Interaction of Actin with Carcinoembryonic Antigen-related Cell Adhesion Molecule 1 (CEACAM1) Receptor in Liposomes Is Ca²⁺- and Phospholipid-dependent

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The regulation of binding of G-actin to cytoplasmic domains of cell surface receptors is a common mechanism to control diverse biological processes. To model the regulation of G-actin binding to a cell surface receptor we used the cell-cell adhesion molecule carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1-S) in which G-actin binds to its short cytoplasmic domain (12 amino acids; Chen, C. J., Kirshner, J., Sherman, M. A., Hu, W., Nguyen, T., and Shively, J. E. (2007) J. Biol. Chem. 282, 5749–5760). A liposome model system demonstrates that G-actin binds to the cytosolic domain peptide of CEACAM1-S in the presence of negatively charged palmitoyl-oleoyl phosphatidylserine (POPS) liposomes and Ca²⁺. In contrast, no binding of G-actin was observed in palmitoyl-oleoyl phosphatidylcholine (POPC) liposomes or when a key residue in the peptide, Phe-454, is replaced with Ala. Molecular Dynamics simulations on CEACAM1-S in an asymmetric phospholipid bilayer show migration of Ca²⁺ ions to the lipid leaflet containing POPS and reveal two conformations for Phe-454 explaining the reversible availability of this residue for G-actin binding. NMR transverse relaxation optimized spectroscopic analysis of ¹³C-labeled Phe-454 CEACAM1-S peptide in liposomes plus actin further confirmed the existence of two peptide conformers and the Ca²⁺ dependence of actin binding. These findings explain how a receptor with a short cytoplasmic domain can recruit a cytosolic protein in a phospholipid and Ca²⁺-specific manner. In addition, this model system provides a powerful approach that can be applied to study other membrane protein interactions with their cytosolic targets.

Monomeric G-actin is recruited to the plasma membrane in response to a variety of cell receptor activation signals (1–4). Although much is known about subsequent steps, including actin polymerization, branching, and participation in functions such as cell motility, less is known about the regulation of the initial G-actin recruitment event. Among the many cell surface receptors that bind G-actin when activated, the so-called "short form" of CEACAM1³ stands out as a rather simple example in that it is a single-pass transmembrane protein with a cytoplasmic domain of only 12 amino acids. When a single amino acid phenylalanine 454 in the cytoplasmic domain is mutated to alanine (F454A), it no longer binds G-actin in in vitro assays, and when transfected into MCF7 cells that form a lumen with wild-type CEACAM1-S, it no longer forms a lumen in three-dimensional culture (5). Intrigued by the ability of such a short stretch of amino acids to convey G-actin binding in response to the homotypic cell-cell interaction function of CEACAM1, we speculated that the adjacent membrane environment and Ca²⁺ signaling may play a role in regulating the binding, otherwise binding would be constitutive and irreversible. Given the close proximity of the cytoplasmic domain of CEACAM1 to the lipid bilayer and the inherent asymmetry of the lipid bilayer with respect to charge, we propose that negatively charged phospholipids would attract Ca²⁺ in response to cell-cell interactions because Ca²⁺ signaling almost always accompanies cell-cell interactions (6, 7). Furthermore, Ca²⁺ recruited to the negatively charged inner leaflet of the plasma membrane would effectively promote the interaction of the cytoplasmic domain of CEACAM1 with G-actin. To test this hypothesis, we generated an in vitro model of the peptide-actin interaction in the context of negatively charged liposomes in the presence or absence of Ca²⁺ and analyzed their interactions by a combination of fluorescent bead analysis, NMR TROSY experiment, molecular dynamics (MD) simulation, and surface plasmon resonance analysis.

EXPERIMENTAL PROCEDURES

Preparation of Liposome-coated Glass Beads—Palmitoyl-oleoyl phosphatidylserine (POPS) and palmitoyl-oleoyl phosphatidylcholine (POPC) powders (Avanti Polar Lipids) were stored in chloroform at 25 mg/ml. Five µl of stock phospholipid solution in a glass tube was vaporized under argon gas to form a thin and even membrane on the glass tube. Phospholipid was dissolved in 100 µl of PBS, vortexed, and subjected to extrusion through a 200-nm membrane 11–13 times (Avanti Polar Lipids).

The abbreviations used are: CEACAM1, carcinoembryonic antigen-related cell adhesion molecule; CEACAM1-S, short isoform of CEACAM1; MD, molecular dynamics; MUA, mercaptoundecanoic acid; POPC, palmitoyl-oleoyl-phosphatidylcholine; POPS, palmitoyl-oleoyl-phosphatidylserine; TROSY, transverse relaxation optimized spectroscopy.

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Glass microspheres (4.5 μm; Bangs Laboratories, Inc.) were cleaned in “piranha solution” (30% H₂O₂ and concentrated H₂SO₄ 1/4 (v/v) for 3 min at 80 °C, use extreme caution!) followed by extensive washing with water on a clean sintered glass funnel. Clean glass beads were diluted in water (10⁸ beads/μl) and stored at 4 °C. One to 2 μl of suspended glass beads were incubated with 100 μl of liposome at 37 °C for 2 h. Liposome-coated glass beads were centrifuged at 4000 rpm for 1 min and washed three times with PBS to remove unbound phospholipid. Glass bead-supported phospholipid membranes were incubated with MUA (mercaptoendocaneoic acid)-CEACAM1-S peptide (0.2 mg/ml) for 1 h at 37 °C and collected by centrifugation. Peptide-liposome-coated glass beads further incubated with 1% BSA in PBS for 1 h and washed three times using 1% BSA/PBS. G-actin (100 μg/ml) or rhodamine-actin (100 μg/ml) in the presence or absence of 1 mM CaCl₂ was incubated with glass beads for 1 h. In the last step, FITC-conjugated anti-actin antibody was incubated with glass beads. Flow cytometry was used to detect the fluorescence of rhodamine-actin or FITC-conjugated anti-actin antibody on a BD FACS caliber. Single beads were selected for analysis based on forward and side scatter analysis (>80%).

Surface Plasmon Resonance Assay—Surface plasmon resonance assays were performed on a Biacore T100 (BIAcoreAB) instrument. Liposome or liposome-containing CEACAM1-S MUA-peptide was prepared using the same method as above. The HPA sensor chip (GE Healthcare) was washed with 40 mM HEPES, 150 mM NaCl, 3 mM CaCl₂ at a flow rate of 20 μl/min. 0.5M EDTA (10 mM) was used to regenerate the HPA sensor chip surface. Actin (0.2 mg/ml) was injected plus or minus 1 mM CaCl₂ was incubated with the sensor chip for 1 h. In the last step, FITC-conjugated anti-actin antibody was incubated with glass beads. Flow cytometry was used to detect the fluorescence of rhodamine-actin or FITC-conjugated anti-actin antibody on a BD FACS caliber. Single beads were selected for analysis based on forward and side scatter analysis (>80%).

MD System Preparation—A molecular model of the transmembrane and cytoplasmic domain of CEACAM1-S derived previously using homology modeling methods was used in this study (5). The F454A mutant peptide was generated by using the side chain coordinates of the C₇₋₄ of Ala-454 in place of Phe-454. The CEACAM1-S peptide was placed in a simulation box with a pressure of 1 bar. The velocity-rescaling thermostat (16) was used for temperature coupling during the equilibration and a Parrinello-Rahman barostat (17, 18) for pressure coupling. The CEACAM1 peptide was kept in place during these equilibration steps using position restraints of 1000 kJ/mol per nm².

After the systems were equilibrated at 310 K, MD simulations of each of the 10 conformations were performed using the GROMOS 49a5 force field (12) extended with Berger lipid parameters (13). Short range nonbonded interactions were truncated at 1.2 nm with the neighbor list updated every 10 fs. To account for the cutoff in the van der Waals interactions, long range dispersion correction was applied to energy and pressure terms. Long range electrostatics were calculated using the smooth particle mesh Ewald method (14). Bonds were constrained using the LINCS algorithm (15) to allow for a time step of 2 fs. For continuity, periodic boundary conditions were applied in every dimension.

MD simulations were performed on five starting conformations of CEACAM1-S in lipid without Ca²⁺ ions and five conformations of CEACAM1-S in lipid with Ca²⁺ ions. Each system was equilibrated by performing 100 ps of MD at 310 K using a NVT ensemble followed by 5 ns of MD under NPT conditions with a pressure of 1 bar. The velocity-rescaling thermostat (16) was used for temperature coupling during the equilibration and a Parrinello-Rahman barostat (17, 18) for pressure coupling. The CEACAM1 peptide was kept in place during these equilibration steps using position restraints of 1000 kJ/mol per nm².

After the systems were equilibrated at the correct temperature and pressure, MD simulations of 100 ns were performed for each of the 10 conformations, using a NVT ensemble with a Nosé-Hoover thermostat (19). Simulations on the F454A mutant were performed over 40–100 ns for a total of ~600 ns. Data were not collected for the first 5 ns of each simulation.

K-Means Clustering Analysis—The interatomic distances between the C₇₋₄, C₉₋₄, and C₁₀ atoms of the CEACAM1-S peptide were extracted every 10 ps from the MD trajectories. The first 5 ns of each trajectory was left out to avoid not well equilibrated conformations. The distances of all simulations were combined into one distance matrix for clustering analysis, leading to a total of 9510 sampled conformations with each conformation expressed as 4950 interatomic distances. The distance matrix was grouped into two clusters using a K- Means clustering algorithm. The process was repeated 10 times to verify that the clusters were reproducible; grouping into three clusters was attempted but was not reproducible.

Preparation of Samples for NMR—Fmoc (N-(9-fluorenyl)-methoxycarbonyl)-Phe-OH, ¹³C, ¹⁵N 98 atom % ¹³C, 98 atom...
% $^{15}$N was purchased from ISOTEC. C18 Sep-Pak cartridges were purchased from Waters (Ireland). POPS (porcine brain) and POPC (chicken egg) were purchased from Avanti Polar Lipids. Nonmuscle actin (99% pure) was purchased from Cytoskeleton.

To remove the residual TFA from the synthesized peptide, a C18 cartridge was conditioned using 70% acetonitrile solution followed by 1% acetic acid wash. After the peptide solution was loaded onto the cartridge, the column was washed using 1% acetic acid, then eluted with 70% acetonitrile-water. Samples were lyophilized and stored at $-80 \, ^{\circ}C$. The peptide concentration was determined using an NMR method (20). Bulk solutions of POPS and POPC were prepared by dissolving 100 mg of each lipid in 4 ml of chloroform, flushed with argon, and stored at $-20 \, ^{\circ}C$.

For POPS or POPC liposomes, 288 or 268 µl of bulk solution was transferred to a 1.5-ml polypropylene tube, and the solvent was evaporated under argon and further dried overnight under vacuum. To the dry lipid film, 400 µl of 50 mM phosphate buffer (90% D$_2$O, pH 7.0) and 1.5 µl of 166 mM EDTA solution were added, vortexed, and sonicated for 30 min or until the lipid solution became transparent. The 400-µl liposome solution was then mixed with 100 µl of 0.5 mM MUA-13C, $^{15}$N-Phe-CEACAM1-S peptide in 50 mM phosphate buffer (90% D$_2$O, pH 7.0), and 1 mM tris (2-carboxyethyl)phosphine. The sample was then transferred to the NMR tube and flushed with argon gas. For the study of the complex with actin, 460 µl of the MUA-CEACAM1-S peptide-liposome solution was added to 2 mg of actin powder. After vigorous vortexing, the solution was transferred to an argon-flushed Shigemi NMR tube. For the sample in the presence of Ca$^{2+}$, the Ca$^{2+}$ was added after the addition of actin at a final Ca$^{2+}$ concentration of 5 mM. For the mixed POPS/POPC liposome system, 144 µl of POPS and 134 µl of POPC were taken from the bulk POPS and POPC solution, respectively, and the two lipids were mixed well before evaporating the solvent.

NMR Experiments—We used $^{13}$C-labeled Phe in the CEACAM1-S peptide as a probe because the chemical shifts of the aromatic ring are well separated from signals of the lipid. Because the CEACAM1-S peptide is inserted into the liposome through the MUA-aliphatic group, there is significant line broadening of the NMR signal from $^{13}$C-labeled Phe aromatic ring. For 0.1 mM concentration of peptide, the peptide signal is very weak from constant-time $^{13}$C-$^1$H heteronuclear single-quantum coherence experiment (21). Much better signal intensity (>2-fold) was obtained from the aromatic ring using constant time TROSY experiment (22). The experiments were carried out at 35 °C on a 600-MHz Bruker instrument equipped with a cryoprobe. The constant time duration, spectrum width, and total number of free induction decays for the $^{13}$C dimension are 17.6 ms, 4 ppm, and 20, respectively. For the $^1$H dimension, the total number of points and spectrum width are 2048 and 14 ppm, respectively. The recycle delay is 1.2 s. The experimental time is about 18 h with the number of scans ranging from 2200 to 2560. The TROSY data were processed using the NMRPipe software (23) and analyzed using the NMRView program (24).

RESULTS

Lipid Bilayer Model System—CEACAM1 has two cytoplasmic domain isoforms, of which the short isoform (CEACAM1-S) is sufficient to induce lumen formation when expressed in the breast cancer cell line MCF7 and grown in three-dimensional culture (Fig. 1A). When CEACAM1-S is expressed in cells that undergo cell-cell contact, CEACAM1-S...
migrates to the cell-cell contact region along with F-actin (Fig. 1B). Although we have shown that the initial interaction of the short cytoplasmic domain is with G-actin (5), the small size of this domain and its close proximity to the inner leaflet of the plasma membrane prompted us to investigate the role of the microenvironment in its interaction with G-actin in a model system. The model comprises a phospholipid bilayer coated onto 4.5-μm glass beads into which is inserted an MUA version of the 12-amino cytoplasmic domain of CEACAM1-S (Fig. 2, A and B). The MUA-peptide spontaneously inserts into the lipid bilayer in a saturable manner that can be quantitated by fluorescently tagged anti-peptide antibodies (mean fluorescence intensity versus concentration of peptide, C).

FIGURE 2. Generation of lipid-embedded cytoplasmic domain of CEACAM1-S. The cytoplasmic domain of CEACAM1-S has 12 amino acids that when synthesized with an N-acyl-MUA (A) spontaneously inserts into 4.5-μm glass beads coated with POPS or POPC liposomes used in actin binding experiments (B) and can be quantitated with fluorescent-tagged anti-peptide antibodies (mean fluorescence intensity versus concentration of peptide, C).

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MD Simulations—To investigate any direct effect Ca2+ ions may have on the conformation of the CEACAM1-S peptide in its native lipid environment we performed a total of 1 μs of MD simulations on CEACAM1-S embedded in an asymmetric lipid bilayer. To reduce bias caused by the choice of an initial conformation we have initiated simulations from five unique start-
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FIGURE 4. G-actin does not bind F454A mutant and binds more strongly to K456A mutant of CEACAM1-S. A, same experiment as in Fig. 3A except F454A mutated peptide was used. B, Same experiment as in A except K456A mutated peptide was used.
decreased by 15% upon the addition of Ca\(^{2+}\)/H\(_{11001}\) to the complex of actin and peptide.

To address whether Ca\(^{2+}\)/H\(_{11001}\) affects the interaction between G-actin and CEACAM1-S peptide by perturbation of the equilibrium between two conformations of Phe-454, we carried out an NMR experiment on free peptide in the POPS environment with different concentrations of Ca\(^{2+}\)/H\(_{11001}\) in the absence of actin. We found that [Ca\(^{2+}\)] per se does not affect the equilibrium.

FIGURE 5. Two major conformation ensembles predicted by MD on the CEACAM1-S peptide in an asymmetric POPS/POPC lipid bilayer and two conformations of Phe-454 in CEACAM1-S peptide inserted into POPS liposomes in the presence of actin and Ca\(^{2+}\) observed by NMR. A–C, MD simulations reveal two conformations that represent different environments for Phe-454. A, in one conformational ensemble Phe-454 is exposed to solvent surrounding the lipid bilayer. B, the other ensemble has Phe-454 buried in the surrounding lipid head groups. C, representative (nearest to mean) conformations are shown for both ensembles aligned and with Phe-454 in a stick representation. D–F, \(^{13}\)C-labeled Phe-454 N-acyl-MUA-peptide (0.1 mM) in POPS (18 mM) liposomes gives two sets of cross-peaks, C1-a and C1-b, corresponding to conformation 1, and C2-a and C2-b corresponding to conformation 2 (total percentages of each conformation indicated) as analyzed by two-dimensional NMR TROSY (D). The total percent of conformation 2 increases with the addition of actin (E) and Ca\(^{2+}\) (F).
between the two conformers (supplemental Fig. S5 and supplemental Table S2). Thus, most likely, Ca\(^{2+}\) is first recruited to the negatively charged phospholipid, which in turn, enhances the peptide-G-actin interaction. In other work, although G-actin has been shown to interact directly with negatively charged liposomes (28), the additional effect of inserted peptides has not been studied.

The NMR analysis also indicates that pH affects the equilibrium of the two observed conformations of the peptide embedded in the lipid (supplemental Fig. S3, A and B). Because Ca\(^{2+}\) is a better studied signal transducer at the plasma membrane compared with pH, the effects of pH were not studied further.

**DISCUSSION**

Three completely different experimental approaches, together with MD simulations, demonstrate a requirement for negatively charged phospholipid POPs and Ca\(^{2+}\) in the regulation of the binding of G-actin to the cytoplasmic domain peptide of CEACAM1-S. In the first approach, POPS or POPC liposomes mimicking the inner or outer leaflet of the plasma membrane, respectively, were coated onto 4.5-μm glass beads, N-acyl-MUA-peptide was inserted into the outer leaflet, and the binding of actin was monitored in the presence or absence of Ca\(^{2+}\). This approach has the advantage that it creates a cell-sized lipid surface that allows specification of interacting components present at either the inside or outside of the cell membrane and measurement of interactions by flow cytometry using fluorescent labeling of components of interest. This approach validated our earlier finding that Phe-454 plays an essential role in actin binding (5). A disadvantage of the approach is that it provides no direct evidence of the molecular state of the inserted peptide. To assess this information, we...
performed NMR analysis on the \( ^{13} \text{C} \)-labeled peptide (at the residue of interest) inserted into liposomes under a variety of conditions. This approach demonstrated that \( \text{Ca}^{2+} \) played a major role in the peptide-actin interaction and not in the peptide-phospholipid interaction. An advantage of this approach is the use of liposomes that more accurately reflect the lipid environment in the cell as opposed to the conventional use of peptides embedded in micelles.

In our model system 1 mM \( \text{Ca}^{2+} \) was required to effect actin binding. Although the global concentration of \( \text{Ca}^{2+} \) in the cytoplasm during signaling peaks at about 1.4 \( \mu \text{M} \), the local concentration of \( \text{Ca}^{2+} \) in the microenvironment around the cytoplasm adjacent to the cell membrane would be in the millimolar range due to the effect of calcium ion channel proteins and/or calcium transporters that produce a spike in the local \( \text{Ca}^{2+} \) concentration. Because our liposomes lack the presence of these \( \text{Ca}^{2+} \) transporters, a millimolar bulk concentration of \( \text{Ca}^{2+} \) was used to mimic the local environment in the cell. The difference between local and global concentrations of \( \text{Ca}^{2+} \) can be visualized in our molecular simulations shown in Fig. 5, A–C, where we have added only two \( \text{Ca}^{2+} \) ions to the lipid bilayer-cytosol box (7 \( \times \) 7 \( \times \) 10 nm). This leads to a local \( \text{Ca}^{2+} \) concentration of about 8 mM. If we simulated the physiological global concentration of \( \text{Ca}^{2+} \) inside activated cells we would either have to use a box 10\(^3\) times larger or assume \( 10^{-3} \) \( \text{Ca}^{2+} \) ions in our box, assumptions that are illogical. We believe that these considerations justify our use of 1 mM \( \text{Ca}^{2+} \) in our experiments.

A potential disadvantage of the NMR method is that the overall molecular size (including the lipid bilayer) of the system is quite large, and thus a millimolar sample concentration is needed to compensate for the increased line width of NMR peaks. Thus, we employed a third approach, namely the use of surface plasmon resonance that has the advantage of allowing kinetic measurements of the binding of various components to each other at lower concentrations. This method also confirmed \( \text{Ca}^{2+} \) and lipid specific requirements for G-actin binding to lipid-embedded CEACAM1-S peptide. However, this method suffers from the lack of structural information. Thus, we performed MD simulations to gain access to changes in the entire peptide embedded in the lipid bilayer at the atomic level. This approach allowed us to provide a theoretical basis for our findings and to make predictions that could be validated by further experiments. A combination of MD simulations and NMR experiments reveal two reversible conformational states of Phe-454. The population of these states is not affected by varying the \( \text{Ca}^{2+} \) concentration but does change on actin binding. MD simulations predict that in one conformation Phe-454 is solvent-exposed whereas in the other conformation Phe-454 is buried in the lipid head groups. Furthermore, MD simulations show that the conformation with Phe-454 buried in the lipid head groups may be stabilized by a cation-\( \pi \) interaction (25, 30) of Lys-456 with Phe-454. This predicted cation-\( \pi \) interaction, and its role in conformational selection, could explain why mutation of Lys-456 affects actin binding in a positive manner. Finally, we performed MD simulations for the F454A mutant peptide and found that Ala-454 was unable to adopt two confirmations in the lipid bilayer, strengthening our use of MD as a method to examine the structural constraints of the model system.

Our findings help explain why many receptor cytoplasmic domain peptides, especially those as short as the CEACAM1-S peptide, may have highly specific and tunable interactions with components of the cytosol. Previously, receptor aggregation was proposed as a major mechanism for activation of cytoplasmic domains in downstream signaling (31, 32). However, this mechanism is inadequate to account for the cooperative effect of the local phospholipid environment and \( \text{Ca}^{2+} \) signaling. In terms of the downstream interacting protein G-actin, it has been shown previously that it is attracted to negatively charged phospholipids (28), but this interaction alone is insufficient to confer membrane site specificity or temporal control by \( \text{Ca}^{2+} \) signals. Indeed, for actin to polymerize at the right location and time at the membrane, it must find a suitable binding partner that responds to rapid signals from both the outside (say receptor clustering) and inside (say \( \text{Ca}^{2+} \) signaling). CEACAM1-S provides the outside activation step by receptor clustering during its cell-cell interactions (31) and the inside activation step via \( \text{Ca}^{2+} \)-generated interactions at Phe-454 in the negatively charged phospholipid environment. In the case of CEACAM1-S, the source of the intracellular \( \text{Ca}^{2+} \) is likely from other receptors that become activated during cell-cell interactions, including G protein-coupled receptors (33, 34). In this respect, both CEACAM1 (35–37) and G protein-coupled receptors (38) reside within the same lipid microenvironments, emphasizing their local ability to coordinate signals. Finally, for actin to polymerize, it must be correctly oriented to form the characteristic double helix of F-actin. In the case of CEACAM1-S, the polymerization would occur directly adjacent to the phospholipid bilayer, exactly the situation seen for the formation of cortical actin. Thus, it is likely that cortical actin formation is dynamic, responding to cell-cell interactions that allow local changes in the distribution of receptors at the cell surface (for an example of the redistribution of CEACAM1-S and actin during cell-cell adhesion, see Fig. 1).

In summary, these findings explain how receptors with short cytoplasmic tails can recruit cytosolic proteins in a phospholipid- and calcium-specific manner. In addition, these models provide a powerful approach that can be applied to other membrane protein interactions.

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