Concentration of an Integral Membrane Protein, CD43 (Leukosialin, Sialophorin), in the Cleavage Furrow through the Interaction of Its Cytoplasmic Domain with Actin-based Cytoskeletons

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Abstract. In leukocytes such as thymocytes and basophilic leukemia cells, a glycosilated integral membrane protein called CD43 (leukosialin or sialophorin), which is defective in patients with Wiskott-Aldrich syndrome, was highly concentrated in the cleavage furrow during cytokinesis. Not only at the mitotic phase but also at interphase, CD43 was precisely colocalized with ezrin-radixin-moesin family members (ERM), which were previously reported to play an important role in the plasma membrane–actin filament association in general. At the electron microscopic level, throughout the cell cycle, both CD43 and ERM were tightly associated with microvilli, providing membrane attachment sites for actin filaments. We constructed a cDNA encoding a chimeric molecule consisting of the extracellular domain of mouse E-cadherin and the transmembrane/cytoplasmic domain of rat CD43, and introduced it into mouse L fibroblasts lacking both endogenous CD43 and E-cadherin. In dividing transfectants, the chimeric molecules were concentrated in the cleavage furrow together with ERM, and both proteins were precisely colocalized throughout the cell cycle. Furthermore, using this transfection system, we narrowed down the domain responsible for the CD43-concentration in the cleavage furrow. Based on these findings, we conclude that CD43 is concentrated in the cleavage furrow through the direct or indirect interaction of its cytoplasmic domain with ERM and actin filaments.

THE mechanism of cytokinesis has attracted increasing interest among cell biologists (for review see Mabuchi, 1986; Rappaport, 1986; Satterwhite and Pollard, 1992). Before cytokinesis, an unknown signal is thought to be transferred from the mitotic apparatus to the equatorial cortex, inducing the formation of a bundle of aligned actin filaments called the contractile ring just beneath the plasma membrane (Perry et al., 1971; Schroeder, 1973). At cytokinesis, the constriction of this ring results in the formation of the cleavage furrow and finally in the cell division. The mechanical force for the constriction is generated through the interaction between actin filaments and myosin-II inside the ring (Fujiwara and Pollard, 1976; Mabuchi and Okuno, 1977; Yumura and Fukui, 1985; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Karess et al., 1991). So far, various types of proteins, including actin and myosin-II, are reportedly concentrated in the cleavage furrow (Sanger et al., 1987, 1989; Ishidate and Mabuchi, 1988; Schweisguth et al., 1990; Earnshaw and Cooke, 1991; Yonemura et al., 1991). However, despite these intensive studies, knowledge of the molecular architecture of the contractile ring remains fragmentary. Especially, information on the way actin filaments associate with the plasma membrane in the cleavage furrow is limited at the molecular level. This information is indispensable in understanding the molecular basis for the formation of cleavage furrows.

To clarify the molecular linkage from the actin filaments to the plasma membrane in the cleavage furrow, the first protein that should be identified is an actin-modulating protein. Many actin-modulating proteins reportedly show high affinity for the plasma membranes; for example, gelsolin (Janmey et al., 1987; Hartwig et al., 1989), lipocortin-85 (Ikebuchi and Waisman, 1990), myosin-I (Adams and Pollard, 1989; Miyata et al., 1989; Mooseker et al., 1989), actin-binding protein (Fox, 1985; Ohta et al., 1991), spectrin (for review see Bennet, 1989), the myristoylated, alanine-rich C kinase substrate (Hartwig et al., 1992), and ponticulin (Shariff and Luna, 1990). However, none of them are reportedly concentrated in the cleavage furrow during cytokinesis. Recently, an 82-kD protein called radixin, a barbed end–capping, actin-modulating protein, has been reported to be highly concentrated in the cleavage furrow in various types of cells (Tsukita et al., 1989; Sato et al., 1991). Sequence analysis of radixin cDNA has revealed that radixin is highly homologous to ezrin and moesin (Gould et al., 1989; Turunen et al., 1989; Funayama et al., 1991; Lankes

and Furthmayr, 1991), and that there is a gene family consisting of ezrin, radixin, and moesin, which we call the ERM[1] family (Sato et al., 1992; Tsukita et al., 1992). Recent close immunofluorescence microscopic analyses have shown that the ERM family plays a crucial role in the association of actin filaments with the plasma membrane in general. Considering that our anti-radixin pAb and mAb more or less crossreacted with ezrin and moesin, we should strictly say that ERM family members are concentrated at the cleavage furrow (see details in Sato et al., 1992). Interestingly, the NH_2-terminal half of ERM significantly resembles that of band 4.1 protein, a major undercoat constituent of the erythrocyte membrane (Sato et al., 1992). In erythrocyte membranes, band 4.1 protein binds to an integral membrane protein called glycophorin C through its NH_2-terminal domain (Leto et al., 1986). Therefore, it is tempting to speculate that in the cleavage furrow, ERM directly binds actin filaments to a membrane protein (glycophorin-like?) at its NH_2-terminal domain.

Since actin filaments are recruited preferentially into the cleavage furrow by migration of preexisting filaments in the cell cortex and are not all assembled de novo in the furrow (Cao and Wang, 1990*a*), the membrane protein such as the above putative ERM-anchoring protein, which is responsible for linking actin filaments to the plasma membrane, is likely to accumulate in the cleavage furrow. However, the occurrence of such a cleavage furrow–accumulated membrane protein has not often been discussed in the field of cytokinesis. We recently noticed that de Petris (1984), during the course of his studies on capping of the cell surface glycoproteins in lymphoid cells, described the concentration of W3/13 antigen in the cleavage furrow in dividing rat thymocytes. The W3/13 antigen is a rat homologue of human CD43 (leukosialin or sialophorin) (Killeen et al., 1987), which is an integral membrane protein expressed in most leukocytes and platelets, and is defective in patients with the X-chromosome-linked immunodeficiency disorder, Wiskott-Aldrich syndrome (for review see Remold-O'Donnell and Rosen, 1990). Considering that CD43 is heavily sialoglycosylated just like glycophorin C (Brown et al., 1981) and that one anti-CD43 mAb (L10) triggers T lymphocyte division in the presence of accessory cells (Mentzer et al., 1987), we are led to speculate that CD43 plays a crucial role in the formation of the cleavage furrow, at least in leukocytes, through direct or indirect interaction with ERM.

In this study, we first analyzed the distribution of CD43 in rat thymocytes and basophilic leukemia cells (RBL-2H3) by both immunofluorescence microscopy and immunoelectron microscopy. In these cells, both at the mitotic phase and at interphase, CD43 was colocalized with ERM, suggesting their direct or indirect binding in vivo throughout the cell cycle. Second, a cDNA encoding an E-cadherin/CD43 chimeric molecule containing the cytoplasmic domain of CD43 was introduced into mouse L cells lacking CD43, and we found that this molecule was colocalized with ERM in the cleavage furrow during cytokinesis. Finally, by means of a transfection system using L cells, we identified the domain responsible for the CD43 concentration in the cleavage furrow; in other words, for the CD43-colocalization with ERM. The results of this study will lead us to a better understanding of the molecular mechanism of the cleavage furrow formation.

1. *Abbreviations used in this paper*: ERM, ezrin-radixin-moesin; PCR, polymerase chain reaction.

Materials and Methods

Cells and Antibodies

Thymocytes from 2-wk-old Wistar rats were collected by centrifugation, resuspended in RPMI-1640 supplemented with 10% FCS, and incubated for 30–60 min at 37°C. RBL-2H3 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and cultured in RPMI-1640 with 10% FCS. Mouse fibroblastic L cells (Earle, 1943) and their E-cadherin transfectants, EL8 cells (Nagafuchi et al., 1987), were cultured in DME with 10% FCS.

The following antibodies were used: anti-rat CD43 mAb, W3/13 (Cedarlane Laboratories Limited, Hornby, Ontario, Canada); anti-radixin pAb, pAb-II (Tsukita et al., 1989); anti-radixin mAb, CR22 (Sato et al., 1991); anti-E-cadherin pAb (Nagafuchi et al., 1987); and anti-E-cadherin mAb, ECCD-2 (Shirayoshi et al., 1986). Since pAb-II and mAb-CR22 were recently found to more or less crossreact with ezrin and moesin (Sato et al., 1992), we tentatively call these antibodies anti-ERM pAb and mAb, respectively. Rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) was used to stain actin filaments. Chromosomes were visualized with 4′6-diamidino-2-phenylindole dihydrochloride.

Immunofluorescence Microscopy

All procedures were carried out at room temperature. Cells were fixed with 4% fresh formaldehyde in 0.1 M Hepes buffer (pH 7.5) for 15 min. After washing three times with PBS containing 30 mM glycine (glycine-PBS), cells were soaked in blocking solution (glycine-PBS containing 2% normal goat serum) for 5 min and incubated with W3/13 or anti-E-cadherin pAb diluted with the blocking solution for 30 min. They were then washed with glycine-PBS three times, treated with 0.2% Triton X-100 in glycine-PBS for 15 min, and washed with glycine-PBS three times. After being soaked in the blocking solution for 5 min, cells were incubated with anti-ERM pAb or mAb diluted with the blocking solution for 1 h, washed three times, and soaked in the blocking solution for 5 min. They were then incubated with secondary antibodies diluted with the blocking solution for 30 min. FITC-conjugated sheep anti-mouse Ig antibody and rhodamine-conjugated donkey anti-rabbit Ig (Amersham International plc, Amersham, UK) were used as secondary antibodies. For some studies, rhodamine-phalloidin and/or 4′6-diamidino-2-phenylindole dihydrochloride were mixed with the secondary antibody. Cells were washed three times and then mounted in 90% glycerol-PBS containing 0.1% para-phenylendiamine. Some samples were not treated with detergent or with anti-ERM antibody. Specimens were observed using a Zeiss Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany). Photographs were taken on T-Max 400 film (Eastman Kodak Co., Rochester, NY).

Since some membrane glycoproteins such as *Helix pomatia* agglutinin receptors are not immobilized completely by formaldehyde fixation (de Petris, 1984), we occasionally fixed specimens in glutaraldehyde. Cells were fixed with 1% glutaraldehyde and 4% formaldehyde in 0.1 M Hepes buffer (pH 7.5) for 1 min, followed by 4% formaldehyde fixation for 15 min. After washing with glycine-PBS three times, cells were incubated with 20 mM sodium borohydride in isotonic carbonate-buffered saline (pH 9.0) for 30 min at 37°C to quench the autofluorescence of glutaraldehyde. No significant difference in the distribution of CD43 was detected between formaldehyde- and glutaraldehyde-fixed cells.

Immunoelectron Microscopy

Cells were fixed and incubated with primary antibodies according to the method used for immunofluorescence microscopy, except that 1% Triton X-100 was used for permeabilization. AuroProbe EM GAM IgG G10 and AuroProbe EM GAR G15 (Amersham International plc) were used as secondary antibodies. After incubation with these antibodies, cells were washed and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature, followed by postfixation with 1% OsO_4 in the same buffer for 1 h on ice. The samples were then dehydrated with ethanol and embedded in Epon 812. Thin sections were cut with a diamond knife, doubly stained with uranyl acetate and lead citrate,

and then examined using an electron microscope, JEM 1200 EX (JEOL, Tokyo, Japan), at an accelerating voltage of 100 kV.

Plasmid Constructs

A pair of primers (5′ primer: 5′-GGTGACCCCAGTCTTCAGTACAAA-AATATC-3′; 3′ primer: 5′-GATCGATGAATACCGTTACTCATAG-3′) was used to amplify the desired portion of rat CD43 cDNA (Killeen et al., 1987) from RBL-2H3 cells by the reverse transcriptase polymerase chain reaction (PCR). Several base mismatches were introduced into each primer to create a unique BstEII (5′ primer) or ClaI cloning site (3′ primer). PCR was performed using a thermal cycler, ZYMOREACTOR AB-1800 (ATTO Corporation, Tokyo, Japan), according to the conditions used by Singer-Sam et al. (1990) with a slight modification. The PCR products were cloned into pBluescriptSK(−) by replacing an EcoRV-ClaI fragment with blunt-ended PCR products that were digested with ClaI. For the following experiments, one clone (LSP1) was used after its sequence was checked using the 7-deaza Sequenase Version 2.0 kit (United States Biochemical Corp., Cleveland, OH).

A plasmid (pBATCLS1) encoding an E-cadherin/CD43 chimera (CLS1) was constructed by replacing the BstEII-SalI fragment of a mouse E-cadherin expression vector (pBATEM2) (Nagafuchi et al., 1987; Nose et al., 1988) with the BstEII-SalI fragment of LSP1 (see Fig. 6). A plasmid (pBATCLS1-A) encoding a CLS1-mutant (CLS1-A) was constructed by replacing the BstEII-KpnI fragment of pBATEM2 with the BstEII-SacI fragment of LSP1. Both the KpnI and the SacI sites were blunt-ended before replacement. A plasmid (pBATCLS1-B) encoding another CLS1-mutant (CLS1-B) was constructed by replacing the BstEII-KpnI fragment of pBATEM2 with the BstEII-KpnI fragment of LSP1 after deleting the ApaI-SacI fragment (encoding region A). Both the ApaI and SacI sites were blunt-ended and then connected with a BglII linker (5′-CAGATCTG-3′).

Transfection of L Cells

To isolate L cells stably transformed with plasmids pBATCLS1, pBATCLS1-A, or pBATCLS1-B, cells were cotransfected with a plasmid for neomycin resistance (pSTneoB) and selected with G418. L cells (1×10^5) were cultured in dishes 35 mm in diameter with or without coverslips, and then transfected by calcium-phosphate coprecipitation for 18 h (Xie, 1984). The DNA-containing medium was then replaced by fresh medium. The cells were cultured another 48 h and the cells on coverslips transiently expressing the chimera were immunofluorescently stained using anti-E-cadherin pAb to check the efficiency of the transfection. If the transfection efficiency was high, cells were transferred to dishes 100 mm in diameter and cultured in the presence of 0.4 mg/ml G418. After 10–20 d, G418-resistant clones were isolated and their reactivity to the anti-E-cadherin pAb was checked by immunocytochemistry. The positive cells were recloned.

Gel Electrophoresis and Immunoblotting

Samples were separated by SDS-PAGE using 7.5% polyacrylamide gels. After electrophoresis, proteins were transferred to a nitrocellulose sheet and the sheet was incubated with ECCD-2. For antibody detection, a blotting detection kit (Amersham International plc) was used.

Results

Concentration of CD43 in the Cleavage Furrow in Thymocytes and Basophilic Leukemia Cells

When rat thymocytes were immunofluorescently stained with anti-rat CD43 mAb specific for the extracellular domain of CD43 (W3/13; Cyster et al., 1991), an intense signal was emitted from the cleavage furrow of dividing cells (Fig. 1, *a* and *b*). Also in a rat basophilic leukemia cell line (RBL-2H3), CD43 was exclusively concentrated in the cleavage furrow during cytokinesis (99.1% of 214 dividing cells; Fig. 1, *c* and *d*; see Table I). Using RBL-2H3 cells, we analyzed the dynamic behavior of CD43 throughout the cell cycle in more detail. At interphase, numerous fine spikes or spots were stained all over the cell surface (see Fig. 3 *d*). When cells entered the mitotic phase and began to round up, the intensity of the staining on the cell surface appeared to markedly increase. At metaphase and early anaphase, spike- or spotlike staining was almost evenly distributed along the cell surface (Fig. 2, *a–d*). At late anaphase, just before the onset of cytokinesis, CD43 started to concentrate at the equa-

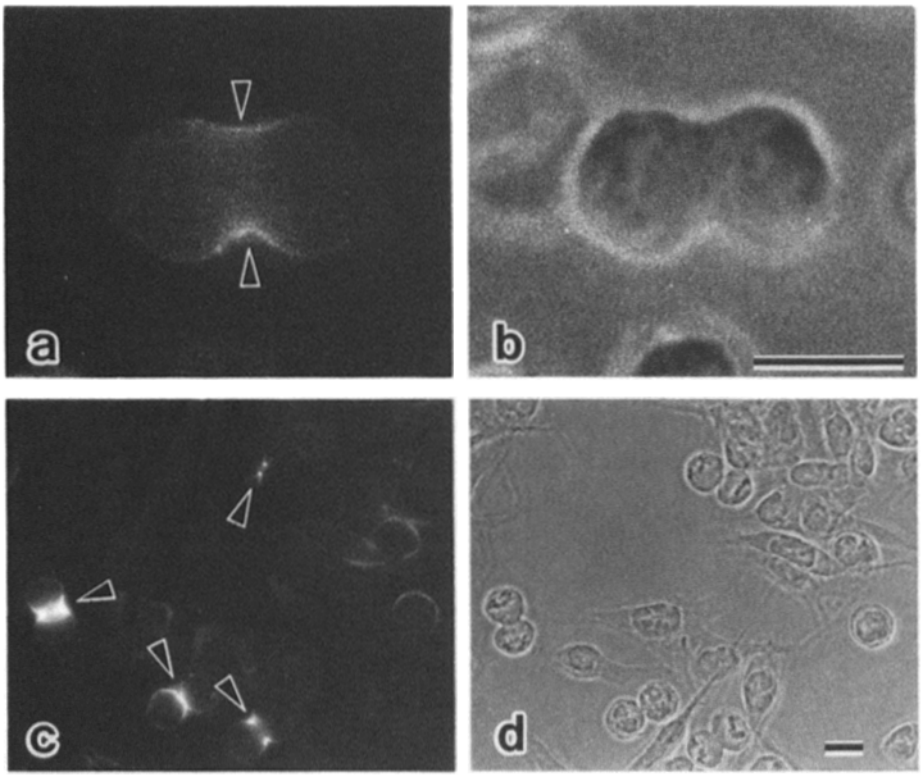


Figure 1. Concentration of CD43 in the cleavage furrow of a dividing rat thymocyte (*a* and *b*) and RBL-2H3 cells (*c* and *d*). Immunofluorescent staining for CD43 using mAb W3/13 (*a* and *c*) and phase-contrast images (*b* and *d*). CD43 is highly concentrated in the cleavage furrow (*arrowheads*) of these cells. Bars, 10 μm (*a* and *b*); 20 μm (*c* and *d*).

Table I. Concentration of CD43 and Its Mutants in the Cleavage Furrow

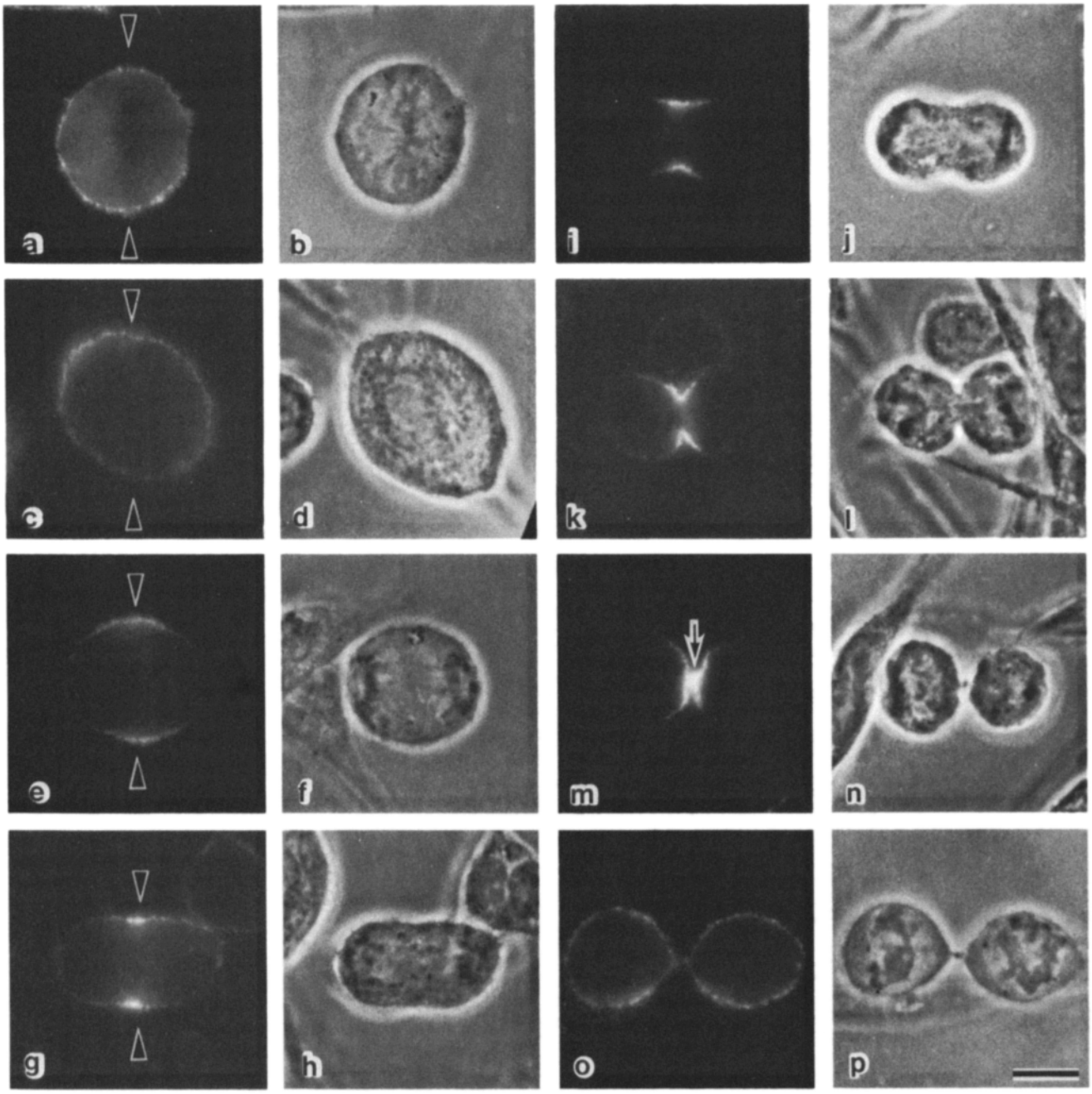
Cell	Protein	Concentration in the cleavage furrow	
		%	*n*
RBL-2H3	CD43	99.1	214
L-CLS1	CLS1	97.8	136
L-CLS1-A	CLS1-A	94.7	343
L-CLS1-B	CLS1-B	4.9	184

Cells grown on coverslips were fixed and stained with appropriate antibodies and 4'6-diamidino-2-phenylindole dihydrochloride as described in Materials and Methods. Dividing cells were identified with reference to the chromosomes or nuclei stained with 4'6-diamidino-2-phenylindole dihydrochloride. Among these dividing cells, the percentage of cells where CD43 or its mutants were concentrated in the cleavage furrow was determined.

torial regions of ellipsoidal cells (Fig. 2, *e* and *f*), and throughout cytokinesis CD43 continued to concentrate in the cleavage furrow (Fig. 2, *g–l*). At the end of cytokinesis, the midbody remained characteristically stained (Fig. 2, *m* and *n*), eventually returning to the interphase spike- or spotlike staining profile (Fig. 2, *o* and *p*).

Colocalization of CD43 with ERM in RBL-2H3 Cells

Since the changing pattern of the distribution of CD43 through the cell cycle highly resembles those of actin (Sanger et al., 1989) and ERM (Sato et al., 1991, 1992), we compared their distribution in RBL-2H3 cells by double staining immunofluorescence microscopy. As shown in Fig. 3, *a–c*, CD43 was coconcentrated with ERM and actin in the cleavage furrow of dividing cells: the coincidence between CD43 and ERM stain-



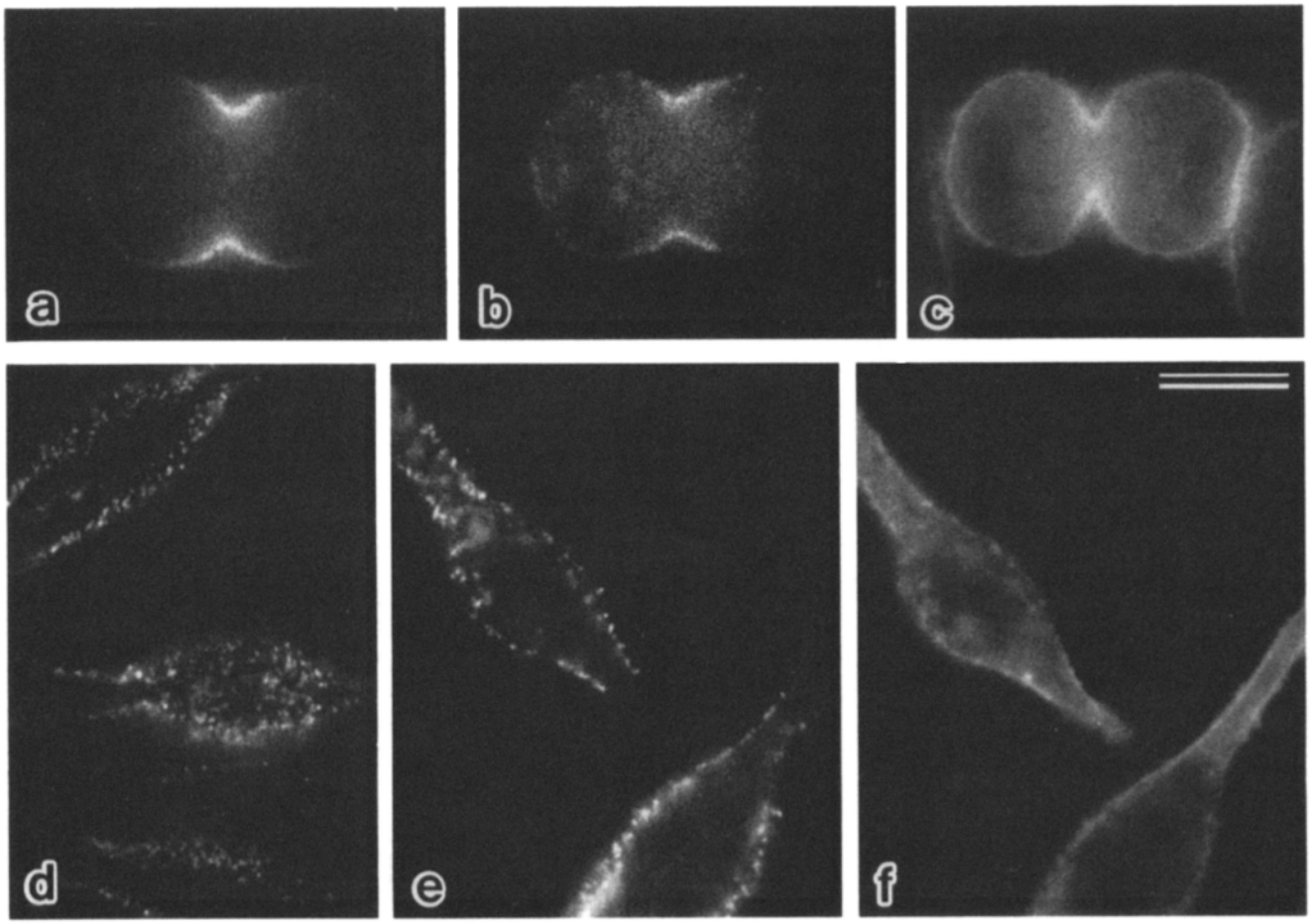


Figure 3. Comparison of the distribution of CD43, ERM, and actin filaments in RBL-2H3 cells. When dividing cells are doubly stained with anti-CD43 mAb (*a*) and anti-ERM pAb (*b*), CD43 is precisely coconcentrated in the cleavage furrow with ERM. In rhodamine-phalloidin staining (*c*), only a fraction of total actin filaments appears to be concentrated in the cleavage furrow. At interphase, both CD43 (*d*) and ERM (*e*) are localized in numerous fine spikes or spots all over the cell surface. When cells are doubly stained with ERM (*e*) and rhodamine-phalloidin (*f*), their patterns do not coincide precisely. Bar, 10 μm.

ing was complete at least at the light microscopic level, whereas only a fraction of the total actin filaments appeared to be colocalized with CD43 and ERM to form the contractile ring in the cleavage furrow. Even at interphase, CD43 and ERM were localized at spike- or spotlike structures (Fig. 3, *d* and *e*), and in double staining both were colocalized (data not shown). Actin filaments were not precisely colocalized with either CD43 or ERM (Fig. 3, *e* and *f*).

Next, the colocalization of CD43 with ERM was further examined at the electron microscopic level using colloidal gold–conjugated secondary antibodies. To ensure the preservation of the morphological integrity of cells and the immobilization of membrane proteins, in single staining with anti-CD43 mAb, the cells were fixed with glutaraldehyde before immunodetection. In dividing RBL-2H3 cells, the number of microvilli in the cleavage furrow was significantly larger than that in the polar region, so that the plasma membrane in the cleavage furrow was divided into microvilli and nonmicrovilli domains (Fig. 4, *a* and *b*). As shown in Fig. 4 *b*, the gold particles indicating the CD43-localization were preferably associated with the microvilli domain. The number of gold particles per each microvillus in the polar region was smaller than that in the cleavage furrow (Fig. 4 *c*). In control experiments, no nonspecific labeling was detected on the cell surface. For double staining immunoelectron microscopy with anti-CD43 mAb and anti-ERM pAb, formaldehyde was used instead of glutaraldehyde, to allow antibodies access to ERM inside cells. During cytokinesis, in the cleavage furrow, both CD43 and ERM were preferably localized in the microvilli domain (Fig. 5 *a*). Also at interphase, both proteins were pre-

Figure 2. Behavior of CD43 during mitosis in RBL-2H3 cells. Immunofluorescence (*a*, *c*, *e*, *g*, *i*, *k*, *m*, and *o*) and phase-contrast (*b*, *d*, *f*, *h*, *j*, *l*, *n*, and *p*) micrographs. At metaphase (*a* and *b*) and early anaphase (*c* and *d*), CD43 is localized on spike- or spotlike structures evenly distributed along the cell surface. At late anaphase (*e* and *f*), CD43 begins to be concentrated in the equatorial region (*arrowheads*). Throughout cytokinesis (*g–l*), CD43 is clearly concentrated in the cleavage furrow. At the end of cytokinesis (*m* and *n*), the midbody (*arrow*) was initially stained and the pattern returned to that at interphase (*o* and *p*). Bar, 10 μm.

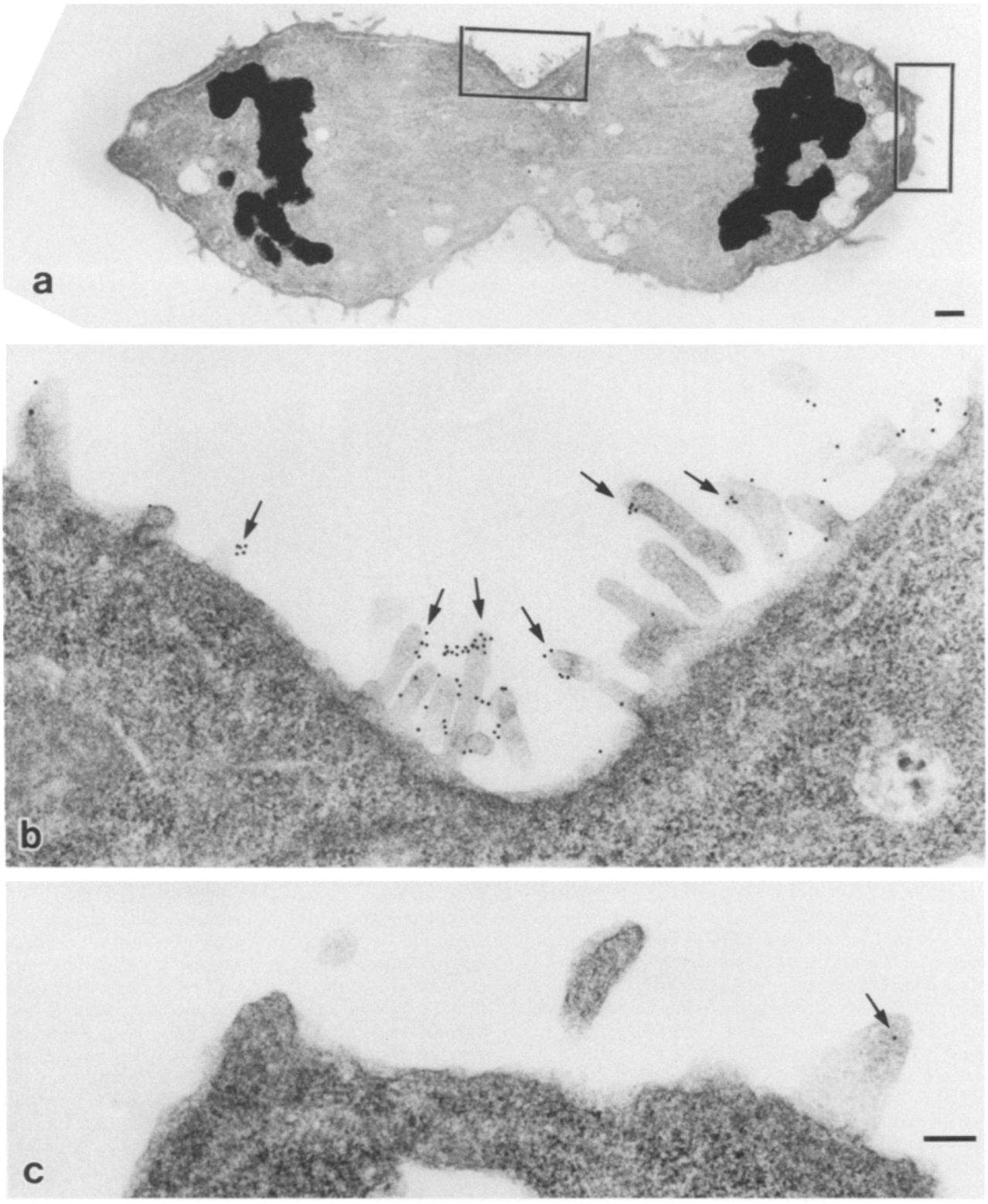


Figure 4. Immunoelectron micrographs of a dividing RBL-2H3 cell with anti-CD43 mAb. Colloidal gold-conjugated secondary antibody (*arrows*) was used. To ensure the preservation of the morphological integrity of cells and the immobilization of membrane proteins, cells were fixed with glutaraldehyde. Semi-thin sections (~200 nm thick) were used to detect as many gold particles as possible in one image. The cleavage furrow (*b*), squared in *a*, has more microvilli than the polar region (*c*), squared in *a*, and the gold particle labeling is preferably associated with microvilli in the cleavage furrow. Note that the number of gold particles per microvillus in the cleavage furrow is significantly larger than that in the polar region. Bars: 1 μm (*a*); 0.2 μm (*b* and *c*).

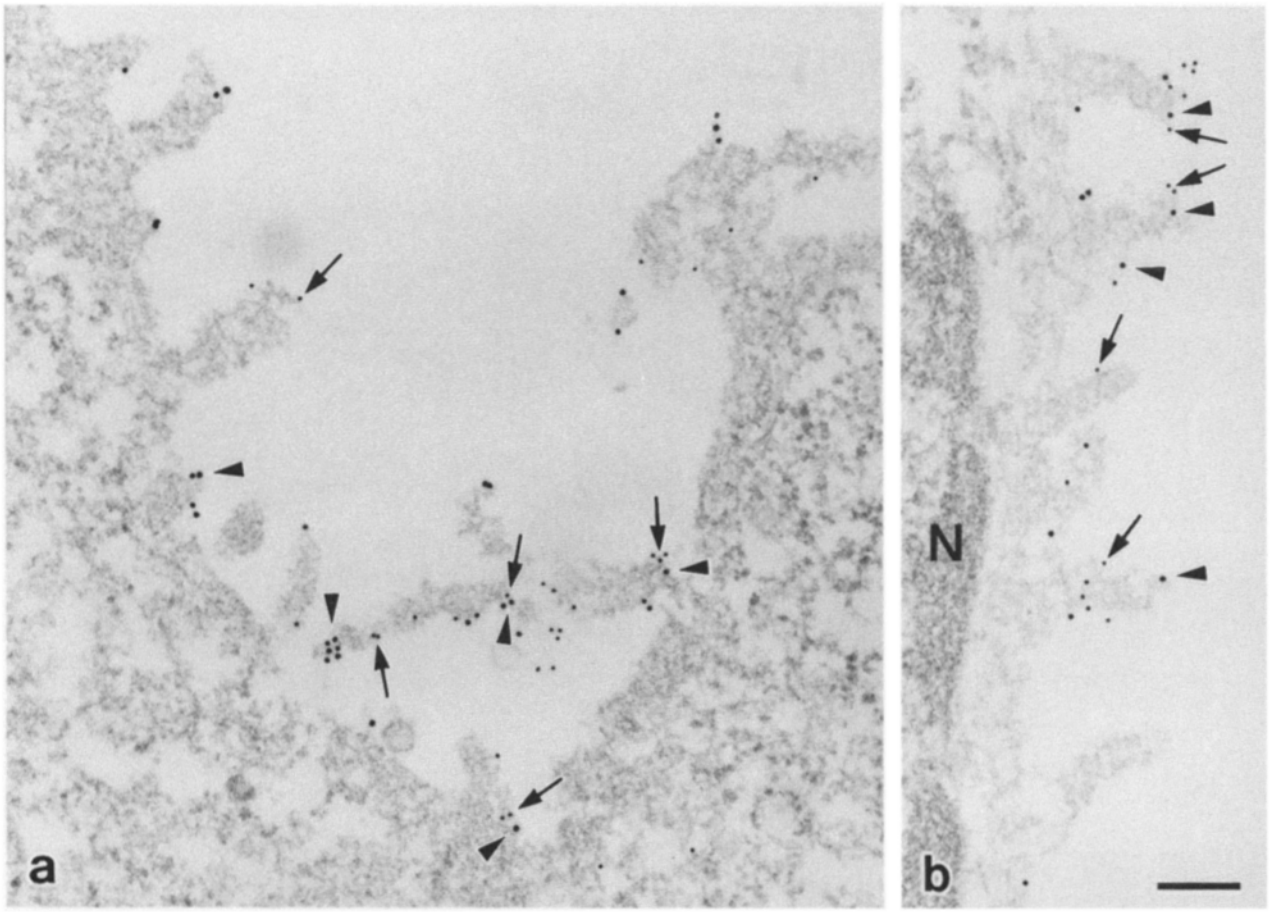


Figure 5. Double labeling immunoelectron micrographs of RBL-2H3 cells with anti-CD43 mAb and anti-ERM pAb. CD43 and ERM are indicated by 10-nm (*arrows*) and 15-nm (*arrowheads*) gold particles, respectively. To allow antibodies to access to ERM inside cells, only formaldehyde was used for fixation. In the cleavage furrow of a dividing cell (*a*) and on the cell surface at interphase (*b*), both CD43 and ERM are preferably localized on microvilli. *N*, nucleus. Bar, 0.2 μm.

cisely colocalized in microvilli (Fig. 5 *b*). This intimate spatial relationship between CD43 and ERM in the RBL-2H3 cells suggests their direct or indirect association through the cytoplasmic domain of CD43.

Behavior of the E-Cadherin/CD43 Chimera Introduced into L Cells

The localization of CD43 in dividing RBL-2H3 cells led us to speculate that in leukocytes CD43 is involved in the formation of the cleavage furrow through a basic molecular mechanism that is commonly shared by various types of cells. To evaluate this speculation, we first attempted to check whether, when a cDNA encoding CD43 is introduced

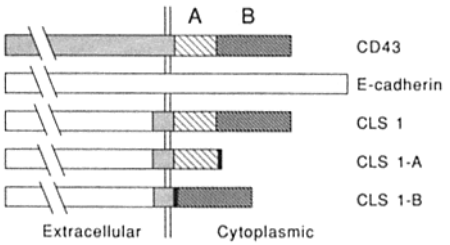


Figure 6. Structure of CD43, E-cadherin, and their chimeras. CLS1 consists of most of the extracellular domain of mouse E-cadherin (amino acids 1–654) and the COOH-terminal half of rat CD43 (amino acids 198–371). CLS1-A and CLS1-B are truncated forms of CLS1 with a deletion of 77 amino acids at the carboxy terminus (295–371 of CD43; region B) and of 40 amino acids (257–296 of CD43, region A), respectively. *Solid boxes*, 3–4 amino acids added to the mutant as a result of the constructions.

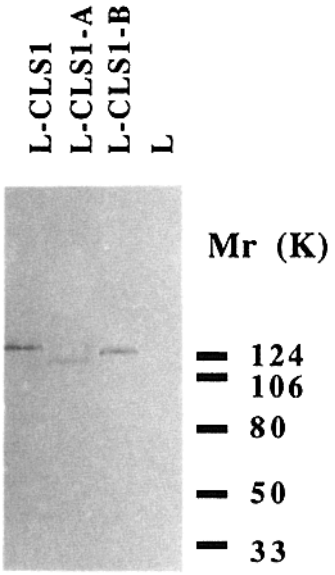


Figure 7. Immunoblot analysis of the chimeric molecules in different transfectants (L-CLS1, L-CLS1-A, and L-CLS1-B) with anti-E-cadherin mAb (ECCD2). Each transfectant cell line expresses an E-cadherin/CD43 chimera that is roughly proportional to the putative length of deletion.

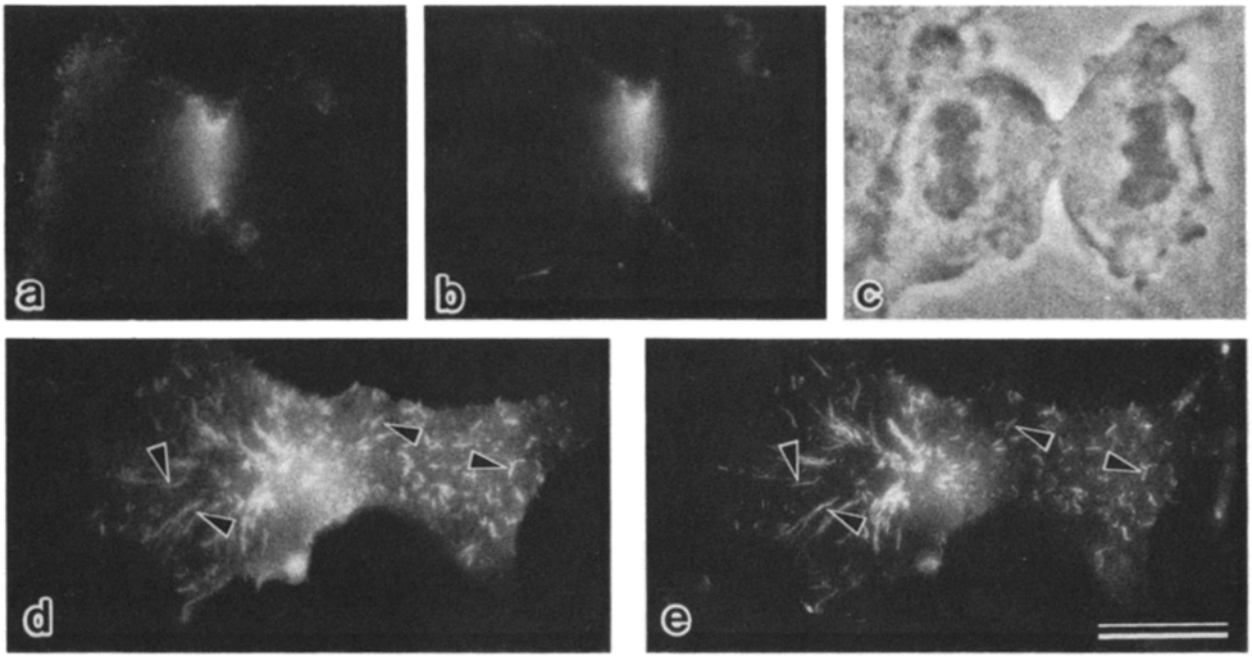


Figure 8. Double staining immunofluorescence (*a*, *b*, *d*, and *e*) and phase-contrast (*c*) micrographs of L-CLS1 cells at cytokinesis (*a–c*) and interphase (*d* and *e*) with anti-E-cadherin pAb (*a* and *d*) and anti-ERM mAb (*b* and *e*). At cytokinesis, both CLS1 and ERM are coconcentrated in the cleavage furrow. Also at interphase, they are precisely colocalized mainly in spikes (*arrowheads*) on the cell surface. Bar, 10 μm.

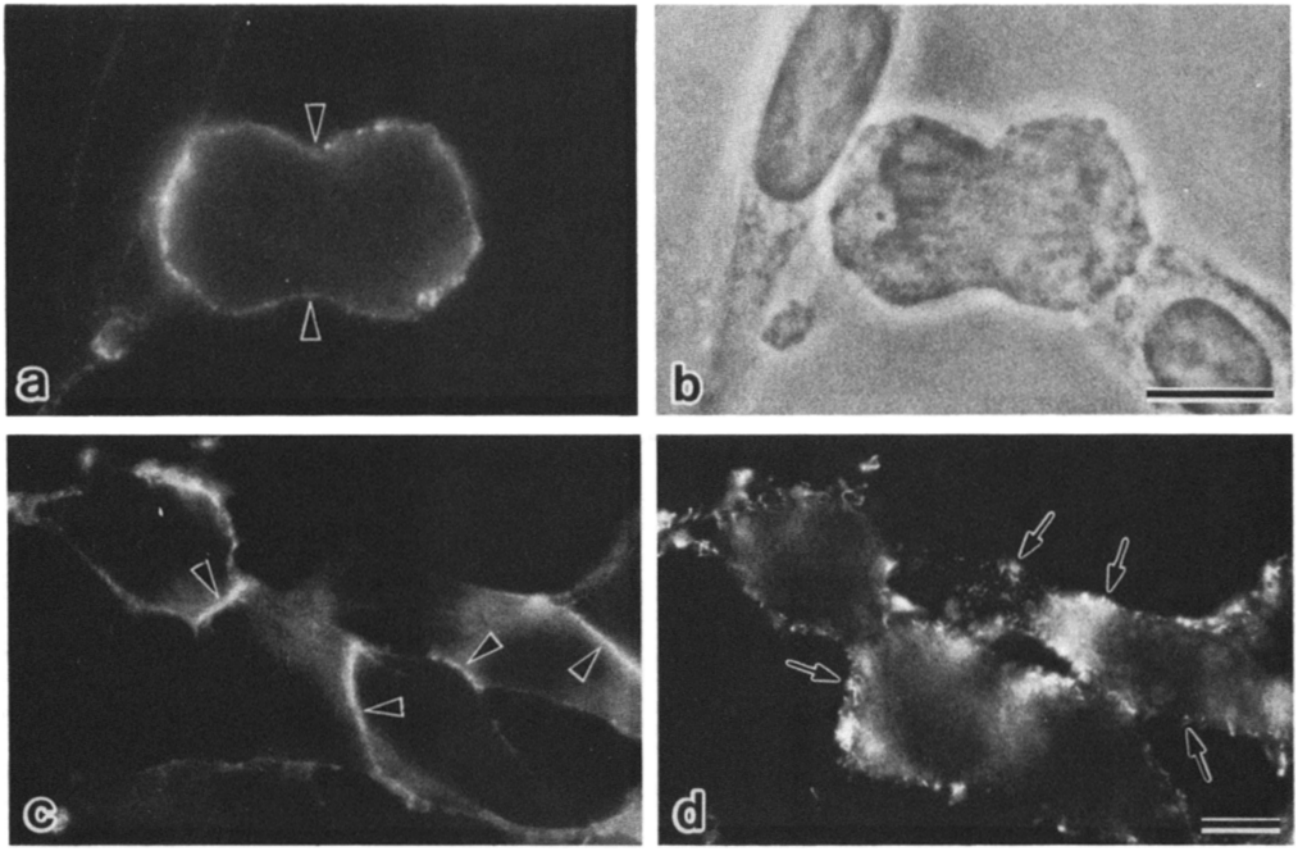


Figure 9. Immunofluorescence (*a*, *c*, and *d*) and phase-contrast (*b*) micrographs of EL8 cells expressing normal E-cadherin with E-cadherin pAb (*a*, *c*) and anti-ERM mAb (*d*). At cytokinesis (*a* and *b*), E-cadherin is not concentrated in the cleavage furrow (*arrowheads*), but is diffusely distributed on the cell surface. At interphase (*c* and *d*), E-cadherin (*c*) is accumulated along boundaries of cells (*arrowheads*), whereas ERM is localized in numerous spikes on cells (*arrows*). Bars, 10 μm.

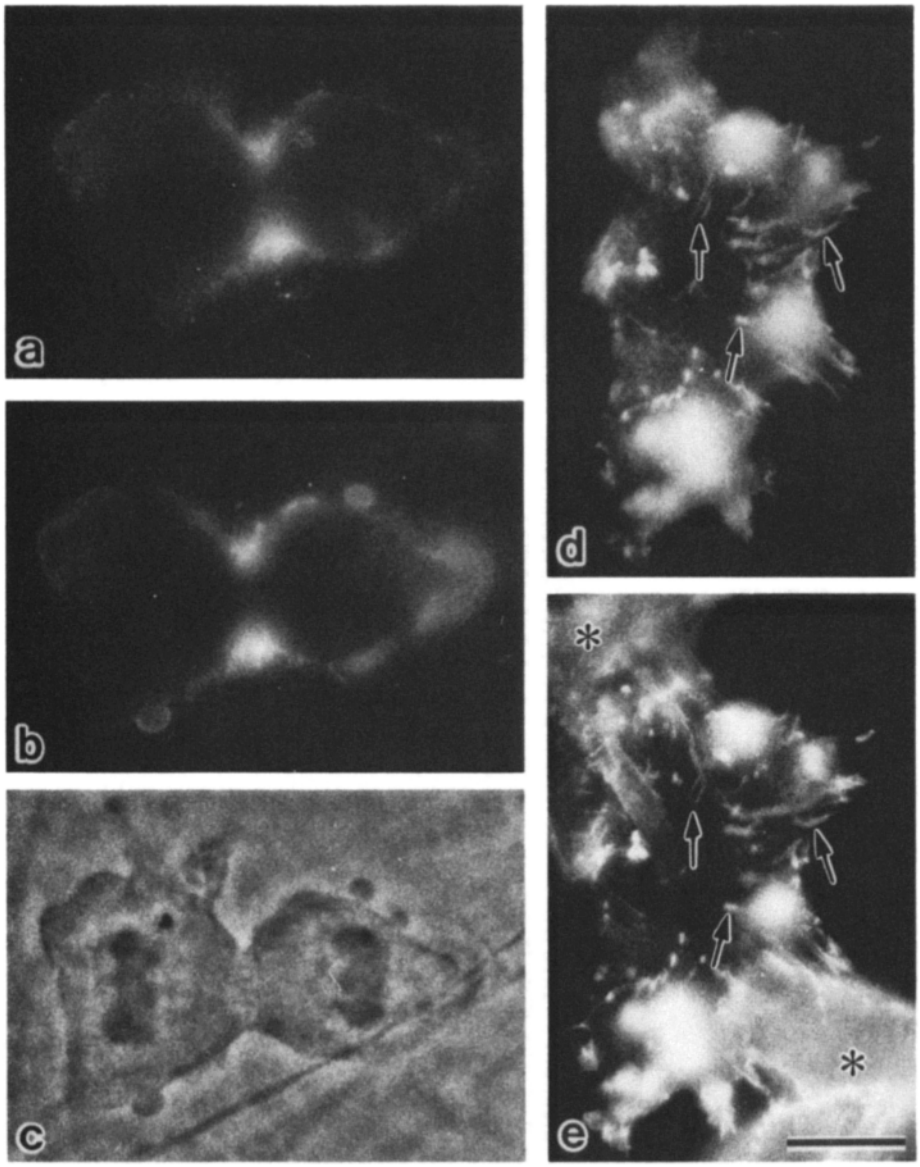


Figure 10. Double staining immunofluorescence (*a*, *b*, *d*, and *e*) and phase-contrast (*c*) micrographs of L-CLS1-A cells at cytokinesis (*a–c*) and interphase (*d* and *e*) with anti-E-cadherin pAb (*a* and *d*) and anti-ERM mAb (*b* and *e*). At cytokinesis, both CLS1-A (*a*) and ERM (*b*) are concentrated in the cleavage furrow. At interphase, they are precisely colocalized mainly in spikes (*arrows*). Some cells (*asterisks*) do not express CLS1-A, since cells transiently expressing CLS1-A are observed in this case. Bar, 10 μm.

into CD43-deficient cells, the expressed CD43 is concentrated in the cleavage furrow through the common mechanism. For this purpose, we obtained a cDNA encoding rat CD43 transmembrane/cytoplasmic domain by PCR, and then constructed a cDNA encoding a chimera (CLS1) consisting of the extracellular domain of mouse E-cadherin and the transmembrane/cytoplasmic domain of rat CD43 (Fig. 6). This cDNA was then introduced into mouse L fibroblasts which lack both CD43 and E-cadherin (Nagafuchi et al., 1987; Beacher et al., 1990). The extracellular domain of E-cadherin was used to eliminate the possibility that the extracellular domain of CD43 is responsible for the CD43 concentration in the cleavage furrow, as well as to report the localization of introduced chimeric molecules in transfectants by the use of an anti-E-cadherin pAb specific for the extracellular domain of E-cadherin (Nagafuchi et al., 1987). Considering that the cytoplasmic domain of E-cadherin is required for its cell adhesion function (Nagafuchi and Takeichi, 1988), it is unlikely that the distribution of CLS1 in transfectants is interfered with, by the direct interaction of CLS1 on adjacent cells through their extracellular domain derived from E-cadherin. Actually, no sign of the cell-to-cell adhesion was detected among cells into which CLS-1 or its mutant was introduced.

The expression of CLS1 in the transfectants obtained (L-CLS1) was first confirmed by immunoblotting with an anti-E-cadherin mAb (Fig. 7). A single band with a molecular mass of ~130 kD was detected. The distribution of CLS1 in transfectants was analyzed by immunofluorescence microscopy with anti-E-cadherin pAb (Fig. 8). Even after transfectants were cloned twice, the expression of CLS1 varied from cell to cell. Therefore, cells expressing a large amount of CLS1 were selected for immunolocalization analysis. During cytokinesis, CLS1 was clearly concentrated in the cleavage furrow in almost all dividing cells (97.8%; see Table I). Double staining immunofluorescence microscopy with anti-E-cadherin pAb and anti-ERM mAb revealed that CLS1 was precisely colocalized with ERM in the cleavage furrow (Fig. 8,

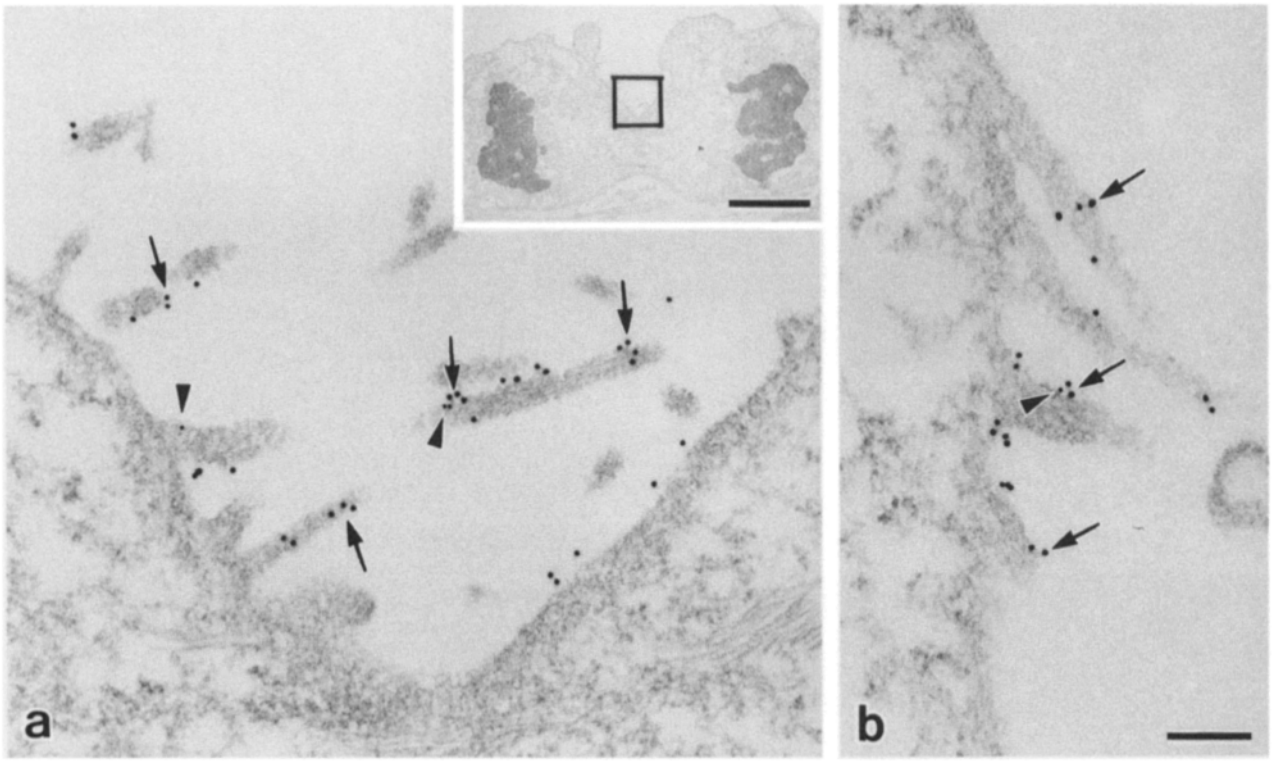


Figure 11. Double labeling immunoelectron micrographs of L-CLS1-A cells with anti-E-cadherin pAb and anti-ERM mAb. CLS1-A and ERM are indicated by 15- (*arrows*) and 10-nm (*arrowheads*) gold particles, respectively. The fixative contained only formaldehyde. (*a*) The cleavage furrow of a dividing cell squared in inset. Both CLS1-A and ERM are preferably localized on microvilli, although ERM were not heavily labeled in transfected cells under the conditions used in this study. (*b*) The surface of the interphase cell. CLS1-A and ERM are localized on microvilli. Bars, 0.2 μm (*a* and *b*); 5 μm (*inset*).

a–c). Also at interphase, CLS1 was colocalized with ERM mainly at spikes on the cell surface (Fig. 8, *d* and *e*). In the L cell transfected with the full-length E-cadherin cDNA, E-cadherin was not concentrated in the cleavage furrow, but diffusely distributed on the cell surface of dividing cells, and was not colocalized with ERM at interphase (Fig. 9). Therefore, we concluded that the colocalization of CLS1 with ERM, including their coconcentration in the cleavage furrow, is not ascribed to the extracellular domain of either E-cadherin or CD43 but to the transmembrane/cytoplasmic domain of CD43.

Region of the Cytoplasmic Domain of CD43 Responsible for Its Concentration in the Cleavage Furrow

We next constructed expression vectors containing cDNAs encoding mutants of CLS1, with deletions in the cytoplasmic domain, and introduced them into L cells (see Fig. 6). In this experiment, the cytoplasmic domain was tentatively divided into regions A and B. When region B was deleted from CLS1, leaving the region A intact, this mutant molecule (CLS1-A) was immunofluorescently shown to be concentrated together with ERM in the cleavage furrow in almost all dividing cells (94.7%; see Table I) (Fig. 10, *a–c*). Also at interphase, CLS1-A and ERM were precisely colocalized (Fig. 10, *d* and *e*). Similar to the localization of endogenous CD43 in RBL-2H3 cells, at the electron microscopic level CLS1-A was preferentially associated with the microvilli structures together with ERM throughout the cell cycle (Fig. 11).

In sharp contrast, the chimeric molecule lacking region A (CLS1-B) was not concentrated in the cleavage furrow during cytokinesis but was diffusely expressed on the surface of transfectants (L-CLS1-B), while intense ERM-staining was detected in the cleavage furrow (Fig. 12, *a–c*; see Table I). The distribution of CLS1-B at interphase did not coincide with that of ERM (Fig. 12, *d* and *e*). These data indicated that region A of the cytoplasmic domain of CD43 plays an essential role in concentrating CD43 molecules in the cleavage furrow and in direct or indirect binding CD43 to ERM.

Discussion

In this study we demonstrated that, in leukocytes such as rat thymocytes and basophilic leukemia cells (RBL-2H3), an integral membrane protein called CD43 (leukosialin or sialophorin) is highly concentrated in the cleavage furrow during cytokinesis. When a cDNA encoding a chimeric molecule containing the extracellular domain of E-cadherin and the transmembrane/cytoplasmic domain of CD43 was introduced into L cells deficient in CD43, this chimera was concentrated in the cleavage furrow in dividing transfectants. Finally, close analyses with deletion mutants of this chimeric molecule led us to conclude that a part of the cytoplasmic domain of CD43 (region A) is responsible for the concentration of CD43 in the cleavage furrow. The other characteristic feature of the distribution of CD43 revealed in this study is that the molecules containing region A are precisely colocalized with ERM not only at the mitotic phase but also at inter-

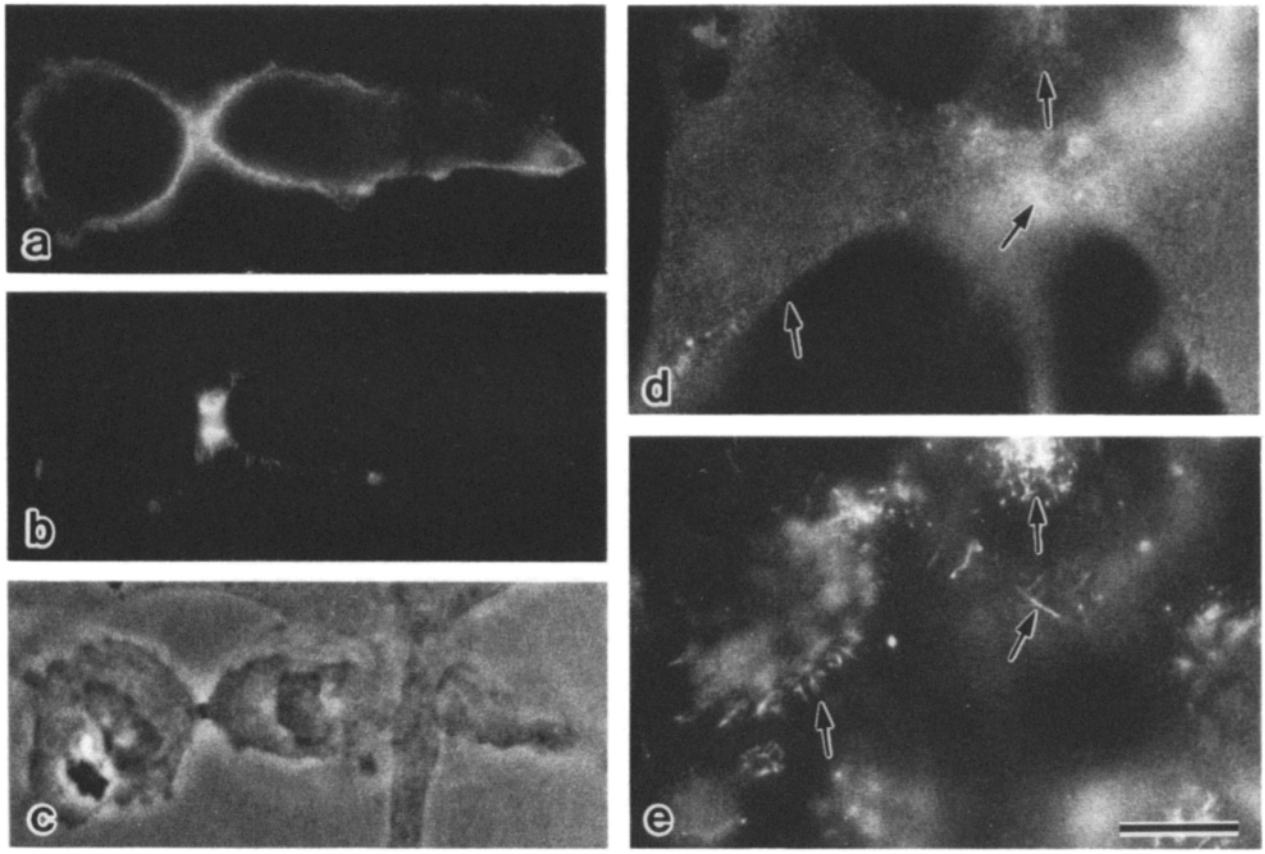


Figure 12. Double staining immunofluorescence (*a*, *b*, *d*, and *e*) and phase-contrast (*c*) micrographs of L-CLS1-B cells at cytokinesis (*a–c*) and interphase (*d* and *e*) with E-cadherin pAb (*a* and *d*) and anti-ERM mAb (*b* and *e*). At cytokinesis, CLS1-B (*a*) is diffusely distributed on the cell surface and not highly concentrated in the cleavage furrow, whereas ERM (*b*) is clearly concentrated in the cleavage furrow. At inter phase, CLS1-B (*d*) is diffusely distributed over the cell surface, whereas ERM (*e*) is localized mainly in spikes (*arrows*). Bar, 10 μm.

phase. ERM are highly concentrated at specific regions such as adherens junctions, ruffling membranes, cleavage furrows, etc., where actin filaments are densely associated with plasma membranes (Sato et al., 1992). As a result of cDNA cloning, ERM are thought to be directly associated with an integral membrane protein. Therefore, we speculate that CD43 is associated with actin filaments through an interaction between its region A and ERM, and that before and during cytokinesis this molecular complex is moved to the equatorial cortex to form the contractile ring. Of course, this study has not provided evidence for the direct interaction of CD43 with ERM. It is also possible that CD43 interacts indirectly with ERM through some other cytoskeletal proteins. Chemical crosslinking, coimmunoprecipitation, or direct binding assay will be required to prove this point.

If CD43 is essentially involved in cytokinesis, the question naturally arises as to why cells lacking CD43, such as L cells, can form the cleavage furrow and proliferate. It could be that there is a gene family of membrane proteins that contains a region A–like sequence in its cytoplasmic domains. In leukocytes, CD43 would be expressed as one member of this family, but as another member in L cells, resulting in their concentration in the cleavage furrow. Another possibility is that there is a ubiquitously expressed integral membrane protein that is directly associated with ERM, and that in leukocytes, CD43 is laterally associated with this putative membrane protein through its region A. In this case, this putative ERM-anchoring protein should be expressed in L cells, so that introduced chimera containing region A are associated with this ERM-anchoring protein to be concentrated in the cleavage furrow. Identification and characterization of the putative CD43 gene family or the putative ubiquitously expressed ERM-anchoring membrane protein would shed light on the molecular mechanism of the cleavage furrow formation in general.

The intriguing aspect of the localization of CD43 (or chimeras containing region A) and ERM is that they are tightly associated with microvilli throughout the cell cycle. In dividing cells analyzed in this study, the number of microvilli in the cleavage furrow was significantly larger than that in the polar region, and the amount of CD43 (or chimeric molecules containing region A) and ERM per microvillus appeared to be larger in the cleavage furrow than in the polar region. Using normal rat kidney cells, Cao and Wang (1990*a*,*b*) have shown at the light microscopic level that membrane-associated actin filaments evenly distributed on the cortex migrate toward the cleavage furrow during cytokinesis. It is thus predicted that microvilli containing ERM and CD43 move to the equatorial cortex to form a cleavage furrow. In fact, using the same cell line (normal rat kidney cells), we confirmed the concentration of microvilli as well as ERM in the furrow. The number of microvilli in the cleavage furrow varies greatly among cell types (Knutton et al., 1975; Sanger et al., 1984). For example, in some

types of cultures such as PtK2 cells, the number of microvilli in the cleavage furrow does not differ from that in nonfurrow areas (Sanger et al., 1984). In PtK2 cells we also observed the concentration of ERM-rich microvilli in the cleavage furrow. Thus, it is likely that the microvilli in dividing cells can be subclassified into ERM-rich and -poor types, and that the former (probably CD43-like membrane protein-rich) microvilli are generally concentrated in the cleavage furrow of cultured mammalian cells. This issue is of interest in that lymphocytes from patients with Wiskott-Aldrich syndrome, where CD43 is defective, are relatively devoid of microvilli (Kenney et al., 1986). Until now, the physiological function of the microvilli in cultured cells has not been fully understood. However, our present observations strongly suggest that these microvilli work as major actin filament–plasma membrane association sites throughout the cell cycle. Actually, microvilli are reportedly enriched in the cap structure of lymphocytes and the posterior region of moving lymphocytes where plasma membrane–associated actin filaments are thought to accumulate by the cortical flow of actin filaments (Yahara and Kakimoto-Sameshima, 1977; Haston and Shields, 1984; Bray and White, 1988).

CD43 is defective in the X-chromosome-linked immunodeficiency disorder, Wiskott-Aldrich syndrome. Among patients with this syndrome, it has been reported that the extracellular domain of CD43 is affected so that the proper signal is not transduced into the cytoplasm, resulting in the abnormal functioning of lymphocytes (Piller et al., 1988; Remold-O'Donnell and Rosen, 1990; Higgins et al., 1991). Our study suggested that CD43 is involved in cytokinesis through the direct or indirect binding of its region A with actin-based cytoskeletons. We thus speculated upon the physiological function of CD43 as follows. The binding of a ligand such as intercellular adhesion molecule-1 (Rosenstein et al., 1991) with the extracellular domain of CD43 may change the association ability of region A in the cytoplasmic domain with actin-based cytoskeletons. Through this mechanism, CD43 may regulate the process of cytokinesis and the proliferation of leukocytes.

Although it is of central importance in cell biology to understand the molecular mechanism of cytokinesis, knowledge of how the cleavage furrow is formed remains limited. This may be partly due to the difficulty in identifying the proteins responsible for the association of contractile ring actin filaments with the plasma membrane. In previous studies, we described the concentration of ERM in the cleavage furrow (Sato et al., 1991, 1992). In this study, we found that an integral membrane protein called CD43 was concentrated at the cleavage furrow through the direct or indirect interaction of its cytoplasmic domain with ERM. Therefore, the molecular mechanism of the cleavage furrow formation can be directly analyzed by gene technology with cDNAs encoding these proteins. Studies are currently being conducted along these lines.

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