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Lactadherin binds selectively to membranes containing phosphatidyl-L-serine and increased curvature

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Abstract

Lactadherin, a milk protein, contains discoidin-type lectin domains with homology to the phosphatidylserine-binding domains of blood coagulation factor VIII and factor V. We have found that lactadherin functions, in vitro, as a potent anticoagulant by competing with blood coagulation proteins for phospholipid binding sites [J. Shi and G.E. Gilbert, Lactadherin inhibits enzyme complexes of blood coagulation by competing for phospholipid binding sites, Blood 101 (2003) 2628–2636]. We wished to characterize the membrane-binding properties that correlate to the anticoagulant capacity. We labeled bovine lactadherin with fluorescein and evaluated binding to membranes of composition phosphatidylserine/phosphatidylethanolamine/phosphatidylcholine, 4:20:76 supported by 2 μm diameter glass microspheres. Lactadherin bound saturably with an apparent K_D of 3.3±0.4 nM in a Ca^{++}-independent manner. The number of lactadherin binding sites increased proportionally to the phosphatidylserine content over a range 0–2% and less rapidly for higher phosphatidylserine content. Inclusion of phosphatidylethanolamine in phospholipid vesicles did not enhance the apparent affinity or number of lactadherin binding sites. The number of sites was at least 4-fold higher on small unilamellar vesicles than on large unilamellar vesicles, indicating that lactadherin binding is enhanced by membrane curvature. Lactadherin bound to membranes with synthetic dioleoyl phosphatidyl-L-serine but not dioleoyl phosphatidyl-D-serine indicating stereoselective recognition of phosphatidyl-L-serine. We conclude that lactadherin resembles factor VIII and V with stereoselective preference for phosphatidyl-L-serine and preference for highly curved membranes.
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Keywords: Lactadherin; Phosphatidylserine; Membrane; Phospholipid vesicle; Blood coagulation; MFG-E8

1. Introduction

Lactadherin is a MW 47,000 glycoprotein of milk fat globules. It has also been known as PAS-6/7, indicating the two glycosylation variants [1], bovine-associated mucoprotein, BA-46, P47, and MFG-E8 [2]. Lactadherin has a domain structure of EGF1–EGF2–C1–C2 in which EGF indicates epidermal growth factor homology domains, and the C domains share homology with the discoidin family including the lipid-binding "C" domains of blood coagulation factor VIII and factor V [2]. The second EGF domain displays an Arg–Gly–Asp motif [3], which binds to the $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins [1,4–6]. The second C domain binds to phospholipids [6].

In milk fat globules, lactadherin lines the surface of the phospholipid bilayer which surrounds the central triglyceride droplet, apparently stabilizing the bilayer [7]. In tissue sections, lactadherin is found within the milk ductules and localized on the apical portion of secretory epithelium in the breast [7]. Abundant expression by breast carcinoma makes lactadherin a potential target for antigen-guided radiation therapy [8]. Lactadherin also lines the apical surface of epithelia in the biliary tree, the pancreas, and sweat glands [7] and is synthesized by aortic medial smooth muscle cells [9]. Lactadherin has been identified as a zona pellucida-binding protein on the acrosomal cap of sperm where it

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mediates fusion with an oocyte [10,11]. Stimulated macrophages, but not quiescent macrophages, synthesize and secrete lactadherin in vitro [12]. Lactadherin then binds to apoptotic cells expressing phosphatidylserine and mediates phagocytosis of the dying cells via interaction of the lactadherin EGF domain with macrophage integrin(s). Mice lacking lactadherin develop splenomegaly and immune complex glomerulonephritis, apparently because of impaired phagocytosis of apoptotic lymphocytes [13].

We have recently found that, in vitro, lactadherin functions as a potent anticoagulant [14]. Homology between the lactadherin and factor VIII C domains correlates with efficient competition for membrane binding sites recognized by both factor VIII and factor V. Lactadherin inhibits the factor Xase complex, in which factor VIII functions, and the prothrombinase complex, in which factor V functions. Lactadherin also inhibits the factor VIIa-tissue factor complex and competes with vitamin K-dependent factor IXa for membrane binding sites. The inhibitory properties of lactadherin contrasted with those of annexin V. Although annexin V binds to phosphatidylserine-containing membranes with high affinity, it functions well as an anticoagulant only when the phosphatidylserine content exceeds 4% and the membrane curvature is very limited. By contrast, lactadherin was an efficient anticoagulant on membranes with <4% phosphatidylserine and regardless of curvature. The in vitro anticoagulant properties of lactadherin suggest the possibility that it may have an anticoagulant function during some physiologic or pathologic conditions [14].

Blood coagulation factor VIII and factor V bind to phospholipid membranes via “C” domains which share homology with lactadherin “C” domains [15–17]. Remarkable features of membrane binding include high affinity (K_D approx. 2 nM) [18] and sufficient specificity so that no plasma proteins compete for membrane binding sites [19]. Factor VIII binds via stereoselective interaction with the phospho-L-serine motif of phosphatidylserine (Ptd-L-Ser) [20]. Factor V also exhibits stereoselective interaction with Ptd-L-Ser [21]. Binding of factor VIII is enhanced by the presence of phosphatidylethanolamine (PE) in the membrane [22], by unsaturated phospholipid acyl chains [23], and by membrane curvature [22]. The crystal structures of the C2 domains of factors VIII and V suggest that membrane binding is mediated by two pairs of hydrophobic residues displayed at the tips of β-hairpin turns [24,25]. Mutagenesis studies have confirmed the role of these residues in phospholipid binding [26,27]. The homology of the lactadherin C domains with those of factors VIII and V suggests that similar phospholipid binding properties may be mediated by hydrophobic residues on putative β-hairpin turns. Indeed, lactadherin has been found to bind Ptd-L-Ser adsorbed to plastic [28] and to utilize primarily the C2 domain in its lipid binding [6]. Furthermore, the capacity of lactadherin to quantitatively compete with both factor VIII and factor V for membrane binding sites [14] suggests that it has high affinity for these membranes and a similar mechanism of binding.

The present study was undertaken to characterize the membrane-binding properties of lactadherin in the context of the properties of factor VIII and factor V. Our results indicate that lactadherin resembles factor VIII and factor V in a high affinity, stereoselective recognition of Ptd-L-Ser and in preferential binding to highly curved membranes. Lactadherin differs from factor VIII in requiring fewer Ptd-L-Ser molecules per binding site and PE-independent binding.

2. Materials and methods

2.1. Materials

Bovine brain phosphatidylserine (Ptd-L-Ser), egg yolk PE, bovine liver phosphatidylinositol, egg sphingomyelin, egg phosphatidylcholine (PC) and dioleoyl PC were from Avanti Polar Lipids (Alabaster, AL). Phospholipase D from streptomyces, phospho-L-serine, phospho-D-serine, L-serine and D-serine were from Sigma (St. Louis, MO).

2.2. Purification and fluorescein labeling of proteins

Lactadherin was purified from bovine milk as previously described [1]. Lactadherin, 0.2 mg in 0.2 ml phosphate-buffered saline, was concentrated 10-fold by ultrafiltration with a Centricon YM-10 (Millipore, Bedford, MA) followed by dilution to the starting concentration into 0.1 M sodium carbonate buffer, pH 9.0. Fluorescein isothiocyanate (Molecular Probes, Eugene, OR) 6 μl of 10 mg/ml solution in DMSO, was added to lactadherin and the mixture was incubated for 1 h at RT in the dark. Free fluorescein was removed by gel filtration using a microspin column equilibrated with 0.1 M sodium carbonate, 0.1 M betaine, 0.004% Tween-80, pH 9.0 (FluoReporter FITC Protein Labeling Kit, Molecular Probes). Fluorescein-labeled lactadherin was concentrated approx. 10-fold by ultrafiltration using a Centricon YM-10, and diluted to approximately the original concentration in 0.15 M NaCl, 50 mM Tris–HCl, pH 7.5. Labeling efficiency was judged by comparison of absorbance at 490 nm to corrected absorbance at 280 nm (corrected $A_{280}=A_{280}-0.35\times A_{490}$, compensating for fluorescein absorption at 280 nm).

2.3. Synthesis of Ptd-L-Ser and Ptd-D-Ser

Ptd-L-ser and Ptd-D-ser were synthesized by enzymatic transphosphatidylation of dioleoyl PC by phospholipase D and purified as previously described [29]. Briefly, 50 mg of dioleoyl PC was suspended in 2 ml of 50% w/v L-serine or D-serine, 5% w/v octyl glucoside, 0.1 M $CaCl_2$, and 0.1 M Na acetate and stirred for 3 h at 45 °C. The reaction was stopped by addition of EDTA and phospho-

lipids were extracted with a 20:1 ratio of chloroform/methanol 1:1. Ptd-L-ser or Ptd-D-ser was purified from phosphatidic acid and residual PC by carboxymethylcellulose column chromatography [29]. Fractions containing Ptd-L-ser or Ptd-D-ser were identified and analyzed for purity by thin layer chromatography on silica plates in a solvent system of chloroform/methanol/acetic acid/water 25:15:4:2. Phospholipids were visualized by spraying the plate with a 1:1 solution of molybdenum blue (Sigma) with 4.2 M sulfuric acid.

2.4. Preparation of phospholipid vesicles

Phospholipid vesicles were prepared by evaporating chloroform from the desired phospholipids, resuspending in methylene chloride and re-evaporating twice under argon. Large multilamellar vesicles (LMV) were prepared by gently swirling tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4) over the dried lipid suspension until all lipid was suspended [30]. Small unilamellar vesicles (SUV) were prepared from a portion of the LMV by sonication in a high intensity bath sonicator under an argon atmosphere (Laboratory Supplies, Inc. Hicksville, NY) as described (www.avantilipids.com/PreparationOfLiposomes.html). Large unilamellar vesicles (LUV) were prepared by extruding LMV suspensions 20 times through two stacked polycarbonate membranes with 0.1 μm diameter laser-etched pores (Millipore) in a High Pressure Extrusion Device (Sciema Technical Services, Vancouver, BC, Canada) under argon as described previously [31]. Phospholipid concentration was determined by phosphorus assay [32]. Vesicles were used fresh, or 1 ml aliquots were quick-frozen in liquid nitrogen, stored at −80 °C, and thawed at 37 °C. Storage at 4 °C before usage did not exceed 24 h.

Evaluation of phospholipid vesicle homogeneity and approximate diameter was performed by size exclusion chromatography on a 1×30 cm closed column of Sephacryl S-1000 (Amersham-Pharmacia) as described [33]. The elution buffer was 0.15 M NaCl, 0.02 M Tris–HCl, pH 7.5 at 1 ml/min provided by an Akta Prime pump (Amersham-Pharmacia). Tween 80 at 0.01% vol/vol was added to the elution buffer for chromatography of size standards but not for vesicles. Vesicle and microsphere elution was monitored by scattered light at 280 nm. LMV eluted first with a retention volume of 7.7 ml. Latex microspheres of 0.55 μm diameter (Polysciences, Inc., Warrington, PA) eluted at 8.4 ml. LUV eluted with a retention volume of 12.0 ml and SUV with a retention volume of 18.8 ml. The hydrodynamic diameters of vesicles were determined utilizing dynamic light scattering with a Brookhaven 90 Plus particle size analyzer equipped with software ver. 3.60 (Brookhaven Instruments Corp, Holtsville, NY). The phospholipid concentrations were 0.1 mM for LMV, 1 mM for LUV and SUV. For LUV the electronic “dust filter” was utilized (setting=30) to exclude very large statistical outliers.

2.5. Flow cytometry phospholipid binding assay

Glass microspheres of 1.6 μm nominal diameter (Duke Scientific, Palo Alto, CA) were cleaned, size-restricted and covered with a phospholipid bilayer as previously described [19]. Briefly, the cleaned microspheres were incubated with sonicated vesicles while additional sonication was applied. Membranes supported by glass microspheres (lipospheres) were washed three times in 0.15 M NaCl, 0.02 M Tris–HCl, 0.1% defatted bovine albumin, 10 μM egg PC as sonicated vesicles; stored at 4 °C; and used within 8 h of synthesis. This procedure was performed on 150 μl aliquots with an approximate liposphere concentration of 1×10^6/ml using a Becton Dickinson FACSCalibur flow cytometer. Data acquisition was triggered by forward light scatter with all photomultipliers in the log mode. Noise was reduced during analysis by eliminating events with forward and side scatter values different from those characteristic of the lipospheres. Mean log fluorescence was converted to linear fluorescence

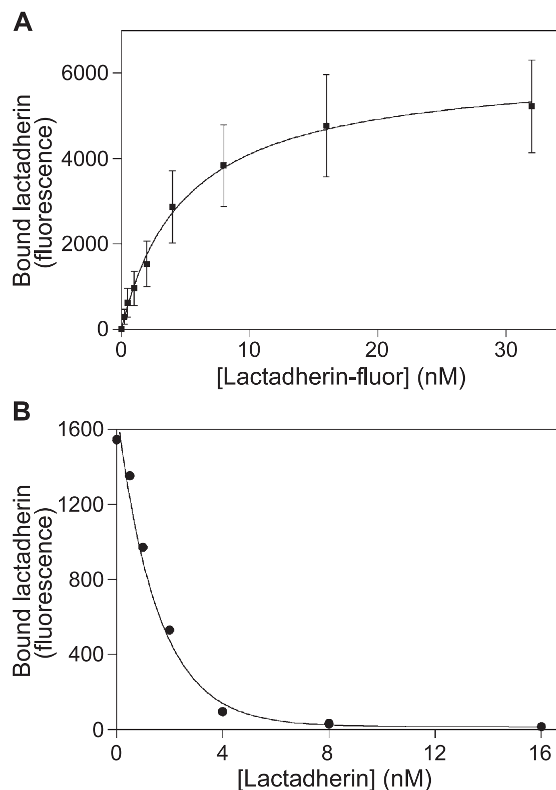

Fig. 1. The binding of lactadherin to lipospheres. Fluorescein-labeled lactadherin was incubated with 10^6 lipospheres/ml in Tris-buffered saline, 1.5 mM $CaCl_2$, 0.1% BSA for 10 min prior to evaluation of bound lactadherin by flow cytometry. (A) The final concentrations of lactadherin were varied to estimate the affinity and stoichiometry of bound lactadherin. Non-linear, least squares curve fitting (smooth line) indicated an apparent K_D of 3.3±0.4 nM. (B) The specificity of phospholipid binding sites for fluorescein-labeled lactadherin binding was evaluated in a competition experiment in which varying concentrations of lactadherin were mixed with 1 nM fluorescein-lactadherin prior to the addition of lipospheres. Displayed data are mean±S.D. for three experiments with different batches of lipospheres (A) or a single experiment representative of three experiments (B).

for values depicted in the figures. Only experiments in which the fluorescence histogram indicated a log normal distribution, as judged by inspection, were analyzed quantitatively. Flow cytometry experiments were performed in 0.14 M NaCl, 0.02 M Trizma–HCl, 0.1% bovine albumin, pH 7.5 with $CaCl_2$ as specified for individual experiments.

2.6. Data analysis

The binding of lactadherin to lipospheres was fitted by non-linear, least squares data analysis using the standard binding model (assuming a single class of binding sites and that the fraction of bound lactadherin was small at each concentration evaluated). For experiments depicted in Figs. 3 and 4, data were normalized, then subtracted from 1 (to prepare curves representing the increasing fraction of bound lactadherin as a function of phospholipid concentration). This treatment is justified by assuming that the liposphere-bound lactadherin is proportional to the free lactadherin concentration over a range of 0–1 nM (see Fig. 1). The derived phospholipid binding curves were fitted to the equation: $\mathrm{lacterin_{Bound}}/\mathrm{lacterin_{Total}}=(\mathrm{phospholipid}/n)/(K_D+\mathrm{phospholipid}/n)$, rearranged as

$$= \mathrm{phospholipid}/((K_D \times n) + \mathrm{phospholipid})$$

using the Prism 3.0 software package. "phospholipid" represents the total phospholipid concentration, "n" is the ratio of phospholipid monomers per binding site, and K_D is the dissociation constant between a binding site and lactadherin. Curves were fitted for the composite variable, $K_D \times n$.

3. Results

We hypothesized that lactadherin would exhibit membrane binding properties that resemble those of factor VIII, e.g. recognition of specific phospholipid binding sites and stereoselective interaction with Ptd-L-Ser. To test this hypothesis, we prepared lactadherin labeled with fluorescein isothiocyanate as described under Section 2.3. The molar ratio of fluorescein to lactadherin was 1.2 in the first labeling and 1.3 in the second labeling. Fluorescein-labeled lactadherin interacted with phospholipid bilayers supported by glass microspheres (lipospheres). The phospholipid composition was 4% Ptd-L-Ser, 20% PE, with the balance as PC. After 10 min, we measured bound lactadherin using flow cytometry (Fig. 1A). Lactadherin bound saturably to lipospheres with half-maximal binding at a lactadherin concentration of approximately 3 nM. We fitted binding data with the standard binding model, which assumes equilibrium between a ligand and a single class of binding sites (smooth line). The best fit indicated a K_D of 3.3±0.4 nM. Non-labeled lactadherin competed with fluorescein-labeled lactadherin (Fig. 1B) for membrane binding sites. These data indicate that lactadherin binds to Ptd-L-Ser containing membranes with high affinity and that binding is not substantially altered by labeling lactadherin with fluorescein isothiocyanate.

In order to evaluate the relationship between membrane Ptd-L-Ser content and the binding of lactadherin, we evaluated binding of fluorescein-labeled lactadherin to membranes containing 1% Ptd-L-Ser vs. 4% Ptd-L-Ser (Fig. 2). We observed saturable binding of lactadherin to both membrane types. The apparent K_D for membranes of 1% Ptd-L-Ser was within 2-fold of membranes with 4% Ptd-L-Ser while the fluorescence plateau of membranes with 4% Ptd-L-Ser was 2.3-fold higher than for membranes with 1% Ptd-L-Ser. This suggests that the major effect of increased Ptd-L-Ser content is to increase the number of Ptd-L-Ser binding sites rather than the affinity of lactadherin for membranes.

To explore the effect of membrane curvature on formation of binding sites for lactadherin, we prepared phospholipid vesicles of various sizes. LMV had a mean hydrodynamic diameter of 810 nm (polydispersity index—0.44). LUV had a mean hydrodynamic diameter of 101 nm (polydispersity index—0.20). SUV had a mean hydrodynamic diameter of 39 nm (polydispersity index—0.32).

In competition binding experiments, SUV exhibited the highest number of binding sites for lactadherin (Fig. 3A). Only 20% of lactadherin remained free to bind lipospheres when vesicles of 16% Ptd-L-Ser were at a phospholipid concentration of 0.05 μM. The number of phospholipid monomers/binding site is <60 under these conditions because the number of binding sites must exceed the bound lactadherin concentration of 0.8 nM. This relationship places limits on the apparent dissociation constant and binding site size for the phospholipid vesicles. Because $n<60$ for SUV of 16% Ptd-L-Ser, the corresponding K_D must be >0.2 nM to preserve the constraints on these parameters imposed by the standard binding model. We discuss these constraints further under Section 4.

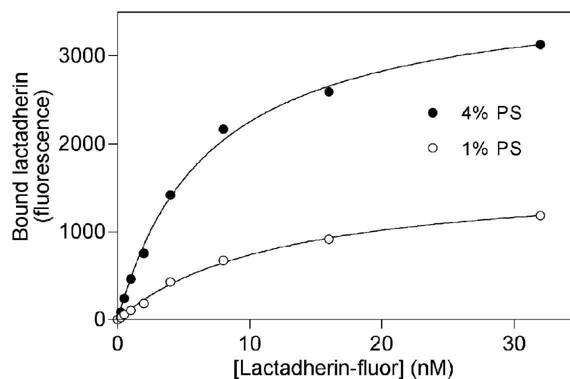

Fig. 2. Relationship between Ptd-L-Ser content and number of lactadherin binding sites. Fluorescein-labeled lactadherin was incubated with lipospheres with membranes of 1% (○) and 4% (●) Ptd-L-Ser at the final concentrations indicated. After 10 min the mean fluorescence/liposphere was evaluated by flow cytometry. Fitted curves indicated a plateau 2.3-fold higher for lipospheres of 4% Ptd-L-Ser. Displayed data is representative of three such experiments.

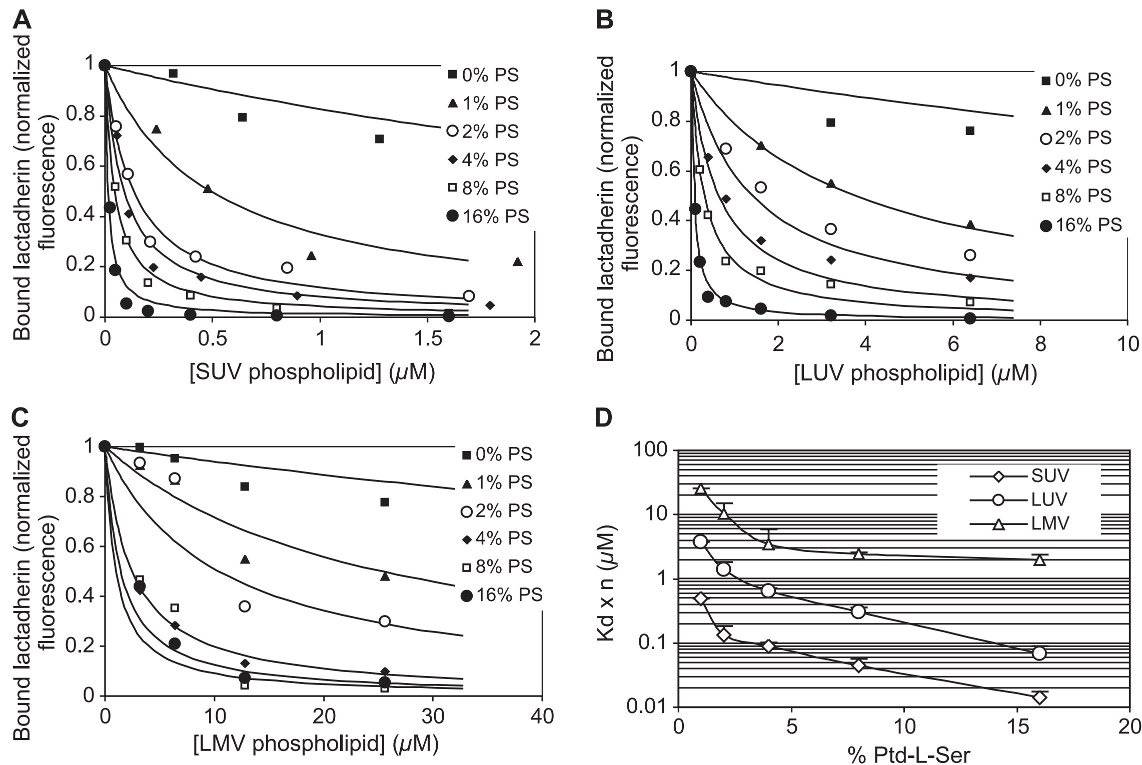

Fig. 3. Relationship of membrane curvature and Ptd-L-Ser content to binding of lactadherin. SUV (A), LUV (B), and LMV (C) with the indicated Ptd-L-Ser content were mixed with 1 nM fluorescein-labeled lactadherin for 30 min. Liposphere were added and bound lactadherin was evaluated after 10 min by flow cytometry. Liposphere-bound lactadherin was interpreted as a measure of free lactadherin. The reduction in free lactadherin with increasing vesicle concentrations was utilized to fit vesicle-binding curves according to the formula described under Section 2.6 (smooth lines). The products of K_D and phospholipid/binding site stoichiometry (n) obtained from curve fitting are plotted as a function of Ptd-L-Ser content (D). These values are approximately 10-fold lower for extruded vesicles (○) vs. LMV (△) and 10-fold again for SUV (◇) vs. extruded vesicles over the range of 1–16% Ptd-L-Ser. Displayed results are from a single set of experiments.

LUV exhibited at least 4-fold fewer binding sites and the minimum possible K_D was 4-fold higher (Fig. 3B). LMV (Fig. 3C) exhibited the fewest binding sites. The ratio of phospholipid monomers/binding site was 30-fold higher than extruded vesicles ($n \leq 6000$). However, the maximum dissociation constant remained in the same range as the K_D's for sonicated and extruded vesicles. This result suggests that, like factor VIII and factor V, lactadherin has a similar affinity for membranes with greatly varying curvature but the number of phospholipid molecules per site can vary substantially.

We used non-linear least squares curve fitting to model our competition binding data (Fig. 3A–C smooth curves). A composite parameter, $K_D \times n$, was utilized to fit the dissociation binding curves (Fig. 3D). Assuming that K_D does not vary substantially with membrane curvature, as discussed above, the fitted curves suggest that sonicated vesicles may have as many as 10-fold more lactadherin bindings sites per phospholipid molecule than larger extruded vesicles. Extruded vesicles have approximately 10-fold more sites/phospholipid monomer than LMV. For all vesicle types, the number of phospholipid monomers per binding site increases approximately 50-fold as the Ptd-L-Ser content drops from 16% to 1% with the steepest drop when Ptd-L-Ser≤2%.

We asked whether PE enhances binding of lactadherin to membranes containing Ptd-L-Ser, as it does for factor VIII. Accordingly, we utilized extruded vesicles containing 0–20% PE and 1% Ptd-L-Ser for competition binding experiments. The PE did not influence the apparent number of binding sites or affinity for lactadherin. Thus, lactadherin differs from factor VIII in exhibiting high affinity binding that is independent of membrane PE content.

We asked whether a negatively charged phospholipid other than Ptd-L-Ser will support binding of lactadherin. Phosphatidylinositol was selected because it is the second most abundant anionic phospholipid of eukaryotic cells. The experiment utilized extruded vesicles with phosphatidylinositol as competitors with lipospheres containing Ptd-L-Ser for binding of lactadherin. The results indicated that, although phosphatidylinositol is a negatively charged lipid, it does not support high affinity binding of lactadherin. PE did not alter the lack of efficacy for phosphatidylinositol in supporting high affinity binding of lactadherin.

All vesicles containing phosphatidylinositol supported low affinity binding of lactadherin. Control vesicles containing only PC or a mixture of PC and sphingomyelin also supported low affinity lactadherin binding. This indicated that neither phosphatidylinositol or PE have a specific effect on membrane binding of lactadherin. Curve-fitting analysis

for vesicles lacking Ptd-L-Ser indicated that the product of $K_D \times n$ was at least 20-fold greater than for vesicles of comparable curvature containing 2% Ptd-L-Ser.

Because factor VIII (unpublished observations) and factor V [34] exhibit Ca^{++}-independent membrane binding, we asked whether Ca^{++} influences membrane binding of lactadherin. The results indicated equivalent binding of lactadherin to membranes containing 4% Ptd-L-Ser in the presence of 1.5 mM Ca^{++} vs. no Ca^{++}. These results indicate that membrane binding of lactadherin is also Ca^{++}-independent.

We asked whether lactadherin, like factor VIII and factor V, exhibits stereoselective preference for Ptd-L-Ser vs. Ptd-D-Ser (Fig. 4). Dioleoyl Ptd-D-Ser and control dioleoyl Ptd-L-Ser were synthesized by transphosphatidylation of dioleoyl PC. Synthesized Ptd-L-Ser and Ptd-D-Ser were purified by carboymethylcellulose chromatography and purity confirmed by TLC migration of synthetic Ptd-L-Ser and Ptd-D-Ser vs. bovine brain Ptd-L-Ser. We mixed the synthetic Ptd-D-Ser or Ptd-L-Ser as 2% of total phospholipid with 20% PE and the balance as PC. The phospholipid mixture was hydrated and sonicated to form SUV as described under Section 2. These vesicles and control vesicles lacking Ptd-L-Ser or Ptd-D-Ser were used as a lipid source to prepare lipospheres with which to evaluate the selectivity of lactadherin for Ptd-L-Ser.

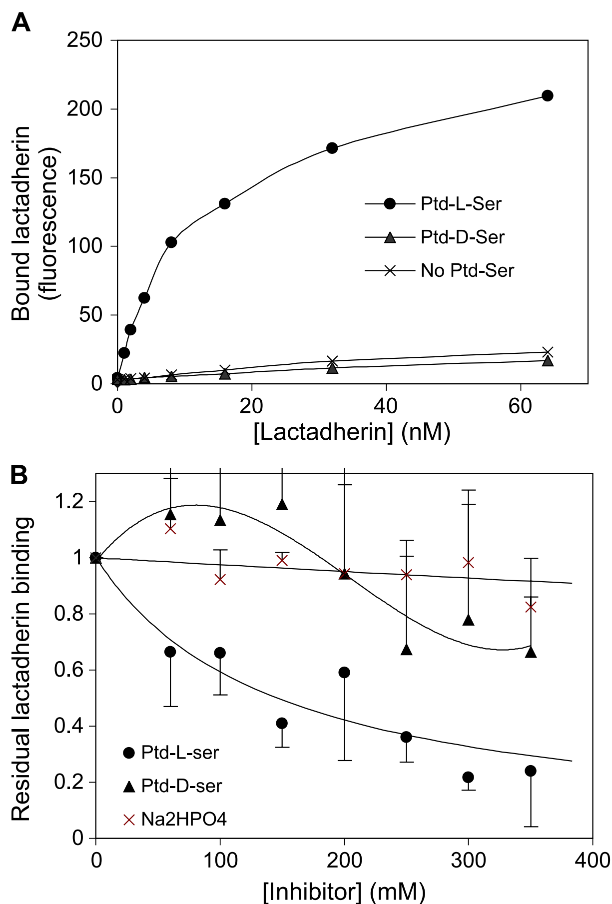

Fig. 4. Relationship of lactadherin binding to the stereochemistry of Ptd-L-Ser. (A) Binding of lactadherin to lipospheres displaying 2% synthetic dioleoyl Ptd-L-Ser (●) vs. 2% synthetic Ptd-D-Ser (▲) vs. no phosphatidylserine (×) was evaluated by flow cytometry. Lactadherin bound with high affinity to membranes containing Ptd-L-Ser but not Ptd-D-Ser. (B) The affinity of lactadherin for the isolated head group of Ptd-L-Ser or Ptd-D-ser was estimated in competition binding experiments with phospho-L-serine (●) vs. phospho-D-serine (▲) vs. phosphate (×). Phospho-L-serine inhibited binding with a K_i of 160 mM (smooth line), whereas neither phospho-D-serine or phosphate inhibited binding more than 25% at concentrations as high as 0.35 M. Displayed results are from a single experiment representative of three experiments (A), and mean±S.D. for three experiments (B).

Lipospheres with membranes containing 2% synthesized dioleoyl Ptd-L-Ser bound lactadherin with high affinity, comparable to Ptd-L-Ser from biologic sources. Lipospheres displaying synthesized 2% Ptd-D-Ser did not support binding of lactadherin above the level supported by control membranes of PE/PC 20:80 (Fig. 4A). These results indicate that lactadherin has a stereoselective affinity for Ptd-L-Ser but that the affinity for Ptd-D-Ser does not exceed the affinity for PE.

To estimate the stereoselective affinity of lactadherin for the L-serine containing head group of Ptd-L-Ser we performed binding inhibition studies (Fig. 4B). Phospho-L-Ser inhibited binding to lipospheres with half-maximal concentration of 160 mM. In contrast, phospho-D-serine caused a modest increase in membrane binding at low concentrations and was at least 4-fold less effective as an inhibitor. These results are consistent with stereoselective binding of lactadherin to Ptd-L-Ser but indicate that additional lipid component(s) are necessary to support high affinity binding.

4. Discussion

Our data identify membrane-binding properties of lactadherin that are both similar to and different from those of blood clotting factors VIII and V. Lactadherin exhibits specificity for Ptd-L-Ser and high affinity for membrane binding sites, similar to factors VIII and V. Also, lactadherin recognizes many more binding sites on membranes that are highly curved as opposed to the more planar membranes found in larger vesicles [22,35]. In contrast to factor VIII, but with similarity to factor V, lactadherin recognizes many binding sites on membranes with a Ptd-L-Ser content of less than 4%. Lactadherin differs from factor VIII in binding which is independent of membrane PE content [22].

This report builds upon published data demonstrating that lactadherin binds preferentially to phosphatidylserine. Prior reports indicated that lactadherin binds to purified Ptd-L-Ser absorbed to a plastic surface [4,6,28] but does not bind to phosphatidic acid prepared in the same manner [28]. Our data demonstrates binding to Ptd-L-Ser that is in membrane bilayers. In addition, we report the high affinity of lactadherin for Ptd-L-Ser when it is dispersed in a membrane bilayer at concentrations that exist in cell

membranes. Furthermore, we report that lactadherin exhibits stereoselective preference for the L-serine diastereomer of phosphatidylserine. Thus, our data indicate that lactadherin recognizes the three-dimensional structure of Ptd-L-Ser rather than interacting on the basis of a physical property such as negative charge [19,36].

Ptd-L-Ser is a diastereomer rather than a stereoisomer of Ptd-D-Ser and we considered the possibility that different physical properties of the diastereomers might influence phospholipid affinity of lactadherin. Ptd-L-Ser has chiral centers at both the α-carbon of serine and the β-carbon of the glycerol backbone. As such, Ptd-D-Ser has different physical properties than Ptd-L-Ser. For example, pure preparations of the two phospholipids undergo phase transitions between Lβ and Lα phases at temperatures that differ by 0.4 °C [37]. Thus, lactadherin's selectivity for membranes containing Ptd-L-Ser could be a consequence of the physical properties of Ptd-L-Ser rather than chemical recognition of the phospho-L-serine head group by lactadherin. This explanation seems unlikely for two reasons. First, when Ptd-L-Ser and Ptd-D-Ser are dispersed in phospholipid bilayers the measurable physical properties are not easily distinguishable. For example, clustering of Ptd-L-Ser in response to Ca^{++} is not measurably different than clustering of Ptd-D-Ser [37]. Second, our experiments with phospho-L-serine vs. phospho-D-serine indicated that lactadherin interacts selectively with the phospho-L-serine moiety (Fig. 4B). Thus, we believe the most likely explanation for selective binding of lactadherin to membranes with Ptd-L-Ser is a stereoselective binding of lactadherin to Ptd-L-Ser that includes selective binding to the phospho-L-serine moiety.

The physiologic relevance of lactadherin's preference for curved membranes may become evident in future studies. We speculate that lactadherin may bind preferentially to convex cell surfaces such as pseudopods or plasma membrane vesicles such as those that enclose milk fat droplets. Another possibility is that in vivo lactadherin may induce membrane curvature rather than respond to it. For example, lactadherin that is secreted into the mammary duct could bind to plasma membrane Ptd-L-Ser or to a protein receptor of mammary epithelial cells and induce regions of convexity.

Lactadherin's membrane-binding requirements contrast sharply with those of annexin V. While both proteins bind preferentially to Ptd-L-Ser-containing membranes, the relationship between Ptd-L-Ser content and number of binding sites is distinct. Lactadherin exhibits a steep positive relationship between Ptd-L-Ser content and number of binding sites over the range of 0–2% Ptd-L-Ser. In contrast, the relationship for Annexin V is sigmoidal with very few binding sites for a composition of less than 4% Ptd-L-Ser at physiologic Ca^{++} concentrations and a steep rise with Ptd-L-Ser content above that level [38,39]. Lactadherin binds preferentially to regions of sharp curvature while Annexin V binds preferentially to flat membrane patches [40]. Annexin V requires Ca^{++} and has enhanced binding when PE is present [41]. Lactadherin is independent of Ca^{++} and PE. Annexin V molecules self-associate on the membrane leading to a cooperative binding [42]. A consequence of the different membrane requirements is that annexin V exhibits limited capacity to inhibit the factor Xase or the prothrombinase complexes unless the Ptd-L-Ser content is high and the phospholipid vesicles have a large diameter [14]. In contrast to annexin V, lactadherin inhibits both enzyme complexes over the range of phospholipid compositions and vesicle curvatures which support enzymatic activity.

Annexin V has found usage as a detector of cells that express surface Ptd-L-Ser [43,44]. Our data predict that lactadherin might serve as a Ptd-L-Ser probe that is complementary to annexin V. The two proteins might bind to different cells or regions of cells that express Ptd-L-Ser. Annexin V binds to apoptotic cells where the phospholipid asymmetry between the inner and outer leaflets of the plasma membrane has collapsed. In contrast, annexin V does not bind to undifferentiated cells or malignant cells, that express sufficient Ptd-L-Ser to support activity of the prothrombinase and factor Xase complexes [45]. If lactadherin successfully identifies immature cells, malignant cells, or certain cellular appendages then it is also plausible that lactadherin or the lectin domain(s) of homologous proteins could serve as pharmacologic targeting modules to identify or direct therapeutic agents to the Ptd-L-Ser-displaying cells.

Phospholipases, like lactadherin, bind to Ptd-L-Ser or other negatively charged lipids in highly curved membrane regions to initiate membrane digestion [46]. Thus, it is possible that occupation of curved membrane regions with Ptd-L-Ser by lactadherin may protect milk fat globules from premature digestion by phospholipases or lipases in the epithelial cell, in milk, in the lumen of the GI tract, or in blood.

A recent report demonstrated that stimulated macrophages bind and engulf apoptotic cells by a mechanism in which lactadherin functions as a bridge ligand [12]. Lactadherin binds to phosphatidylserine on apoptotic cells. The macrophages recognize bound lactadherin via the RGD motif and use lactadherin to engulf the apoptotic cells. Our data suggest that in vivo lactadherin might bind to cells, cell membrane vesicles, and possibly lipoproteins that display Ptd-L-Ser and are curved. The immobilized cell, vesicle or particle could then be engulfed by phagocytes. In this way lactadherin may assist in engulfment of fat globules, apocrine secretory vesicles, and cellular microparticles. Alternatively, these data suggest that lactadherin might be used as a pharmacologic bridge ligand, targeting drug-laden liposomes to macrophages or other integrin-displaying cells.

In summary, the data in this report indicate that lactadherin binds selectively to Ptd-L-Ser-containing membranes. Like factors VIII and V, it binds preferentially to highly curved membranes. These properties provide a

rational explanation for the anticoagulant function of lactadherin and lead to speculation about physiologic functions.

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