Self-association of PAR-3-mediated by the Conserved N-terminal Domain Contributes to the Development of Epithelial Tight Junctions*

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PAR-3 is a scaffold-like PDZ-containing protein that forms a complex with PAR-6 and atypical protein kinase C (PAR-3-atypical protein kinase C-PAR-6 complex) and contributes to the establishment of cell polarity in a wide variety of biological contexts. In mammalian epithelial cells, it localizes to tight junctions, the most apical end of epithelial cell-cell junctions, and contributes to the formation of functional tight junctions. However, the mechanism by which PAR-3 localizes to tight junctions and contributes to their formation remains to be clarified. Here we show that the N-terminal conserved region, CR1-(1–86), and the sequence 937–1,024 are required for its recruitment to the most apical side of the cell-cell contact region in epithelial Madin-Darby canine kidney cells. We also show that CR1 self-associates to form an oligomeric complex in vivo and in vitro. Further, overexpression of CR1 in Madin-Darby canine kidney cells disturbs the distribution of atypical protein kinase C and PAR-6 as well as PAR-3 and delays the formation of functional tight junctions. These results support the notion that the CR1-mediated self-association of the PAR-3-containing protein complex plays a role during the formation of functional tight junctions.

Cell polarity plays essential roles not only in cell functions but also in development and tissue maintenance. Recent studies have revealed that PAR-3, PAR-6, and their binding partner, atypical protein kinase C (aPKC),1 cooperate together to establish cell polarity in a variety of biological contexts (1). PAR-3 and PAR-6 are products of the par (partitioning-defective) genes required to establish anterior/posterior cell polarity of the Caenorhabditis elegans zygote. In the C. elegans one-cell embryo, PAR-3, PAR-6, and aPKC (PKC-3) become localized asymmetrically to the anterior periphery in a cell cycle-dependent manner. In Drosophila embryos and mammalian epithelial cells, PAR-3, aPKC, and PAR-6 are also required to establish cell polarity (1).

PAR-3 is an evolutionarily conserved scaffold-like protein. PAR-3, Bazooka (Drosophila), and mammalian PAR-3 share three conserved regions (CR), CR1, CR2, and CR3 (Fig. 1A). CR3 is involved aPKC-binding sequences (2, 3). The deletion of a part of CR3 or a point mutation at one of the conserved Ser in CR3 causes defects in binding to aPKC (3, 4). CR2 contains three PDZ domains, which are protein-interacting modules initially recognized as a repeat of 80 amino acids in PSD-95, Drosophila Dlg-A, and ZO-1 (5, 6), suggesting their contribution to protein-protein interactions. In fact, in addition to PAR-6, the C terminus of type B ephrin (7) and the junctional adhesion molecule (JAM) (8, 9) have been reported to bind to the PDZ domains of PAR-3. In addition to CR2 and CR3, PAR-3 contains a unique amino-terminal domain, whose amino acid sequences (PAR-3, 69–152 aa; Bazooka, 1–81 aa; mammalian PAR-3, 1–83 aa) are 37, 52, and 45% identical to one another and show no homology to any other proteins (2). Although the conservation of the CR1 domain implies its importance, its function remains to be clarified.

The function of mammalian PAR-aPKC is well characterized in epithelial cells, which have apico-basal polarity and an asymmetric junctional complex including adhering junctions (AJ) and tight junctions (TJ) that cap the most apical end of cells. In polarized epithelial cells, aPKC forms a ternary complex with PAR-3 and PAR-6, which co-localize at TJ (2, 10). Studies using cultured epithelial cells, such as MDCK cells, have revealed the importance of cell-cell junctions for the establishment and maintenance of cell polarity (11–13). The molecular mechanism underlying the biogenesis of epithelial-specific junctional structures has been analyzed by observations of wound healing processes or calcium-dependent reorganization of cell-cell adhesion of epithelial cells (calcium switch). At the initial stage of cell-cell contact of epithelial cells, primordial spot-like junctions are formed at the tips of thin cellular protrusions radiating from adjacent cells where E-cadherin and ZO-1 are concentrated (14). JAM, a transmembrane protein identified in TJ, co-localizes with E-cadherin at the tips of primordial spot-like junctions (8). Then, as cellular polarization proceeds, E-cadherin and ZO-1 are completely sorted into epithelial-specific beltlike AJ and TJ, respectively. Occludin and then PAR-3 and aPKC accumulate gradually at the ZO-1-positive spotlike junctions during this process (15). Overexpression of a dominant negative form of aPKCα (aPKCαkn) in MDCK cells results in mislocalization of PAR-3 and ZO-1 and a decline in transepithelial electrical resistance (TER), indicating that the formation of functional TJ is impaired under these conditions.

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Furthermore, in the process of wound healing, the expression of aPKC in MTD1-A epithelial cells does not inhibit the formation of primordial spotlike AJ, but it blocks the development into beltlike AJ and the formation of TJ. These results clearly indicate that the kinase activity of aPKC is required for the maturation of TJ from primordial AJ. In

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addition, the kinase activity of aPKC is regulated by PAR-6; association of PAR-6 with aPKC suppresses its kinase activity, but this suppression is overcome by the binding of activated GTP-bound Cdc42 to PAR-6 (4, 10, 16–20). The overexpression of a PAR-6 mutant lacking the aPKC-binding domain also disrupts the formation of functional TJ in MDCK (16). Furthermore, overexpression of PAR-3, but not PAR-3 lacking the aPKC-binding domain, promotes TJ assembly (21). These observations suggest that in mammalian epithelial cells, the PAR-aPKC complex, which is recruited into the cell-cell contact region during the initial phase of cell polarization, contributes to the asymmetric development of initial cell-cell contacts to mature junctional complexes. However, the precise mechanisms by which PAR-3 and the PAR-aPKC complex localize to the cell-cell contact region during the maturation of epithelial junctional structures remains to be clarified.

In this study, we show the self-association of PAR-3 mediated by the CR1 domain. We also show that CR1 is required for the correct recruitment of not only PAR-3 but also aPKC and PAR-6 to the cell-cell contact region in MDCK cells. Further, the ectopic CR1 fragment delays the development of functional TJ. These results suggest that the CR1-mediated self-association of PAR-3...
biss-maleimidohexane were purchased from Pierce. Antibodies were obtained from Zymed Laboratories. Mouse anti-aPKC \( \text{Inc.} \) (Santa Cruz, CA). Rabbit anti-claudin-1 and occludin polyclonal monoclonal antibody were purchased from Santa Cruz Biotechnology, Kyoto, Japan. Rabbit anti-Myc (A-14), anti-T7 (Omni probe), and anti-\( \text{ZO-1} \) (red), and analyzed under a confocal microscope. Top, \( x \) \( y \) images of T7-PAR-3. Bottom, \( z \) sectional views. The data shown are representative of 3–6 experiments.

plays a role during the development of epithelial junctional complexes and cell polarity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemical cross-linkers, \( m \)-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), bis-(sulfosuccinimidyl)suberate (BS3), and bis-maleimidohexane were purchased from Pierce.

Rabbit anti-PAR-3 (C2), aPKC (\( \lambda \)), and PAR-6 (GW2AP) polyclonal antibodies were previously described (10). Mouse anti-ZO-1 monoclonal antibody was kindly provided by Dr. S. Tsukita (Kyoto University, Kyoto, Japan). Rabbit anti-PAR-3 (\( C2 \)), aPKC (\( \lambda \)), and E-cadherin monoclonal antibodies were purchased from Transduction Laboratories. Mouse anti-Myc and T7 monoclonal antibodies were previously described (10). Mouse anti-ZO-1 monoclonal antibody was obtained from Calbiochem and Novagen, respectively. Rabbit anti-PAR-3 (\( C2 \)) polyclonal antibodies and mouse anti-T7 (Omni probe) monoclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-claudin-1 and occludin polyclonal antibodies were obtained from Zymed Laboratories. Mouse anti-aPKC, and E-cadherin monoclonal antibodies were purchased from Transduction Laboratories. Mouse anti-Myc and T7 monoclonal antibodies were obtained from Calbiochem and Novagen, respectively. Rabbit anti-PAR-3 and PAR-6 antibodies were raised against glutathione S-transferase (GST) fusion proteins of the N-terminal domain of PAR-3 (N2; amino acid residues 1–115), the PAR-3-specific C-terminal domain (A2; amino acid residues 1124–1137), and a synthetic peptide against the C terminus of human PAR-6 (BC32; amino acid residues 359–372).

Alexa488-conjugated secondary antibodies and rhodamine-phalloidin were obtained from Molecular Probes, Inc. (Eugene, OR), and Cy3- and horseradish peroxidase-conjugated secondary antibodies and ECL Western blotting detection reagents were purchased from Amersham Biosciences.

**Cell Cultures and Transfections**—COS1 cells, CaCo-2 cells, and MDCK II cells were maintained in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical Co., Japan) supplemented with 10% fetal bovine serum (JRH Biosciences), penicillin, and streptomycin (Invitrogen). For immunoprecipitation, COS1 cells were transfected by an electroporation method using 16 \( \mu \)g of expression plasmids for \( 6 \times 10^5 \) cells as previously described (22) and maintained in growth medium for 48 h. For immunofluorescence, Caco-2 cells and MDCK cells were transfected using LipofectAMINE PLAS reagent (Invitrogen) according to the manufacturer’s instructions.

Expression Plasmids—To obtain cDNA clone(s) encoding mouse PAR-3, we screened a mouse liver cDNA library (Clontech) using the entire coding region of rat PAR-3 (2) as a probe. We identified cDNA clones encoding PAR-3 splice variants.

T7-PAR-3 constructed with SRHis vector was previously described (2, 10). The same coding region of rat PAR-3 (2) was inserted into the \( Xba \) (485–86) was constructed with a \( Nae \) restriction site of pEF-BOS to express PAR-3 without the tag sequence. Another rat PAR-3 clone coding the sequence corresponding to amino acids 93–1,337, mouse PAR-3 short type splicing isoform (1,034 amino acids) lacking amino acids 741–743 and 827–856, a DNA fragment corresponding to amino acids 258–708 of rat PAR-3 amplified by PCR, and the Nael-NcoI fragment of mouse PAR-3 encoding amino acids 1–86 were inserted into the \( Nae \) restriction site of T7-sPAR-3. For the preparation of PAR-3(1–115)-Myc, a DNA fragment corresponding to amino acids 1–115 of rat PAR-3 was amplified by PCR and cloned into the XhoI restriction site of pcDNA3 vector (Invitrogen). pTER-T7-PAR-3Δ1/92 was constructed by changing the \( Bgl \) II restriction site of T7-PAR-3Δ1/92 including the deletion site to that of T7-sPAR-3. For the preparation of PAR-3(1–115)-Myc, a DNA fragment corresponding to amino acids 1–115 of rat PAR-3 was amplified by PCR and cloned into the XhoI restriction site of pcDNA3 vector (Invitrogen). pTER-T7-PAR-3Δ1/92 was constructed by changing the \( Bgl \) II restriction site of T7-PAR-3Δ1/92 including the deletion site to that of T7-sPAR-3.
Self-association of PAR-3 Mediated by the CR1 Domain

Fig. 4. Distribution of endogenous PAR-3, aPKCa, PAR-6, and ZO-1 in T7-PAR-3(1–86)-expressing cells. MDCK cells were transiently transfected with T7-PAR-3(1–86) (A and C) or T7-PAR-3(258–708) (B) and maintained for 43 h to form confluent monolayers. Cells were fixed, double-stained with antibodies for T7 tag (green) and endogenous proteins (red; for PAR-3, C2; for aPKCa, a1; for PAR-6, GW2AP) and analyzed under a confocal microscope. Weak staining of PAR-3, aPKCa, and PAR-6 is seen in T7-PAR-3(1–86)-expressing cells (A). The data shown are representative of 3–5 data experiments. C, among cells expressing T7-PAR-3(1–86), the percentage of cells showing weaker staining of the indicated proteins compared with surrounding cells not expressing ectopic proteins was quantified (n = 105–120). Containing 20 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM EDTA, 2 mM Na2VO4, 50 mM NaF, 10 μg/ml of leupetin, 1 μg/ml of aprotinin, 2 mM phenethylsulfonfonyl fluoride, and 1% Nonidet P-40. After a 30-min incubation on ice, the lysates were clarified by centrifugation at 14,000 rpm for 30 min and incubated with antibodies preabsorbed on Protein G- or Protein A-Sepharose (Amersham Biosciences) for 2 h at 4 °C. After washing five times with lysis buffer, the immunoprecipitants were eluted with Laemmli’s SDS-sample buffer.

For 5 min.

The expression of T7-PAR-3(1–86) was induced by decreasing the concentration of the tetracycline analogue doxycycline (Sigma) in the culture medium. Calcium switch and measurement of TER were performed as previously described (10).

RESULTS

Self-association of PAR-3 through the Evolutionarily Conserved N-terminal Domain, CR1—Mammalian PAR-3 has several splicing isoforms. One of the major isoforms, sPAR-3, shares its N-terminal 1,024 residues with PAR-3 and has an additional 10-residue sequence at the C terminus (Fig. 1A) (21, 23). When low levels of PAR-3 or sPAR-3 were expressed in Caco-2 cells, sPAR-3 as well as PAR-3 distributed to the cell-cell contact region in a manner similar to endogenous PAR-3 (data not shown). However, in cells expressing large amounts of ectopic proteins, PAR-3 distributed as punctate clusters and co-localized with PAR-3 (Fig. 1C) or T7-envelope sPAR-3 distributed difusely in the cytosol (Fig. 1C). Interestingly, when T7-sPAR-3 was co-transfected with PAR-3, T7-sPAR-3 distributed as punctate clusters and co-localized with PAR-3 (Fig. 1D, data not shown). The percentage of cells, in which T7-sPAR-3 forms punctate clusters, among the cells expressing excess amounts

Immunofluorescence Labeling and Microscopy—Transfected Caco-2 cells, MDCK cells, and MDCK Tet-Off cell lines were plated on glass coverslips in 24-well plates or on 12-mm diameter Transwell filters with a pore size of 0.4 μm (Corning Cornerstone Corp.). Immunofluorescence labeling was performed as previously described (10), and the results were observed under a fluorescence microscope (BX60; Olympus) and IP Lab (Photometrics) or analyzed by laser-scanning microscopy (μRadiance; Bio-Rad).

Generation of MDCK Cell Lines Expressing T7-CR1—MDCK Tet-Off cell lines expressing T7-PAR-3(1–86) were produced as described (16) using a Tet-Off gene expression system and MDCK Tet-Off cell lines (Clontech). The expression of T7-PAR-3(1–86) was induced by decreasing the concentration of the tetracycline analogue doxycycline (Sigma) in the culture medium. Calcium switch and measurement of TER were performed as previously described (10).

Yeast Two-hybrid Assay—Yeast expression vectors for PAR-3(1–115) were constructed in pAS2–1C (29) or pGAD424 (Clontech) and were simultaneously transformed into the yeast strain Y190 (MATa ura3::52 his3::200 ade2::101 lys2::901 trp1::901 leu2::3,112 gal4::gal80–cyh2::LYS2::GAL1ΔJN3::HIS3::ATA::HIS3::URA3::GAL1ΔJN3::GAL1ΔJN3::LacZ; Clontech) as previously described (10). The transformants were restreaked onto synthetic plates lacking uracil, leucine, tryptophan, and histidine in the presence or absence of 30 mM 3-amino-1,2,4-triazole and by a filter assay for prey proteins was assayed by the growth on the plate in the presence of 200 μM 3-aminotriazole and by a filter assay for prey proteins was assayed by the growth on the plate in the presence of 200 μM 3-aminotriazole and 30 μM 3-amino-1,2,4-triazole and 30 mM 3-amino-1,2,4-triazole.
of T7-sPAR-3 depends on the ratio of the amounts of T7-sPAR-3 cDNA versus co-transfected PAR-3 cDNA (Fig. 1E). Although the physiological significance of the differences between sPAR-3 and PAR-3 remains unknown, their co-localization suggests that sPAR-3 interacts with PAR-3 in vivo. Interestingly, the diffused cytoplasmic distribution of a deletion mutant of sPAR-3 lacking CR1 (T7-sPAR-3 Δ1/92) was not affected by co-transfection with PAR-3 (Fig. 1E). This suggests the importance of CR1 for the interaction.

The importance of CR1 for the interaction between PAR-3 and sPAR-3 was confirmed by immunoprecipitation experiments using COS cells expressing various tagged PAR-3 dele-
Fig. 6. Induced expression of T7-PAR-3-(1–86) delays the initial TER peak in MDCK cells after calcium switch. Confluent monolayers of MDCK stable cell lines expressing T7-PAR-3-(1–86) (Clone 5 and Clone 9) and parental cell lines (control) were cultured with or without 20 ng/ml doxycycline (DC) for 48 h on Transwell filters. The cells were then incubated in low calcium medium containing 5% fetal bovine serum and 3 μM CaCl₂ for 15 h and subjected to calcium switch by replacement with normal calcium medium. TER was measured, and the background resistance obtained from empty filters was deduced. The data shown are the means with S.D. of three determinations and representative of four independent assays.

The cross-linked T7-PAR-3-(1–86) shows a diffused distribution in the cytosol in addition to weak staining throughout the cell surface (Fig. 3D, suggesting that residues 937–1,024 are required for the localization of PAR-3/sPAR-3 on the apical side of the cell-cell contact region. However, residues 937–1,034 are not sufficient for the correct localization of PAR-3/sPAR-3 on the apical side of the cell-cell contact region, since GST-PAR-3-(937–1,034) distributes throughout the whole plasma membrane (data not shown). The first PDZ domain (residues 270–359) of PAR-3 binds to the C-terminal sequence of JAM, a membrane protein at TJ, and this binding is required for recruiting PAR-3 to the cell-cell contact region during cell polarization (8, 9). However, PAR-3 3267/709, which lacks the PDZ domain, distributes to the most apical part of the cell-cell contact region, as in the case of the wild type (Fig. 3G), suggesting that the anchoring of the first domain of PAR-3 to JAM is not required for the recruitment of ectopic PAR-3 to mature TJ. Considering that PAR-3-(258–708) does not concentrate at the cell-cell contact region (Fig. 4B), PAR-3-JAM interaction is not sufficient for the stable localization of PAR-3 to TJ. Furthermore, the binding to aPKC is not required for localization, since deletion of the aPKC-binding domain, 709–928, does not affect the localization (Fig. 3H). On the other hand, PAR-3 Δ1/92 and sPAR-3 Δ1/92 showed diffuse distributions in the cytosol and on the cell surface (Fig. 3, E and F). Taken together with the fact that PAR-3-(1–86) did not concentrate on the apical side of the cell-cell contact region (Fig. 4A), these results suggest that the CR1 domain is not sufficient but is indispensable for the stable localization of PAR-3/sPAR-3 at TJ. Similar results were obtained when a series of T7-PAR-3 proteins were expressed in mouse epithelial MTD1-A cells (data not shown).

CR1 Is Also Required for the Distribution of aPKC and PAR-6 into the Most Apical Part of the Cell-Cell Contact Region of MDCK—To evaluate the significance of CR1 in the correct localization of PAR-3 in epithelial cells, we examined the effects of transiently overexpressed T7-PAR-3-(1–86) on the distribution of endogenous PAR-3 in polarized MDCK cells. PAR-3-(1–86) shows a diffused distribution in the cytosol in addition to weak condensation at the cell-cell contact region (Fig. 4A). Importantly, PAR-3 staining on the apical side of the cell-cell contact region is greatly reduced by the overexpression of PAR-3-(1–86) (Fig. 4A). Among cells expressing PAR-3-(1–86), 81% showed weaker PAR-3 staining than surrounding cells not expressing ectopic proteins (Fig. 4C). In contrast, the overexpression of PAR-3-(258–708), corresponding to the PDZ domain, had little effect on the localization of PAR-3 (Fig. 4B), and weaker PAR-3 staining was observed in only 7% of cells expressing PAR-3-(258–708). Thus, ectopic PAR-3-(1–86) is thought to affect the correct localization of endogenous PAR-3 in a dominant negative manner. Consistent with the observation that PAR-3 forms a ternary complex with aPKC and PAR-6 (10, 16), PAR-3-(1–86) also disturbed the concentration of aPKCs and PAR-6 in the cell-cell contact region (Fig. 4, A and C). Considering the self-association ability of CR1, these results suggest that the self-association of PAR-3 through CR1 is required for the localization of the PAR-3-aPKC-PAR-6 complex to the apical side of the cell-cell contact region in polarized MDCK cells.

In contrast to the effect on the localization of the PAR-aPKC complex, PAR-3-(1–86) hardly affects the localization of TJ components including ZO-1 (Fig. 4, A and C), occludin, and Claudins (data not shown). We previously demonstrated that the overexpression of aPKCΔkin or PAR-3 in MDCK cells affects the formation of TJ (10, 21), although these effects were ob-
served only when ectopic proteins were expressed during the course of cell polarization but not after the cells were fully polarized. Thus, the present findings support the notion that the PAR-aPKC complex is not critically required for the maintenance of TJ in polarized cells and also led us to examine the dominant negative effect of PAR-3-(1–86) on the process of TJ formation.

CR1-mediated Recruitment of the PAR-aPKC Complex to the Cell-Cell Contact Region during the Development of Cell-Cell Junctions—To analyze further the dominant-negative effect of PAR-3-(1–86) in detail, we generated stable MDCK cell lines that express T7-PAR-3-(1–86) under the control of doxycycline using a tetracycline-repressive system. Western analysis and immunofluorescence demonstrate the expression of PAR-3-(1–86) in the absence of doxycycline and the suppression of its expression in the presence of 20 ng/ml doxycycline (Fig. 5). In addition, we could not find any difference in the amounts and solubilities of endogenous PAR-3, aPKC, and PAR-6 before and after the depletion of doxycycline (Fig. 5A). Fig. 5B shows that the induced PAR-3-(1–86) binds to endogenous PAR-3 but does not have significant effects on the binding between PAR-3 and aPKC. It is also confirmed that the phosphorylation of PAR-3

![Fig. 7. Induced expression of T7-PAR-3-(1–86) delays the relocalization of endogenous PAR-3 (A) and PAR-6 (B) to the cell-cell contact region after calcium switch.](image-url)
at Ser-827 by aPKC (3, 4) was not affected in these cell lines (data not shown).

The stable cell lines cultured in the presence or absence of doxycycline were reseeded and further maintained on the filters for 5 days for polarization to occur. In the absence of PAR-3-(1–86) expression, endogenous PAR-3 concentrated in the most apical part of the cell-cell contact region, as observed in control cell lines (Fig. 5C). In the presence of PAR-3-(1–86) expression, however, the staining of PAR-3 at the cell-cell contact region decreased, and the staining in the cytosol appeared (Fig. 5D). In these cells, co-localization of PAR-3-(1–86) and endogenous PAR-3 was observed on the lateral surface with weak condensation in the most apical part. These results suggest that ectopically expressed PAR-3-(1–86) interacts with endogenous PAR-3 and that this inhibits the recruitment of PAR-3 to the most apical part of the cell-cell contact region. Importantly, the colocalization of PAR-3-(1–86) and PAR-3 was not clearly observed when the expression of PAR-3-(1–86) was induced after the cells reached confluence (Fig. 5E). In these cells, the PAR-3 staining was mostly concentrated at the most apical part of the cell-cell contact region as observed in the absence of PAR-3-(1–86) expression, whereas PAR-3-(1–86) was distributed in the cytoplasm and nucleus. These results suggest that the ectopic expression of CR1 affects the localization of endogenous PAR-3 during the course of cell polarization. Taken together, the CR1-mediated self-association of PAR-3 is required for its correct recruitment to the apical side of the cell-cell contact region during cell polarization.

**Overexpression of CR1 Affects the Development of Functional TJ**—We next examined the effect of CR1 expression on functional TJ formation after calcium switch in terms of TER, which reflects the selective permeability barrier for paracellular ion flow regulated by TJ. In the absence of ectopic CR1 (DC+), all cell lines showed a similar TER profile; the TER peaked at 4 h after calcium switch (Fig. 6) and then fell to a steady-state value after 24 h (data not shown). Ectopic expression of CR1 (DC−) resulted in a delay of the initial peak by 2–4 h in both cell lines but not in control lines (Fig. 6), although the steady-state value of TER was not affected (data not shown). Since total cell numbers did not differ with or without CR1 expression in either cell line, the delay in the TER peak is not caused by a difference in cell density (data not shown). These results suggest that ectopic CR1 impairs the formation of TJ assembly only in the early phase of TJ development, and the effect can be overcome during the whole process.

To confirm this possibility, we observed the effect of ectopic CR1 on the distribution of junctional components after calcium switch. As shown in Fig. 7A, the induction of CR1 disrupted the concentration of PAR-3 at the cell-cell contact region as shown by 1) weak staining at the cell boundary in addition to cytosolic...
staining and 2) lack of beltlike staining encircling the apex of each cell. These effects lasted until 2 h after calcium switch, whereas only the former effect was observed after 4 h. In control parental cell lines, neither effect was observed. A transient lack of beltlike staining encircling each cell after calcium switch was also observed in cells expressing CR1 and immunocyto-stained with anti-aPKC (data not shown) and PAR-6 antibodies (Fig. 7B). Further, the distribution of other junctional components and F-actin were affected only in the early phase of cell polarization. As shown in Fig. 8A, in the absence of CR1 (DC+), beltlike staining of ZO-1 encircling the cells appears within 1 h after calcium switch. However, the expression of CR1 clearly delays the appearance of the beltlike staining of ZO-1. Furthermore, in these CR1-expressing cells, primordial spotlike staining of ZO-1 was frequently observed even 1 h after calcium switch (yellow arrow). Similar results were obtained for occludin and claudin-1, which was most severely affected (Fig. 8B). Moreover, the ectopic expression of CR1 also delayed the development of the beltlike distribution of E-cadherin from the spotlike staining (Fig. 8B, yellow arrow). These results indicate that ectopic CR1 affects the early phase of TJ development (i.e. it affects the progression of primordial spotlike AJ to mature TJ). It has been demonstrated that the maturation of epithelial-specific AJ from primordial spotlike AJ is coupled to the reorganization of F-actin (14, 15). Thick F-actin bundles running circularly along the cell boundary were observed in CR1-expressing cells (Fig. 8B, yellow arrow), supporting the notion that ectopic CR1 also affects F-actin reorganization indispensable for beltlike AJ maturation.

**DISCUSSION**

In this report, we show that the evolutionarily conserved N-terminal region of PAR-3, CR1, contributes to the self-association of PAR-3 through direct interaction between CR1 domains (Figs. 1 and 2). The CR1 domain also contributes to the localization of PAR-3 on the apical side of the cell-cell contact region in polarized cells (where TJ is), because a PAR-3 mutant lacking CR1 failed to concentrate in the cell-cell contact region (Fig. 3, E and F), and the overexpression of the CR1 domain attenuated the localization of endogenous PAR-3 in the cell-cell contact region (Figs. 4 and 5D). Taking into account the ability of CR1 to self-associate, the CR1-mediated self-association of PAR-3 is required for the localization of PAR-3 at TJ. Indeed, in polarized MDCK cells, ectopic CR1 colocalized with endogenous PAR-3 in the cytosol and lateral domain (Fig. 5D). Interestingly, this inhibitory effect of CR1 was not clearly observed when CR1 expression was induced after the cells had reached confluence (Fig. 5E), suggesting that the self-association of PAR-3 at mature junctional structures is more stable than that of PAR-3 distributed diffusely in the cytosol.

Furthermore, the overexpression of CR1 inhibited the recruitment of aPKC and PAR-6 to the apical side of the cell-cell contact region in polarized cells (Figs. 4 and 7B) without inhibiting the formation of the PAR-3-aPKC complex (Fig. 5B). These results suggest a requirement for the CR1-mediated self-association of PAR-3 for the recruitment of the PAR-aPKC complex to TJ and support the idea that PAR-3 plays a role as a scaffold protein. At the junctional structures, self-multimerized PAR-3 and PAR-aPKC complexes could associate with other junctional proteins and form supracomplexes. Indeed, type B ephrin (7) and nectin (24) have been identified as PAR-3-binding proteins. PAR-6 has been demonstrated to interact with PALS1, another TJ component (25). Further binding proteins remain to be identified. Their ability to form supracomplexes could stabilize the self-association of PAR-3 and the PAR-aPKC complex itself and may help organize signaling cascades into large supramolecular complexes, in which each individual scaffold molecule can bind to a distinct set of structural and signaling molecules. Many proteins that contain multiple PDZ domains, such as PSD-95 (26, 27) and hDlg (28), form large signaling complexes mediated by self-multimerization in addition to acting as scaffold proteins targeting appropriate proteins to sites of cellular signaling (5, 6). Taken together, the self-association of PAR-3 might contribute to the construction of large signaling complexes to support signal transduction mediated by the PAR-aPKC signaling cassette and promote the maturation of epithelial-specific junctional structures.

The ability of PAR-3 to self-associate suggests the possibility of controlling the signaling switch. As a result of alternative splicing, multiple forms of PAR-3 have been identified (4, 23). One form has a variation at the carboxyl terminus shown in sPAR-3 (4, 21, 23), and another has a deletion of a core sequence of the aPKC-binding domain (3, 23). Their specific functions are assumed by their differential expression patterns in many tissues (4, 21, 23) and the conservation of splicing events across species. A reverse transcriptase-PCR strategy identified four transcripts derived from a combination of both splicing events in several tissues and cell lines, although all of the protein products were concentrated in the most apical part of the cell-cell contact region when they were transfected into MDCK cells (Fig. 3 and data not shown). Importantly, all of these transcripts contain an N-terminal CR1 domain, and alternative splicing isoforms of PAR-3 can form various heterocomplexes through CR1 (Fig. 1). Consistent with this idea, when MDCK cell lysates were fractionated by gel filtration, sPAR-3 and PAR-3 were eluted together at 400–600 kDa (data not shown). The ability of PAR-3 to self-associate modifies the constituents of the signaling complex in response to changes in spliced variants and thereby prevents or enables specific signal transduction at that site. Further studies are required to clarify the functions of these splice variants of PAR-3 and the mechanisms regulating splicing events in various tissues and cell lines.

It has been reported that the kinase activity of aPKC is required for the formation of functional TJ (10) and that PAR-6 plays a role in the formation of TJ through modification of aPKC activity dependent on Cdc42/Rac1 (16). In this regard, it is interesting that the overexpression of CR1 delayed the formation of functional TJ (Fig. 8) and epithelial-specific junctional structures (Figs. 7 and 8) after calcium switch. Compared with the effect of aPKCΔkn, the inhibitory effect on the formation of newly synthesized junctional assembly by CR1 was limited in the early stages (Figs. 6–8). However, it is important that the inhibition of TJ formation resulting from the overexpression of CR1 is similar to the previous results in the following two points: 1) little inhibitory effect is observed when overexpression is induced in cells with mature TJ, and 2) overexpression of a dominant negative mutant blocks the progression from dotlike AJ to beltlike AJ. On the other hand, the overexpression of CR1 does not affect expression of endogenous proteins, binding between them, or the phosphorylation of PAR-3 by aPKC (Fig. 5, A and B). Thus, the inhibitory effect of CR1 on TJ formation is thought to result from a dominant negative effect of CR1 on the recruitment of PAR-aPKC complexes to junctional structures in the epithelial cell polarization process. That is, the CR1-mediated self-association of PAR-3 and the exact localization of the PAR-aPKC complex to the junctional region are required for the formation of functional TJ. These results are supported by the previous observation that the overexpression of full-length PAR-3, but not a PAR-3 isoform lacking the aPKC-binding domain, promotes TJ assem-

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K. Mizuno and S. Ohno, unpublished results.
and PAR-3 was clearly detectable in the polarization process. Although the binding between JAM and PAR-3 at the cell-cell junctional region during the polarization process. The evolutionary conservation of the CR1 domain of PAR-3 suggests that the contribution of PDZ domains to the localization of PAR-3 at cell-cell junctions could be restricted to the early stages of epithelial cell polarization and that CR1-mediated self-association of PAR-3 and amino acids 937–1024 are required for the further stable incorporation of PAR-3 into mature junctional structures. This idea is supported by the observation that PAR-3-(258–708) concentrates at the immature cell-cell boundary region in early phase of polarization of MDCK cells (data not shown).

The evolutionary conservation of the CR1 domain of PAR-3/Bazooka implies that self-association mediated by the CR1 domain is required for the regulation of cellular polarity in C. elegans and Drosophila embryos. Further studies are needed to examine the possibility that the self-association of PAR-3 is regulated in vivo.

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Self-association of PAR-3-mediated by the Conserved N-terminal Domain Contributes to the Development of Epithelial Tight Junctions

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