruit and tether leukocytes to the vessel wall and then provide firm adhesion to and subsequent migration through the endothelial cell layer. The selectins are a three-member family of adhesion molecules expressed by leukocytes (L-selectin), platelets (P-selectin), and endothelial cells (E- and P-selectin), which in collaboration with their carbohydrate-presenting ligands execute leukocyte tethering and rolling along the luminal surface of venules that surround peripheral lymphoid organs and sites of inflammation (3).

The three selectins share analogous extracellular domains, including an N-terminal carbohydrate-binding lectin domain known to facilitate the observed rolling behavior of leukocytes despite the hydrodynamic shear force of the bloodstream (Fig. 1A). In contrast, the intracellular tails of these proteins are not conserved, a fact that suggests different modes of regulation and intracellular binding partners for each of the selectins. Although only 17 amino acids long, the cytoplasmic tail of L-selectin has been implicated in concentrating the protein to the tips of microvilli through its interaction with membrane-cytoskeleton cross-linking proteins α-actinin and the ezrin/radixin/moesin (ERM) family (4). The tail is phosphorylated by protein kinase C isozymes as well as Src-tyrosine kinase p56Lck (5, 6) and has a key role in the down-regulation of L-selectin by mediating ectodomain shedding (7).

Upon leukocyte activation through the engagement of G-protein-coupled receptors (GPCRs) in vivo by cytokines and in vitro by phorbol esters, the extracellular domains of L-selectin are rapidly cleaved at a membrane-proximal cut site by tumor necrosis factor alpha-converting enzyme (TACE) (also known as A disintegrin and metalloprotease-17 (ADAM-17)) (8). This regulatory mode is unique in the selectin family to L-selectin. Once cleaved, the extracellular domains remain attached to their ligands or circulate as a soluble fraction in the plasma, whereas the cytoplasmic and transmembrane domains...
and 11 amino acid residues of the extracellular portion remain attached to the cell. A key player in the shedding response to leukocyte activation is the ubiquitous calcium (Ca\(^{2+}\))-binding protein calmodulin (CaM). Known to regulate numerous effectors involved in growth, proliferation, and movement (9, 10), CaM appears to associate constitutively with the L-selectin tail in resting leukocytes and thereby protects the extracellular domains from proteolytic cleavage (11, 12). Artificial activation of leukocytes with phorbol 12-myristate 13-acetate induces the release of CaM from L-selectin and the shedding of the extracellular domains. It has been proposed that CaM exerts its effects by inducing a conformational change in the extracellular domains that renders the cleavage site resistant to proteolysis, a hypothesis supported by the relaxed sequence specificity but length prerequisite displayed by the cleavage site (13, 14).

To further understand the function of CaM in regulating L-selectin ectodomain shedding, we have examined the interaction between these two proteins at the structural level, in turn studying the requirement for Ca\(^{2+}\) as well as the role of the transmembrane domain and juxtamembrane region. We have found that both Ca\(^{2+}\) and a limited region of the L-selectin cytoplasmic domain, including a portion of the predicted membrane-spanning region and critical hydrophilic residues therein, are required for tight binding between CaM and L-selectin. A solution-based NMR structure explains the molecular details of this interaction.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Unlabeled and isotopically enriched CaM was recombinantly expressed in *Escherichia coli* BL21(DE3) cells containing the pET30b(+) expression vector as described previously (15). For isotope labeling, minimal medium containing \(^{15}\)N and either \(^{1}\)H,\(^{13}\)C- or \(^{1}\)H,\(^{15}\)N-labeled glucose in H\(_2\)O or [\(^{2}\)H,\(^{12}\)C]glucose in 99.9% \(^{2}\)H\(_2\)O was used. To create (\(^{1}\)H/\(^{13}\)C-methyl-Met)/\(^{2}\)H/\(^{15}\)N-labeled CaM, 100 mg/liter \(^{1}\)H,\(^{14}\)N,\(^{15}\)N-labeled methionine was added to the culture 1 h prior to induction with isopropyl \(\beta\)-D-1-thiogalactopyranoside (15). CaM samples were purified to homogeneity by ultracentrifugation (90% H\(_2\)O plus 10% \(^{2}\)H\(_2\)O or 99.9% \(^{2}\)H\(_2\)O, and either 4 mM CaCl\(_2\) or 5 mM EGTA. Samples for RDC measurements contained an extra 200 mM KCl and 16 mg/ml filamentous phage PF1 (Asla Biotech Ltd.) to achieve partial alignment. The increased KCl concentration is required to prevent CaM-phage interactions (15, 18) and did not have an effect on the binding affinity of LSEL for Ca\(^{2+}\)-CaM (supplemental Table S1). To avoid the peak broadening that characterizes NMR spectra of the 1:2 interaction, samples of the 1:1 complex, a protocol analogous to that published by Gifford et al. (15) was employed. Briefly, the PALES software was used to determine the domain orientation of CaM in the complex by fitting 109 measured H\(_{\alpha}\)-N RDCs to those back-calculated from CaM-peptide crystal structures deposited in the Protein Data Bank (22). Following the procedure outlined by Ikura and co-workers (18), only RDCs from structured regions of CaM (as defined by backbone chemical shifts)

**NMR Spectroscopy**—NMR experiments were performed on a Bruker Avance 500-MHz NMR spectrometer equipped with a triple resonance, inverse cryogenic probe with a single axis \(z\) gradient. Resonance assignments of the backbone and side chain atoms for CaM in complex with LSEL (L-selectin long peptide) were obtained using through-bond heteronuclear scalar couplings with the standard pulse sequences (15). For assignment of the side chain methyl group of the methionines, three-dimensional HMBC and LRCH experiments that record the long range correlations between the H\(_{\beta}\)/Ce and H\(_{\gamma}\)/Cy atoms were used (16). Resonance assignments as well as intrapeptide NOEs for LSEL were performed on a MicroCal VP-ITC microcalorimeter. Intermolecular NOEs for the (\(^{1}\)H/\(^{13}\)C-methyl-Met)/\(^{2}\)H/\(^{15}\)N-labeled CaM-LSEL complex were obtained from three-dimensional \(^{13}\)C-edited NOEY-HSQC spectra. A mixing time of 100 ms was employed for all NOEY spectra. \(^{1}\)D\(_{\text{NH}}\) RDCs were measured using an IPAP-HSQC (17). NMR samples contained 0.2–0.8 mM \(^{13}\)N-\(^{13}\)C-\(^{15}\)N-\(^{15}\)N, \(^{2}\)H/\(^{15}\)N, or \(^{1}\)H/\(^{13}\)C-methyl-Met)/\(^{2}\)H/\(^{15}\)N-labeled CaM in 20 mM BisTris (pH 6.8), 100 mM KCl, 0.03% NaN\(_3\), 0.5 mM 2,2-dimethyl-2-silapentane-5-sulfonate, 90% H\(_2\)O plus 10% \(^{2}\)H\(_2\)O or 99.9% \(^{2}\)H\(_2\)O, and either 4 mM CaCl\(_2\) or 5 mM EGTA. Samples for RDC measurements contained an extra 200 mM KCl and 16 mg/ml filamentous phage PF1 (Asla Biotech Ltd.) to achieve partial alignment.

The increased KCl concentration is required to prevent CaM-phage interactions (15, 18) and did not have an effect on the binding affinity of LSEL for Ca\(^{2+}\)-CaM (supplemental Table S1). To avoid the peak broadening that characterizes NMR spectra of the 1:2 interaction, samples of the 1:1 complex, a protocol analogous to that published by Gifford et al. (15) was employed. Briefly, the PALES software was used to determine the domain orientation of CaM in the complex by fitting 109 measured H\(_{\alpha}\)-N RDCs to those back-calculated from CaM-peptide crystal structures deposited in the Protein Data Bank (22). Following the procedure outlined by Ikura and co-workers (18), only RDCs from structured regions of CaM (as defined by backbone chemical shifts)
were chosen for analysis: residues 6–72 from the N-terminal domain and 85–144 from the C-terminal domain (18, 23). The structure of bound LSEL15 was solved using CYANA (version 2.0) (24) and subsequently used to derive upper distance limit restraints as well as hydrogen bonding and backbone dihedral angle restraints. CaM-specific information included H_{\alpha}-N RDCs, hydrogen bond restraints based on chemical shift index-derived secondary structure prediction, backbone dihedral angle restraints both obtained from the RDC-selected starting model and calculated from chemical shifts using TALOS (25), and Ca^{2+} ligand restraints. Distances between CaM and LSEL15 were provided by intermolecular NOEs between the methionine methyl groups of CaM and amino acid side chains of the bound peptide. Due to the lack of a suitable internal standard through which to calibrate these spectra, peak overlap, and the non-linear intensity of potential methyl-methyl NOEs, the intermolecular NOE distance restraints were binned into one distance class of 1.8–6.0 Å.

The structure calculation of the Ca^{2+}-CaM-LSEL15 complex was performed using a previously established two-step, low temperature torsion angle simulated annealing protocol in the program Xplor-NIH (version 2.24) (15, 26, 27). Briefly, in the first step, the starting model built from the homologous crystal structure underwent Powell energy minimization and torsion angle dynamics at 200 K followed by simulated annealing in which the temperature was decreased from 200 to 20 K in ΔT = 10 K steps. The lowest energy structure was selected as the starting model for step 2. In the second step, the structure from step 1 underwent torsion angle dynamics at 20 K followed by simulated annealing in which the temperature was decreased from 20 to 1 K in ΔT = 1 K steps. 100 structures were generated in step 2, and the lowest energy structure was selected for further analysis. The structures were validated by the program PROCHECK (28). Molecular graphics were created using the program MOLMOL (29) or PyMOL (version 1.3).

**Molecular Modeling of the CaM-L-selectin-ERM Complex**—A model of this heterotrimer was built by overlapping L-selectin residues Arg^{356}–Lys^{363} shared between the CaM-LSEL15 complex structure presented here and a pre-existing model of the L-selectin cytoplasmic tail bound to the N-terminal domain of moesin (also known as the FERM (band four-point one, ezrin, radixin, moesin homology) domain) and the C-terminal lobe of CaM (30). The overlay produced an ~1-Å backbone root mean square deviation (RMSD) between the shared residues (supplemental Fig. S1). The resulting heterotrimeric complex was energy-minimized for steric clashes using discrete molecular dynamics in the program Chiron (31).

**RESULTS**

**Identification of the L-selectin CaM Binding Site and Ca^{2+} Specificity of the Interaction**—To characterize the interaction between L-selectin and CaM at the molecular level, the roles of Ca^{2+} as well as the juxta- and transmembrane regions of L-selectin were examined. ITC was used to determine binding affinities as well as other thermodynamic parameters for the interactions between both Ca^{2+}-free and Ca^{2+}-bound CaM and two peptides: one encompassing solely the cytoplasmic tail of L-selectin (LSELs) and the second the cytoplasmic tail plus a segment of L-selectin thought to reside in the transmembrane domain (LSELl) (Fig. 1A). Four different titrations were performed: LSEL or LSELs into Ca^{2+}-CaM and LSELl or LSELs into apo-CaM (Fig. 1B). From these titrations, the requirement for Ca^{2+} as well as a portion of L-selectin predicted to be in the membrane-spanning region becomes apparent. The binding curve of LSEL with Ca^{2+}-CaM is characterized by a two-step process, similar to that reported for other CaM-binding peptides (32, 33). The first, which describes the binding of the first peptide to CaM, has a dissociation constant on the order of 10^{-7} M and is driven predominantly by entropic factors (Fig. 1C and supplemental Table S1). The second step, corresponding to the binding of a second LSELl peptide to Ca^{2+}-CaM, has a K_{d} on the order of 10^{-6} M, indicating a significantly weaker affinity, and is an enthalpically favorable event. Interestingly, despite different energetic driving factors and affinities, both LSELl binding sites on Ca^{2+}-CaM are insensitive to an increased salt concentration, suggesting that these interactions are predominantly hydrophobic and are not driven by charge-charge interactions (supplemental Table S1). The occurrence of two binding events is supported by surface plasmon resonance experiments (data not shown). The titration of LSELl into apo-CaM produced a complex endothermic binding curve with multiple LSELl peptides interacting with CaM. Unlike the titration with the Ca^{2+}-bound protein, however, the number and identity of the thermodynamic interactions are not clear. Modeling of these data was largely unsuccessful, although a dissociation constant on the order of 10^{-6} M obtained for the first binding event is not unreasonable (data not shown). The results for LSELl are contrasted with those for LSELs. No significant amount of heat was released or absorbed from the titration of either Ca^{2+}-bound or -free CaM with this peptide that represents solely the cytoplasmic tail of L-selectin.

A similar outcome was seen when the interactions of both Ca^{2+}-bound and apo-CaM with the L-selectin peptides were examined through 15N HSQC titrations, a technique that provides information on ligand-induced global conformational shifts as well as chemical exchange. A 15N HSQC titration again points to two independent LSELl binding sites on Ca^{2+}-CaM that differ significantly in affinity (supplemental Fig. S2A). Up until the 1:1 stoichiometric ratio, this titration is characterized by slow chemical exchange as the 1H(N) backbone resonances corresponding to free Ca^{2+}-CaM disappear and those for complexed CaM appear. In contrast, the binding of the second LSELl peptide to Ca^{2+}-CaM leads to broadening of the 1H(N) resonances often to the point of disappearance. This effect is characteristic of intermediate chemical exchange on the NMR time scale and indicates a significantly weaker equilibrium constant for this interaction. Because slow exchange typically corresponds to an intermolecular dissociation constant on the order of 10^{-7} M or less and intermediate exchange often occurs when K_{J} is ~10^{-6} M, the type of chemical exchange observed for each of the binding events corresponds with the Ca^{2+}-CaM-LSELl affinities obtained through calorimetry. Spectra characteristic of an intermolecular interaction in fast exchange on the NMR time scale were produced with the titration of LSELl into apo-CaM as well as LSELs into either Ca^{2+}- or apo-CaM; the observed backbone 1H(N) resonances are an average
of the free CaM and complexed CaM chemical shifts (supplementary Fig. S2, B–D). Fast chemical exchange is typically observed for intermolecular interactions with a dissociation constant on the order of $10^{-5}$ M or higher. Surface plasmon resonance experiments support an affinity of this magnitude for the binding of LSELs to Ca$^{2+}$-CaM (data not shown).

Next, backbone CSPs were measured to characterize the regions of both apo- and Ca$^{2+}$-CaM affected by the addition of either LSELl or LSELs to the sample. The CSPs were then plotted as a function of residue number for the various CaM and peptide combinations (Fig. 1D). In agreement with the ITC results, the degree and extent of the CSPs observed was the most significant for the complex between Ca$^{2+}$-CaM and LSELl. This combination exhibited significant chemical shift changes throughout CaM with the greatest extent observed in the central linker, a trend seen in other Ca$^{2+}$-CaM complexes (34). In contrast, the other combinations produced CSPs of a much smaller magnitude and located predominantly in the C-terminal lobe of CaM. Unlike the CSPs measured for Ca$^{2+}$-CaM-LSELl, which represent the 1:1 complex, the CSPs for Ca$^{2+}$-CaM-LSELs, apo-CaM-LSELl, and apo-CaM-LSELs were derived from peptide-saturated complexes.

Taken together, the biophysical data for this interaction highlight the roles of Ca$^{2+}$ and a portion of L-selectin’s predicted membrane-spanning region in creating a significant interaction between CaM and this protein. Although binding between CaM and L-selectin was detected in the absence of Ca$^{2+}$ or with solely the cytoplasmic tail (using apo-CaM or LSELs, respectively) these interactions are in fast exchange on the NMR time scale, produce small and localized CSPs in the CaM backbone 1H(N) resonances, and either release/absorb very little heat as detected by ITC or suggest nonspecific binding. These observations are characteristic of significantly weaker interactions that are electrostatically driven. As such,
we suspect that the interaction between Ca\(^{2+}\)-CaM and LSEL\(\text{I}\) is the functionally important form of this complex.

**Structure Determination of Ca\(^{2+}\)-CaM Complexed 1:1 with L-selectin**—To understand the interaction between CaM and L-selectin at the molecular level, a solution NMR-based structure determination of Ca\(^{2+}\)-CaM bound 1:1 with LSEL\(\text{I}\) was carried out. Standard NMR-based structure determination of protein complexes proceeds via a series of experiments that first assign the resonances of CaM and the bound peptide and then independently determine the secondary and tertiary structures of CaM and the peptide through NOE-based distance restraints and finally solve the complex through the collection and analysis of intermolecular NOEs between CaM and the peptide. However, due to peak broadening observed even for the 1:1 complex between Ca\(^{2+}\)-CaM and LSEL\(\text{I}\), this method was not viable. Instead, a reduced structure determination protocol was employed, in which the importance of methionine and the role that it plays in Ca\(^{2+}\)-CaM-target interactions is exploited and combined with the use of backbone RDCs to solve the structure of the CaM-L-selectin complex (15).

A comparison of solved CaM-target peptide structures reveals that it is through variation in domain orientation and not in backbone conformation that CaM binds its numerous targets (23). Consequently, RDC analysis can be used to determine the orientation of the two lobes of CaM when bound to LSEL\(\text{I}\) and provide a homologous starting model for the structure of CaM in the complex (18, 35). Because RDCs are a function of bond vector orientation in relation to the external magnetic field, they are particularly useful for orienting two domains of a protein with respect to each other (36). Here, the correlations between measured H\(_{\alpha}\)-N RDCs for CaM bound to LSEL\(\text{I}\) and theoretical RDCs back-calculated from crystal structures deposited in the Protein Data Bank were determined (supplemental Table S2). The closest agreement obtained was to those back-calculated from the CaM-Ca(v)I.L2 Ca\(^{2+}\)-channel (Protein Data Bank code 2F3Y) (37). This published structure was subsequently used as the starting model for CaM in complex with L-selectin.

To facilitate the collection of NOE-based distance restraints required to determine the tertiary structure of the complex, the synthetic peptide employed was shortened from a 24-mer to a 15-mer. This peptide, LSEL\(\text{I}\), bound to CaM in the same manner as LSEL\(\text{I}\) (Fig. 2, A and B, and supplemental Table S1) (results not shown) and produced spectra with narrower line widths, easing the process of resonance assignment. In total, 189 non-degenerate intrapeptide NOEs provided the distance restraints for calculation of the bound LSEL\(\text{I}\) structure by the automatic assignment and structure calculation program CYANA (version 2.0). The 20 lowest structures have a backbone RMSD of 0.71 Å (Fig. 2C). From the lowest energy structure, 171 distance restraints were supplied for the full complex structure calculation (Table 1).

The standard experiments used to assign the key aliphatic side chain methyls and collect intermolecular NOEs (a NOEY-\(\text{H}^{-1}\text{H},\text{HSQC}\) and its F1\(-1\text{H},\text{C}\),\(^{15}\text{N}\) isotope-filtered version, respectively) produced ambiguous resonance assignments and difficulties in assigning NOE cross-peaks. Unambiguous intermolecular NOEs are required to tie the complex together, providing distance restraints as well as domain translational information not supplied by RDCs. To solve this problem, a labeling scheme analogous to that of methyl-labeling isoleucine, leucine, and valine against a perdeuterated background was employed (38). Using CaM \(\text{H}^{-1}\text{H},\text{C}^{13}\), labeled on the methyl groups of methionine but otherwise isotopically \(\text{H}^{-1}\text{H},\text{C}^{12}\) \((\text{H}^{-1}\text{H},\text{C}^{-1}\text{C}-\text{methyl-Met})/\text{H}^{-1}\text{H},\text{N}-\text{labeled CaM}\), the high number of methionine residues in CaM (9 of 148 amino acid residues) and their location in the hydrophobic pockets were exploited to collect intermolecular NOEs between CaM and the bound peptide (15, 39). Altogether, 69 intermolecular NOEs between CaM and LSEL\(\text{I}\) as well as six intermethylene NOEs were measured (Fig. 2, D and E, and Table 1).

The solution structure of the 1:1 interaction between Ca\(^{2+}\)-CaM and LSEL\(\text{I}\) was calculated using a two-step, low temperature torsion angle simulated annealing protocol (15, 26). In this protocol, the RDC-selected homologous CaM starting structure was refined using backbone H\(_{\alpha}\)-N RDCs and experimentally determined dihedral angle restraints, peptide-specific CYANA-derived distance and dihedral angle restraints, hydrogen bonding restraints for both molecules, and intermolecular NOEs (Table 1). The 30 lowest energy structures calculated in this manner have a backbone and heavy atom RMSD in the folded regions of 0.318 and 0.378 Å, respectively.

**Solution Structure of Ca\(^{2+}\)-CaM Complexed 1:1 with the LSEL\(\text{I}\) Peptide and Comparison with Other Complexes**—The lowest energy solution structure of the 1:1 complex of Ca\(^{2+}\)-CaM-LSEL\(\text{I}\) is presented in Fig. 3A. The bound peptide is \(\alpha\)-helical over almost its entire length (Phe\(^{356}\)-Lys\(^{862}\)), retaining the conformation calculated by CYANA (Fig. 2C). The N- and C-terminal lobes of CaM interact with the N- and C-terminal portions of the LSEL\(\text{I}\) peptide, respectively, to form a complex in parallel orientation relative to the bound peptide. The aliphatic side chain of LSEL\(\text{I}\) residue Ile\(^{352}\) projects into the methionine-lined hydrophobic pocket of the N-lobe of CaM, anchoring the N-terminal portion of the peptide to this lobe of CaM (Fig. 3, B and C). In an analogous manner, the side chains of both Leu\(^{354}\) and Leu\(^{358}\) point into the hydrophobic pocket of the C-terminal lobe of CaM, anchoring the C-terminal portion of the peptide through a “double-anchor” motif (40). For all three key residues, a significant number of NOEs were collected between their side chains and the methionine probes that line the hydrophobic pockets of CaM. The basic residues downstream from Leu\(^{358}\) interact with the exit channel of the C-terminal lobe of CaM. This channel is more negatively charged than the entrance channel formed by the CaM N-terminal lobe; thus, the parallel orientation observed is energetically favorable because the more hydrophobic or more positively charged halves of the peptide interact with the corresponding lobes of CaM. The combination of hydrophobic and electrostatic interactions between CaM and LSEL\(\text{I}\) probably explains the apparent insensitivity of this complex to increased salt concentration (supplemental Table S1).

Due to the spacing of the peptide anchors, the LSEL\(\text{I}\) peptide binds Ca\(^{2+}\)-CaM with a “1–3/7” motif. Although this particular spacing is novel, the motif is a hybrid of the 1–3 and 1–7 anchor positions seen in the MARCKS and NMDA receptor Ca\(^{2+}\)-CaM complexes, respectively (Protein Data Bank codes...
Counterintuitively, the side chain of Trp353 does not seem to serve as an anchor residue for either lobe of CaM and instead resides between them near the central linker (Fig. 3A), a position supported by fluorescence spectroscopy studies using selenomethionine-substituted CaMs (results not shown) (41). In comparison with the canonical 1–14 spacing seen for CaM bound to the myosin light chain kinase peptides, this 1–3/7 anchor spacing is much tighter, the consequence of which is seen in the close proximity of the two lobes of CaM. If this proximity is measured as the distance between the centers of mass for each lobe, the LSEL_{15} complex is more compact not just compared with myosin light chain kinase or the other kinase complexes, but also more compact than the NMDA receptor or MARCKS complexes (19.7 Å versus 20.6 and 21.1 Å, respectively) (supplemental Table S3). The entire backbone RMSD of Ca^{2+}/H11001-CaM/LSEL_{15} is quite large when compared

FIGURE 2. NMR-based structure determination of 1:1 Ca^{2+}-CaM-L-selectin complex. A, bar graph comparing the determined thermodynamic parameters for the calorimetric titration of Ca^{2+}/H11001-CaM with LSEL_{1} or LSEL_{15}. Data are represented as mean ± S.E. (error bars) for three independent experiments. B, overlays of ^1H HSQC spectra of Ca^{2+}-CaM complexed 1:1 with LSEL and LSEL_{15}. C, NOE-based solution structure of LSEL_{15} when bound to Ca^{2+}-CaM. The final 20 structures generated by CYANA (version 2.0) are aligned over residues 350–356. LSEL_{15} is an ^α-helix from Phe350 to Lys356 in the lowest energy structure. The anchor residues of Ile352, Leu354, and Leu358 are indicated in hot pink. D, ^13C HSQC spectrum of Ca^{2+}/H11001-bound (1H/13C-methyl-Met)/2H/15N-labeled CaM either peptide-free or complexed 1:1 with LSEL_{15}. Due to the selective labeling scheme, only the methyl groups of the CaM methionines are detected. Eight of the nine methionine amino acid residues of CaM line the hydrophobic binding pockets of CaM, and changes in both line width and chemical shift of the methyl group of these residues point to the involvement of both lobes in the interaction with LSEL_{15}. E, representative strip plots from ^13C-edited NOESY-HSQC spectra of the 1:1 complex of (1H/13C-methyl-Met)/2H/15N-labeled CaM and LSEL_{15}. The intermolecular NOEs between the methyl groups of Met-72 (left) and Met-144 (right) and identified protons on LSEL_{15} are indicated. Also seen are intramethionine NOEs between the He and Ha protons.
with other known structures; however, on a domain by domain basis, this difference is much smaller, and the tertiary structure is in quite good agreement, reflecting the versatility of the flexible linker in accommodating widely different CaM-binding domains.

**Molecular Model for the Simultaneous, Non-competitive Binding of CaM and Ezrin/Moesin FERM Domain to the Tail of L-selectin**—In addition to and concurrent with CaM, members of the ERM family of membrane-cytoskeleton cross-linkers bind the cytoplasmic tail of L-selectin. By linking L-selectin's cytoplasmic tail with the actin cytoskeleton, the ERM family—In addition to and concurrent with CaM, members of the ERM family of membrane-cytoskeleton cross-linkers bind the cytoplasmic tail of L-selectin. By linking L-selectin's cytoplasmic tail with the actin cytoskeleton, the ERM family

**TABLE 1**

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- Predicted from TALOS (25).
- Obtained from the lowest-energy bound LSEL15 structure calculated by CYANA-2.0.
- Based on CSI-defined secondary structure.
- Based on secondary structural elements common to the 20 lowest energy structures of bound LSEL15 calculated by CYANA-2.0.
- Performed using PROCHECK on structure regions 6–144 and 350–362.

Calmodulin-regulated L-selectin shedding

**Phosphorylation of the L-selectin Tail Does Not Directly Regulate the Interaction with CaM**—It has been proposed that for L-selectin, as for many other proteins, the PKC phosphorylation and CaM-binding sites overlap and that phosphorylation of serine residues in L-selectin's cytoplasmic tail by PKC leads to disassociation of CaM and thus shedding of the extracellular domains (11, 12). GPCR stimulation activates PKC pathways, and in phosphol-ester- or chemoattractant-activated leukocytes, PKC isoforms have been found interacting with the L-selectin tail, and this sequence is phosphorylated on evolutionarily conserved serine residues (Ser\(^{364}\) or Ser\(^{367}\)) (Fig. 5A) (5). However, binding studies performed through both ITC and \(^{15}\)N HSQC titrations indicate that phosphorylation on these residues does not impede the ability of CaM to bind L-selectin (Fig. 5B, supplemental Fig. S3, and supplemental Table S1). This observed insensitivity to phosphorylation agrees with our presented structural data on the 1:1 CaM-L-selectin complex because both Ser\(^{364}\) and Ser\(^{367}\) are outside of L-selectin’s CaM-binding domain. Instead, our model of the heterotrimeric complex suggests that phosphorylation could affect the interaction between L-selectin and the N-terminal domain of ezrin/moesin. Both Ser\(^{364}\) and Ser\(^{367}\) are close to and involved in the interaction with moesin (Fig. 5C), and phosphorylation on either residue could lead to a change in the ability of ezrin/moesin to bind L-selectin and indirectly influence CaM binding.

**Structural Characterization of the 1:2 Ca\(^{2+}\)-CaM/LSEL15 Interaction**—Due to significant peak broadening observed upon the addition of a second molar equivalent of LSEL15 to Ca\(^{2+}\)-CaM (supplemental Fig. S2), the 1:2 complex is not amenable to NMR-based structure determination. However, on the basis of observed signal broadening in \(^{15}\)N HSQC spectra, we have determined regions of Ca\(^{2+}\)-CaM affected by the interaction with the second LSEL15 peptide. The broadening of backbone cross-peak signals in \(^{15}\)N HSQC spectra of

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**TABLE 1**

<table>
<thead>
<tr>
<th>Experimental restraints and structural statistics of the lowest energy structure of Ca(^{2+})-CaM-LSEL15</th>
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<th>Values</th>
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<td>No. of experimental restraints</td>
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<tr>
<td></td>
<td>Residues in disallowed regions (%)</td>
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</tbody>
</table>

- Predicted from TALOS (25).
- Obtained from the lowest-energy bound LSEL15 structure calculated by CYANA-2.0.
- Based on CSI-defined secondary structure.
- Based on secondary structural elements common to the 20 lowest energy structures of bound LSEL15 calculated by CYANA-2.0.
- Performed using PROCHECK on structure regions 6–144 and 350–362.
peptide-saturated Ca\(^{2+}\)/H\(_{11001}\)-CaM (a 1:2.5 molar ratio) is not uniform, and analysis of normalized peak height (peak height as it compares to that in the 1:1 spectra) can quantify the degree of signal loss on a per residue basis (Fig. 6 and supplemental Fig. S2A). Many \(^{15}\)N HSQC cross-peaks originating from backbone resonances in the second and third EF-hands as well as the central linker are broadened to the extent that they have disappeared (0% normalized peak height). As mentioned earlier, this observation is characteristic of an intermediate exchange regime and suggests that these residues are participating in a medium strength interaction. When the degree of signal loss is mapped onto the 1:1 structure of Ca\(^{2+}\)/H\(_{11001}\)-CaM, those residues most affected by the binding of the second peptide suggest a secondary interface on the top of the CaM molecule away from the Ca\(^{2+}\)/H\(_{11001}\)-binding EF-loops (Fig. 6B). This secondary interface is made up of a negatively charged face on the CaM C-lobe and a hydrophobic groove found on the back of the N-lobe. It is conceivable that the more hydrophobic portion of LSEL\(_{15}\) or LSEL\(_{1}\) could bind to the groove while the positively charged sequences of these peptides bind the negative face. Although this second binding site involves both lobes of CaM, it is distinct from the hydrophobic pockets that create the “classical” Ca\(^{2+}\)/CaM binding site through which the first LSEL\(_{1}\)/LSEL\(_{15}\) peptide interacts.

**DISCUSSION**

The presented study examines at a biophysical and structural level the interaction between CaM and L-selectin. We have determined the solution structure of Ca\(^{2+}\)/CaM bound 1:1 to a synthetic peptide representing the cytoplasmic/transmembrane domain of L-selectin. Due to broad and missing signals, particularly from critical side chain methyl groups, initial attempts to determine a high resolution structure of this complex through a traditional NOE-based strategy were unsuccessful. Therefore, a novel structure determination protocol was employed in which backbone H\(_{2}\)N-RDCs were used to determine the domain orientation of the two lobes of CaM, and intermolecular NOEs between the CaM methionine methyl groups of CaM (boxed) and the LSEL\(_{15}\) peptide. Key interacting residues Ile\(^{352}\), Phe\(^{353}\), and Leu\(^{358}\) of LSEL\(_{15}\) are indicated in red.

**FIGURE 3. Structural basis for the 1:1 interaction of Ca\(^{2+}\)/CaM with L-selectin.** A, ribbon representation of the lowest energy structure of the complex. The N- and C-terminal lobes of CaM are shown in purple and gold, respectively, and the LSEL\(_{15}\) peptide is indicated in green. The side chains of LSEL\(_{15}\) amino acid residues Ile\(^{352}\), Leu\(^{354}\), and Leu\(^{358}\) are highlighted in red. Together, these three side chains anchor the LSEL\(_{15}\) peptide to CaM with a “1–3/7 motif.” The side chain of Trp\(^{353}\) is in teal. The four Ca\(^{2+}\) ions are indicated as yellow spheres. B, closer view of the interface between CaM and the anchoring amino acid side chains of LSEL\(_{15}\). Right, interaction of the CaM N-lobe with Ile\(^{352}\) of LSEL\(_{15}\); left, the CaM C-lobe with Leu\(^{354}\) and Leu\(^{358}\) of LSEL\(_{15}\). In each panel, the helices and side chain carbons are colored purple/navy, gold/black, or green/red for the N- or C-lobe of CaM or LSEL\(_{15}\), respectively. Methionine methyl group protons that serve as probes for intermolecular NOEs between CaM and LSEL\(_{15}\) are colored green. C, schematic showing the observed intermolecular NOEs between the methionine methyl groups of CaM (boxed) and the LSEL\(_{15}\) peptide. Key interacting residues Ile\(^{352}\), Phe\(^{353}\), and Leu\(^{358}\) of LSEL\(_{15}\) are indicated in red.
proteins, as suggested by both ITC and CSPs (Fig. 1). It is through the hydrophobic patches exposed in the Ca\(^{2+}\)/H11001-replete state that the two lobes of CaM bind the hydrophobic side chain anchors of L-selectin (Fig. 3). Two of these anchors, Ile\(^{352}\) and Leu\(^{354}\), are located in the predicted membrane-spanning region of L-selectin (the third, Leu\(^{358}\), is in the established cytoplasmic tail), indicating that CaM must either pull this region out of the membrane, perturb the membrane bilayer structure, or do a combination of both to bind L-selectin (discussed below).

At the physiological level, the requirement for Ca\(^{2+}\) is a bit unexpected. The interaction between CaM and L-selectin is thought to exist in resting leukocytes, a state in which the cytoplasmic Ca\(^{2+}\) concentration is 10\(^{-7}\) M and CaM is in either an apo or partially Ca\(^{2+}\)-bound form. However, both the absence of the communoprecipitation of CaM with L-selectin in the presence of the Ca\(^{2+}\)-chelator EDTA (12) and the directly induced shedding of L-selectin from both neutrophils and lymphocytes upon the addition of pharmacological Ca\(^{2+}\)-CaM inhibitors (11) point to the in vivo Ca\(^{2+}\) requirement of this interaction. The contemporary belief in spatial/temporal aspects of Ca\(^{2+}\) signaling, conceptualized by microdomains, could help to explain this discrepancy. As Ca\(^{2+}\) enters the cytoplasm, it produces a local plume, a restricted domain of increased Ca\(^{2+}\) concentration that on its own or in combination to produce larger microdomains can regulate specific cellular processes in different regions of the cell (45). The time scale of the Ca\(^{2+}\) influx is also critical because brief transients lead to a more restricted increase influencing effectors solely in the nearby environment, contrasting with the global concentration change and universal effector activation that results from a sustained influx. The engagement of ligands by L-selectin’s lectin domain has been shown to cause an internal store-derived increase in cytosolic Ca\(^{2+}\) through a pathway independent of heterotrimeric G-proteins (46). However, the magnitude and duration of this Ca\(^{2+}\) influx is smaller and shorter than that seen with GPCR engagement. The Ca\(^{2+}\) “puffs” produced could lead to a concentration high enough to allow Ca\(^{2+}\)-CaM to bind L-selectin but localized and of a duration short enough to distinguish the effects from GPCR- or immunoreceptor-based cell activation characterized by a global Ca\(^{2+}\) increase that lasts for hours and triggers L-selectin ectodomain shedding (47, 48). Furthermore, CaM, like other members of the EF-hand superfamily, exhibits a strong increase in affinity for Ca\(^{2+}\) in the presence of a target protein (49). Therefore, it is reasonable to expect that in the presence of L-selectin, a significant portion of CaM may be Ca\(^{2+}\)-bound even at 100 nM Ca\(^{2+}\), interacting with L-selectin in its Ca\(^{2+}\)-bound state in the resting cell.

The solution structure highlights the role of L-selectin’s juxtamembrane region in the interaction between L-selectin and CaM; the C-lobe of CaM binds predominantly to this sequence
that includes the anchor residue Leu358. This finding is supported by both in vitro and in vivo experiments. Coimmunoprecipitation studies with either mapping antibodies or truncation mutants indicate that the eight C-terminal amino acids of the tail are not required for binding (11, 12), a fact mirrored in the similar CaM-binding abilities of the LSEL15 peptide, and LSEL15 peptides (Fig. 2 and supplemental Table S1). In vitro peptide binding array and in vivo mutant-based shedding studies as well as L-selectin splice variants found in mice further define cytoplasmic amino acid residues 356-RLRLKKG-361 as critical to CaM binding (Fig. 5A) (12, 13, 50, 51). Communoprecipitation experiments on transfected L-selectin mutants support the role of Leu358 and Lys359 in anchoring the C-lobe of CaM and forming favorable interactions with this lobe’s negatively charged exit channel, respectively (11).

Due to large degrees of error associated with the derived constants and related to the small magnitude of the observed enthalpy change (the data are significantly scattered off the smooth theoretical curve), we did not report curve fitting of the ITC data for the binding of LSEL15 to CaM (Fig. 1B); however, an affinity on the order of $10^{-7}$ M was obtained using surface plasmon resonance (data not shown). A recent publication by Deng et al. reported a dissociation constant on the order of $10^{-8}$ M for the interaction between CaM and the cytoplasmic tail of L-selectin (52). This value, similar for both Ca$^{2+}$- and apo-CaM, was determined using ITC and a peptide representing the cytoplasmic domain plus one amino acid of the putative transmembrane helix (L-selectin residues Ala355–Tyr377). Both the peptide studied by Deng et al. (52) and LSELs exhibit an exothermic enthalpy change of similar magnitude upon binding CaM; however, differences in chemical exchange behavior suggest that the peptide studied by Deng et al. binds CaM with higher affinity than our peptide LSELs; the binding between LSELs and CaM occurs in fast chemical exchange on the NMR time scale (supplemental Fig. S2), whereas the peptide studied by Deng et al. (52) bound in intermediate exchange. The addition of hydrophobic Ala355 to the cytoplasmic domain peptide most likely explains the difference in affinity and exchange behavior of the two peptides.

Despite significant research focused on the ability of L-selectin’s cytoplasmic juxtamembrane region to bind CaM, the predicted membrane-spanning region has largely been ignored, and yet the present study provides structural evidence that this sequence is critical for the interaction of these two proteins. Amino acid residues Ile352 and Leu354 that serve to anchor the N- and C-lobes of CaM originate in the first turn of the proposed transmembrane helix, and the neighboring bulky, hydrophobic amino acid residues found in this sequence of L-selectin form favorable interactions with the hydrophobic patches exposed in Ca$^{2+}$-CaM. It is likely that these hydrophobic patches create an environment of characteristics similar to those found in the membrane, a fact highlighted by the lipid binding abilities of CaM (23). In vitro support for this structure is provided by Deng et al. (52), who observed FRET between a fluorescence probe attached to CaM and Trp353 when a peptide representing the external cleavage site, transmembrane helix, and cytoplasmic domain of L-selectin was reconstituted in a phosphatidylcholine bilayer. The interaction of CaM with a sequence predicted to extend by ~6 amino acids into L-selectin’s putative membrane-spanning region is not a unique...
occurrence. Ca$^{2+}$- and integrin-binding protein 1 binds the cytoplasmic tail of the αIIB subunit of the platelet integrin αIIBβ3 through a sequence both juxtamembrane to and extending by six amino acids into the predicted membrane-spanning region (53–58). Furthermore, bioinformatic analysis presented here indicates that several other transmembrane receptors down-regulated through Ca$^{2+}$/CaM-dependent ectodomain shedding have CaM-binding motifs that extend into the transmembrane domain (Fig. 7) (59–61). This suggests a common mode of CaM-mediated regulation that, to date, has yet to be studied in vivo.

The CaM-L-selectin solution structure provides insight into a mechanism through which CaM regulates L-selectin ectodomain shedding. The interaction between the C-lobe of CaM and the cytoplasmic tail is driven through electrostatic forces, as suggested by the ITC and NMR titration data and by the fact that both apo- and Ca$^{2+}$-CaM bind this region (Fig. 1D) (11, 30, 52). CaM would be electrostatically attracted to this part of

![Diagram](https://via.placeholder.com/150)
L-selectin dangling down from the plasma membrane, and in the Ca\(^{2+}\)/H\(_{11001}\)-bound state, it would bind both the positive charges and Leu\(_{358}\) found in the juxtamembrane region. The importance of this event is seen by the fact that CaM does not co-immunoprecipitate with L-selectin\(_{358}\)LKK\(_{360}\)/358EEE\(_{360}\) cytoplasmic or Arg\(_{356}\) truncation mutants (12). Once bound, due to the dynamic nature of the lipid membrane, CaM would “sense” anchor residues Ile\(_{352}\) and Leu\(_{354}\) present in the first helical turn of L-selectin’s proposed membrane-spanning region. By binding these residues and their neighboring hydrophobic amino acids, CaM could effect a conformational change on the extracellular side of the membrane, preventing proteolytic cleavage. Upon cell activation, CaM disassociation would release this portion of L-selectin enacting a structural rearrangement, exposing the TACE cleavage site (Fig. 8).

This inside-out signaling hypothesis is supported by both in vivo and in vitro evidence. First, the sequence of L-selectin’s transmembrane domain and juxtamembrane region is conserved throughout evolution (Fig. 5A). Second, considerably less CaM is associated with the 6-kDa trunk of L-selectin retained in the membrane following cleavage, pointing to its dissociation as a regulatory mechanism (12). Third, cytoplasmic mutations that abrogate the interaction with CaM affect the binding of an antibody against L-selectin’s extracellular membrane-proximal cleavage site (11). This antibody can bind when CaM is bound to the cytoplasmic tail, suggesting that interacting partners of the cytoplasmic portion of L-selectin affect the conformation of its extracellular sequence. Finally, studies on L-selectin’s membrane-proximal truncation mutants point to the relaxed sequence specificity but length and conformational requirement of the TACE protease because both truncations as well as proline substitutions prevented proteolysis (51). It appears that a membrane-proximal stalk of sufficient length is required to permit TACE access to the substrate, and the bind-
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ing of CaM to the cytoplasmic tail could cause a conformational change on the extracellular side of the membrane that shortens the amount of stalk available, preventing cleavage.

A key unanswered component of this proposed mechanism is the cause of CaM dissociation from the cytoplasmic tail of L-selectin. Our results suggest that the cause is neither a Ca\(^{2+}\)-induced conformational change in CaM nor, as has been widely speculated, phosphorylation of serine residues found in the cytoplasmic tail of L-selectin (Fig. 5, supplemental Fig. S3, and supplemental Table S1). Although ITC experiments indicate that L-selectin binds Ca\(^{2+}\)-CaM with an initial dissociation constant on the order of 10\(^{-7}\) M (supplemental Table S1), it is possible that the in vivo affinity is not as high due to other energetic considerations, such as potential membrane interactions and the presence of other proteins bound concurrently to the cytoplasmic tail. As a result, the sustained Ca\(^{2+}\) influx and resulting global concentration increase mediated by CRAC (calcium release-activated calcium) channels upon GPCR or immunoreceptor engagement may expose more abundant and higher affinity CaM binding sites, such as calcineurin and the CaM kinases, two families of proteins up-regulated upon cell activation. Alternatively, the induced close proximity of phosphatidylserine or phosphorylated phosphatidylinositol has been suggested to play a role in CaM disassociation (52).

Treatment of leukocytes with lineage-specific activating stimuli induces PKC-dependent phosphorylation of Ser\(^{364}\) and/or Ser\(^{367}\) in the cytoplasmic domain of L-selectin. The result of this phosphorylation is 2-fold. First, an increase in L-selectin’s ligand binding activity is observed (62, 63). This response is immediate and precedes the ectodomain shedding of L-selectin that occurs minutes following cell activation. Second, mutagenesis-based studies have shown serine phosphorylation to be key to cell activation-linked ectodomain shedding (43). The introduction of one or two highly negatively charged groups significantly alters the local surface characteristics of that region of the protein, typically inducing or preventing intra- or intermolecular interactions. Because serine phosphorylation does not directly affect the ability of CaM to bind L-selectin (Fig. 5B, supplemental Fig. S3, and supplemental Table S1), it probably plays a role in the regulation of L-selectin’s other cytoplasmic binding partners. Through both in vitro binding experiments and in vivo fluorescence lifetime imaging microscopy, members of the ERM family of cytoskeleton-membrane cross-linkers have been found to co-localize with CaM to the cytoplasmic domain of L-selectin (Fig. 4B) (30) and play a significant role in both tethering of the adhesion receptor to its ligands as well as in PKC-dependent shedding (64, 65). The side chains of both Ser\(^{364}\) and Ser\(^{367}\) (although to a lesser extent) are at the interface of the FERM domain of ezrin/moesin and L-selectin, and it is likely that their phosphorylation would affect this binding interaction (Figs. 4C and 5C). PKC-dependent phosphorylation could mediate the exchange of ezrin for moesin once the cell is activated (of particular importance if their roles do not overlap), induce a conformational rearrangement in the CaM-L-selectin-ERM heterotrimeric complex triggering CaM dissociation, or affect the binding of additional partners to the cytoplasmic domain of L-selectin. Whether or not CaM must dissociate for phosphorylation to occur remains to be determined.

A second, weaker binding site on CaM was observed for L-selectin (Figs. 1 and 6). This site maps to the “top” of the CaM molecule and could play a role in the clustering of L-selectin observed during leukocyte rolling in vivo or following triggering by antibodies or glycomimetics in vitro (66). By linking the cytoplasmic tails of two L-selectin receptors in cis, CaM could help to create a signaling platform that leads from the phosphorylation of L-selectin amino acid residue Tyr\(^{372}\) by the Src-tyrosine kinase p\(^{56}\) to the activation of Ras signaling pathways and the production of O\(_2\)\(^{•}\) (30, 67). This clustering pathway occurs prior to L-selectin ectodomain shedding in response to cell activation (6) and is thought to promote outside-in signals that lead to integrin activation and chemokine receptor activation and eventually leukocyte arrest.

L-selectin’s rapid ectodomain shedding in response to cell activation is an anti-adhesive process that has an immediate influence on the accumulation of leukocytes along the vasculature wall and is required for neutrophil transendothelial migration into inflamed tissue (68, 69). Furthermore, shedding prevents antigen-activated T-cells from re-entering peripheral lymph nodes (70), and the released domains hinder leukocyte recruitment by reducing ligand availability, thus diminishing inflammation (71). Underscoring the physiological consequences of shedding, there are numerous clinical settings in which this phenomenon may either serve as a protective feedback mechanism or exacerbate existing pathologies (72, 73). By better understanding the cellular and molecular mechanisms of this process, including the role of CaM in activation-induced cleavage, it may be possible to produce therapies that manipulate shedding of the extracellular domains of L-selectin. The NMR solution structure presented here provides the first molecular details for a mechanism through which CaM negatively regulates the shedding of the extracellular domains of L-selectin. Furthermore, it highlights the role of calcium-signaling pathways in the observed shedding response to GPCR- or immunoreceptor-based cell activation.

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