Phosphatidylinositol 4,5-Bisphosphate Clusters the Cell Adhesion Molecule CD44 and Assembles a Specific CD44-Ezrin Heterocomplex, as Revealed by Small Angle Neutron Scattering*

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Background: The mechanism by which the conserved CD44 cytoplasmic tail (CD44ct) functions is not well understood.

Results: The disordered CD44ct interacts with FERM only in the presence of phosphatidylinositol 4,5-bisphosphate (PIP2).

Conclusion: PIP2 clusters CD44ct and facilitates the assembly of a specific CD44-Ezrin heterotetramer complex.

Significance: The study reveals the role of PIP2 in clustering CD44 and in assembling multimeric CD44-Ezrin signaling complexes.

The cell adhesion molecule CD44 regulates diverse cellular functions, including cell-cell and cell-matrix interaction, cell motility, migration, differentiation, and growth. In cells, CD44 co-localizes with the membrane-cytoskeleton adapter protein Ezrin that links the CD44 assembled receptor signaling complexes to the cytoskeletal actin network, which organizes the spatial and temporal localization of signaling events. Here we report that the cytoplasmic tail of CD44 (CD44ct) is largely disordered. Upon binding to the signaling lipid phosphatidylinositol 4,5-bisphosphate (PIP2), CD44ct clusters into aggregates. Further, contrary to the generally accepted model, CD44ct does not bind directly to the FERM domain of Ezrin or to the full-length Ezrin but only forms a complex with FERM or with the full-length Ezrin in the presence of PIP2. Using contrast variation small angle neutron scattering, we show that PIP2 mediates the assembly of a specific heterotetramer complex of CD44ct with Ezrin. This study reveals the role of PIP2 in clustering CD44 and in assembling multimeric CD44-Ezrin complexes. We hypothesize that polyvalent electrostatic interactions are responsible for the assembly of CD44 clusters and the multimeric PIP2-CD44-Ezrin complexes.

CD44 is a single transmembrane cell adhesion molecule (1–3). Binding of the CD44 extracellular domain to the extracellular matrix component hyaluronan facilitates the sensing and response of cells to their microenvironment (4). CD44 also interacts with c-Src kinase and co-immunoprecipitates with growth factor receptors, such as members of the EGF family of receptors and the c-Met tyrosine kinase receptor (5–7). The CD44 molecules are considered to act as scaffolds or platforms to recruit transmembrane receptor tyrosine kinase complexes, thereby increasing the signaling potency of the receptor complexes (2). The CD44 assembled receptor complexes integrate cell surface signaling events for cross-talk and influence cell growth, survival, and differentiation. Overexpression of certain CD44 isoforms is a hallmark in many types of invasive cancers that are resistant to anti-cancer therapies (8–12).

The highly conserved cytoplasmic tail of CD44 is essential in cell signaling (3) and is required for CD44 binding to the extracellular matrix. Oligomerization of the CD44 cytoplasmic tail is necessary for the effective binding of the CD44 extracellular domain to macromolecular hyaluronan (13, 14). Moreover, the CD44 cytoplasmic tail interacts with membrane-cytoskeleton adapter proteins, including ankyrin, Ezrin, and other members of the Ezrin-Radixin-Moesin (ERM)^2 family proteins (15–17). The CD44-ERM complexes anchor the CD44-associated transmembrane receptor complexes to the actin cytoskeleton, so as to coordinate the spatial and temporal localization and the signaling of receptor complexes (18). Disrupting the interaction of CD44 cytoplasmic tail and ERM proteins interrupts the signal transduction from the c-Met receptor to MEK-Erk (7). Enhancing the association of CD44 with Ezrin increases cell migration (19). Phosphorylation regulates the association of CD44 with Ezrin and affects cell motility (20). Thus, the dynamic interaction between the cytoplasmic tail of CD44 and Ezrin is crucial
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for CD44 ligand binding, for the sensing and response of cells to their microenvironment, and for the localization and assembly of signaling complexes that regulate cell motility, migration, survival, growth, and proliferation.

The functions of Ezrin and the other ERM proteins are known to be regulated by a conformational autoinhibition mechanism, with the inactive protein being held in a closed conformation by head to tail intramolecular interactions (21, 22). Binding to the phosphatidylinositol 4,5-bisphosphate (PIP2) lipid disrupts the autoinhibition and activates ERM proteins (23). In the activated ERM proteins, the N-terminal 4.1 Ezrin-Radixin-Moesin (FERM) domain binds to target proteins, which include the multi-PDZ scaffolding protein NHERF1 that in turn binds to a variety of membrane receptors and ion channels, whereas the C-terminal tail binds to cytoskeletal actin. ERM proteins organize a variety of signaling events at the interface between the cell membrane and cytoskeleton (22).

Previous biochemical studies have shown that PIP2 is involved in the interaction of CD44 with the ERM proteins (24). Biochemical and x-ray crystallography studies have determined that the FERM domain, which mimics an activated ERM protein, binds directly to positively charged amino acid residues in the juxtamembrane peptide of the cytoplasmic tail of CD44 (16, 17). The role of PIP2 in CD44-Ezrin association has generally been attributed to the activation of ERM proteins. Here we report that the entire cytoplasmic tail of CD44 of 73 amino acid residues (CD44ct), which adopts a disordered “collapsed coil” conformation in solution, is unable to bind directly to the FERM domain of Ezrin (which mimics an activated Ezrin). Instead, PIP2 is necessary for CD44ct to form a complex with FERM. Using selective deuteration and contrast variation small angle neutron scattering (SANS), we show that PIP2 mediates the assembly of a discrete heterotetramer complex of CD44ct both with the FERM domain of Ezrin and with the full-length Ezrin. Additionally, PIP2 causes CD44ct to aggregate. These results reveal an important role of PIP2 in clustering CD44ct and in the assembly of multimeric CD44-Ezrin complexes by polyvalent electrostatic interactions.

MATERIALS AND METHODS

Protein Expression and Purification—The proteins used in this study were expressed in bacterial cells. The entire cytoplasmic domain including amino acid residues 293–365 of mouse CD44 transcript variant 3 (CD44ct) was subcloned into a PET-32a vector that expresses a fusion protein of a His6 plus thioredoxin domain including amino acid residues 293–365 of mouse CD44. Additionally, PIP2 causes CD44ct to aggregate. These results reveal an important role of PIP2 in clustering CD44ct and in the assembly of multimeric CD44-Ezrin complexes by polyvalent electrostatic interactions.

Protein Concentration Determination—The concentrations of all proteins except CD44ct were determined by UV absorption at 280 nm, using the extinction coefficient calculated from ProtParam on the ExPASy Server (32). The extinction coefficient of the cytoplasmic tail of CD44 (CD44ct) is zero at 280 nm. The concentration of CD44ct was thus measured at 205 nm, using the Protein concentration (mg/ml) = (Absorbance at 205 nm)/31 (33, 34).

Static and Dynamic Light Scattering Experiments—Static light scattering experiments were performed using a DynaPro NanoStar (Wyatt Technology Corporation) with a laser of wavelength 824.7 nm at a fixed 90° scattering angle. Before light scattering experiments, the sample was centrifuged at 10,000 rpm for 5–10 min. Protein concentrations were varied from 0.5 to 2 mg/ml during light scattering measurements. Light scattering experiments were performed at 20 °C.

CD Spectroscopy Experiments—CD experiments were performed with an Aviv 400 spectropolarimeter (Aviv Biomedical, Inc., Lakewood, NJ). The protein samples were dissolved at 0.1–0.2 mg/ml in buffer containing 20 mM phosphate (pH 7.5), 150 mM NaCl, and 1 mM DTT. Quartz cuvettes of 1-mm path-length (Hellma USA, Plainville, NY) were used for CD experiments. The CD measurements performed at 20 °C. The secondary structure content of each spectrum was calculated using a multilinear regression method that does not depend so much on accurate determination of protein concentration.

Gel Filtration Analysis of CD44ct Binding to Ezrin in Solution and in PIP2—A Superdex 200 10/300 GL gel filtration column was used to analyze the binding of CD44ct with Ezrin(T567D) or with FERM in solution and in PIP2, respectively. The buffer used for these gel filtration analyses is 25 mM Tris (pH 7.5), 300 mM NaCl, 0.5 mM DTT, and 0.1 mM EDTA. To determine the binding in solution, 50 μM Ezrin(T567D) or FERM is incubated with equal molar CD44ct for 1 h. The complexes were prepared by first mixing 50 μM CD44ct with FERM or Ezrin(T567D) at 1:1 molar ratio, and then PIP2 at 10 molar ratio was added. The complex was incubated on ice for 1 h before the gel filtration to examine the shift in elution peak position.

Pulldown Experiments to Determine CD44ct Binding to Ezrin in Solution and in PIP2—The pulldown experiments were performed by incubating 15 μl of 10 μM of His6-Trx-CD44ct with 15 μM of 10 μM FERM, without PIP2 or in the presence of 10 molar ratio PIP2 in binding buffer, containing 20 mM imidazole, 20 mM phosphate (pH 7.0), 300 mM NaCl for 1 h on ice. Approximately 15 μl of Ni-Magbeads (Genscript) were added to the incubation. After the beads were washed with binding buffer three times, the protein or protein complex was eluted in 100 μl of buffer containing 500 mM imidazole, 20 mM phosphate buffer (pH 7.0), and 300 mM NaCl. The eluted protein or protein complex was analyzed by SDS-PAGE.

Isothermal Titration Calorimetry—Isothermal titration calorimetry (ITC) experiments were run in a MicroCal™ Auto- iTC system at 25 °C. All proteins were dialyzed for 12–18 h against the ITC buffer containing 10 mM HEPES (pH 7.5), 300 mM NaCl, 0.5 mM EDTA, and 0.5 mM β-mercaptoethanol. All samples were degassed for 10 min prior to experiments, and all
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measurements were carried out in duplicate. The heat of ligand dilution was obtained from the average heat of the last few injections after complete saturation of binding protein. This value was subtracted from all of the injection points during data analysis. All data were fit to a single site binding model using Bindworks to yield the stoichiometry (n), the association constant (K_a), and the enthalpy change (ΔH) for the binding reaction.

Analytical Ultracentrifugation Analysis of the PIP2-CD44ct-FERM Complex—Both sedimentation velocity and sedimentation equilibrium experiments were performed on the PIP2-CD44ct-FERM complex. Before the ultracentrifugation experiment, the complex was prepared by first mixing 50 μM CD44ct with 500 μM PIP2, and 50 μM FERM was added. The complex was purified by gel filtration using a Superdex 200 column. Samples were loaded into a six-chamber centerpiece at 2.1 μM concentration and centrifuged at two rotor speeds (10,300 and 18,000 rpm) at 20 °C and allowed to reach equilibrium in an Optima XL-I centrifuge (Beckman Coulter, Fullerton, CA). We used an absorbance optical data acquisition system and measured the sedimentation equilibrium data at 260-nm wavelength. Buffer viscosity, density, and specific volume were calculated using program SEDNTERP (35). The program SEDFIT (36) was used to sort and prepare equilibrium data for further analysis in program SEDPHAT (37). The sedimentation equilibrium data at two speeds were fit globally in SEDPHAT using a model that describes a single species of interacting systems.

Solution Small Angle X-ray Scattering Experiments—Solution x-ray scattering experiments were performed at Beamline X9 at the National Synchrotron Light Source, Brookhaven National Laboratory (38). Protein samples were loaded in a 1-mm diameter glass capillary. Triplicates of scattering data were acquired by flowing 20 μl of protein sample or buffer during a 30-s exposure. Analysis of the simultaneous SAXS/WAXS data acquisition was performed using the program pyXS (39). Standard Guinier plots were calculated to identify aggregates (40).

SANS Experiments and Data Analyses—SANS experiments were performed on the extended Q range SANS instrument at the Spallation Neutron Source located at Oak Ridge National Laboratory (41). All measurements used a sample to detector distance of 4 m. Two wavelength settings were used: 60 Hz with a wavelength, λ, band of 2.5–6.1 Å and 30 Hz (frame-skipping mode) with two wavelength bands of 2.5–6.1 Å and 9.4–13.4 Å. The former configuration provides a useful Q range (the wave vector transfer, Q = 4π sin(θ/2)/λ, where 2θ is the scattering angle) of ~0.009 to ~0.22 Å⁻¹, whereas the latter provides additional low Q data (down to ~0.005 Å⁻¹) with the same practical upper limit. The choice of configuration was determined by the expected size of the particles being studied.

Before SANS experiments, the proteins or protein-lipid complexes were dialyzed against buffers of different D_2O concentrations. The samples were loaded into 1-mm-path length circular quartz cells (Hellma USA). SANS experiments were performed at 10 °C. More details of the SANS experimental conditions and the SANS data reduction are described elsewhere (31, 42).

SANS data reduction followed standard procedures that are implemented in MantidPlot to correct for dark current (background radiation and electronic noise), the detector sensitivity, and the scattering contribution from the solvent and empty cells before being azimuthally averaged to produce I(Q) versus Q. The data were scaled into absolute units of cm⁻¹ using a calibrated standard (43).

The length distribution function P(r), radius of gyration R_g, the forward scattering intensity I(0) that is the neutron scattering intensity extrapolated to Q = 0 Å⁻¹, and the maximum dimension D_max were calculated from the scattering data using both the program GNOM (44). The molecular mass of a protein or protein complex can be determined by contrast variation SANS. Based a reference by Jacrot and Zaccaci (45), an equation was derived to show that I(0)⁻⁰.⁵ versus the neutron scattering length density of the buffer ρ_o yields (27),

\[
\left( \frac{I(0)}{N} \right)^{0.5} = \frac{\bar{U}}{N_A} \left[ \rho_o M_D + \rho_h M_h + \rho_{lipid} M_{lipid} \right] - \rho_o (M_D + M_h + M_{lipid})
\]

where N is the number of the complexes in a volume of 1 cm³, V_H = M_H/ρ_H/NA, V_D = M_D/ρ_D/NA, and V_{lipid} = M_{lipid}/ρ_{lipid}/NA are the molecular volumes of the hydrogenated and deuterated components and the PIP2 lipid, respectively. M_H and M_D are the molecular masses of the hydrogenated and deuterium labeled components, respectively, and ρ_H = ρ_D = ρ_{lipid} is the partial specific volume. According to Equation 1, the slope of the [I(0)/N]⁻⁰.⁵ versus ρ_o plot gives the molecular mass and thus the stoichiometry of the complexes. The partial specific volume was assumed to be ρ = 0.73 cm³/g. Other details of contrast variation small angle scattering have been described previously (46, 47).

The three-dimensional shapes of “dummy bead” coordinates were generated using the program DAMMIN (48). Multiple calculations were performed using DAMMIN, and the generated 10–20 structures were averaged and filtered using the program DAMAVER and DAMFILT (49). The three-dimensional density map was generated from the averaged coordinates using the program Situs (50). The fitting and docking of the high resolution structure to the density map were performed using Situs or UCS Chimera (51). For complexes of multiple components, the multiphase program MONSA restores the three-dimensional shape of the complex (48). The fitting and docking of the high resolution structure to the density map were performed using Situs (50) or UCSF Chimera (51).

RESULTS

The Cytoplasmic Tail of CD44 Is Disordered and Adopts a “Collapsed Globule” Conformation

The entire cytoplasmic tail of CD44 (CD44ct) has 72 amino acid residues (Fig. 1A). Because the extinction coefficient of CD44ct at 280 nm is zero, it is difficult to detect CD44ct alone by gel filtration. We thus have first analyzed the fusion protein of CD44ct with an N-terminal thioredoxin tag (Trx-CD44ct). Gel filtration shows that Trx-CD44ct migrates as a single peak (Fig. 1B). Static light scattering shows that the measured molec-
ular mass of Trx-CD44ct is close to the theoretical molecular mass of a monomer (Fig. 1C and Table 1). The gel filtration and light scattering results thus indicate that Trx-CD44ct is a monomer in solution at protein concentration lower than 2 mg/ml. Above protein concentration of 2 mg/ml, static light scattering indicates that Trx-CD44ct has weak self-association (Fig. 1C).

A comparison of the SANS data from the deuterated CD44ct (dCD44ct) and deuterated fusion protein Trx-CD44ct provides an estimation of the shape and the oligomer state of CD44ct in solution (Fig. 1, E–H). SANS shows that the $R_g$ and $D_{\text{max}}$ of dTrx-CD44ct are larger than those of dCD44ct (Table 1 and Fig. 1G). The three-dimensional shape of dCD44ct reconstructed from SANS can be docked into the three-dimensional envelope of monomeric dTrx-dCD44 (Fig. 1H). These comparisons suggest that dCD44ct is a monomer in solution.

CD spectroscopy shows that CD44ct is largely disordered without apparent secondary structure features (Fig. 1D). The disorder in CD44ct is also exhibited in the SANS data. The relatively large $R_g$ and $D_{\text{max}}$ of the 72-residue dCD44ct as compared with a well folded thioredoxin of 109 residues indicate that dCD44ct is not a compact globular structure (Fig. 1G and Table 1). The docking experiment also suggests that CD44ct is not as compact as the thioredoxin globular domain amino acid residues (Fig. 1H).

The Kratky plot from SANS can be used to show whether a protein is a compact globular structure, a random coil-like chain, or a partially folded protein (52, 53). The Kratky plot of a folded globular protein is a bell-like curve because of the presence of a clear interface between the measured particle and surrounding solvent, whereas that of a random coil chain tends to increase linearly with $Q$ (52). The plateau behavior of the Kratky plot suggests that, in solution, dCD44ct is neither a well folded globular protein nor a true random coil chain-like structure (Fig. 2). Thus, altogether, the static light scattering, CD, and SANS results indicate that CD44ct is a monomer that is somewhat collapsed but without significant secondary structure.

**Binding to PIP2 Unfolds CD44ct and Causes CD44ct to Aggregate**

Upon adding PIP2 to CD44ct, the CD spectrum shows that CD44ct remains largely disordered with no apparent changes in secondary structure (Fig. 1D). SANS, performed at the contrast-matching point of PIP2 in 20% D$_2$O, indicates that dCD44ct forms aggregates in PIP2 solution (Fig. 2). The protein concentration normalized forward scattering $I(0)/c$ of dCD44ct in PIP2 solution is ~14 times higher than that of dCD44ct in solution, suggesting that ~14 dCD44ct molecules reside in the

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**FIGURE 1.** The cytoplasmic domain of CD44 is a disordered monomer in solution. A, amino acid sequence of CD44ct. The multiple basic residues that bind PIP2 are highlighted in red. B, gel filtration chromatogram of Trx-CD44ct. C, static light scattering measurements of the molecular mass of Trx-CD44ct at different protein concentrations. D, CD spectra of CD44ct in solution (black line) and in PIP2 (red line). The protein concentration used for CD experiments is 0.1 mg/ml, and PIP2 concentration is 125 $\mu$M. E, comparing SANS data of dTrx-CD44ct (open black circle) and dCD44ct (filled blue square). The concentrations of dCD44ct and dTrx-CD44ct are 1.5 mg/ml, respectively. F, Guinier plot of dTrx-CD44ct (open black circle) and dCD44ct (filled blue square). G, $P(r)$ function of dTrx-CD44ct and dCD44ct in H$_2$O buffer. As a comparison, $P(r)$ of the crystal structure of thioredoxin (black line, PDB code 2TRX) is shown. H, the three-dimensional shape of dCD44ct is docked to that of dTrx-CD44ct. The three-dimensional shapes of dCD44ct and dTrx-CD44ct are reconstructed ab initio from SANS data, respectively, using the program DAMMIN (41). The crystal structure of thioredoxin (PDB code 2TRX) is docked in dTrx-CD44ct envelope.
aggregates (Fig. 2A and Table 1). \( R_g \) and \( D_{\text{max}} \) of \( d\)CD44ct in PIP2 are significantly larger than that of \( d\)CD44ct in solution (Table 1 and Fig. 2B), also indicating that \( d\)CD44ct aggregates upon binding to PIP2.

Unlike \( d\)CD44ct alone in solution (Fig. 2C), the Kratky plot of \( d\)CD44ct in PIP2 has a peak at \( QR_g \approx 1.8 \text{ Å}^{-1} \), suggesting that the \( d\)CD44ct aggregates are globular-like clusters (Fig. 2D). However, at \( QR_g > 3.5 \), in which SANS probes the internal structure of the aggregates, the Kratky plot increases with \( Q \), a behavior that is typical of a random coil. The SANS results thus indicate that upon binding to PIP2, \( d\)CD44ct unfolds into an open and random coil-like conformation and that the opened \( d\)CD44ct aggregates into large globular clusters.

FIGURE 2. Comparing the conformation of CD44ct in solution and in complex to PIP2. A, protein concentration normalized SANS data \( I(Q)/c \) of \( d\)CD44 in buffer solution (black filled square) and in PIP2 solution (black open circle). The SANS experiments were performed in 20% \( D_2O \) at the contrast-matching point of PIP2. The concentration of \( d\)CD44ct is 1.5 mg/ml, and the concentration of PIP2 is 1.2 mM. B, \( P(r) \) function of the SANS data shown in A. C, the normalized Kratky plots of \( d\)CD44ct in solution. D, the normalized Kratky plots of \( d\)CD44ct in PIP2 solution. The comparison suggests that, upon binding to PIP2, the conformation of CD44ct changes from a collapsed and disordered globule to aggregates composed of random coil-like chains.
consistent with the contrast-matching SANS results. Comparing the gel filtration results of CD44ct in PIP2 and in DHPC2 thus indicates that the aggregation of CD44ct is specific to PIP2.

**PIP2 Is Necessary for CD44ct Binding to Ezrin**

Using gel filtration and pulldown experiments, we have analyzed the binding of CD44ct to the full-length Ezrin (Fig. 3A). A phosphomimetic Ezrin(T567D) mutant was selected in this experiment, based on previous findings that a phosphomimetic Ezrin(T567D) mutant is more susceptible to adopt an open conformation than the wild-type full-length Ezrin upon binding to PIP2 and is thus more active in binding to target proteins than the wild-type Ezrin (23, 31).

The gel filtration results show that CD44ct does not interact with Ezrin(T567D) in solution but forms a complex with Ezrin(T567D) only in the presence of PIP2 (Fig. 3A). This result is consistent with previous findings that PIP2 is involved in the interaction of Ezrin with CD44 (24). Previous studies have attributed the role of PIP2 in the CD44-Ezrin interaction as disrupting the head to tail autoinhibition in ERM proteins and opening the molecular conformation of ERM. The exposed FERM domain of an active ERM protein is generally considered to be capable of binding directly to CD44.

Nevertheless, to our surprise, we find that in solution CD44ct does not bind to the FERM domain of Ezrin, which is a mimic of active Ezrin (54). Gel filtration, pulldown, and ITC experiments all show that CD44ct does not interact with the FERM domain in solution (Fig. 3, B, D, and E). Instead, only in the presence of PIP2, do CD44ct and FERM form a complex, as shown by gel filtration and pulldown experiments (Fig. 3, C and D). These results suggest that PIP2 not only serves as a conformational opener for the full-length Ezrin but is also necessary to mediate the interaction of CD44ct with FERM or with the full-length Ezrin.

**Contrast Variation SANS Reveals the Assembly of a 2CD44ct-2FERM or a 2CD44ct-2Ezrin Heterotetramer Complex in PIP2**

The Stoichiometry and the Structure of the PIP2\(^4\)-CD44ct\(^4\)-FERM Complex—To determine how PIP2 mediates CD44-ERM interaction, we have first performed contrast variation SANS on CD44ct and with PIP2 (Fig. 4A). With selective deuteration of a subunit in a multicomponent complex, contrast variation SANS can resolve the conformation of each component, as well as the stoichiometry and architecture of the whole complex (27, 31, 46). The molar ratio of incubation is PIP2\(^4\)-CD44ct\(^4\)-FERM = 10:1:1 based on our gel filtration and pulldown experiments, which show that the interaction of CD44ct with FERM
is more complete at this molar ratio of incubation than at lower molar ratios of PIP2.

SANS shows that the PIP2-CD44ct-dFERM complex has well defined \( P(r) \) functions at each contrast (Fig. 4B). The normalized forward scattering intensity \( I(0) \) versus the neutron scattering length density of the buffer \( \rho_b \) shows a straight line (Fig. 4C). These results suggest that \(^4\)CD44ct and \(^4\)FERM form a discrete complex in the presence of PIP2. According to Equation 1, the molecular mass of a protein complex can be determined by contrast variation SANS from the slope of Fig. 4C to be \( M_w = 107,826 \pm 2,770 \) g/mol, which corresponds to the molecular mass of a \(^2\)CD44ct-\(^2\)FERM heterotetramer plus 8–9 PIP2 lipid molecules (Table 1).

The Stuhrmann plot, defined as \( R_g^2 \) plotted versus \( 1/\Delta \rho \), where \( \Delta \rho \) is the neutron scattering length density contrast of the whole complex against solvent, can be used to estimate the distribution of scattering length density of the complex (55). The dependence of the \( R_g^2 \) on the contrast is approximated by the following expression,

\[
R_g^2 = R_s^2 + \frac{A}{\Delta \rho} - \frac{B}{\Delta \rho^2}
\]

(Eq. 2)

where \( R_s \) is the \( R_g \) of the complex at infinite contrast, \( A \) relates to the distribution of density as a function of distance from the center of mass, and \( B \) relates to the distance separating the center of mass of the particle shape from the center of mass of neutron density. A straight line with a positive slope in the Stuhrmann plot (Equation 2), as shown in Fig. 4D, indicates that the distribution of the hydrogenated PIP2 with respect to the deuterated \(^4\)CD44ct-\(^4\)FERM component is symmetric and that the hydrogenated PIP2 is located in the interior of the deuterated complex. In addition, the \( R_g \) at infinite contrast can be obtained from the Stuhrmann plot to be \( R_g = 56.2 \pm 0.5 \) Å. The \( I(0) \) and the Stuhrmann plot analyses thus suggest that \(^4\)CD44ct and \(^4\)FERM form a heterotetramer in PIP2 with a 2-fold symmetry.

In 20% D\(_2\)O at the contrast-matching point of PIP2, the scattering is from \(^4\)CD44ct-\(^4\)FERM. The \(^4\)CD44ct-\(^4\)FERM complex has \( R_g = 59.4 \pm 0.4 \) Å and \( D_{max} = 200 \pm 5 \) Å (Fig. 4B). The three-dimensional shape of the \(^4\)CD44ct-\(^4\)FERM complex was reconstructed from SANS using the program DAMMIN (48) (see Fig. 8A). A 2-fold symmetry was imposed when reconstructing the three-dimensional shape of the complex based on the information obtained from molecular mass analysis of the complex.

The Stoichiometry and the Structure of the PIP2-\(^4\)CD44ct-\(^4\)FERM Complex—We have then performed contrast variation SANS on the complex of \(^4\)CD44ct in complex with the hydrogenated FERM (\(^4\)FERM) in PIP2 at the incubation molar ratio of PIP2-\(^4\)CD44ct-\(^4\)FERM = 10:1:1 (Fig. 5). Again, the well behaved \( P(r) \) functions and the straight line of the normalized \( I(0)^{0.5} \) versus \( \rho_b \) plot suggest that the PIP2-\(^4\)CD44ct-\(^4\)FERM complex is a discrete complex with defined molecular mass (Fig. 5, B and C). The slope of Fig. 5C yields the molecular mass of the complex to be \( M_w = 97,772 \) g/mol, corresponding to the molecular mass of two \(^4\)FERM and two \(^4\)CD44ct plus 7–8 PIP2 lipid molecules in the complex (Fig. 5C and Table 1). The number of PIP2 lipids in the PIP2-\(^4\)CD44ct-\(^4\)FERM complex is consistent with that in the PIP2-\(^4\)CD44ct-\(^4\)FERM complex within experimental error.
With selective deuteration, the SANS data at different contrasts reveal the structures of PIP2\textsuperscript{dCD44ct}/H18528\textsuperscript{dCD44ct}, PIP2\textsuperscript{hFERM}, and the whole PIP2\textsuperscript{dCD44ct}/H18528\textsuperscript{hFERM} complex, respectively. In 40% D\textsubscript{2}O buffer that is the contrast-matching point of hydrogenated FERM, the Kratky plot shows that PIP2\textsuperscript{dCD44ct} adopts an open, random coil-like conformation (Fig. 5\textsuperscript{D}). In 100% D\textsubscript{2}O, the three-dimensional shape of the PIP2\textsuperscript{hFERM} component indicates that FERM is a dimer, and the Kratky plot indicates that PIP2\textsuperscript{hFERM} is a multidomain entity (Fig. 5\textsuperscript{D}). In 0% D\textsubscript{2}O in which all components in the complex contribute to scattering, the bell-like Kratky plot suggests a compact globular structure of the whole PIP2\textsuperscript{dCD44ct}/H18528\textsuperscript{hFERM} complex (Fig. 5\textsuperscript{D}).

In addition to SANS, we have performed light scattering on the PIP2\textsuperscript{dCD44ct}/H18528\textsuperscript{hFERM} complexes (Fig. 6). The dynamic light scattering data suggest that both the PIP2\textsuperscript{dCD44ct} and PIP2\textsuperscript{hFERM} complex are monodispersed, suggesting that both types of complexes are discrete entities (Fig. 6, A and B, inset, and the % polydispersity values). The molecular mass of PIP2\textsuperscript{dCD44ct} complex, as measured from static light scattering is 200 kDa. The hydrodynamic radius \(R_h\) of the PIP2\textsuperscript{dCD44ct} complex, as measured from dynamic light scattering, is 72 \pm 1 \text{ Å}. For the PIP2\textsuperscript{dCD44ct}/H18528\textsuperscript{hFERM}, the molecular mass is 133 kDa, and \(R_h\) is 65 \pm 2 \text{ Å}. Additionally, analytical ultracentrifugation experiments indicate that complex migrates as a discrete species. The best fit of the sedimentation velocity data of the major peak (Fig. 7\textsuperscript{D}) gives a frictional ratio of \(f/f_o = 1.359\). The higher value of \(f/f_o\) suggests that the sedimented species likely assumes an elongated shape. Sedimentation equilibrium experiments were performed at two speeds, 10,300 and 18,000 rpm. The sedimentation equilibrium data could fit into a "single species of interacting system" model and gave a molecular mass of \(\approx 134.9 \pm 6.7\) kDa (Fig. 7\textsuperscript{B}). The size and the molecular mass of the complex measured from light scattering and analytical ultracentrifugation experiments are larger than the SANS result. This is because light scattering and analytical centrifugation experiments measure the protein-lipid complex, whereas contrast-matching SANS measures protein assembly in the protein-lipid complex.

It is of interest to point that when preparing the protein/lipid complex for the light scattering and analytical centrifugation experiments, we first mixed the PIP2 and CD44ct at a 10:1 molar ratio, and FERM was added at 1 molar ratio. The complex was incubated overnight and separated by gel filtration before the centrifugation experiments. The sequence of mixing the different components for centrifugation experiments is different from the sample preparation for SANS experiments when we first mixed the CD44ct and FERM at equal molar ratio, and PIP2 of 10 molar ratio was added to form the complex. With either method of sample preparation, similar molecular mass
and size of the complex are obtained. These results suggest that the PIP2-CD44ct aggregate is reversible and that the PIP2-CD44ct aggregates dissociate on the time scale of incubation to form a stable tetramer with FERM. The exact kinetics of PIP2-CD44ct association and dissociation is a subject for future investigation.

The three-dimensional shape of the PIP2\textsuperscript{H18528d}CD44ct\textsuperscript{H18528d}FERM complex was reconstructed from SANS using the program MONSA (48) at different contrasts, assuming that the complex has three phases in terms of neutron density distribution (Fig. 8). A 2-fold symmetry was imposed when reconstructing the three-dimensional shape of the complex, based on the stochiometry and symmetry information obtained from the Stuhrmann plot and the molecular mass analyses. The reconstructed three-dimensional shape of the PIP2\textsuperscript{d}CD44ct\textsuperscript{d}FERM complex is shown in Fig. 8B.

Comparing the three-dimensional shapes of the PIP2\textsuperscript{d}CD44ct\textsuperscript{d}FERM and PIP2\textsuperscript{d}CD44ct\textsuperscript{h}FERM shows that the two differently deuterated complexes have similar overall architecture (Fig. 8). Further, the three-dimensional shape of PIP2\textsuperscript{d}CD44ct\textsuperscript{d}FERM reveals the internal structure and the relative position of each component in the heterotetramer complex. In this heterotetrameric complex, two \textsuperscript{h}FERM domains, shown in green, assemble into a dimer that are flanked by two \textsuperscript{d}CD44ct molecules shown in gold. The dimeric FERM is confirmed by the SANS data from the PIP2\textsuperscript{d}CD44ct\textsuperscript{h}FERM complex at 100% D\textsubscript{2}O, which is the contrast-matching point of the deuterated \textsuperscript{d}CD44ct component (Fig. 8E).
In the PIP2-dCD44ct-hFERM complex (Fig. 8B), the two dCD44ct molecules (gold) adopt an open conformation and are in contact with hFERM (green) and with the PIP2 lipid (red). The two dCD44ct are also in contacts with each other only at the lower half of the complex. In the PIP2-dCD44ct-hFERM complex, the red blobs representing PIP2 clusters are sandwiched between FERM and dCD44ct, likely mediating the interaction between hFERM and dCD44ct. Another patch of the PIP2 red blob is also shown at the upper tip of each of the two dCD44ct molecules, likely contributing to stabilizing the open conformation of PIP2 and mediating the interaction with FERM. These observations suggest that PIP2 contributes to stabilizing the open conformation of CD44ct and to mediating the binding of CD44ct with FERM.

**PIP2 and CD44ct Molecules Assemble the Activated Full-length Ezrin into a Dimer—Using SANS, we have compared the conformation and molecular mass of the full-length deuterated phosphomimetic mutant dEzrin(T567D) in solution, in PIP2,
and in PIP2 and dCD44 (Fig. 9). All the SANS experiments shown in Fig. 9 were performed in 20% D₂O, which is the contrast-matching point of PIP2. The SANS data of ⁴Ezrin(T567D) in solution and in PIP2 are taken from our previous publication (31). In that study, we showed that Ezrin or Ezrin(T567D) adopts a closed conformation, whereas the PIP2-bound Ezrin or Ezrin(T567D) adopts an open and monomer conformation. We also showed that upon binding to PIP2, Ezrin(T567D) is more likely to adopt an open conformation than the wild-type Ezrin. We thus chose ⁴Ezrin(T567D) in this study for determining the effects of CD44ct and PIP2 on the assembly of Ezrin.

Comparing the protein concentration normalized forward scattering I(0)/c indicates that the PIP2-⁴CD44ct-⁴Ezrin(T567D) complex has a molecular mass 2.15 times that of ⁴Ezrin(T567D) in solution, whereas I(0)/c of Ezrin(T567D) in PIP2 is approximately the same as that of ⁴Ezrin(T567D) in solution (Fig. 9A and Table 1). The comparison indicates that ⁴CD44ct assembles ⁴Ezrin(T567D) into a dimer in PIP2, although it is difficult to estimate with certainty how many ⁴CD44ct molecules are in the complex because of the relatively small molecular mass of ⁴CD44ct as compared with ⁴Ezrin(T567D). However, SANS on both the PIP2-⁴CD44ct-hFERM and PIP2-⁴CD44ct-FERM complexes has shown that two ⁴CD44ct molecules are in the heterotrimer complex (Figs. 5C and 6C). Combining the contrast-matching SANS experiments thus suggests that two CD44ct molecules assemble two full-length Ezrin molecules into a dimer in PIP2. Without CD44ct, the PIP2-bound Ezrin is an open monomer (31).

Fig. 9B compares the P(r) functions of ⁴Ezrin(T567D) in solution, ⁴Ezrin(T567D) in PIP2, and the ⁴CD44ct-⁴Ezrin(T567D) complex. We have previously shown that in solution, ⁴Ezrin(T567D) adopts a closed conformation, whereas the PIP2-bound ⁴Ezrin(T567D) adopts an open conformation (31). The radius of gyration of the ⁴CD44ct-⁴Ezrin(T567D) complex is significantly larger than the open, monomeric form of ⁴Ezrin(T567D) in PIP2 (Fig. 9B and Table 1). D_max of the ⁴CD44ct-⁴Ezrin(T567D) complex is 450 Å, as compared with D_max = 140 Å of ⁴Ezrin(T567D) in solution and D_max = 250 Å of ⁴Ezrin(T567D) in PIP2.

The three-dimensional shape of the ⁴CD44ct and PIP2 assembled ⁴Ezrin(T567D) dimer reconstructed using the program DAMMIN is shown Fig. 9C. An open model of Ezrin is docked into the three-dimensional envelope. Because the size of ⁴CD44ct is quite small as compared with the ⁴Ezrin(T567D) dimer, the ⁴CD44ct is not located in the map. However, the position and the conformation of ⁴CD44ct are shown in the PIP2-⁴CD44ct-hFERM complex in Fig. 8B. These results thus show that in the presence of PIP2, ⁴CD44ct assembles a dimer ⁴Ezrin(T567D) complex.

DISCUSSION

Our results first show that PIP2 binding to CD44ct unfolds CD44ct, and the unfolded CD44ct molecules either cluster into large aggregates among themselves or assemble with Ezrin into a specific heterotetramer complex. The study thus reveals an autoregulation mechanism in the cytoplasmic tail of CD44, as well as the important roles of PIP2 in CD44 clustering.

Second, the results further reveal that PIP2 assembles CD44ct and Ezrin into a heterotetramer complex. Previous studies have shown Ezrin and other ERM proteins are regulated by a self-inhibition mechanism, with the inactive ERM protein being held in a closed conformation by head to tail intramolecular interactions (21, 22, 56–58). Binding to PIP2 and phosphorylation disrupt the autoinhibition and activate ERM proteins (23). It is generally accepted that in the activated ERM proteins, the exposed FERM domain binds directly to CD44. In addition to CD44, the FERM domain of activated Ezrin also binds to multi-PDZ scaffolding protein NHERF1 that in turn binds to a variety of transmembrane receptors and ion transport proteins, whereas the exposed actin-binding site interacts with cytoskeletal actin (25–30). Previous studies also show that the basic residues in the juxtamembrane region of CD44ct are involved in direct binding to the FERM domain of ERM proteins (16, 17). Indeed, we find that a truncated CD44 juxtamembrane peptide can bind directly to the FERM domain. Nevertheless, our experiments show that the entire CD44ct does not bind to FERM. Instead, PIP2 is required for the entire CD44ct tail to form a complex with FERM. Moreover, SANS shows that in the PIP2-CD44ct-FERM complex, PIP2 clusters are sandwiched between CD44 and FERM. Thus, PIP2 molecules are involved in mediating the interaction of CD44 with FERM in the heterotetramer assembly shown in Fig. 10.

The above results of PIP2 mediating the assembly of multimeric CD44ct-FERM complexes may be explained by earlier findings that the polybasic residues in a peptide sequester PIP2...
into clusters (59, 60) and thatPIP2 can also sequester into clusters of transmembrane proteins that possess positively charged polybasic residues in the juxtamembrane (61). Polyvalent electrostatic interactions are responsible for such clustering of PIP2 and cytoplasmic tails of transmembrane proteins (61). The juxtamembrane region of CD44ct contains a stretch of multiple basic residues (Fig. 1A), similar to those basic residues in the juxtamembrane region of syntaxin-1A (61). The FERM domain of ERM proteins possesses two PIP2-binding motifs that are also composed of multiple basic residues (23, 31, 62, 63). We hypothesize that the negatively charged PIP2 molecules function as molecular fasteners that bridge specific heterocomplex formation of CD44ct with Ezrin by polyvalent ionic interactions. Future experiments could use mutagenesis and high resolution structural studies to test this hypothesis.

In cells, PIP2 is localized in the microdomains of the plasma membranes, in lamellipodia of migrating cells, as well as in the apical membrane and at the cell–cell junctions of polarized epithelial cells (60, 64–70). CD44 is also localized in these microdomains of cells. PIP2 is involved in the spatial-temporal regulation of the assembly and disassembly of the transmembrane protein complexes (71), which are required for effective cellular sensing and response to extracellular ligand binding and for regulating the cellular dynamics of cell adhesion, migration, and turnover of subcellular structures (58). Our findings point to the important roles of the PIP2 lipid in dynamically regulating CD44 conformation and clustering and in mediating the assembly of discrete multimeric complex of CD44 with Ezrin. Such membrane multimeric complexes of CD44 and Ezrin may be involved in the assembly of actin cytoskeleton-dependent receptor clusters at the cell surface (18) or in virus-host cell interactions (72).

The regulation of CD44 activity is likely to be multifaceted. CD44 is enriched in the cholesterol-rich lipid raft microdomains (19). Studies have shown that the lipid raft-associated CD44 is inactive to bind to Ezrin. Lipid raft thus negatively regulates cell migration (73, 74). Our study shows that PIP2 activates CD44 and mediates clustering of CD44 and the interaction of CD44 with Ezrin. Thus, it is likely that PIP2 and the cholesterol-rich lipid raft play opposite roles in regulating the function of CD44. At present, the structural mechanism of CD44ct autoinhibition is not known.

In summary, Fig. 8 shows the schematic presentation of the findings of this study that PIP2 induces aggregation of CD44ct and that PIP2 assembles multimeric CD44-Ezrin complexes. Future studies should determine the structure of CD44ct at a resolution higher than the SAXS data presented in this study and address how full-length CD44 binding to ligand triggers the changes in PIP2 localization and the dynamic exchange of CD44 with the lipid raft and with PIP2-associated microdomains.

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