Association of Ezrin with Intercellular Adhesion Molecule-1 and -2 (ICAM-1 and ICAM-2)

REGULATION BY PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE*

(Received for publication, January 16, 1998, and in revised form, May 14, 1998)

Leena Heiska‡, Kaija Alfthan§, Mikaela Grönholm‡, Pekka Viljas‡, Antti Vaheri‖, and Olli Carpen**

From the Departments of ‡Pathology and §Virology, University of Helsinki, Haartman Institute, 00014 Helsinki, VTT Biotechnology and Food Research, FIN-02044 VTT (Espoo), and ‖Medical School, University of Tampere, 33101 Tampere, Finland

Ezrin is a cytoplasmic linker molecule between plasma membrane components and the actin-containing cytoskeleton. We studied whether ezrin is associated with intercellular adhesion molecule (ICAM)-1, -2, and -3. In transfected cells, ICAM-1 and ICAM-2 colocalized with ezrin in microvillar projections, whereas an ICAM-1 construct attached to cell membrane via a glycosphatidylinositol anchor was uniformly distributed on the cell surface. An interaction of ICAM-2 and ezrin was seen by affinity precipitation, microtiter binding assay, colloidnoprecipitation, and surface plasmon resonance methods. The calculated $K_D$ value was $3.3 \times 10^{-7}$ m. Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) induced an interaction of ezrin and ICAM-1 and enhanced the interaction of ezrin and ICAM-2, but ICAM-3 did not bind ezrin even in the presence of PtdIns(4,5)P$_2$. PtdIns(4,5)P$_2$ was shown to bind to cytoplasmic tails of ICAM-1 and ICAM-2, which are the first adhesion proteins demonstrated to interact with PtdIns(4,5)P$_2$. The results indicate an interaction of ezrin with ICAM-1 and ICAM-2 and suggest a regulatory role of phosphoinositide signaling pathways in regulation of ICAM-1 ezrin interaction.

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** To whom correspondence should be addressed: Haartman Institute, University of Helsinki, P. O. Box 21 (Haartmaninkatu 3), FIN-00014 Helsinki, Finland. Tel.: 358-9-19125413; Fax: 358-9-19125690; E-mail: olli.carpen@helsinki.fi.

‡ The abbreviations used are: ERM, ezrin/radixin/moesin; GPI, glycosphatidylinositol; HA, hemagglutinin antigen; ICAM, intercellular adhesion molecule; PC, phosphatidylcholine; PS, phosphatidylserine; PtdIns(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; SPR, surface plasmon resonance; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; RU, resonance unit(s); PBS, phosphate-buffered saline.

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Ezrin and ICAM Fusion Protein Constructs and the Complement precipitation Assay of Yeast Lysates—The cDNAs encoding the cytoplasmic domains of ICAM-1 (amino acids 478–505) and ICAM-2 (amino acids 229–254) or amino- and carboxyl-terminal domains (amino acids 1–309 and 278–585, respectively) of ezrin were introduced to the pgE202 yeast expression vector, which is derived from E. coli. The same ICAM-cDNAs and the full-length ezrin cDNA were introduced to the pgJ4–5 yeast expression vector, which contains the HA epitope tag (41). The sequences of all constructs were verified by sequencing.

Boiling yeast cells transformed with the EG202 fusion construct and the pgJ4–5 fusion construct were grown overnight at 30 °C, washed once with PBS, and resuspended in the presence of 1 M acid-washed glass beads (Sigma) in 0.5 ml of ELB buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA) supplemented with 1% Nonidet P-40 and protease inhibitors. The debris was removed by centrifugation, and the supernatant diluted with ELB plus 0.1% Nonidet P-40 to a final Nonidet P-40 concentration of 0.5%. The protein concentration was measured at A280 nm. 2 μg of total protein lysate was incubated in the presence or absence of 5 μg/ml PtdIns(4,5)P2 for 20 min at room temperature. The 12CA5 (anti-HA) antibody was added at a dilution of 1:1000 and incubated for 30 min on ice, followed by a protein A-Sepharose bead incubation for 2 h. After washes with ELB and 0.1% Nonidet P-40, the bound proteins were eluted by boiling in Laemmli sample buffer and analyzed by subsequent SDS-PAGE and immunoblotting.

Immunoblot Analysis—The samples were separated by 10% SDS-PAGE, blotted onto nitrocellulose sheets, and blocked overnight using 5% nonfat milk powder in PBS plus 0.1% Tween 20. Primary antibodies were incubated for 1 h. Anti-LexA antibody was diluted 1:5000 and 12CA5 mAb 1:1500 in PBS plus 0.1% Tween 20. As secondary antibodies, either sheep peroxidase-conjugated anti-rabbit IgG or sheep peroxidase-conjugated anti-mouse IgG (Boehringer Mannheim GmbH, Mannheim, Germany) was used at a 1:1500 dilution for 30 min. The bound antibodies were detected by enhanced chemiluminescence (Boehringer). The comparison of the immunoprecipitates was done by densitometric analysis option of NIH Image program.

SPR Assays—Real-time analysis of the binding between ezrin and ICAM-1 and ICAM-2 was performed with a BIAcore AB (Uppsala, Sweden). Peptides were immobilized on a sensor chip according to the manufacturer's protocol using the Amine Coupling Kit (BIAcore AB). ICAM-1 and ICAM-2 peptides (IC1 and ICK2) were diluted in 10 mM phosphate buffer (pH 7.0), and ICAM-3 peptide (IC3) was diluted in 10 mM acetate buffer (pH 4.0) to 200 μg/ml. The resonance units (RU) for immobilized ICAM-1, ICAM-2, and ICAM-3 peptides were 229±10 RU. Typically, 600 RU were injected in the running buffer (HKE) to 2.9 μm, and the solution was run over the peptide surfaces in the absence and presence of 9 μM PtdIns(4,5)P2 micelles or 100 μM/ml mixed vesicles. Typically, the lipids were sonicated and preincubated with ezrin for 30 min at room temperature. In some experiments, the preincubation was omitted and PtdIns(4,5)P2 (4.5 μM) was first injected over the peptide surface, followed by an injection of ezrin, with or without prior incubation with LexA fragment, bovine serum albumin (1.45 μM). All samples were run over a control surface (activated and blocked surface). The measurements were performed at 25 °C under a constant flow of 5 μl/min. Kinetic constants for the interaction between the ICAM-2 peptide and ezrin were determined by immobilizing 1224 RU of the peptide on the sensor surface and injecting 2.9 μM ezrin with the flow rate of 15 μl/min at 25 °C over the ICAM-2 peptide surface. The apparent association rate (k+a) and dissociation rate (k−d) constants for the interaction were determined from three parallel runs, and the data were analyzed according to a 1:1 interaction model using the BIAevaluation 2.1 software supplied by the manufacturer. The apparent dissociation constant (KD) was calculated from the ratio k−d/k+a.

RESULTS

Colocalization of Ezrin, ICAM-1, and ICAM-2—Earlier studies have shown that transfection of ezrin into thymoma cells redistributes ICAM-2 on the cell surface and renders them sensitive for killer cell lysis (27). Ezrin also colocalizes with ICAM-2 in these cells suggesting an interaction between the proteins. To extend these colocalization studies, we transfected wild type ICAM-1, and an engineered ICAM-1 form in which the cytoplasmic and transmembrane region is replaced with a GPI anchor, to COS-1 cells and compared their distribution to ezrin by indirect immunofluorescence microscopy. Transfected ICAM-1-wt was concentrated in microvilli where it perfectly

COS-1 cells were transfected with ICAM-1 cDNA subcloned into CMV expression vector (33) or with a construct that replaces the transmembrane and cytoplasmic domains with a glycoporphathidylinositol (GPI) anchor (25). Transfections were performed with the DEAE-dextran method as described (34). Chinese hamster ovary cells stably expressing ICAM-2 (35) were used as a transfection vector, which expresses a truncated and therefore non-functional ICAM-2. In addition, COS-1 cells were transfected with a plasmid containing the ICAM-3 cDNA. The ICAM-3 expression vector (27) using Lipofectamine (Life Technologies, Inc.). The CMV expression vector alone was used as a control for transfections. The transfected cells were grown on glass coverslips for 48 h before fixation with 3.5% paraformaldehyde in PBS at 4 °C for 10 min. The monoclonal antibodies were used at 10 μg/ml in PBS. After washes with PBS, glass coverslips were reacted with fluorescent isothiocyanate-conjugated goat F(ab′)2, anti-mouse IgG (Immunotech, Marseille, France). The cells were then permeabilized with 0.1% Triton X-100 in PBS, stained with rabbit antisera against ezrin (1:50) or a control serum (Universite Montpellier II, Montpellier, France) and is described in Ref. 25. For affinity precipitation, 2 g of placental lysate was passed through the peptide-Sepharose column as described in Ref. 21. Ezrin was incubated in the presence or absence of lipids (50 μl/ml) under nitrogen in HKE buffer at room temperature. The 12CA5 (anti-HA) antibody was added at a dilution of 1:1000 and incubated for 30 min on ice, followed by a protein A-Sepharose bead incubation for 2 h. After washes with ELB and 0.1% Nonidet P-40, the bound proteins were eluted by boiling in Laemmli sample buffer and analyzed by subsequent SDS-PAGE and immunoblotting.

Affinity Chromatography and Affinity Precipitation Assays—The ICAM-1 and ICAM-3 peptides were coupled to Sepharose beads, and placental lysate was passed through the peptide-Sepharose column as described in detail in Ref. 25. For affinity precipitation, 2 μg of placental ezrin was incubated in the presence or absence of lipids (50 μg/ml) in HKE buffer (50 mM HEPES, pH 7.4) for 2 h at room temperature. The 12CA5 (anti-HA) antibody was added at a dilution of 1:1000 and incubated for 30 min on ice, followed by a protein A-Sepharose bead incubation for 2 h. After washes with ELB and 0.1% Nonidet P-40, the bound proteins were eluted by boiling in Laemmli sample buffer and analyzed by subsequent SDS-PAGE and immunoblotting.

Enzyme-linked Immunosorbent Binding Assay—Biotin-conjugated icAM-peptides were coated onto streptavidin-linked microtiter wells (Labsystems, Helsinoki, Finland) at a concentration of 0.01 mg/ml in PBS. The wells were blocked with 2% bovine serum albumin in PBS for 2 h at 37 °C. Recombinant ezrin (500 ng) was incubated with lipids (10 μg/ml) under nitrogen in HKE buffer at room temperature for 30 min. The ezrin-lipid mixture was allowed to react with the peptides in microtiter wells for 2 h at room temperature in a plate shaker. The wells were washed extensively with HKT (20 mM HEPES, pH 7.4, 130 mM KCl, 0.05% Tween 20) buffer and incubated with the primary antibody, mAb 3C12 at 1:6000 dilution in HKT, and the secondary antibody, rabbit horseradish peroxidase-conjugated anti-mouse (Dako, Copenhagen, Denmark), 1:3000 dilution, for 1 h each. After washes, the substrates reaction was performed with o-phenylenediamine (4-phenylidone) (Sigma) and the absorbances were measured by Multiscan Microplate Reader (Labsystems) at the wavelength of 492 nm. Specific values were calculated by subtracting the background absorbance readings (<0.05 absorbance units) from the total values. For inhibition assays, a dilution series of soluble, non-biotinylated IC1 peptide was added to the reaction mixture containing ezrin and PtdIns(4,5)P2.

Pep tide Synthesis and Immobilization—Three peptides of the following sequences were synthesized: IC1 peptide, RPRKI KYKRLQAOQGTPMKPNTAQTP; IC2 peptide, QHLRQRMGTYG- VRAAWRRLPQAFRP; and IC3 peptide, REHQRSGSYHVREEST- KKYRLQQAQKGTPMKPNTQAP. The IC1, IC2, and IC3 peptides were coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) at a concentration of 2 mg/ml according to the protocol provided by the manufacturer. The coupling efficiency was 93–99%.

Purification of Placental and Recombinant Proteins—The purification of placental ezrin was done essentially as described in Ref. 36. The baculovirus ezrin expression construct was a kind gift of P. Mangeat (Universite Montpellier II, Montpellier, France) and is described in Ref. 37. Recombinant ezrin was purified as described in Ref. 13. The vinculin PtdIns(4,5)P2 binding glutathione S-transferase fusion construct, kindly provided by D. Critchley (University of Leicester, Leicester, United Kingdom), and its purification is described in Refs. 38 and 39. The glutathione S-transferase domain of recombinant vinculin was cleaved off by thrombin.

Ezrin Interaction with ICAM-1 and ICAM-2
colocalized with endogenous ezrin (Fig. 1A). In contrast, the ICAM-1-GPI construct, which is attached to the cell membrane via a lipid, showed no preferential localization but was uniformly distributed on the cell surface (Fig. 1A). No apparent codistribution with ezrin was detected. We also analyzed the colocalization of transfected ezrin in adherent Chinese hamster ovary cells that stably express human ICAM-2. The colocalization of these proteins was high (Fig. 1B), concentrating at microvillar structures.

Binding of Ezrin to ICAM Cytoplasmic Domain Peptides—When placental lysate was passed over a column containing ICAM-1 cytoplasmic peptide as an affinity matrix, only a few polypeptides were retained to the peptide beads. One of the most prominent proteins eluted from the column migrated at a molecular mass of 75 kDa and reacted with an ezrin-specific antiserum (Fig. 2A), indicating a linkage between ICAM-1 and ezrin. The eluate did not contain several other cytoskeletal components, talin, vinculin, and spectrin, which were immunoblotted as a control (data not shown) (25). In a similar experiment, in which ICAM-3 cytoplasmic peptide was used instead of ICAM-1, the profile of the bound peptides was different (Fig. 2A, lane 4 versus lane 1) and ezrin was not detected among the proteins eluted from the column.

To study whether a direct association exists between ezrin and ICAM-1, we purified placental ezrin and analyzed its binding to streptavidin-linked microtiter wells coated with biotinylated ICAM-1 peptide. ICAM-1 peptide bound a small amount of ezrin in the absence of phospholipids (Fig. 2B). The binding was not affected when ezrin had been preincubated with PC or PIP2.
A real-time analysis of the binding between ezrin and ICAM peptides was performed with the SPR-based method. The peptides were immobilized on sensor chips, and ezrin or ezrin/PtdIns(4,5)P2 mixture was run over the peptide-containing surfaces. Fig. 4 (A and B) shows overlaid sensorgrams in the absence and presence of added phospholipid, respectively. Analysis of the binding curves of several experiments indicated an interaction between immobilized ICAM-2 peptide and ezrin, whereas no binding to ICAM-1 and ICAM-3 peptides compared with the control surface (activated and blocked sensor chip) was observed. When ezrin was preincubated with micellar PtdIns(4,5)P2, it bound both to ICAM-1 and ICAM-2 peptides, but not to ICAM-3. Ezrin binding to ICAM-2 was enhanced by the presence of PtdIns(4,5)P2. The apparent $K_a$ and $K_d$ values determined for the interaction between the ICAM-2 peptide and ezrin in the absence of phospholipid were $2.9 \pm 0.03 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$ and $9.5 \pm 0.85 \times 10^{-4} \text{ s}^{-1}$, respectively. The apparent $K_D$ calculated was $3.3 \times 10^{-7} \text{ M}$. No significant differences in the kinetic constants were observed, when about 5-fold less of ICAM-2 peptide (237 RU) was immobilized on the sensor surface.

To study the possibility that ezrin was associating to ICAM-1 and ICAM-2 just via PtdIns(4,5)P2 micellar aggregates, the SPR assays were performed also by preincubating ezrin with vesicles consisting of a neutral phospholipid PC as a carrier, mixed with 8% (molar fraction) of PS or PtdIns(4,5)P2. The interaction inducing effect of PtdIns(4,5)P2 was retained under these conditions, whereas the PC/PS vesicles had no effect on ICAM-1-ezrin interaction (Fig. 4C). SPR results confirmed the role of PtdIns(4,5)P2-containing vesicles also in enhancing ezrin binding to ICAM-2 (Fig. 4D).

In sequential analyses, where PtdIns(4,5)P2 was run over ICAM-1 and ICAM-2 peptide surfaces prior to ezrin, a significant increase in the resonance units was induced on both surfaces by PtdIns(4,5)P2 alone (Fig. 5A). This result suggested an interaction between the ICAMs and PtdIns(4,5)P2. In contrast, ICAM-3 peptide surface did not bind PtdIns(4,5)P2 (data not shown). Binding of ezrin on the ICAM-1 or ICAM-2 peptide surfaces pretreated with PtdIns(4,5)P2 was evident (Fig. 5A). As an additional test for the specificity of PtdIns(4,5)P2 effect, the recombinant PtdIns(4,5)P2-binding fragment of vinculin was injected over the surfaces. The vinculin fragment induced only a minor shift in resonance units when run over ICAM-1 (Fig. 5C) or ICAM-2 (data not shown) peptide surfaces after injection of micellar PtdIns(4,5)P2. Bovine serum albumin bound neither surface after the PtdIns(4,5)P2 treatment (data not shown).

Coimmunoprecipitation of Ezrin and ICAM Cytoplasmic Domains—We made fusion protein constructs containing the cytoplasmic domains of ICAM-1 or ICAM-2, and cotransfected them into yeast together with constructs expressing full-length or
NH2-terminal or COOH-terminal fragments of ezrin (Fig. 6A). All the constructs were expressed at expected molecular sizes as analyzed by immunoblotting (Fig. 6B). The full-length ezrin fusion protein was immunoprecipitated and the presence of coimmunoprecipitating ICAM fusion products detected. A weak band of ICAM-1 and a stronger band of ICAM-2 fusion protein were detectable in ezrin immunoprecipitates. The effect of PtdIns(4,5)P2 was analyzed by incubating identical lysates with PtdIns(4,5)P2 before the coimmunoprecipitation. PtdIns(4,5)P2 treatment resulted in a 40-fold increase in the amount of coimmunoprecipitated ICAM-1 and in an 8-fold increase of coimmunoprecipitated ICAM-2 as analyzed densitometrically. A control fusion protein, which lacked ICAM cytoplasmic domains, did not coimmunoprecipitate with ezrin constructs under any conditions (data not shown). When ICAM fusion construct were immunoprecipitated, the carboxyl-terminal ezrin fusion protein did not coimmunoprecipitate either in the presence or absence of PtdIns(4,5)P2. On the other hand, the amino-terminal fusion construct coimmunoprecipitated with both ICAMs, and the presence of PtdIns(4,5)P2 caused a 4-fold enhancement of binding to ICAM-2, but did not affect binding to ICAM-1 (Fig. 6C).

**DISCUSSION**

In this study, we report that the cytoplasmic domain of ICAM-2 interacts with ezrin. The interaction is highly facilitated by PtdIns(4,5)P2, and PtdIns(4,5)P2 induces an interaction between ezrin and ICAM-1 but not ICAM-3. An association was first suggested by transfection studies, which showed a close codistribution of wild-type ICAM-1 or ICAM-2, and control surfaces. 4.5 μM PtdIns(4,5)P2 (PIP2) was injected over sensorchip, followed by subsequent injection of 1.45 μM ezrin. B, overlaid sensorgrams of ICAM-1 surface injected with 4.5 μM PtdIns(4,5)P2, followed by subsequent injection of 1.45 μM vinculin fragment (lower curve) or purified ezrin (upper curve). In control, PtdIns(4,5)P2 and vinculin fragment were injected on a control surface.

**Fig. 4.** Binding of ezrin to ICAM-1 and ICAM-2 peptides studied by surface plasmon resonance. ICAM-1, ICAM-2, and ICAM-3 cytoplasmic peptides (IC1, ICK2, and IC3, respectively) were immobilized on a sensor chip, and purified ezrin was injected on the peptide surfaces under a constant flow. A, overlaid sensorgrams of ICAM-1, ICAM-2, and ICAM-3 peptide surfaces and a control surface (activated and blocked sensor surface). Ezrin (2.9 μM) was injected in the absence of PtdIns(4,5)P2. B, overlaid sensorgrams of ICAM-1, ICAM-2, and ICAM-3 peptide surfaces and a control surface. Ezrin was incubated with 9 μM PtdIns(4,5)P2 for 30 min at room temperature before the injections. C, overlaid sensorgrams of ICAM-1 peptide surface and a control. Ezrin was incubated for 30 min with lipid vesicles consisting of PC as a carrier mixed with 8% (molar fraction) PS or PtdIns(4,5)P2 (PIP2) before injection over ICAM-1 peptide. In control, ezrin preincubated with PC/PIP2 was injected over control surface. The total lipid concentration was 100 μg/ml. D, overlaid sensorgrams of ICAM-2 peptide surface as in C.

**Fig. 5.** Binding of PtdIns(4,5)P2 to ICAM-1 and ICAM-2 cytoplasmic domains and comparison of ezrin and vinculin binding. A, overlaid sensorgrams of sequential injections over ICAM-1, ICAM-2, and control surfaces. 4.5 μM PtdIns(4,5)P2 (PIP2) was injected over sensorgram, followed by subsequent injection of 1.45 μM ezrin. B, overlaid sensorgrams of ICAM-1 surface injected with 4.5 μM PtdIns(4,5)P2, followed by subsequent injection of 1.45 μM vinculin fragment (lower curve) or purified ezrin (upper curve). In control, PtdIns(4,5)P2 and vinculin fragment were injected on a control surface.
Ezrin Interaction with ICAM-1 and ICAM-2

FIG. 6. Coimmunoprecipitation of ICAM-1, ICAM-2, and ezrin fusion proteins. A, a schematic picture of ezrin molecule and the constructs used in this assay. The structure of ezrin consists of a globular amino terminus (oval), an α-helical region (striped bar) and a COOH-terminal domain (white bar) containing a charged actin-binding region (black box). The fusion protein constructs contain either an HA epitope tag or sites detected by LexA antibody. The numbers depict the amino acid residues of ezrin, ICAM-1, and ICAM-2 contained in the constructs. B, yeast cells were cotransfected with either an ICAM-1 or ICAM-2 and an ezrin fusion protein cDNA construct. The expression of fusion proteins was verified by immunoblotting of the yeast cell lysates with anti-HA-tag or anti-LexA antibody. Lysate of yeast cells transfected with HA-ezrin-wt (lane 1), ICAM-1 (lane 2), HCAM-2 (lane 3), LexA-ICAM-1 (lane 4), LexA-ICAM-2 (lane 5), LexA-ezrin N (lane 6), or LexA-ezrin C (lane 7). Lanes 1–3 are blotted with an anti-HA antibody, and lanes 4–7 with an anti-LexA antibody. The positions of molecular size markers (kDa) are indicated by lines and numbers. C, the yeast cell lysate was incubated in the absence or presence of added PtdIns(4,5)P2. In the left panel, the fusion proteins were immunoprecipitated with an anti-HA-tag antibody recognizing HA-ezrin-wt and coprecipitating fusion proteins detected by immunoblotting with an anti-LexA antibody. Lysate of yeast cells transfected with HA-ezrin-wt (lanes 1–4), ICAM-1 (lanes 5–8), and ICAM-2 (lanes 9–12), and the fusion proteins were immunoprecipitated with an anti-HA-tag antibody recognizing HA-ICAM-1 or HA-ICAM-2, respectively, and coprecipitating fusion proteins detected by immunoblotting with an anti-LexA antibody recognizing LexA-ezrin N or LexA-ezrin C.

The SPR measurements demonstrated that PtdIns(4,5)P2 interacts with the cytoplasmic tails of ICAM-1 and ICAM-2. Previously, a variety of cytoplasmic polypeptides, particularly actin-binding proteins, have been shown to bind PtdIns(4,5)P2, and PtdIns(4,5)P2 is an important modulator of the actin-containing cytoskeleton (28, 29). However, ICAM-1 and ICAM-2 are the first adhesion proteins reported to interact with PtdIns(4,5)P2. The consensus binding sequences for PtdIns(4,5)P2 binding are (R/K)(R/K)(R/K) or XXXXXXKKKKKK(R/K)/(R/K) or XXXXXXXXKKK (49), although other binding sequences exist. The binding of phosphoinositides is not simply electrostatic, but also secondary structure and hydrophobic segments are thought to be important (49–52). The cytoplasmic domains of ICAM-1 and ICAM-2 contain both basic and hydrophobic residues resembling the described PtdIns(4,5)P2 binding sites, and in particular the juxtamembrane sequence of ICAM-1 is highly homologous with the PtdIns(4,5)P2 binding consensus motif. ICAM-3 did not bind PtdIns(4,5)P2 in SPR measurements, and its amino acid sequence also differs from the typical PtdIns(4,5)P2 binding site pattern. The differences in sequences suggest that the cytoplasmic interactions of ICAM-3 are possibly not regulated the same way as the interactions of ICAM-1 and -2.

Phosphoinositides promote conformational changes of the peptides to which they are bound (52, 53) and may increase or stabilize the formation of higher form oligomers, as suggested in the dimerization of the protein kinase Akt (54). PtdIns(4,5)P2 is implicated in adhesion; it is needed for focal adhesion formation (45), and the levels of PtdIns(4,5)P2 are increased by integrin clustering, which activates cytoskeleton-associated PtdInsP kinase locally within focal adhesion complex (55). It is tempting to speculate that increased phosphoinositide levels caused by preadhesion signaling or by initial

the few proteins that were retained. The lysate apparently contained PtdIns(4,5)P2. With all different methods used, the interaction of ICAM-1 or -2 and ezrin was either induced or highly enhanced by PtdIns(4,5)P2. This is in analogy with the interaction between ezrin and the transmembrane adhesion protein CD44 (13). The microtiter and SPR assays with phospholipid vesicles consisting of a neutral carrier lipid PC mixed with a small portion of PtdIns(4,5)P2, resembling the natural composition of cellular membranes, showed that the binding is not just an aggregation of PtdIns(4,5)P2 micelles to the peptides, but a specific effect. Another cytoskeleton-associated protein, vinculin, is regulated by PtdIns(4,5)P2 (39, 44, 45), but compared with ezrin the phosphoinositide binding fragment of vinculin did not show significant binding to ICAM peptide surfaces pretreated with PtdIns(4,5)P2. This result further emphasizes the specificity of ezrin binding to ICAM peptides in the presence of PtdIns(4,5)P2.

ICAM-3 did not bind ezrin even in the presence of added phospholipids. When T-cells produce cellular uropods in response to cytokines and cAMP, ICAM-3 is redistributed to the uropod region together with myosin, not with the actin-based cytoskeleton (46–48). Moesin has been shown to interact with ICAM-3 in polarized T-cells, and stimulation of T-cells redistributed ICAM-3 and moesin, but not ezrin to the formed uropods (48). In these cells, the expression level of ICAM-1 is low. It is possible that ICAM adhesion molecules and ERM proteins, with partly overlapping and redundant functions, display variable combinations in different physiological and cellular environments. In the present study, we have not analyzed whether other ERM family members interact with ICAM-1 and ICAM-2 in a fashion similar to ezrin. Considering the high sequence homology and a similar binding ability to CD44 (13), it is likely that radixin and moesin can also associate with ICAM-1 and -2.

The SPR measurements demonstrated that PtdIns(4,5)P2 interacts with the cytoplasmic tails of ICAM-1 and ICAM-2. Previously, a variety of cytoplasmic polypeptides, particularly actin-binding proteins, have been shown to bind PtdIns(4,5)P2, and PtdIns(4,5)P2 is an important modulator of the actin-containing cytoskeleton (28, 29). However, ICAM-1 and ICAM-2 are the first adhesion proteins reported to interact with PtdIns(4,5)P2. The consensus binding sequences for PtdIns(4,5)P2 binding are (R/K)(R/K)(R/K) or XXXXXXKKKKKK(R/K)/(R/K) or XXXXXXXXKKK (49), although other binding sequences exist. The binding of phosphoinositides is not simply electrostatic, but also secondary structure and hydrophobic segments are thought to be important (49–52). The cytoplasmic domains of ICAM-1 and ICAM-2 contain both basic and hydrophobic residues resembling the described PtdIns(4,5)P2 binding sites, and in particular the juxtamembrane sequence of ICAM-1 is highly homologous with the PtdIns(4,5)P2 binding consensus motif. ICAM-3 did not bind PtdIns(4,5)P2 in SPR measurements, and its amino acid sequence also differs from the typical PtdIns(4,5)P2 binding site pattern. The differences in sequences suggest that the cytoplasmic interactions of ICAM-3 are possibly not regulated the same way as the interactions of ICAM-1 and -2.

Phosphoinositides promote conformational changes of the peptides to which they are bound (52, 53) and may increase or stabilize the formation of higher form oligomers, as suggested in the dimerization of the protein kinase Akt (54). PtdIns(4,5)P2 is implicated in adhesion; it is needed for focal adhesion formation (45), and the levels of PtdIns(4,5)P2 are increased by integrin clustering, which activates cytoskeleton-associated PtdInsP kinase locally within focal adhesion complex (55). It is tempting to speculate that increased phosphoinositide levels caused by preadhesion signaling or by initial
adhesion would modulate the adhesive functions and intracellular interactions of ICAM-1 and ICAM-2, maybe by changing the conformation of the short cytoplasmic tails or by oligomerization of the molecules. Alternatively, local accumulation of PtdIns(4,5)P\(_2\) in the plasma membrane could lead to bridging of ICAMs and ezrin.

PtdIns(4,5)P\(_2\) acts as a precursor for the secondary messenger molecules inositol triphosphate and diacylglycerol and is one of the substrates of PI 3-kinase, but also has independent signaling properties. It can interact with a number of actin-binding proteins and thereby regulate actin polymerization and intermolecular association (10, 58). The mechanism of ezrin activation is unknown but is speculated to involve phosphorylation or dimerization/oligomerization of the molecules (59, 60). A PtdIns(4,5)P\(_2\) binding site is contained in the NH\(_2\)-terminal region of ezrin through uncovering binding sites for F-actin and other interactions. This type of regulation has been reported for vinculin, in which PtdIns(4,5)P\(_2\) dissociates an intramolecular head-to-tail joining (44) rendering the masked talin and actin binding sites accessible, and exposing a phosphorylation site for protein kinase C (39, 45). Our commounprecipitation results support the concept that the binding site for transmembrane proteins is in the amino-terminal part of ezrin. The finding that PtdIns(4,5)P\(_2\) enhances ICAM-2 binding also to the NH\(_2\)-terminal fragment of ezrin shows a complex role for PtdIns(4,5)P\(_2\) possibly modulating ICAM-2 activity in addition to ezrin regulation.

Ezrin is a prominent constituent of microvilli and other cellular protrusions and the leading edge. It is located at cell membrane regions that primarily come into contact with other cells. Thus ezrin, as well as other ERM-members, serve as ideal cytoplasmic binding partners for adhesion molecules. The ability of ezrin to redistribute ICAM-2 (27), and probably also ICAM-1, to microvilli or concentrated patches on uropod-like structures increases the accessibility of these molecules and could also increase their avidity by clustering (Fig. 7). Concentration of ICAMs would benefit the cells during conjugation between lymphoid cells and their target cells, and during migration of lymphocytes through endothelium to tissues. An interaction between ezrin and ICAMs also facilitates post-binding events, as demonstrated by experiments in which relocalization of ICAM-2 to the uropod region after transfection of ezrin rendered target cells susceptible for NK cell killing (27). A similar mechanism may be involved in killing of viral infected cells, when ezrin redistributes from the cytoplasm into newly formed microvilli (61). The finding that PtdIns(4,5)P\(_2\) can be sequestered into lateral domains in the membrane upon binding to specific proteins (62) raises the possibility that the interplay of all three components, ezrin, ICAM-1 or ICAM-2 and PtdIns(4,5)P\(_2\), is regulated by their local distribution. In this manner, the versatile interactions between adhesion molecules and the cytoskeleton could be linked to the signals that cells obtain from their environment and from inside the cell.

Acknowledgments—We thank E. Golemis for anti-LexA mAb, R. Brent for plasmids, D. Critchley and P. Mangeat for the vinculin and ezrin expression constructs, P. Janney for fruitful discussions, F. Zhao and M. Sainio for help with computer programs, and T. Halmesvaara and M.-L. Mantylä for skillful technical assistance.

Note Added in Proof—While this article was under review, another paper describing an interaction between ICAM-2 and ezrin was published (63).

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Association of Ezrin with Intercellular Adhesion Molecule-1 and -2 (ICAM-1 and ICAM-2): REGULATION BY PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE
Leena Heiska, Kaija Alfthan, Mikaela Grönholm, Pekka Vilja, Antti Vaheri and Olli Carpén

doi: 10.1074/jbc.273.34.21893

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