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Activation of CEA-CAM-1-mediated cell adhesion via CD98: involvement of PKCδ

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Abstract CD98 is a multifunctional protein involved in amino acid transport and regulation of integrin-mediated cell adhesion. Herein, we demonstrated that CD98 stimulation by anti-CD98 antibodies induced CEA-CAM-1-mediated cell adhesion in BaF3 cells expressing CEA-CAM-1, and suggest that this might be responsible for compact clumping of F9 embryonic carcinoma cells by CD98 stimulation. CEA-CAM-1 was co-immunoprecipitated by anti-CD98 antibody. CD98 stimulation induced the translocation of cytoplasmic protein kinase Cδ (PKCδ) to the cell adhesion sites, and rottlerin that inhibited the PKCδ translocation abolished the cell aggregation without affecting integrin activation. The results suggested that CD98 stimulation could activate CEA-CAM-1-mediated cell adhesion independently of integrins.

Key words: CD98; CEA-CAM-1; Cell adhesion; Protein kinase Cδ; Embryonic carcinoma

1. Introduction

CD98 is a heterodimeric membrane protein consisting of a heavy (H) chain and one of at least six alternative light (L) chains [1]. Four of the L chains are shown to have L-type amino acid transport activity [2], while a common H chain plays a role in guiding the heterodimeric complex to the plasma membrane [3]. CD98 is expressed in various normal cells and most tumor cells [4]. In addition to the amino acid transport activity, diverse functions have been ascribed to CD98, such as regulation of cell adhesion, cell fusion, and proliferation [5]. Accumulating evidence indicates that CD98 plays a significant role in regulating integrin-mediated cell adhesion [6]. Cross-linking CD98 by anti-CD98 monoclonal antibodies (mAbs) was shown to promote α3β1-integrin-dependent syncytium formation associated with virus infection [7]. It was reported that transfection of CD98 H chain cDNA could reverse the dominant negative effect of the chimeric cytoplasmic domain of β1 integrin, and that cross-linking CD98 enhanced β1-integrin-mediated cell adhesion [8]. Several reports also indicated that CD98 was associated with β1-integrins [9–11], and we reported that CD98 stimulation by anti-CD98 mAbs induced the activation of β2-integrin (LFA-1) via phosphatidylinositol 3-kinase (PI3K) and Rap1 activation [12]. Recently, Rintoul et al. reported that cross-linking CD98 induced the clustering of β1-integrins and proposed that CD98 acted as a 'molecular facilitator' in the plasma membrane [13]. In the present study, we show that CD98 stimulation can activate CEA-CAM-1-mediated cell adhesion via protein kinase Cδ (PKCδ) activation. The results reveal that CD98 can regulate different types of adhesion molecules through distinct mechanisms, reinforcing the notion that CD98 acts as a 'molecular facilitator' in the plasma membrane.

2. Materials and methods

2.1. Cells and culture

F9, EL and BaF/3 cell lines were maintained in 10% fetal calf serum, Dulbecco's modified Eagle's medium and RPMI 1640, respectively, with additional interleukin-3 (100 U/ml) for the latter. The BaF/3 cell line transfected with human LFA-1 (B/LFA-1) has been reported before [14].

2.2. Antibodies and reagents

Anti-mouse CD98 H chain (14.37, 10.10) and L chain (10.7) mAbs as well as rabbit anti-mouse CEA-CAM-1 antibodies (#2456 and #231) have been reported before [3,15]. In some experiments, the $F(ab')_2$ fragment of mAb 14.37 was used. Anti-PKCδ antibody (Santa Cruz), rottlerin (Sigma) and wortmannin (Sigma) were purchased commercially.

2.3. cDNA transfection

BaF/3 cells were transfected with cDNA of CEA-CAM-1 long (L) or short (S) isoform in a pLXSN vector by electroporation and selected in medium containing 1.2 mg/ml G418 (B/C-CAM-1-L, B/C-CAM-1-S).

2.4. Immunoprecipitation, immunoblotting and flow cytometry

Cells were lysed with buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.4, inhibitors of proteases and phosphatases) containing varying concentrations of NP-40. The lysates were incubated overnight with anti-CD98 mAb at 4°C followed by precipitation with protein A-Sepharose beads. Immunoblotting and flow cytometric analysis were done as before [3,16].

2.5. Immunostaining

B/C-CAM-1 cells cultured in the presence of mAb 14.37 (2 μg/ml) were fixed by drop-wise addition of 2 volumes of 2×fixing solution (7.4% formaldehyde, 0.4% Triton X-100 in phosphate-buffered saline). After 10 min, the cells were cytospun gently and two-color-stained with biotinylated mAb 10.10 and rhodamine–streptavidin followed by rabbit anti-CEA-CAM-1 antibody and FITC-anti-rabbit IgG. F9 cells were cultured on coverslips in the absence or presence of mAb 14.37 and similarly immunostained. The samples were analyzed with a confocal microscope (Olympus).

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Abbreviations: mAb, monoclonal antibody; PI3K, phosphatidylinositol 3-kinase; CD98-H, -L, CD98 heavy and light chains

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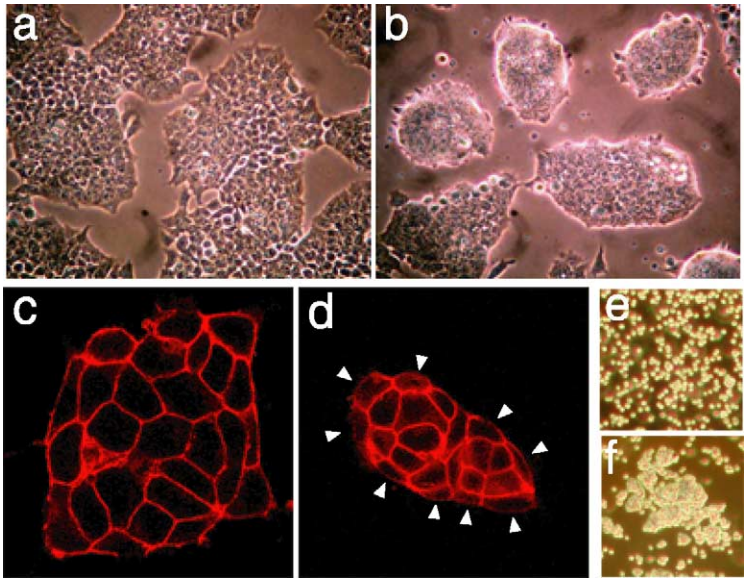

Fig. 1. Anti-CD98 mAb induces Ca^{2+}-independent cell clumping of F9 cells. F9 cells were cultured in the absence (a,c,e) or presence (b,d,f) of mAb 14.37 (2 μg/ml) for 24 h. a,b: Phase-contrast pictures (original magnification, ×200). c,d: Immunostaining with biotinylated mAb 10.10 and rhodamine–streptavidin (original magnification, ×600). e,f: Phase-contrast pictures after treatment with trypsin/EDTA for 5 min. Arrowheads indicate the cell contact-free surface.

3. Results

3.1. Anti-CD98 mAb induces Ca^{2+}-independent cell clumping in F9 embryonic carcinoma cells

F9 cells cultured in the presence of anti-CD98 H chain mAb (14.37) for over 24 h formed highly compact cellular clumps resembling embryonic bodies, while they normally grew in a flat monolayer (Fig. 1, b vs. a). To examine the localization of CD98, we immunostained the F9 cells with biotinylated mAb 10.10 recognizing a different epitope of CD98 H chain. While CD98 was detected evenly around the entire cell surface including the cell contact-free surface of control F9 cells, it was localized exclusively at the cell–cell adhesion sites of those cultured with mAb 14.37 (Fig. 1, c vs. d). The cell size of the latter was smaller than that of the former, reflecting the tight three-dimensional packing of the cells. F9 cells cultured with mAb 14.37 remained aggregated significantly after brief treatment with trypsin/EDTA, while control F9 cells were dispersed completely (Fig. 1, f vs. e). L cells expressing E-cadherin (EL) showed no such clumping by mAb 14.37 in spite of the comparable expression of CD98 (data not shown).

3.2. CD98 stimulation by anti-CD98 mAb activates CEA-CAM-1-mediated cell adhesion

Cell adhesion independent of Ca^{2+} is a typical feature of the adhesion molecules of the immunoglobulin superfamily (ISF). As shown in Fig. 2A, F9 cells expressed significant CEA-CAM-1, a member of the ISF, while EL cells did so only marginally. While CEA-CAM-1 was expressed around the entire cell surface of control F9 cells, it was localized at the contact sites of packed F9 cells cultured with mAb 14.37 (Fig. 2B, a vs. b). We then examined the effect of a blocking anti-CEA-CAM-1 antibody (#231) on the clumping of F9 cells. Inclusion of antibody #231 up to a final 1:100 dilution either preceding or together with mAb 14.37 in the culture, however, did not affect the clumping of F9 cells at 24 h. The development of cell clumping became microscopically evident

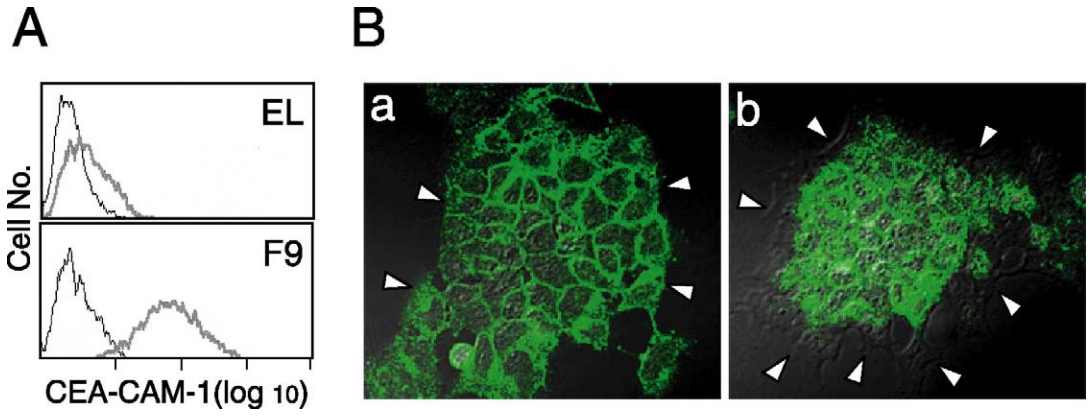

Fig. 2. Expression of CEA-CAM-1 in F9 cells. A: Flow cytometric analysis of CEA-CAM-1 in EL and F9 cells. B: F9 cells cultured in the absence (a) or presence (b) of mAb 14.37 for 24 h were immunostained with anti-CEA-CAM-1 antibody. Merged pictures with Nomarski are shown. Arrowheads indicate the cell contact-free surface.

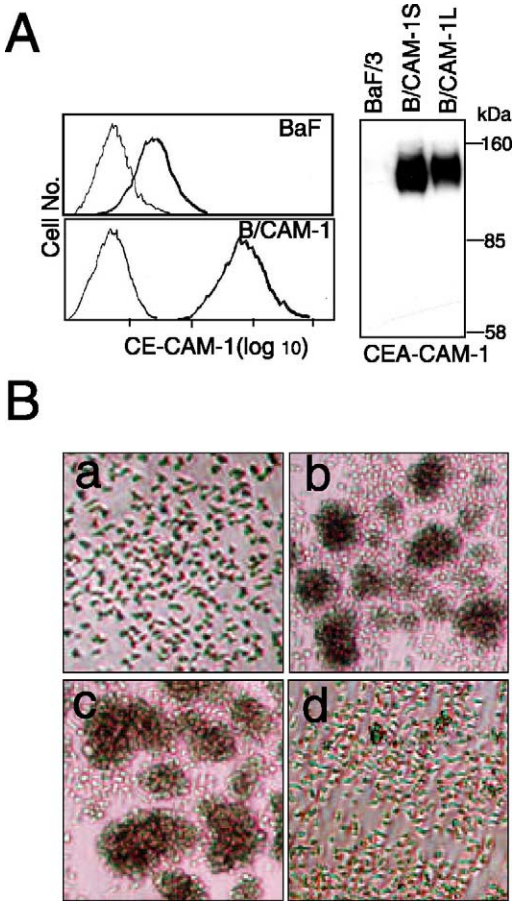

Fig. 3. Anti-CD98 mAb activates CEA-CAM-1-mediated cell adhesion. A: Expression of CEA-CAM-1 in BaF/3 cells transfected with cDNA of CEA-CAM-1S or CEA-CAM-1L. B: Parental BaF/3 (a), B/C-CAM-1S (b) or B/C-CAM-1L (c,d) cells were cultured in the presence of mAb 14.37 alone (a–c) or together with a blocking anti-CEA-CAM-1 antibody (d).

only after 24 h, and it was possible that turnover of cell surface CEA-CAM-1 occurred during the period. We therefore intended to examine the effect of CD98 stimulation on CEA-CAM-1-activation more directly by establishing BaF/3 cells stably expressing CEA-CAM-1 (B/C-CAM-1) of either the long (L) or the short (S) isoform (Fig. 3A). Neither B/C-CAM-1L nor B/C-CAM-1S was aggregated spontaneously (data not shown). However, both cell lines, but not the parental BaF/3 line, showed marked cell aggregation within as early as 1 h after the addition of mAb 14.37 (Fig. 3B, a–c). This rapid aggregation was inhibited completely by the inclusion of antibody #231 at a final dilution of 1:200 (Fig. 3B, d), while the preimmune serum was without effect (data not shown).

3.3. Association of CD98 with CEA-CAM-1

We then examined the possible association of CD98 with CEA-CAM-1. F9 cells were lysed with buffer containing varying concentrations of NP-40. As shown in Fig. 4A, a significant proportion of CEA-CAM-1 was co-immunoprecipitated by mAb 10.10 at 0.1% NP-40, while the efficiency was reduced at higher NP-40 concentrations. The extent of co-immunoprecipitation was enhanced when F9 cells were cultured in the presence of mAb 14.37. Similarly, CEA-CAM-1 was co-immunoprecipitated in B/C-CAM-1L cells by either anti-CD98-H (10.10) or anti-CD98-L mAb (10.7) (Fig. 4A), indicating that a portion of CEA-CAM-1 was associated with heterodimeric CD98 complex. To confirm the results, F9 and B/C-CAM-1L cells cultured in the presence of mAb 14.37 were two-color-stained with mAb 10.10 and anti-CEA-CAM-1 antibody. As shown in Fig. 4B, CD98 and CEA-CAM-1 were co-localized at the cell adhesion sites in both cell types.

3.4. Probable involvement of PKCδ in the activation of CEA-CAM-1-mediated cell adhesion by anti-CD98 mAb

Among a number of metabolic inhibitors examined, rottlerin, a specific inhibitor of PKCδ, completely inhibited the aggregation of B/C-CAM-1L cells by mAb 14.37 (Fig. 5A). The inhibitory effect was specific for CEA-CAM-1-mediated cell aggregation, because the same dose of rottlerin did not affect the LFA-1-mediated aggregation of B/LFA-1 cells by mAb 14.37 (Fig. 5A). In contrast, a PI3K inhibitor, wortmannin, which inhibited the aggregation of B/LFA-1 cells, hardly affected the aggregation of B/C-CAM-1L cells (Fig. 5A). Trypsin/EDTA-resistant aggregation of F9 cells cultured with mAb 14.37 for 24 h was also inhibited by rottlerin (Fig. 5A). Cytochalasin D inhibited the aggregation of both cell types (data not shown). Immunostaining analysis revealed that endogenous PKCδ, which was present diffusely in the cytoplasm of control F9 cells, was translocated to the cell adhesion sites 5 h after the culture with mAb 14.37 (Fig. 5B). In the additional presence of rottlerin, this translocation of PKCδ was inhibited significantly (Fig. 5C). Concomitantly, shrinkage in the cell size of F9 cells cultured in the presence of mAb 14.37 was inhibited by rottlerin (Fig. 5B), suggesting that the PKCδ translocation played a critical role in the cell clumping of F9 cells by anti-CD98 mAb.

4. Discussion

While CEA-CAM-1 mediates homophilic cell adhesion, the precise mechanisms for its regulation remain largely unknown. The present results demonstrated that anti-CD98 mAbs induced rapid cell aggregation in the BaF3 cell lines expressing CEA-CAM-1, but not in the parental BaF3 cells with little endogenous CEA-CAM-1, and the aggregation was blocked by anti-CEA-CAM-1 antibody. A similar effect was observed when using two anti-CD98 H mAbs with different epitope specificity, implying that the effect was due to the cross-linkage of CD98 antigen. A significant portion of CEA-CAM-1 could be co-immunoprecipitated by anti-CD98 mAb, although it remained to be seen whether they were associated directly or just coexisted in the same membrane domain. Consistent with this, CEA-CAM-1 and CD98 were co-localized at the cell adhesion sites in the B/C-CAM-1 cells stimulated by anti-CD98 mAb. These results indicate that CD98 stimulation could activate the cell adhesion mediated by CEA-CAM-1.

The aggregation of B/C-CAM-1 cells by anti-CD98 mAb was inhibited specifically by rottlerin, an inhibitor of PKCδ. Rottlerin has been reported to be fairly specific for PKCδ [17], which is a member of the novel PKCs activated by diacylglycerol independently of Ca^{2+} and translocated to membranes in response to phorbol esters [18]. Among a number of suggested functions, recent reports indicated the involvement of

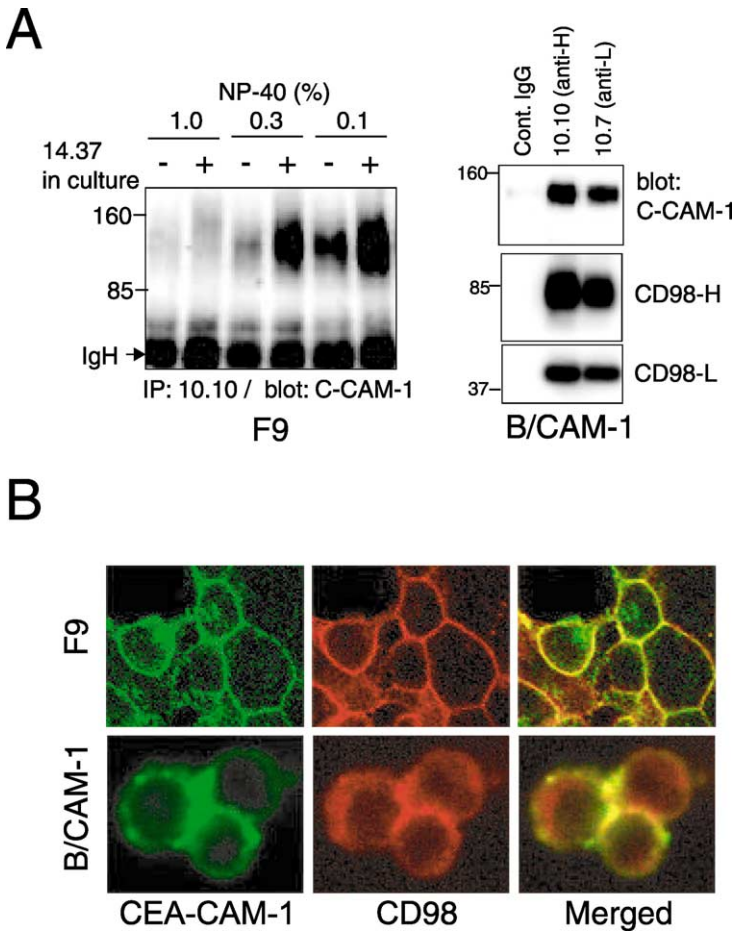

Fig. 4. Co-immunoprecipitation and co-localization of CEA-CAM-1 and CD98. A: (left) F9 cells cultured with or without mAb 14.37 were lysed with buffer containing varying concentrations of NP-40, immunoprecipitated with mAb 10.10, and immunoblotted with anti-CEA-CAM-1 antibody. (right) B/CAM-1S cells were lysed (0.1% NP-40), immunoprecipitated with control IgG, mAb 10.10, or 10.7 (anti-CD98-L chain) followed by immunoblotting with the indicated antibodies. B: F9 and B/C-CAM-1L cells were cultured in the presence of mAb 14.37 for 24 and 2 h respectively, and two-color-stained with mAb 10.10 (red) and anti-CEA-CAM-1 antibody (green).

PKCδ in the control of cell adhesion. For instance, PKCδ was shown to regulate E-cadherin-mediated cell adhesion by interacting with DF3/MUC1 [19] or RACK1 scaffolding protein [20]. It was also reported that the aggregation of U937 cells by anti-CD98 mAb was inhibited significantly by rottlerin, although adhesion molecules involved in the aggregation remained to be determined [21]. On the other hand, we reported that anti-CD98 mAb activated LFA-1/ICAM-1-mediated adhesion via PI3K and Rap1 activation in an LFA-1-dependent manner [12], and recently it was indicated that PI3K activation by cross-linking CD98 was dependent on integrins [13]. Activation of CEA-CAM-1-mediated adhesion by anti-CD98 mAb, however, was unaffected by a PI3K inhibitor. In contrast, activation of LFA-1-mediated cell adhesion by the same mAb was not affected by rottlerin. Thus, the present results strongly suggested that CD98 stimulation could activate CEA-CAM-1-mediated cell adhesion via PKCδ independently of integrins.

In F9 cells that endogenously expressed significant CEA-CAM-1, anti-CD98 mAbs induced compact cell clumping reminiscent of embryonic bodies after 24 h in the culture. Anti-CD98 mAb induced the translocation of PKCδ to the cell adhesion sites in F9 cells, and rottlerin that inhibited this translocation suppressed the cell clumping, strongly suggesting the critical role of PKCδ activation. An attempt to block the F9 cell clumping by anti-CEA-CAM-1 antibody was without success. However, even B/C-CAM-1 cells developed significant cell aggregation in the presence of both mAb 14.37 and anti-CEA-CAM-1 antibody, when they were cultured for as long as 24 h (Kakugawa et al., unpublished data). The results implied that membrane turnover of active CEA-CAM-1 overcame the blocking effect of the antibody. Thus, it is likely that CD98 stimulation activates the endogenous CEA-CAM-1 in F9 cells via PKCδ leading to the characteristic cell clumping, although additional effects of CD98 stimulation on other adhesion systems including E-cadherin and integrins remain to be investigated.

CEA-CAMs are multifunctional proteins involved in not only cell adhesion but also inhibition of tumor growth [22,23]. Interestingly, CD98 stimulation has also been reported to inhibit the growth of certain tumor cells [24]. Our unpublished results indicated that the growth of F9 cells that expressed both L and S isoforms of CEA-CAM-1 was inhibited in the presence of mAb 14.37, while that of B/C-CAM-1L or B/C-CAM-1S was unaffected (Kakugawa et al., unpublished). Growth inhibition mediated by CEA-CAM-1 was shown to require the expression of both L and S forms at certain ratios [25], and thus it may be an intriguing possibility

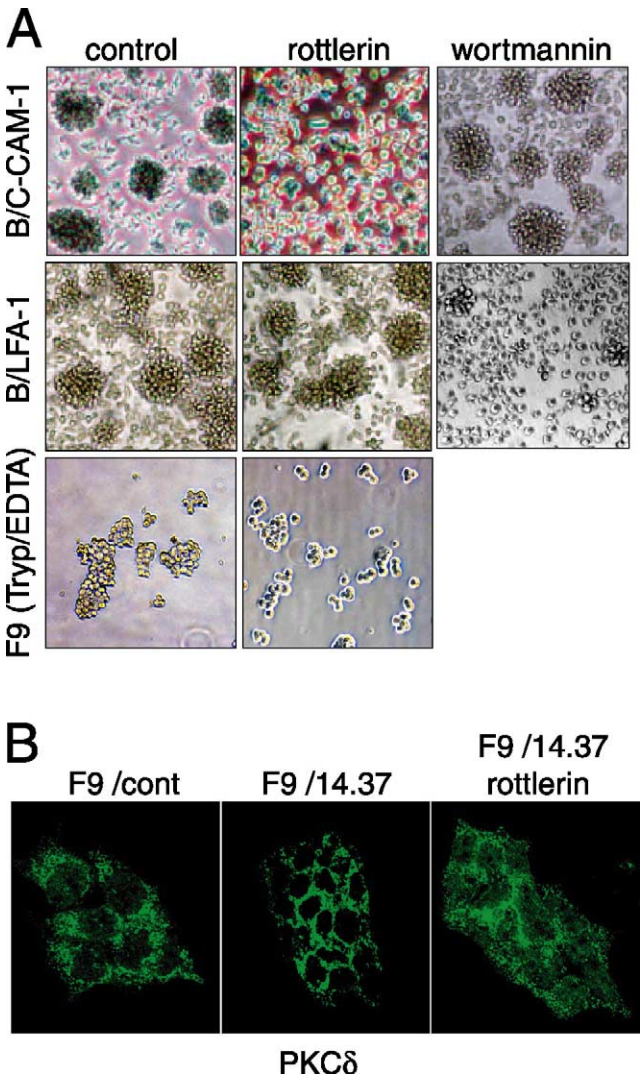

Fig. 5. Inhibition of the cell aggregation and translocation of PKCδ by rottlerin. A: B/C-CAM-1 and B/LFA-1 cells were cultured with mAb 14.37 in the absence or presence of rottlerin (5 μM) or wortmannin (50 nM) for 2 h. F9 cells were cultured in the presence of mAb 14.37 for 24 h with or without rottlerin (5 μM), and treated with trypsin/EDTA. B: F9 cells were cultured in the absence or presence of mAb 14.37 for 5 h with or without rottlerin, and immunostained with anti-PKCδ antibody (original magnification, ×600). Note the difference in cell size among the three groups.

that CD98 stimulation inhibits tumor cell growth in part through CEA-CAM-1 activation. CEA-CAMs are suggested to play significant roles in the control of tumor growth and invasion, and CD98 may provide a unique molecular target to control malignancy.

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