Cell-Adhesion Activity of the Cadherin-Related Neuronal Receptor (CNR) Family

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Contents

Abstract

Cadherin-related neuronal receptor (CNR) family proteins are a group of diversified synaptic protocadherins linked to the Fyn tyrosine kinase. CNR protein has six well-conserved cadherin motifs; however, its adhesion activity has not been defined. I found overexpressed CNR1 was transported to the plasma membrane in HEK293T cells. A cell aggregation assay using HEK293T cells revealed that calcium-dependent aggregation activity was increased by full-length CNR1 expression. To examine whether the CNR1 molecule binds homophilically, CNR1 transfectant and Mock cells were tested for the ability to interact with the recombinant Fc-fusion protein of the CNR1 extracellular domain immobilized on culture plates. Interestingly, whether or not they expressed CNR1, HEK293T cells adhered to the CNR1 extracellular domain, in a calcium ion dependent manner. I showed that the CNR1 EC1 domain was sufficient to induce the cell adhesion activity. This heterophilic adhesion activity was blocked by the RGDS peptide and anti-β1 integrin antibodies. Furthermore, the expression patterns of CNR and β1 integrin overlapped in the molecular layer of the developing mouse cerebellum. RGD-sensitive integrins are reported to play significant roles in brain development and synaptic plasticity. These results suggest that the CNR family could mediate a heterophilic, calcium-dependent adhesion with integrin superfamily members at the synapse.

Introduction

Millions of neurons form complicated and highly organized networks for communication and information processing in higher animals. Within the neural networks, synapses are asymmetric adhesive junctions that are highly specialized for interneuronal attachment. To understand how neuronal networks are generated and regenerated, I sought to elucidate how the specificity and plasticity of the synaptic junction are regulated. Recent reports demonstrated that a large number of molecules involved in cell adhesion are located at synapses. Interactions between these molecules are thought to be involved in morphological dynamics, but this process is not well understood (for review see Benson et al., 2000; Yagi and Takeichi, 2000).

The CNR family consists of synaptic cadherins, which were originally identified as Fyn tyrosine kinase-coupled membrane proteins. Fyn is also localized to synapses, where it regulates NMDA receptor function. The loss of Fyn proteins causes impairments in synaptic function (Yagi and Takeichi, 2000). All known CNR family members are expressed in various regions of the mouse brain. They are enriched in the postsynaptic density (PSD) fraction, and immuno-electron microscopy revealed that they localize to synaptic junctions (Kohmura et al., 1998). In addition, the first cadherin domain (EC1) sequence of the CNR family proteins is well conserved and uniformly associates with Reelin protein, and CNR family proteins act as multiple Reelin receptors in the embryonic cerebral cortex (Senzaki et al., 1999). The genomic structure of the CNR family genes is a gene cluster, similar to those of the immunoglobulin and T-cell-receptor genes (Wu and Maniatis, 1999; Sugino et al., 2000). Furthermore, it was

recently demonstrated that somatic mutagenesis occurs in the mouse *CNR3* transcripts, introducing the possibility of a striking diversification of CNR proteins (Hirayama et al., 2001). Thus, the CNR family is implicated in the regulation of the diversity, specificity, and plasticity of synaptic junctions. However, the adhesive properties of CNR molecules have not been determined.

It is well established that classical cadherins have calcium-dependent homophilic adhesion activity in the L1 and Neuro2A cell lines. However, when CNR1 expression vectors are introduced into these cells, the distribution of the CNR1 protein is different from that of classical cadherins and protocadherins; that is, CNR1 protein is concentrated in the cytoplasmic regions, and there is little or none on the cell surface. Here, I developed an assay for the cell adhesion activity of CNR1 using HEK293T cells, and used it to demonstrate that CNR1 protein elicits a strong Ca^{2+}-dependent cell adhesion activity. CNR1 adhered to non-transfected HEK293T cells, indicating that its cell adhesion activity is heterophilic. This heterophilic adhesion activity between CNR1 protein and HEK293T cells was regulated by integrins. The RGD motif, the consensus for binding to integrins, lies within the EC1 domain of CNR1 and is highly conserved among the CNR family proteins. These data, along with reports (Xiao et al., 1991; Staubli et al., 1998) that the RGDS peptide and anti-integrin antibodies effectively block the physiological function of integrin family proteins in synapses, suggest that cross-talk between the CNRs and integrins play roles in generating and rearranging synaptic junctions.

Materials and Methods

Antibodies

The following primary antibodies were used: (a) monoclonal mouse 6-1B antibody against CNR (Kohmura et al., 1998); (b) rabbit polyclonal antibody against the cytoplasmic domain of anti-CNR1 A (Takei et al., 2001); (c) mAb ECCD-2 against E-cadherin (Takara Shuzo Co., Japan); (d) Rabbit polyclonal antibody against PDI (StressGen Biotechnologies Co., Victoria, BC); (e) mAb 3S3 against human β1 integrin (Cosmo Bio, Co., Japan), for immunoprecipitation and attachment inhibition experiments, and (f) rabbit polyclonal antibody against the cytoplasmic domain of β1 integrin (Chemicon, Rosemont, IL) for immunohistochemistry and western blotting. Secondary antibodies for immunofluorescence microscopy included biotin- or Alexa-488-labeled anti-mouse IgM antibodies (Vector Laboratories, Inc. Burlingame, CA), Texas red-avidin (Sigma Chemical Co., St. Louis, MO), FITC-labeled anti-Rat IgG antibodies (Jackson Immuno Research, West Grove, PA), Alexa-488 anti-Rat IgG, and Alexa-594 anti-rabbit IgG (Molecular Probes, Inc. OR).

Production of Monoclonal Anti-CNR1 N-Terminal Antibody

The antigenic peptide of the CNR1 N terminus (QIHYSIPEEAKHGT) was conjugated with KLH and injected into rats. The spleens were removed from the immunized rats and the splenocytes were fused with P3U1 mouse myeloma cells by the polyethylene glycol method (Kohler and Milstein, 1975). Fused cells were selected by growth in HAT medium and the ELISA-positive cells were cloned. The specificity of

the monoclonal antibody, called 4F11, was confirmed by immunoblots using the lysate from HEK293T cells that had been transiently transfected with mouse *CNR*1, 6, and 12 cDNA. 4F11 recognized these overexpressed CNRs.

Plasmids Expressing CNR1 Variants

The NotI-XhoI fragment of full-length CNR1 (Type A) cDNA (Kohmura et al., 1998; Sugino et al., 2000) was inserted into the NotI-XhoI site of the CMV-driven mammalian expression vector, pcDNA3.1+ (Invitrogen, San Diego, CA). The constructs were confirmed by restriction mapping and DNA sequencing. The mouse E-cadherin expression vector (pBATEM2) was a gift from Drs. M. Takeichi and A. Nagafuchi (Kyoto University).

Cell Culture and Transfection

HEK293T, L1, COS7, CHO, Neuro 2A, CHP212, and MDCKII cells were grown in Dulbecco's modified Eagle's medium: Nutrient mixture F12 (DMEM/F12) (Invitrogen, San Diego, CA) with 10% FCS. For transfection and immunostaining, cells were grown on coverslips, or in the case of MDCKII cells, 0.4-μm pore size Trans-well membranes (Corning Inc., Corning, NY). The MDCKII cells were a gift from Dr. M. Murata (National Institute of Physiology). For each experiment, the cDNAs were transfected into cell lines using Lipofectamine PLUS reagents according to the manufacturer's instructions (Invitrogen, San Diego, CA).

Immunostaining

The cells were transiently transfected with cDNAs at the same time and under the same conditions. Forty eight hours after transfection, the cells were fixed in 4% paraformaldehyde for 20 min at room temperature (RT), washed with PBS 3 times, and permeabilized with 0.05% TritonX-100 for 5 min at RT. For surface staining, the permeabilization step was skipped. Cells were blocked with 3% Goat serum for 30 min. The 6-1B antibody was used to detect overexpressed CNR1, and ECCD-2 was used to identify overexpressed murine E-cadherin. Cells were incubated with these appropriately diluted primary antibodies for 1 hr at RT, washed with PBS three times, and incubated with appropriately diluted secondary antibodies for 1 hr at RT. After washing, cells were mounted in 1% p-Phenylenediamine.

For live staining, another way to stain cell-surface CNR1 protein, transfectants were washed twice with cooled 5% FCS/PBS, and non-specific binding was blocked by incubating with Block Ace (Dainippon Pharmaceutical Co. Japan) for 10 min on ice. The cells were incubated with the appropriately diluted primary antibody in Block Ace for 1 hr on ice, followed by washing with cooled 5% FCS/HMF 3 times and incubation with the appropriately diluted FITC-conjugated secondary antibody for 1 hr on ice. After washing, the cells were fixed with 1% paraformaldehyde in PBS on ice for 30 min and mounted in 1% p-Phenylenediamine. To detect intracellular antigen using this method, cells were first pretreated with 70% ethanol at –20°C for 30 min. After washing, cells were mounted in 1% p-Phenylenediamine and analyzed using a fluorescence

microscope (Olympus BX51/DP50 system) and Zeiss confocal laser scan microscope, Axiovert 135 (Microsystem LSM).

Immunohistochemistry

ICR mice (SLC Japan Inc.) were used in these experiments. In the developmental studies, the day of birth was considered postnatal day (P) 0. Animals used in the studies were both male and female. At least 3 animals were used for preparations at each of the following ages: P7, P10, P14, and P25.

All mice were anesthetized with diethyl ether, then perfused. The mouse brains were removed and postfixed for 1 hr with 4% paraformaldehyde/PBS, and cryoprotected in a 20% sucrose solution. Parasagittal and or horizontal cryostat sections (5-μm thick) were obtained. Sections were blocked with 0.3% TritonX-100/5% goat serum/PBS and stained with appropriately diluted primary antibody with blocking solution overnight at 4°C. These sections were then treated with the secondary antibody (Molecular Probes, Inc. OR). Mounted sections were examined with the fluorescence microscope and the Zeiss confocal laser scan microscope. The control experiment was performed by antigen absorption with an excess amount of the antigen peptide of β1 integrin.

Cell Adhesion Assay

Cell aggregation experiments were carried out according to the method of Resink et al (Resink et al., 1999). A 24-well petri dish (Becton Dickinson Co., Lincoln

Park, NJ) was precoated with 10 mg/ml BSA overnight at 4°C. HEK293T cells were seeded at 2.5×10^6 onto 60-mm culture dishes (Falcon) the day before transfection. DNAs were transfected with Lipofect AMINE 2000 reagents according to the manufacturer's instructions for HEK293T cells. After 36-48 hrs of transfection, the cells were washed twice and collected in PBS containing 10 mM HEPES (pH 7.3). Cells were divided into three aliquots and treated with EGTA (2 mM final), trypsin/EGTA (0.025%, 2 mM final), and trypsin/$CaCl_2$ (0.025%, 1 mM final) respectively. After incubation for 20 min at 37°C, the cells were washed once gently, and the reaction was stopped by adding a solution containing 1 mM $MgCl_2$, 0.1% soybean trypsin inhibitor, and 60 μg/ml DNaseI and incubating for 30 min at 37°C. The cells were then gently washed and resuspended in PBS containing 10 mM HEPES (pH 7.3), 1 mg/ml BSA, and 1 mM EGTA. The cells were resuspended at 5×10^6 cells/ml and maintained at 4°C. Aliquots of the cell suspensions were withdrawn and processed for immunoblot analysis to check for CNR1 expression. Aggregation assays were started by the addition of 50 μl of cell suspension to 500 μl of pre-warmed (37°C) PBS containing 10 mM HEPES (pH 7.3), 1 mg/ml BSA, and either 2 mM $CaCl_2$ or 2 mM EGTA. The dish was placed on a gyratory shaker set to rotate at 70-80 rpm, and incubated at 37°C. Incubations were terminated by the addition of 500 μl of 5% glutaraldehyde/PBS. The suspensions were transferred to 3.5-cm petri dishes by pipette with a cut tip. The dishes were viewed through an Axiovert 135 microscope with a 5×objective and photographed using a Hamamatsu C5810 video system. Five optic fields of each dish were photographed. Particle numbers in the digital images were determined using NIH Image version 1.61.

Unless otherwise stated, aggregation is expressed as the functional loss of particle number, Nt/N_0, where N_0 is the particle number at time 0 and Nt the particle number at a given time point.

The method for on-plate cell adhesion experiments has been previously reported (Lemmon et al., 1989; Yamagata et al., 1999). A 2-cm^2 nitrocellulose filter strip was dissolved in 4.8 ml of methanol, then 250 μl of this nitrocellulose solution was spread onto a 6-well culture plate (Becton Dickinson Co., Lincoln Park, NJ) and dried. To avoid contamination of the test samples, Vaseline rings with a diameter of 1 cm (one ring for each test sample) were drawn on the dried surface of the wells. Twenty-five microliters of 0.2 μg/μl anti-human Fc fragment specific antibody (Jackson Immuno Research, West Grove, PA) were spotted in the Vaseline ring and incubated for 1 hr in a humidified CO_2 incubator at 37°C. The droplets were then removed and the area was washed 3 times with Hank's calcium- and magnesium-free saline solution. The anti-human Ig Fc antibody-coated spots were then treated for 1 hr at 37°C with Fc fusion proteins (0.2 ~ 0.5 μg/spot). Human plasma fibronectin (10 μg/ml) (Chemicon, Rosmont, IL) was spotted on another area of the same dish. To verify that the Fc fusion proteins were in excess amounts in the solution and therefore saturated on the plate, the solution was collected, and checked for the presence of fusion proteins by western blotting. The dishes were washed 3 times with calcium- and magnesium-free PBS and incubated with 0.5 μg/ml BSA/PBS overnight at 4°C to block nonspecific binding. Subconfluent monolayers of transfectant and parent HEK293T cells were washed twice with PBS, then treated with PBS containing 6 mM EDTA for 10 min at 37°C. The cells

were collected and washed with PBS and 10% FCS calcium-free DMEM. The cell pellet was then resuspended in 10% FCS calcium-free DMEM at 2×10^6 cells/ml, and the cells were kept on ice to inhibit aggregation. Aliquots of the cell suspension were placed on the test dishes with 1 mM $CaCl_2$ or 1 mM EGTA. The plated cells were incubated at 37°C for 2 hr then washed four times with PBS containing 10 mM HEPES (pH 7.3), and fixed with 5% glutaraldehyde solution. The dishes were viewed on the video system with a $5 \times$ objective. Five optic fields of each sample spot were photographed, and the digital images were analyzed. The pixels corresponding to the cells that were attached to dishes were measured in each field. Three independent experiments were performed, and the standard deviations calculated.

Statistical evaluation of the differences was performed using Student's paired *t*-test with Stat-view J-4.5. Differences were considered to be significant at $P < 0.05$.

Preparation of The Extracellular Domain of CNR1–Fc Fusion Protein

Sequences encoding the extracellular domain of mouse CNR1 (CNR1 EC-Fc, amino acids 1–692), the EC1 domain of CNR1 (EC1-Fc, amino acids 1–133), the region containing the RGD motif (RGD(+)-Fc, amino acids 1–97), the region not containing the RGD motif (RGD(-)-Fc, amino acids 1–67), and the signal sequence (Sig.-Fc, amino acids 1–39) of CNR1, and the extracellular domain of mouse E-cadherin (Ecad-Fc, amino acids 1–709) were amplified by PCR and inserted into the expression vector pEF-Fc (Mizushima and Nagata, 1990; Senzaki et al., 1999). HEK293T cells were plated at 8 X 10^4 cells /cm^2, and transfected the next day with each plasmid DNA using

Lipofectamine 2000 and OPT1-MEM (Invitrogen, San Diego, CA) following the manufacturer's protocol. Twenty-four hours after transfection, the medium was changed. Forty-eight hours after transfection, the medium was collected and assayed for the presence of the fusion protein by western blot analysis. The protein concentration was determined by western blot analysis using a purified human IgG standard (Sigma Chemical Co., St. Louis, MO).

Preparation of Fc-fusion protein-coated beads and bead aggregation assays.

Latex-sulfate microspheres (6.9×10^9, diameter 6.4 μm; Sigma Chemical Co., St. Louis, MO) were washed, resuspended in 0.2 ml of 25 mM MES, pH 6.1 (MES buffer), and incubated with 100 μg of an anti-human Fc γ fragment-specific antibody, with gentle mixing for 18 hours at room temperature, as reported (Lambert et al., 2000). The beads were then spun at 13,000 x g for 1 min at 4°C, washed 3 times with 2 ml of MES buffer, and incubated with PBS containing 5 mg/ml bovine serum albumin/MES buffer for 1 hr at room temperature. Aliquots of 200 μl of bead suspension (1×10^7 beads) were added to concentrated media containing Fc fusion proteins and incubated for 2 hrs to overnight at 4°C. To verify that the Fc fusion proteins were in excess amounts in the solution and therefore saturated on the beads, the Fc fusion proteins remaining in solution were checked by western blotting.

Sonicated beads (3×10^6 particles in 500 μl of calcium-free DMEM, 10% FCS containing 1 mM $CaCl_2$ or 2 mM EGTA) were placed in a 24-well petri dish (Becton Dickinson Co., Lincoln Park, NJ). The dish was then placed at 37°C in an

incubator (5% CO_2), and the aggregation was evaluated under an Axiovert 135 microscope at regular time points. For quantification, after 2 hrs of aggregation, the bead suspensions were randomly photographed under the microscope and the number and size of the aggregates were scored. The dishes were viewed on the Hamamatsu C5810 video system with a 5×objective. Five optic fields of each sample spot were photographed, and the digital images were analyzed by NIH Image version 1.61. The pixels corresponding to the cells attached to the dishes were measured in each field. Three independent experiments were performed, and the standard deviations were calculated.

Inhibition of Attachment

As inhibitors of attachment, the tetrapeptides RGDS and RGES, the 11-amino-acid peptide from the CNR1 sequence (RGDLLEVNLQN) (Peptide Institute Inc., Japan), and the β1 integrin function-blocking mAbs, clone 3S3 (Cosmo Bio, Co., Japan) and 6S6 (Chemicon, Rosmont, IL), were used. To investigate the effect of peptide dose on attachment, various peptide concentrations were used. Tetrapeptide solutions of 0, 0.01, 0.1, 0.2, and 1 mM were prepared in 10% FCS calcium-free DMEM containing 1 mM $CaCl_2$. In the case of anti-β1 antibody inhibition, the antibody concentration was 10 μg/ml under the same conditions. As a control, mouse IgG (Sigma Chemical Co., St. Louis, MO) was used at the same concentration as the anti-β1 antibody.

Results

Localization of CNR1 protein in several transfected cell lines

In L1 and Neuro2A cells, which are typically used for cell aggregation assays of classical cadherins, the exogenous CNR1 protein is concentrated in the endoplasmic subcellular membrane, and is not present on the plasma membrane (Kohmura et al., 1998). Using L1 cells, I found exogenous E-cadherin accumulated in areas of cell-to-cell contact (Figure 1A, a), whereas exogenous CNR1 protein showed a reticular distribution (Figure 1A, b). These reticular signals overlapped with the endoplasmic reticulum (ER) marker protein disulfide isomerase (PDI), indicating that the overexpressed CNR1 protein was stopped at the ER (Figure 1A, c).

To study the adhesion activity of CNR1, I sought a cell line in which the exogenous CNR1 protein could be transported to the surface of the plasma membrane. To this end, I transformed 7 different cell lines with a CNR1 expression vector: HEK293T, L1, Neuro2A, COS7, CHO, CHP212, and MDCKII. I then assayed for CNR1 localization on the cell surface by immunostaining with an anti-CNR1 antibody (6-1B) that recognizes the extracellular domain of the CNR1 protein. I could detect the CNR1 protein within all the cell lines when the transfectants were permeabilized with Triton X-100. The CNR1 proteins displayed a vesicular and reticular distribution within every cell line (Figure 1B, a-g). However, when immunostaining was performed in the absence of detergent (TritonX-100), the CNR1 protein was seen on the cell surface of the HEK293T cells (Figure 1B, h), but on no other cell line (Figure 1B, h-n). This result suggested that the exogenous CNR1 protein could be transported into the cell surface of

HEK293T cells.

To confirm the surface localization of CNR1 protein in HEK293T cells, live CNR1 transfectants were directly immunostained without fixation using both the 6-1B and CNR-A antibodies. The CNR-A antibody recognizes the cytoplasmic domain of CNR1. In the live HEK293T transfectants, CNR1 staining was detected only with 6-1B (Figure 2A, a). In contrast, EtOH-permeabilized transfectants showed staining with both the 6-1B and CNR-A antibodies (Figure 2A, b). No staining was found in untransfected HEK293T cells, except for a weak nonspecific staining by 6-1B in the EtOH-treated samples (Figures 2A, b and d). These results demonstrated that the CNR1 protein could be expressed on the surface of the HEK293T transfectants. On the other hand, immunoreactivity was detected in live L1 cells when E-cadherin (Figure 2B, a), but not CNR1 (Figure 2B, b), was expressed.

Cell adhesion assay using CNR1-transfected HEK293T cells

I next examined the cell adhesion activity of the CNR1–transiently transfected HEK293T cells. At least 60~80% of the transfected cells were immunoreactive. An aliquot of cells was collected in every experiment to assess the amount of expressed CNR1 protein by western blot analysis. I then compared the aggregation properties of suspensions of CNR1-transfectants (CNR1+) and parental HEK293T cells. For this assay, the cells were first dissociated by incubating them in the presence of 2 mM EGTA (E treatment) followed by gentle pipetting. The cells were then allowed to aggregate in Ca^{2+}-free or Ca^{2+}-containing saline. Both parental and transformed cells

exhibited Ca^{2+}-independent aggregation. A small but insignificant difference in the aggregative properties between the CNR1+ and the parental cells was detected {Nt/No: 0.16 vs. 0.29, $P = 0.30$ at 90 min} (Figure 3A).

Without trypsin treatment (E-treated cells), there was no significant difference between the cell-aggregation activity of CNR1 transfectants and parental cells. Both the E-treated transfectants and parental cells had strong endogenous cell adhesion activity; therefore, the increase in cell adhesion activity due to CNR1 protein might have been undetectable due to the high background (Figure 3A). However, when the cells were treated with Trypsin/Ca^{2+} (TC treatment), the Ca^{2+}-dependent aggregation was more prominent in the CNR1+ cells than in the parental cells {Nt/No: 0.11 vs. 0.34, $P = 0.025$ at 90 min} (Figure 3C). This difference between TC-treated CNR1+ and parental cells was not evident when the aggregation assay was conducted under calcium-free conditions ($P = 0.96$ at 90 min) (Figure 3D). Compared with the TC-treated cell suspensions, the Trypsin/EGTA (TE)-treated CNR1+ and parental cells exhibited a reduced ability to aggregate and there was no significant difference between the cell-aggregation activity of the transfectants and parental cells (Figure 3B).

These results suggest that overexpressed CNR1 increased a calcium-dependent cell-aggregation activity of HEK293T cells. However, the parental HEK293T cells contain various endogenous cell-adhesion molecules. Thus, I did not know whether the increase in CNR1-dependent cell-aggregation activity was due to a homophilic interaction (CNR1-CNR1) or to heterophilic interactions (CNR1 with other endogenous molecules).

Binding features of immobilized CNR1 extracellular domain

To determine the adhesive properties of CNR1, I used a recombinant protein containing its extracellular domain. I constructed a soluble fusion protein that consisted of the CNR1 extracellular domain and the Fc region of human IgG1 (CNR1 EC-Fc). The CNR1 EC-Fc was immobilized on a plastic dish coated with anti-human Fc antibody, and cell binding was examined by the method of Lemmon et al. (Lemmon et al., 1989). Cells were dissociated with EDTA and cultured on the plates containing the immobilized protein.

In the presence of 1 mM Ca^{2+}, CNR1 transfectants adhered to the CNR1 EC-Fc-coated dishes in a time-dependent manner (Figure 4A, a and Figure 4B). In the absence of Ca^{2+}, the CNR1 cells did not bind to the CNR1 EC-Fc (Figure 4A, b). Regardless of the presence of Ca^{2+} ions, the CNR1 transfectants could not bind to the CNR1 signal peptide–Fc fusion protein (Sig.-Fc) (Figure 4A, c). Thus, I could reproduce the Ca^{2+}-dependent cell-adhesion activity of CNR1 in this experiment using recombinant proteins bearing the extracellular domain of CNR1.

I next examined the possibility of a heterophilic binding activity of CNR1 protein by using parental HEK293T cells. Surprisingly, the parental cells could bind to the CNR1 EC-Fc in the presence of Ca^{2+} to a similar extent as did the CNR1 transfectants (Figure 4A, d). No binding was seen in the absence of Ca^{2+} or using the Sig.-Fc protein (Figures 4A, e and f). The similar binding seen with the CNR1 transfectants and the parental cells indicated that the immobilized CNR1 protein could

bind heterophilically to endogenous cell surface ligands of the parental HEK293T cells.

Furthermore, I performed a bead aggregation experiment using 6.4-μm-diameter latex beads on which the CNR1 EC-Fc protein was immobilized, according to the method of Lambert et al (Lambert et al., 2000). The quality and quantity of the proteins on the beads were examined by western blotting (Figure 5A). The aggregation properties of these beads were investigated and compared with the aggregation of beads presenting at their surface either E-cad-Fc as a positive control or Sig-Fc as a negative control. The CNR1 EC-Fc beads did not show significant aggregation, which was similar to that of the Sig-Fc beads. Most of these beads remained unaggregated. In contrast, the E-cad beads showed significant self-aggregation in a Ca^{2+}-dependent fashion, as illustrated by quantitative analysis of the number of beads engaged in aggregates after 2 hr of incubation, respectively (Figure 5B). In the presence of 1 mM calcium, the total aggregation index (Nt/No) was 0.66 ± 0.11 for the CNR1 EC-Fc beads, 0.39 ± 0.03 for the E-cad beads, and 0.70 ± 0.05 for the Sig-Fc beads. In contrast, in the absence of calcium, the total aggregation index was 0.75 ± 0.04 for the CNR1 EC-Fc beads, 0.78 ± 0.04 for the E-cad beads, and 0.83 ± 0.04 for the Sig-Fc beads, respectively. Thus, I could detect the calcium-dependent homophilic binding activity of the E-cad beads, but could not of the CNR1 beads in this experiment. These results suggest that the CNR1 homophilic binding activity was not as strong as the homophilic binding activity of E-cadherin.

Extracellular cell adhesion site of the CNR1 protein

To identify the cell adhesion sites within the extracellular region of the CNR1 protein, a series of deleted recombinant Fc fusion proteins were tested for their cell adhesion activity (Figure 6A and Figure 6C). I first found that CNR1 EC1-Fc (amino acids 1-133, the first cadherin domain of CNR1) could bind to HEK293T cells (Figure 6B, a). Therefore, I used various fusion proteins that were based on differences in Reelin-binding activity (Senzaki et al, 1999). A mutant containing a deletion up to the RGD motif (RGD(+)-Fc, amino acids 1-97) showed binding activity to HEK293T cells (Figure 6B, b), whereas a mutant containing a deletion that included the RGD motif (RGD(-)-Fc, amino acids 1-67) (Figure 6B, c) and Sig.-Fc (amino acids 1-39) did not. These results indicated that sequences critical for the HEK293T-binding domain are located between Ser-68 and Gly-97 of the CNR1 protein. As described by Kohmura et al., this amino acid sequence is completely conserved among CNRs 1 to 8 (Kohmura et al., 1998); therefore, CNRs 1-8 may all have binding activity to HEK293T cells.

Inhibition of the adhesion of HEK293T cells to recombinant CNR1 extracellular domain-Fc fusion protein by the RGDS peptide

To further examine the molecular features of this heterophilic binding activity, I used TE- and TC-treated HEK293T cells. TE-treated cells lost the binding activity to immobilized CNR1 protein. TC-treated HEK293T cells could adhere to the CNR1 EC-Fc, but TE-treated cells could not (Figure 7A and 7B). This suggested that the endogenous CNR1-ligand proteins of HEK293T cells were cleaved by the TE treatment

but not by the TC treatment. It is known that cadherin and integrin proteins have divalent cation-dependent adhesive activity and Ca^{2+}-dependent trypsin resistance (Takeichi et al. 1981; Fujii et al. 1993). I next investigated the properties of these molecules in the cells (Figure 7C and Figure 7D). HEK293T cells were subjected to E, TC, and TE treatment, and the digestion products were analyzed by western blotting with an anti pan-cadherin antibody and an anti β1 integrin antibody. The pan-cadherin immunoreactive band of E-treated HEK293T cells was revealed to be a single band of about 140 kd. The β1 integrin immunoreactive band of the E-treated cells was observed at 130 kd. In the TE-treated cells, the pancadherin and β1 integrin immunoreactive bands were shifted to 60 kd and 100 kd, respectively. However, in the TC-treated cells, the pan-cadherin and β1 integrin immunoreactive bands remained at the same molecular weight as in the E-treated cells. These data suggested that the candidate ligand for CNR1 protein was of the cadherin or integrin family. Of these, I suspected the ligand to be of the integrin family, because L1 cells could bind to immobilized CNR1 EC-Fc protein in the presence of Ca^{2+} (Figure 8A). L1 cells are known to have little cadherin activity (Nose et al. 1988). In addition, an RGD motif is encoded within the EC1 domain of CNR1. The experiments using CNR1 deletion mutants revealed that the sequence including the RGD motif possessed binding activity to HEK293T cells in the presence of Ca^{2+} (Figure 6C). The RGD motif is well known to be a recognition sequence for several integrin family members. Linear peptides modeled on the RGDS tetrapeptide have been shown to inhibit integrin's adhesion to fibronectin or vitronectin (Ruoslahti, 1996).

To determine whether this property extends to CNR1, I tested the effects of the RGDS tetrapeptide on the binding activity of HEK293T cells to CNR1EC-Fc. The RGDS tetrapeptide (10 μM) potently inhibited the binding in a dose-dependent fashion, whereas an RGES control peptide had no effect (Figure 8B). The level of RGDS required to inhibit CNR1 protein binding was similar to the level that inhibited fibronectin binding (Figure 8D). Taken together, these results indicated that an endogenous RGD-sensitive integrin in HEK293T cells was a likely candidate for the heterophilic binding of CNR1 protein.

To confirm the binding activity of the CNR1 protein, I used a peptide consisting of an 11-amino-acid sequence (RGDLLEVNLQN) in the EC1 domain of CNR1. This peptide inhibited the HEK293T cell-binding activity to fibronectin in a dose-dependent fashion (Figure 9A). This result suggested that the CNR sequence could inhibit the adhesion activity of integrin and competitively inhibit the interaction between integrin and fibronectin. This dose-dependent inhibition of attachment was also seen using mouse primary fibroblasts, except that the effective peptide concentration was 10 times higher than that for HEK293T cells (Figure 8C, Figure 8E). Therefore, the CNR1 EC-Fc also bound the mouse RGD-sensitive integrins.

CNR1 and the β1 integrin subfamily are involved in Ca^{2+}-dependent heterophilic interactions

Sixteen alpha and eight beta subunits of integrin have been identified, which form a large number of heterodimeric receptor combinations. Some of the fibronectin

and vitronectin receptors (α5 and α8/β1 integrins and αv/β1, 3, 5, 6, and 8 heterodimers, etc.) are RGDS-peptide sensitive (Hynes, 1992). Several β1 integrin-containing dimers are present on HEK293 cells, but the β3 integrin subunit is not (Caltabiano et al., 1999). Therefore, to examine whether endogenous β1 integrin is involved in the cell adhesion activity of CNR1 protein, I used specific antibodies against β1 integrin. The anti β1 antibodies 3S3 and 6S6 are reactive with the β1 integrin chain and efficiently block integrin's function. Using these antibodies, I confirmed the inhibition of the binding activity of HEK293T cells to fibronectin at a 10 μg/ml dose of antibody (Figure 9B). Interestingly, the adhesion of HEK293T cells to CNR1 EC-Fc protein was significantly inhibited by mAb 3S3 to β1 integrin at an antibody concentration of 10 μg/ml, similar to the level that inhibited fibronectin binding. Overexpressed CNR1 protein and HEK293T endogenous β1 integrin protein appeared to be colocalized at an adhesional junction (Figure 9C). These results strongly demonstrated that CNR1 protein could act as a ligand and/or receptor for integrins. Because conserved amino acids among CNR family members play a role in their binding to integrins, these findings suggest that most of the CNR family proteins interact with integrins at the synaptic junction.

Developmental expression of integrin β1 subunit in mouse brain

Kohmura et al. showed by in situ hybridization that the CNR family is expressed grossly in the forebrain and cerebellum (Kohmura et al. 1998). Furthermore, it has been reported that β1 integrin immunoreactivity is observed in the mouse cerebellar cortex (Murase and Hayashi, 1996). To further investigate the localization of

CNR and β1 integrin in the mouse brain, I performed double labeling immunohistochemistry of the mouse cerebellar cortex using anti-CNR and anti-β1 integrin antibodies. For this study, a monoclonal antibody (4F11) against the N-terminus of CNR1 protein was raised. The specificity of the antibody was confirmed by western blotting using brain lysate and the recombinant CNR proteins overexpressed in HEK293T cells.

The CNR immunoreactivity (CNR-ir) detected with the 4F11 antibody was observed in the molecular layer, Purkinje somata, and glomeruli of the cerebellar cortex. From P7 to P14, the CNR-ir in the molecular layer spread from the bottom to the pial surface. It is known that synapses between the dendrites of Purkinje cells and the parallel fibers of the granule neurons are formed extensively in the molecular layer from P7 to P14. β1 integrin-ir was observed in the molecular layer, Purkinje cell layer, glomeruli, pia mater, and blood vessels. From P7 to P14, the β1 integrin-ir in the molecular layer also spread externally. At P25, integrin-ir was decreased in the molecular layer; however, the signal in the pia mater and blood vessels remained unchanged. CNR-ir was still extensive at P25.

Interestingly, the CNR-ir and β1 integrin immunoreactivity (β1 integrin-ir) overlapped in the molecular layer at P7–P14 during early cerebellar development (Figure 10A). A high-power magnification confocal fluorescence image was recorded from the molecular layer at P14. Both the CNR-ir and β1 integrin-ir were detected as small, intense dots, and most of these dots were colocalized (Figure 10B, a, b, and c).

The spatiotemporal colocalization strongly suggests that the CNR and β1

integrin directly interact in vivo. This observation suggested that molecular interaction and/or adhesion activity between CNRs and integrins play important roles in relatively early synaptogenesis during cerebellar development.

Discussion

Here I present evidence that the CNR1 protein has cell-adhesion activity that is mediated by its interactions with integrins. In particular: 1) CNR1 protein can be transported to the plasma membrane of HEK293T cells. 2) Overexpressed CNR1 protein induces the acquisition of Ca^{2+}-dependent cell-adhesion activity in HEK293T cells. 3) The cell-adhesion activity of the CNR1 extracellular domain is heterophilic and is inhibited by the RGDS tetrapeptide and anti-β1 integrin antibody. 4) Overexpressed CNR1 protein is colocalized with β1 integrin at the adhesional junction of HEK293T cells. 5) The colocalization of CNR1 and β1 integrin is also found in the molecular layer of the cerebellum during early synaptogenesis.

Despite several studies demonstrating that integrins, in particular RGD-sensitive integrins, play significant roles in regulating synaptic maturation and function (Staubli et al., 1998; Chavis and Westbrook, 2001), the integrins' binding partners at the synapse have been unknown. This report, showing the direct interaction and co-localization of CNR and integrin proteins, strongly indicates that CNR family proteins act as binding partners for the integrins at synaptic junctions. This idea is also supported by the previous observations (Kohmura et al., 1998) that the CNR1 protein is localized to synaptic junctions, and that the RGD sequences are conserved in the first cadherin domains of CNR family proteins.

Cell adhesion activity of CNR1

In this study, I first found that HEK293T cells transported a portion of the

CNR1 protein to the plasma membrane. Previous reports have demonstrated that cadherins and protocadherins possess Ca^{2+}-dependent homophilic cell-adhesion activity (for review, Yagi and Takeichi, 2000). This activity has been mainly examined using L1 cells overexpressing cadherins and protocadherins; native L1 cells have no cell-adhesion activity. I first attempted the CNR1 overexpression experiment using L1 cells, but I found that the CNR1 protein is not localized to the plasma membrane in several cell lines, including L1 and Neuro2A. This result indicates that CNR1 protein uses a transport system to reach the plasma membrane that is different from that used by classic cadherins and protocadherins. To overcome this problem, I examined several cell lines for the cell-surface expression of the CNR1 protein.

There is evidence that the polarity of neurons and epithelial cells have common features and that these cell types use similar mechanisms to sort membrane proteins (Dotti and Simons, 1990). My results indicated that the transport of overexpressed CNR1 was generally stopped at the endoplasmic membrane in several cell lines, including MDCKII cells. The origin of MDCKII cells is epithelium, but in these cells CNR1 was localized to the ER and not to either the apical or basolateral plasma membrane (Figure 1). In contrast, I found that a portion of the CNR1 protein was transported to the plasma membrane when it was overexpressed in HEK293T cells (Figure 2). This result suggests that the reason CNR1 protein was stopped at the ER in L1 cells and others was not due to the absence of cell polarity. The difference between L1 and HEK293T cells is not clear; however, it is possible that HEK293T cells have a system for CNR1 plasma membrane transport that other cell types lack.

Therefore, I used HEK293T cells expressing full-length CNR1 and was able to detect Ca^{2+}-dependent cell-adhesion activity mediated by the CNR1 protein. Interestingly, the CNR1 protein has heterophilic adhesion activity for integrins and shows less homophilic than heterophilic activity (Figure 3C, 4B, and 5B).

A major heterophilic adhesion activity has not been reported for classic cadherins and protocadherins. Therefore, CNR family proteins, which possess highly conserved EC1 domains, have different molecular features from classic cadherins and protocadherins. Interestingly, the conserved EC1 domain containing the RGD motif of CNR family proteins has specific molecular features: it binds to Reelin proteins (Senzaki et al., 1999), and shows a high degree of amino-acid replacement in somatic mutations of the *CNR3* transcript (Hirayama et al., 2001). Furthermore, I demonstrated here that the EC1 domain interacts with integrins. Thus, it appears that the function of the CNR-EC1 domain changes during the process of neural differentiation and maturation: it acts as a Reelin receptor at neural layering and positioning, as a binding partner of integrins during synaptogenesis and plasticity, and of diverse proteins in the central nervous system for a variety of later events.

β1 integrin is a ligand for CNR1

The integrin family is a group of heterodimeric adhesion molecules that interact with numerous signaling molecules at their cytoplasmic domain. The extracellular domains of integrin form the ligand-binding sites, which recognize many matrix and membrane proteins. Integrin subunits are expressed in a regionally

differentiated manner in the brain, and some members have been previously shown to be localized to synapses (for review see Benson et al, 2000). Previously, Murase and Hayashi observed the synaptic localization of the most commonly used subunit, β1 integrin, in cerebellar Purkinje cells in adult rat and mouse (Murase and Hayashi 1996), and Grooms et al. reported that the β1 integrin subunit is expressed in the apical dendritic region of the CA1 hippocampus, and occasionally in astrocytes (Grooms et al., 1993). Furthermore, transcripts of β1 integrin increase in an activity-dependent manner (Pinkstaff et al., 1998; Pinkstaff et al., 1999). Nearly half of the over 20 known integrin dimers recognize RGD sequences; therefore, RGD-containing peptides are commonly used to mimic the ligands of these integrins (Ruoslahti, 1996). Several recent studies suggest that integrin-mediated adhesion plays a role in synapse formation. Hippocampal slices exposed to the integrin antagonist GRGDSP peptide showed stabilization of the long-term potentiation (Xiao et al., 1991; Staubli et al., 1998) and in another study, integrin antagonists inhibited synapse maturation (Chavis and Westbrook, 2001). However, despite these functional studies of the integrin side, the most well-known integrin ligands, such as fibronectin and laminin, are sparse in the central nervous system. Thus, the binding partners for the integrins at synapses had not been confirmed. In this study, I propose that CNR proteins work as a binding partner of β1 integrin at synapses.

The cerebellum is one of the best-characterized regions of the brain with respect to development. It undergoes dramatic developmental changes during the first 3 weeks of postnatal life in the mouse. During this period, neuroblasts proliferate in the

external granular layer (EGL) and differentiate into cerebellar granular cells that migrate toward the internal granular layer. This leads to a dramatic increase in the volume of the cerebellum (over 1000-fold) and to the formation of the mature cerebellar structure, including deep fissures and folia. In contrast to the granular cells, Purkinje cells have already stopped proliferating at birth; thereafter (P3-28), they extend their dendrites into the molecular cell layer, where they form synapses with parallel fibers and climbing fibers from granule cells and inferior olivary nucleus cells, respectively (Altman, 1972; (Leclerc et al., 1989; Goldowitz and Hamre, 1998). I observed the co-localization of CNR-ir and β1 integrin-ir in immature synapses before P14 in the mouse cerebellum. Thus, it is possible that interactions between the CNRs and integrins perform temporary functions in brain development, as cell adhesion molecules involved in the initiation of synaptogenesis.

Adhesion molecules might, in turn, directly modulate the properties of glutamate receptor channels, a possibility suggested by the physical association of integrin with NMDA receptors in large, multiprotein complexes (PKC, CAMKII, PP2B, PTK, and PKA). In particular, previous work indicated that CNR is associated with Fyn tyrosine kinase (Kohmura et al., 1998). Fyn has been shown to interact with the NMDA receptor subunit (NR2B) and to regulate its activity (Miyakawa et al., 1997). Furthermore, the integrin cytoplasmic tails interact with a number of proteins (e.g. focal-adhesion kinase, paxillin, integrin-linked kinase), which in turn interact with many classic signaling pathways such as those involving mitogen-activated protein kinase (MAPK), Rho, and PKC. The heterophilic association between CNRs and integrins

proposed here may regulate several signaling pathways and cytoskeletal networks pre- and post-synaptically during synaptic formation and plasticity.

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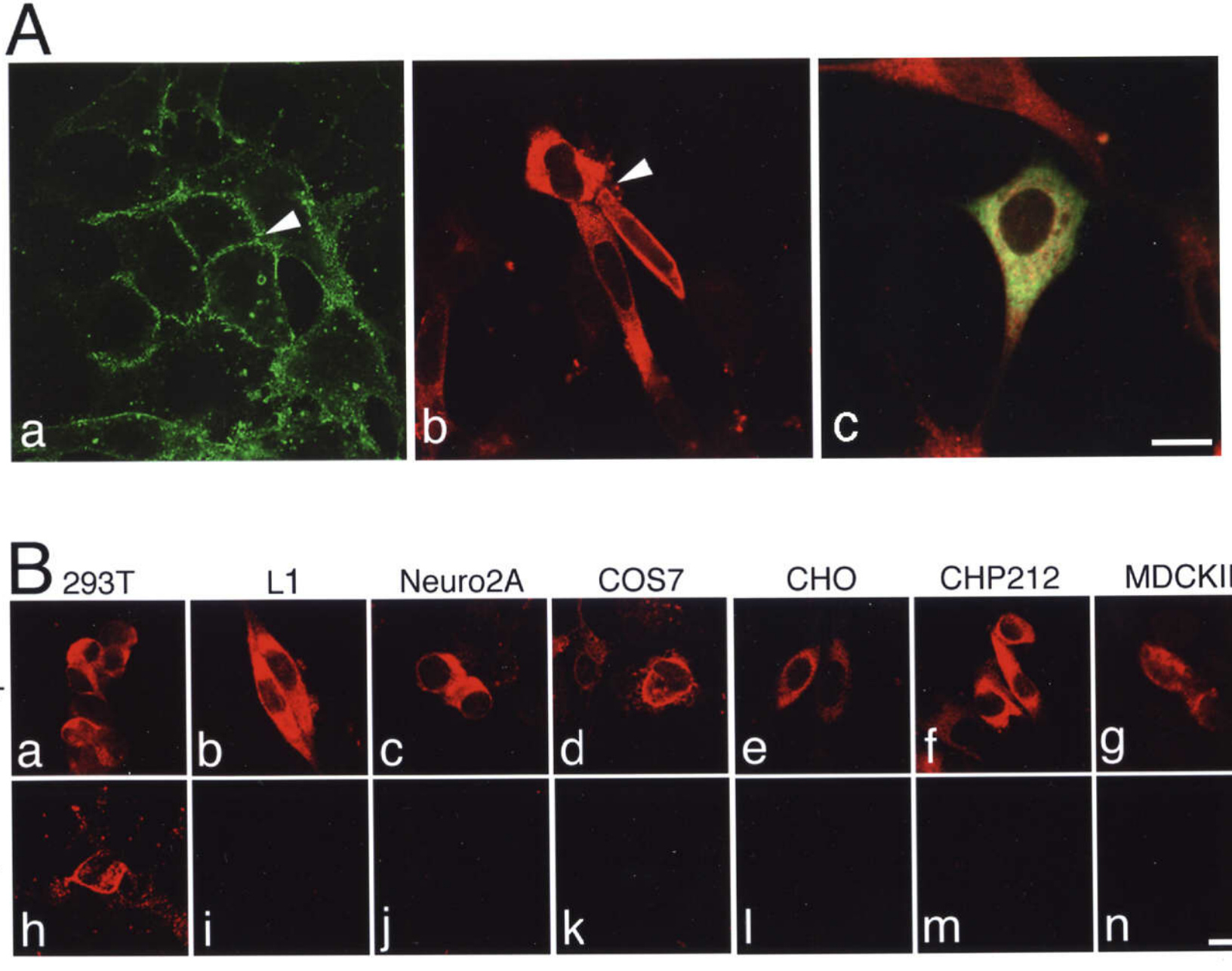
Figure 1


A
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L1
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Figure legends

Figure 1. Subcellular localization of the overexpressed mouse full-length CNR1 (type A).

(A) E-cadherin-transfected L1 cells were stained with anti-E-cadherin (ECCD-2) [a]. CNR1-transfected L1 cells. Anti-CNR antibody (6-1B) was used to detect CNR1 [b]. Arrowheads indicate sites of contact with other cells. Double labeling of CNR1 (Green in [c]) and an endoplasmic reticulum (ER) marker protein (anti-PDI: Red in [c]). The CNR1 and ER marker proteins were co-localized as bright reticular staining throughout the cytoplasm [c]. Scale bar, 25 μm.

(B) CNR1 distribution in seven different cell lines [a-n]. *CNR1* cDNA was transfected into HEK293T (human embryonic kidney) [a and h], L1 (mouse fibroblast) [b and i], Neuro 2A (Rat neuroblastoma) [c and j], COS7 (monkey kidney) [d and k], CHO (Chinese hamster ovary) [e and l], CHP212 (human neuroblastoma) [f and m], and MDCKII (dog kidney) [g and n] cells. These transfectants were fixed and immunostained with 6-1B. To detect all of the overexpressed antigen, cells were stained after permeabilization with Triton X-100 [a–g]. CNR1 protein displayed a vesicular and reticular distribution in each cell line. For antigen detection on the cell surface, the transfectants were immunostained without Triton X-100 [h–n], as described in Materials and Methods. Surface CNR1 on HEK293T transfectants was detected with 6-1B [h]. Scale bar, 50 μm.

Figure 2

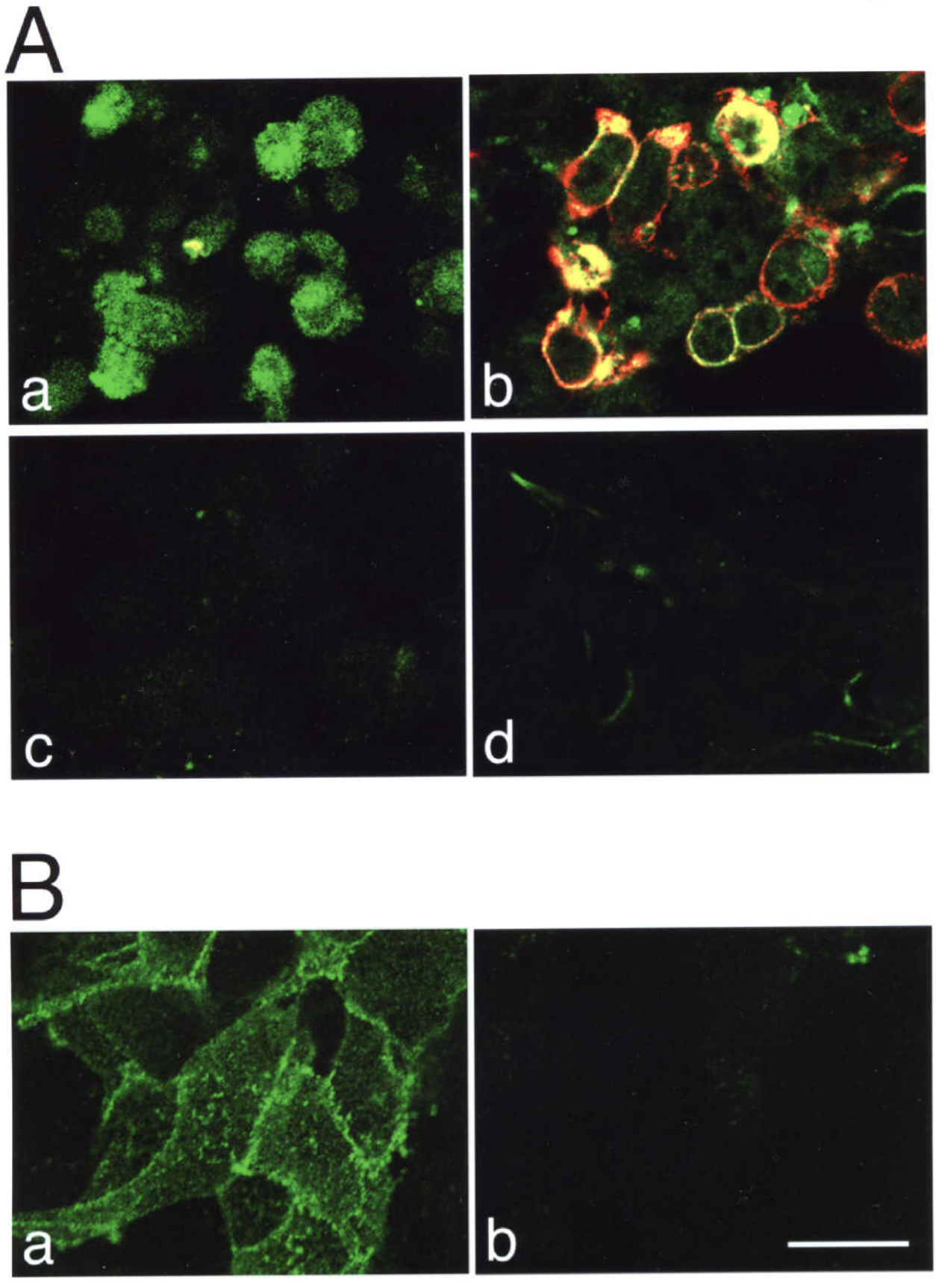
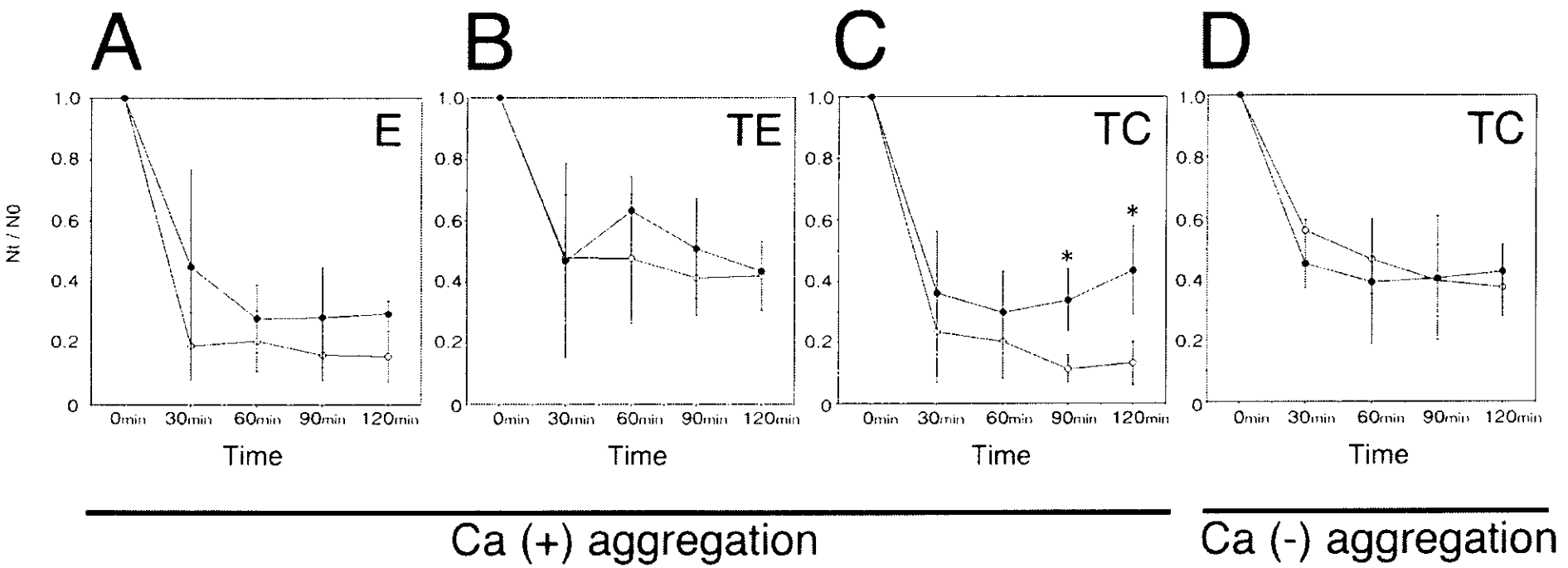


Figure 2. CNR1 was localized to the plasma membrane in HEK293T cells.

(A) Double staining with anti-CNR1 extracellular domain (6-1B: Green) and anti-CNR (type A) C terminal (CNR A: Red) antibodies. CNR1-overexpressing [a and b] and parental [c and d] HEK293T cells were stained alive [a and c] or after permeabilization with EtOH treatment [b and d]. No signal was detected on the membranes of non-transfected samples stained with the same antibody combination [c and d]. Live-stained CNR1-expressing HEK293T cells showed significant staining of the cell surface [a] (the plane of focus in this field is at the surface of the cells).

(B) L1 cells transfected with E-cadherin (a) and CNR1 (b) were also stained live with the ECCD-2 and 6-1B antibodies to specifically label E-cadherin and CNR1 on the surface. E-cadherin was seen on the surface of L1 cells, but CNR1 was not. Scale bar, 50 μm.

Figure 3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D
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Nt / N0
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0.8
0.6
0.4
0.2
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0min
30min
60min
90min
120min
Time
*
*
Ca (+) aggregation
Ca (-) aggregation

Figure 3. Mouse full-length CNR1 (type A)-expressing HEK293T cells (CNR1+ cells) exhibited increased Ca^{2+}-dependent aggregation.

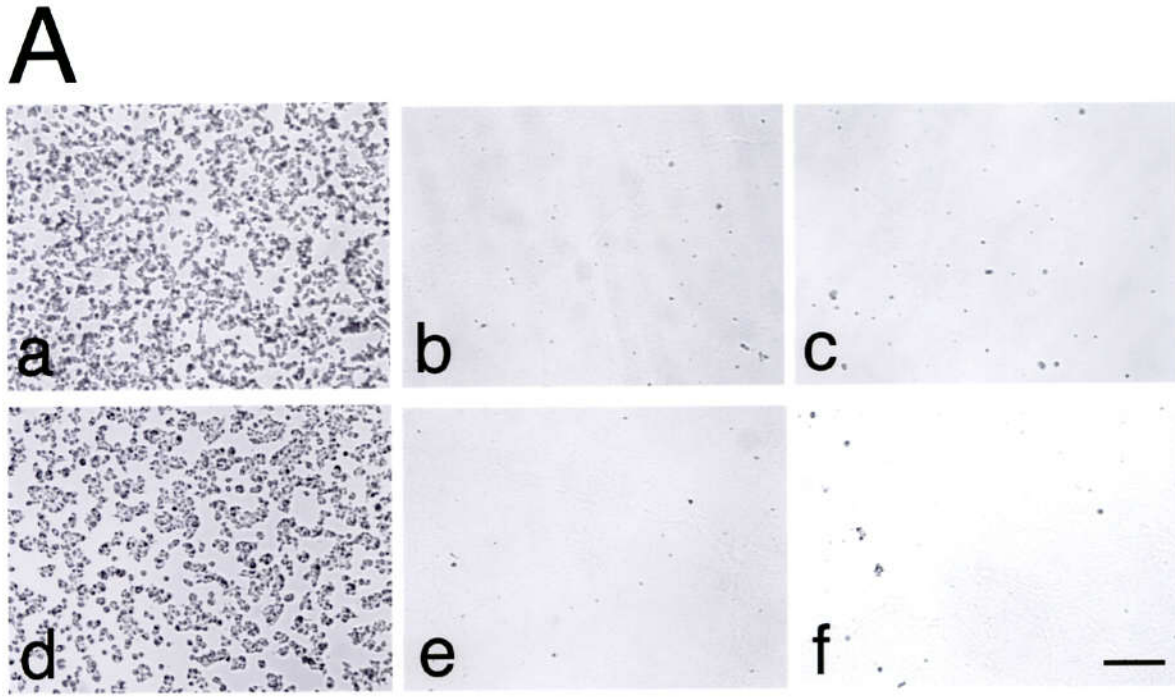
(A) Aggregation of EGTA-treated CNR1+ (open circles; ○) and parental HEK293T (filled circles; ●) cells in the presence of Ca^{2+}. The aggregative property was expressed as a reduction in particle number relative to the number at time zero (Nt/N0) and data represent the means ± standard deviations from three independent experiments.

(B) Aggregation of Trypsin/EGTA-treated CNR+ cells (○) and parental HEK293T cells (●) in the presence of Ca^{2+}.

(C) Trypsin/Ca^{2+}-treated cells were assayed for their aggregation activity in the presence of Ca^{2+}. A significant difference in the aggregative properties between CNR+ cells (○) and parental HEK293T cells (●) was observed. P values calculated by Student's *t*-test comparing the CNR1+ and parental cells are: * $P < 0.05$.

(D) In the absence of Ca^{2+}, no difference in the aggregative property was observed between Trypsin/Ca^{2+}-treated CNR+ (○) and parental HEK293T (●) cells.

Figure 4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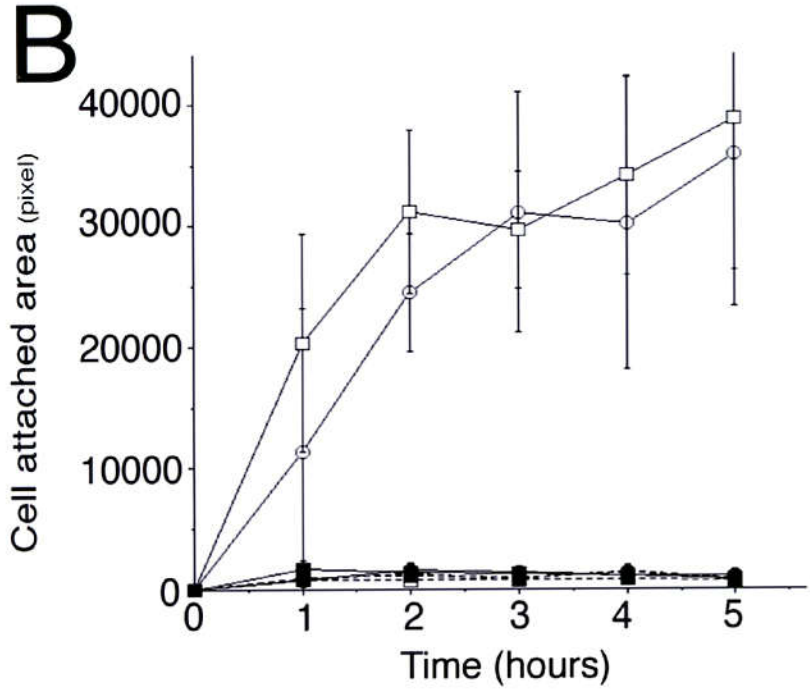
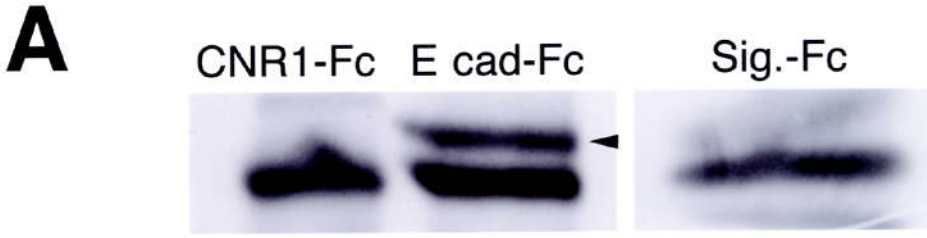

B
Cell attached area (pixel)
40000
30000
20000
10000
0
0
1
2
3
4
5
Time (hours)

Figure 4. Parental HEK293T cells have adhesion activity to the CNR1 EC-Fc fusion proteins.

(A) Fc fusion proteins containing the extracellular domain of CNR1, CNR1 EC-Fc [a, b, d, and e] and the CNR1 signal peptide–Fc fusion protein, Sig.-Fc [c and f] were spotted onto a dish on which anti-Fc antibody was immobilized. The adhesion of CNR1+ cells [a, b, and c] and parental HEK293T cells [d, e, and f] are shown. Cells bound to CNR1 EC-Fc in the presence [a and d], but not in the absence [b and e] of Ca^{2+}. Scale bar, 200 μm.

(B) Kinetics of cell adhesion. Values show the means and standard deviation (S.D.) of the attached cell areas (pixels) from three independent experiments. CNR1 transfectants (circle; ○ and ●) or parental cells (square; □ and ■) were incubated for the indicated times on CNR1 EC full-Fc substrate (empty; ○ and □) or Sig.-Fc (filled; ● and ■)-coated spots. Solid lines indicate the presence of Ca^{2+} and dashed lines indicate the absence of Ca^{2+}. CNR1 transfectants and parental cells adhered to the CNR1 EC-Fc only in the presence of Ca^{2+}.

Figure 5


A
CNR1-Fc
E cad-Fc
Sig.-Fc

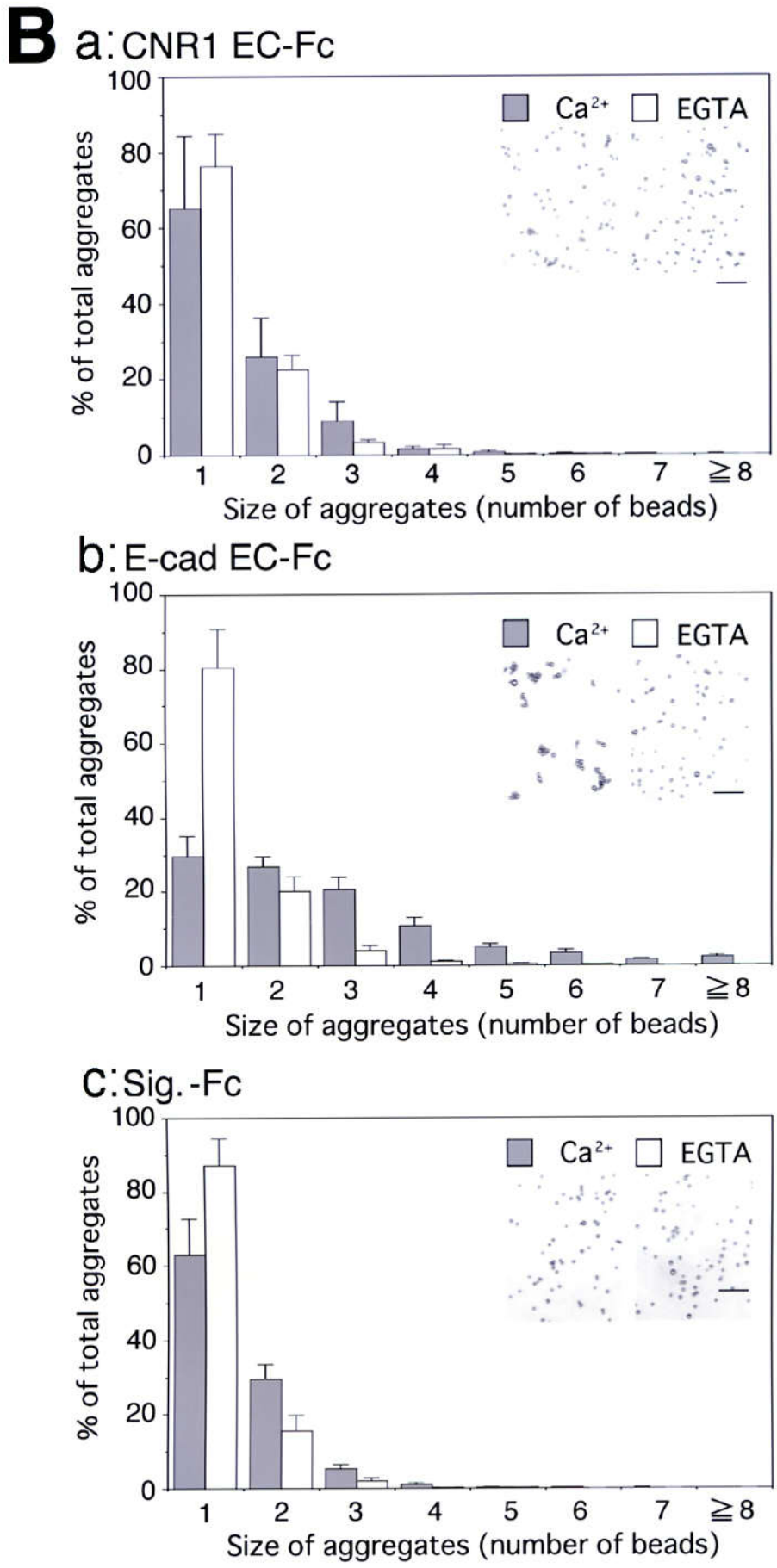
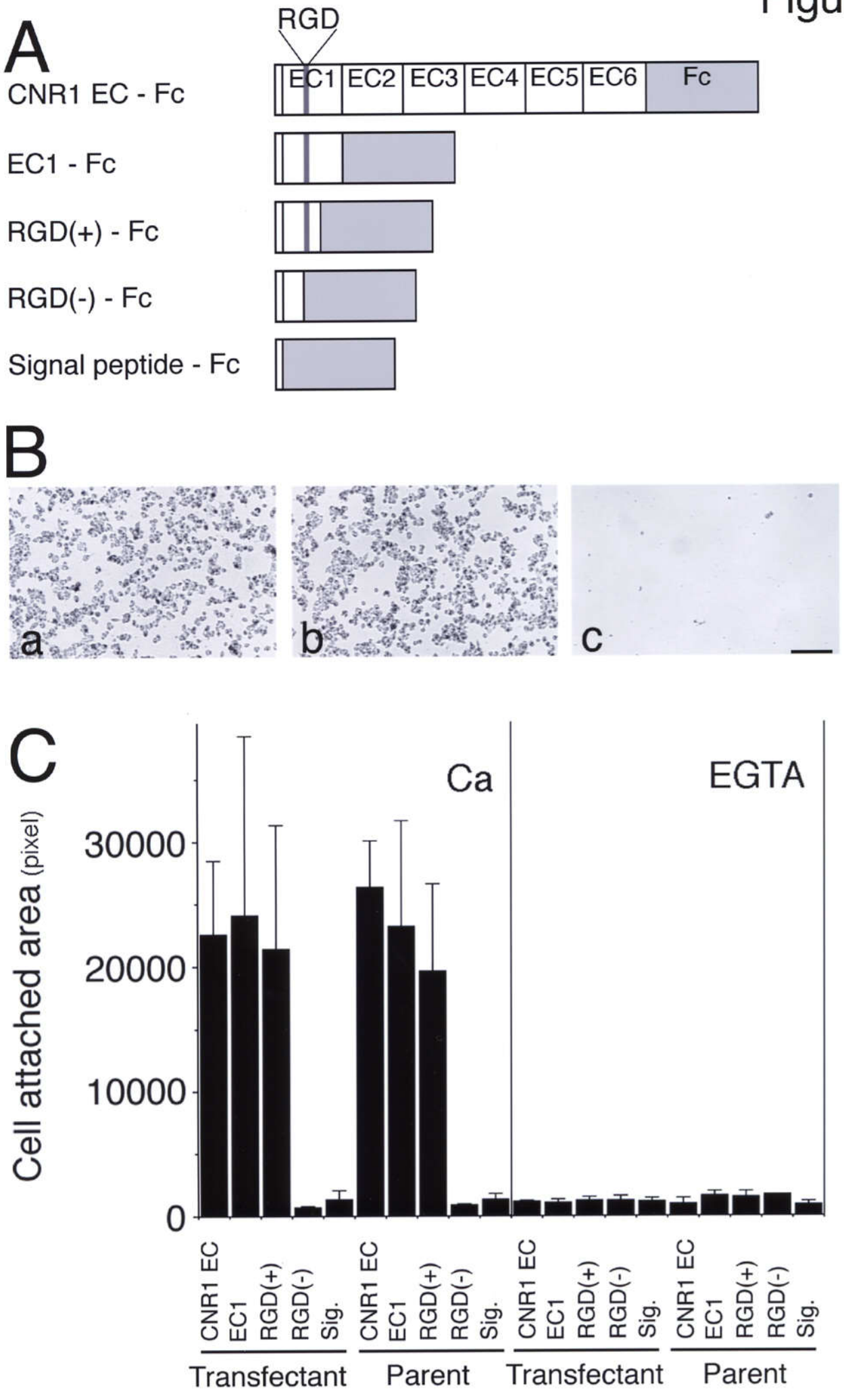

B
a: CNR1 EC-Fc
b: E-cad EC-Fc
c: Sig.-Fc
Ca2+
EGTA
% of total aggregates
Size of aggregates (number of beads)
0
20
40
60
80
100
1
2
3
4
5
6
7
≧8

Figure 5. Specific self-aggregation of Fc-fusion protein beads.

(A) Production of the Fc fusion proteins and beads loaded with each fusion protein. Fifty microliters of beads loaded with Fc fusion proteins were analyzed by anti-human Fc antibody immunoblotting. At the E-cad Fc lane, an arrowhead indicates a weak band running at a higher molecular weight than expected for the E cad-Fc protein, which may correspond to a pre-mature form.

(B) CNR1 EC-Fc (a), E cad–Fc (b) or Sig.-Fc (c) -coated beads were allowed to aggregate for 2 hr in medium containing calcium or EGTA, as described in Materials and Methods. The E-cad-Fc beads were aggregated in the presence of Ca^{2+}, but not in the absence of Ca^{2+} (b). However, the CNR1-Fc beads barely aggregated, despite the presence of Ca^{2+} in the medium (a). The beads were counted and their distribution per size of aggregate was plotted. The values show the mean and S.D. of three independent experiments (beads counted for each condition); each inset shows the photograph of the bead suspension after aggregation. Scale bar, 50 μm. A large number of aggregates of more than 3 beads was detected with the E cad-Fc beads in the presence of 1 mM Ca^{2+}, while the Sig.-Fc beads, CNR1-Fc beads, and E cad-Fc beads in absence of Ca^{2+} mostly remained as single or double particles.

Figure 6


A
RGD
CNR1 EC - Fc
EC1
EC2
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EC4
EC5
EC6
Fc
EC1 - Fc
RGD(+) - Fc
RGD(-) - Fc
Signal peptide - Fc
B
a
b
c
C
Ca
EGTA
Cell attached area (pixel)
30000
20000
10000
0
CNR1 EC
EC1
RGD(+)
RGD(-)
Sig.
Transfectant
Parent
Transfectant
Parent

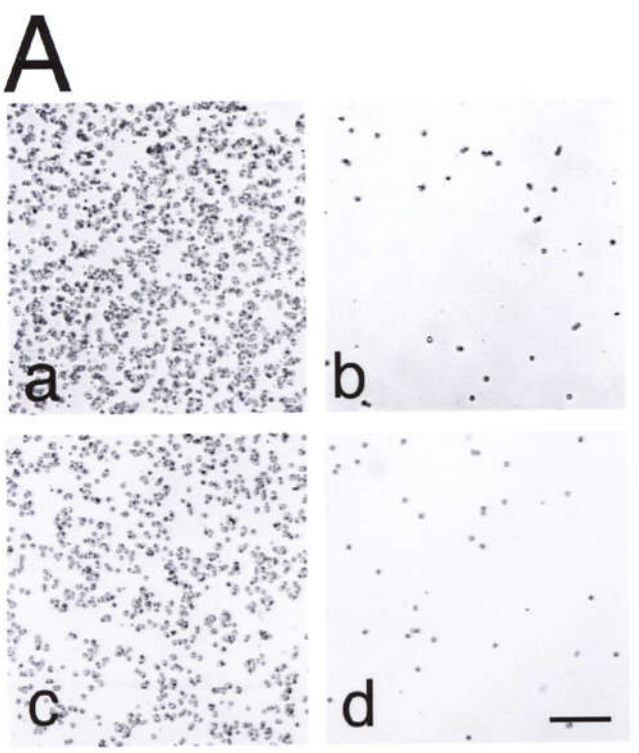
Figure 6. Cell adhesion activity of the series of recombinant CNR1 extracellular domain proteins.

(A) Schematic diagram of the CNR1 extracellular domain–Fc fusion protein variants. The variants included the full-length CNR1 extracellular segment (CNR1 EC-Fc), the EC1 domain of CNR1 (EC1-Fc), the region containing the RGD motif (RGD(+)–Fc), the region lacking the RGD motif (RGD(-)–Fc), and the signal sequence (Sig.-Fc).

(B) The panels show the parental HEK293T cell adhesion of each spot in the presence of Ca^{2+}, the EC1-Fc spot [a], the RGD(+)–Fc spot [b], and the RGD(-)–Fc spot [c], respectively. Scale bar, 200 μm.

(C) Quantitative determination of adhesion activity. The CNR1 transfectants and the parental cells were cultured for two hours on spots containing a series of CNR extracellular domain-Fc fusion proteins, respectively (CNR1 EC, EC1, RGD(+), RGD(-), and Sig.) in the presence of 1 mM $CaCl_2$ or 1 mM EGTA. The attached-cell area was photographed and measured (pixels). The values show the mean and S.D. of three independent experiments.

Figure 7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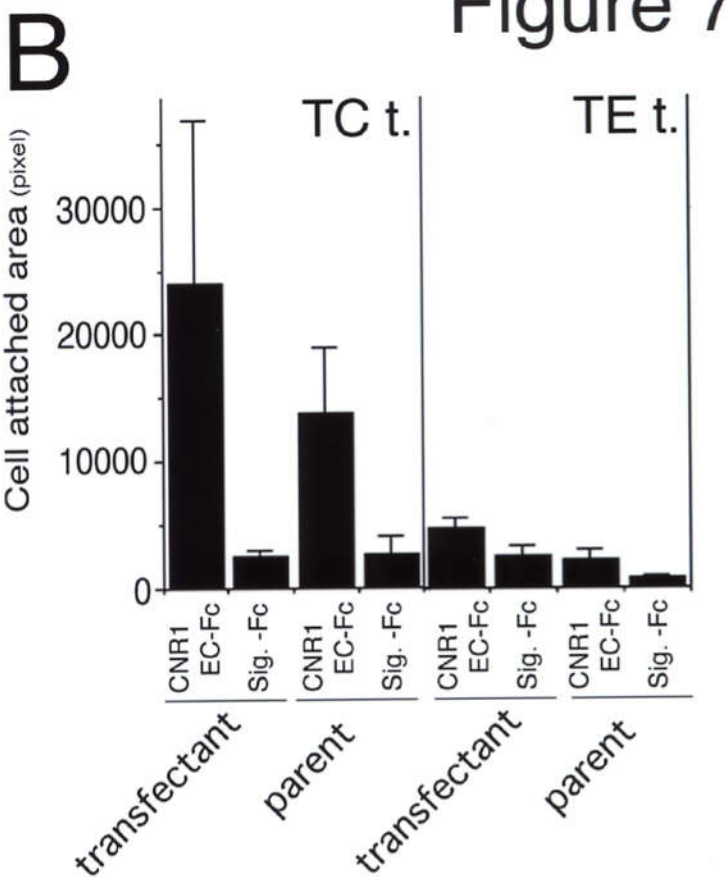
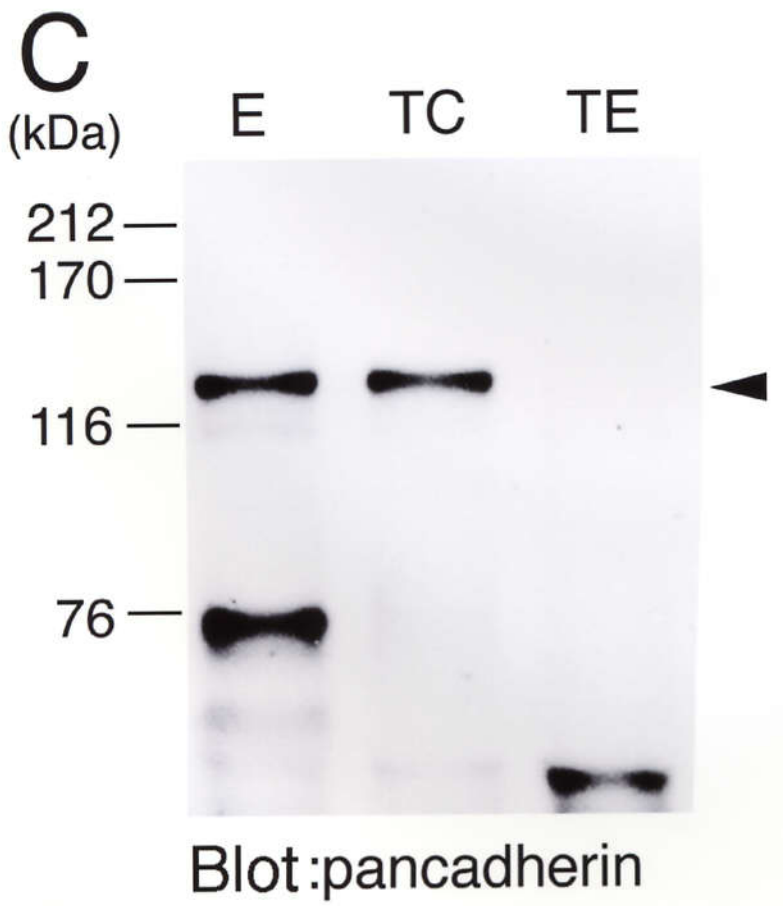
b
c
d


B
TC t.
TE t.
Cell attached area (pixel)
30000
20000
10000
0
CNR1 EC-Fc
Sig. -Fc
transfectant
parent
transfectant
parent


C
(kDa)
E
TC
TE
212
170
116
76
Blot:pancadherin

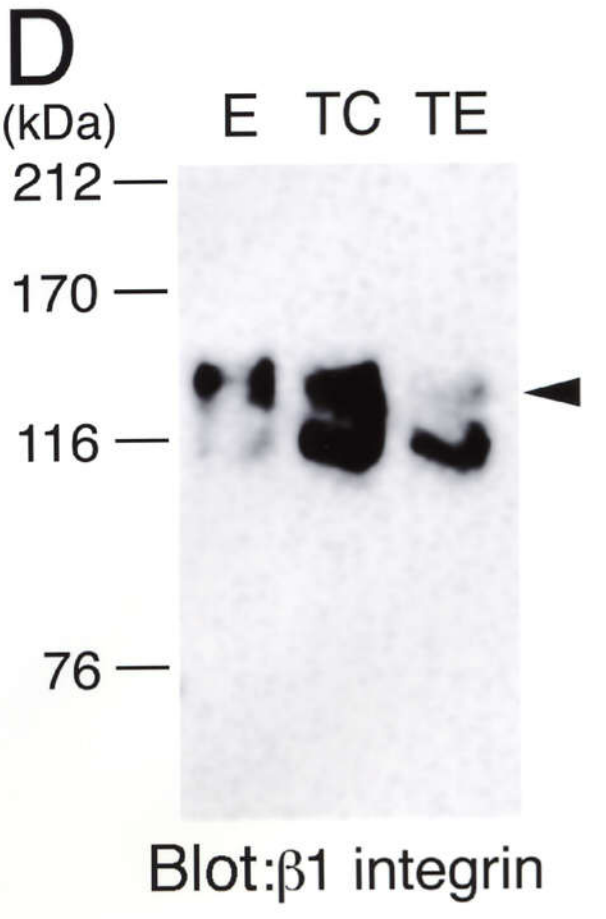

D
(kDa)
E
TC
TE
212
170
116
76
Blot:β1 integrin

Figure 7. Trypsin sensitivity of the adhesion activity of HEK293T cells to CNR1.

(A) The CNR1 transfectants [a and b] and parental HEK293T cells [c and d] were treated with Trypsin/Ca^{2+} (TC) (a and c) and Trypsin/EGTA (TE) [b and d], and cultured for 2 hours on spots containing CNR1 EC-Fc protein, in the presence of 1 mM $CaCl_2$. Scale bar, 200 μm.

(B) The attached-cell area was photographed and measured (pixels). The values show the means and S.D. of three independent experiments. The left side of the graph shows TC-treated samples, and the right side shows TE-treated samples. The CNR1-transfected cells (transfectant) and parental cells (parent) lost their CNR1-binding activity upon TE treatment.

(C) Trypsin sensitivity of endogenous adhesion molecules of HEK293T cells. Classical cadherins were detected with a pan-cadherin antibody. From the left lane, the cells underwent: EGTA treatment (E), Trypsin/Ca^{2+} treatment (TC), and Trypsin/EGTA treatment (TE), respectively. Arrowheads indicate the undigested band sizes.

(D) β1 integrin was detected with an anti-β1−integrin antibody. The band at the original molecular weight of β1 integrin remained in the TC-treated sample.

Figure 8

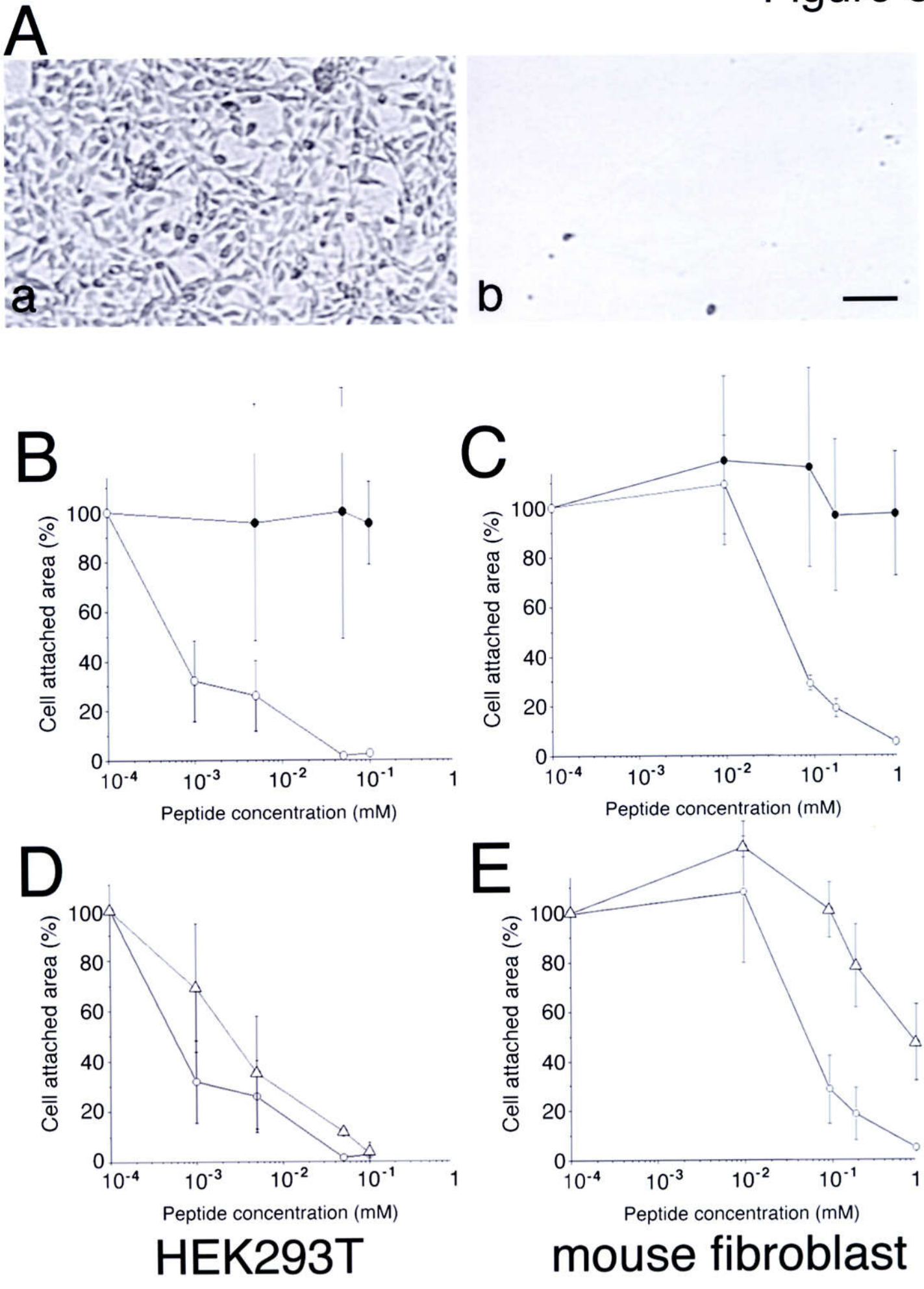

A
a
b
B
C
D
E
Cell attached area (%)
Peptide concentration (mM)
HEK293T
mouse fibroblast

Figure 8. Inhibition of the adhesion activity of HEK293T cells to CNR1 by an integrin antagonist tetrapeptide.

(A) L1 cells have a calcium-dependent adhesion activity to CNR1 EC-Fc. L1 cells adhered to the CNR1 EC-Fc spot [a], but not to Sig.-Fc [b]. Scale bar, 100 μm.

(B) Sequence-dependent inhibition of the cell adhesion to immobilized protein substrates by tetrapeptides. Attachment of HEK293T cells to CNR1 EC-Fc spots was assayed in the presence of increasing concentrations of RGDS (○) or RGES (●) peptide. The percent attachments shown represent the mean (± S.D.) of three observations.

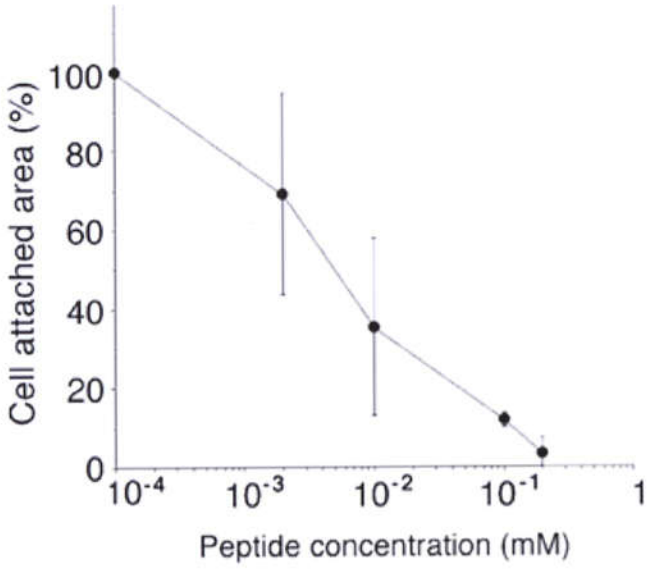
(C) Sequence-dependent inhibition of the cell adhesion to immobilized protein substrates by tetrapeptides. Attachment of mouse fibroblasts to CNR1EC-Fc spots was assayed in the presence of increasing concentrations of RGDS (○) or RGES (●) peptide.

(D) The inhibitory dose of RGDS for attachment of HEK293T cells to CNR1 EC-Fc spots (○) was similar to that for fibronectin spots (△).

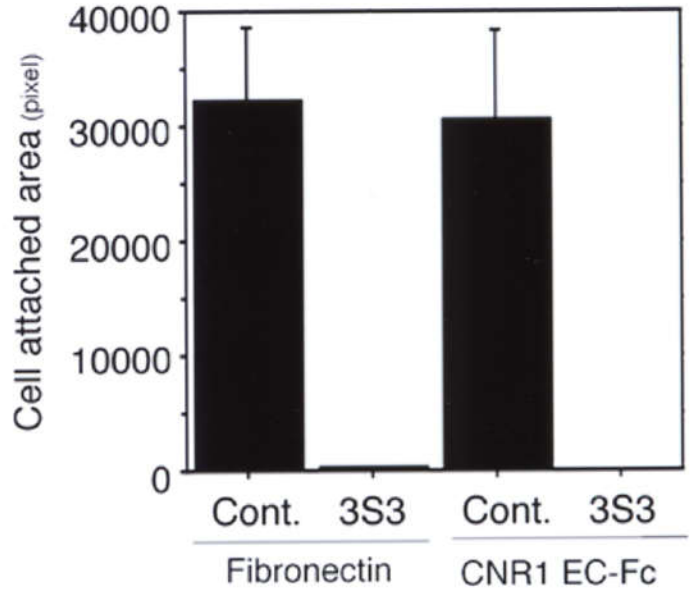
(E) The inhibitory dose of RGDS for attachment of mouse fibroblasts to CNR1 EC-Fc spots (○) was similar to that for fibronectin spots (△).

Figure 9

A


Cell attached area (%)
100
80
60
40
20
0
10-4
10-3
10-2
10-1
1
Peptide concentration (mM)

B


Cell attached area (pixel)
40000
30000
20000
10000
0
Cont.
3S3
Cont.
3S3
Fibronectin
CNR1 EC-Fc

C

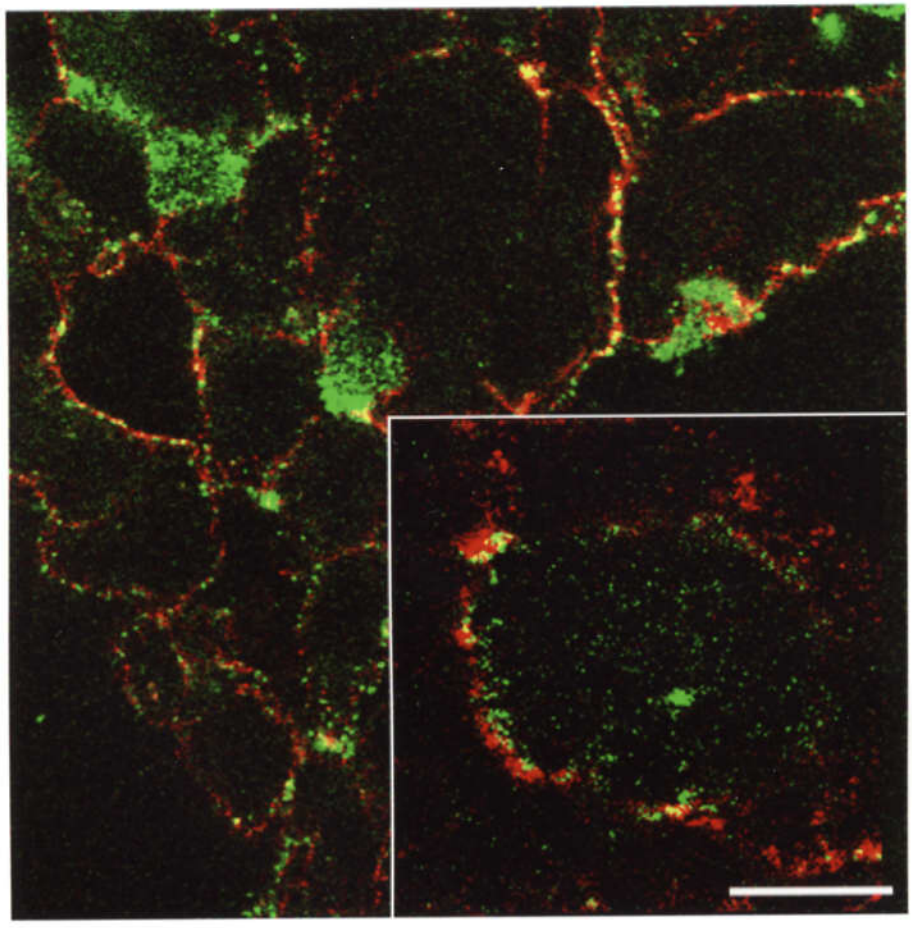


Figure 9. Evidence of protein interaction between β1 integrin and CNR1.

(A) Effect of a peptide containing the sequence around the RGDS motif (RGDLLEVNLQN) of the CNR1 EC1 domain on cell attachment to a fibronectin spot. The percent attachments shown represent the mean (± S.D.) of three observations.

(B) The 3S3 mAb, a β1 integrin-function-blocking antibody (10 μg/ml) altered HEK293T cell adhesion to fibronectin and CNR1 EC-Fc. In contrast, the attachment of HEK293T cells to CNR1 EC-Fc was not affected by the same concentration of control mouse IgG.

(C) Confocal images of an adhesional junction of the HEK293T cells, which overexpress CNR1, live-stained CNR1, and β1 integrin protein. Immunostaining for CNR1 and β1 integrin was localized to the adhesive junction; insets show a cell at greater magnification. Scale bar, 10 μm.

Figure 10

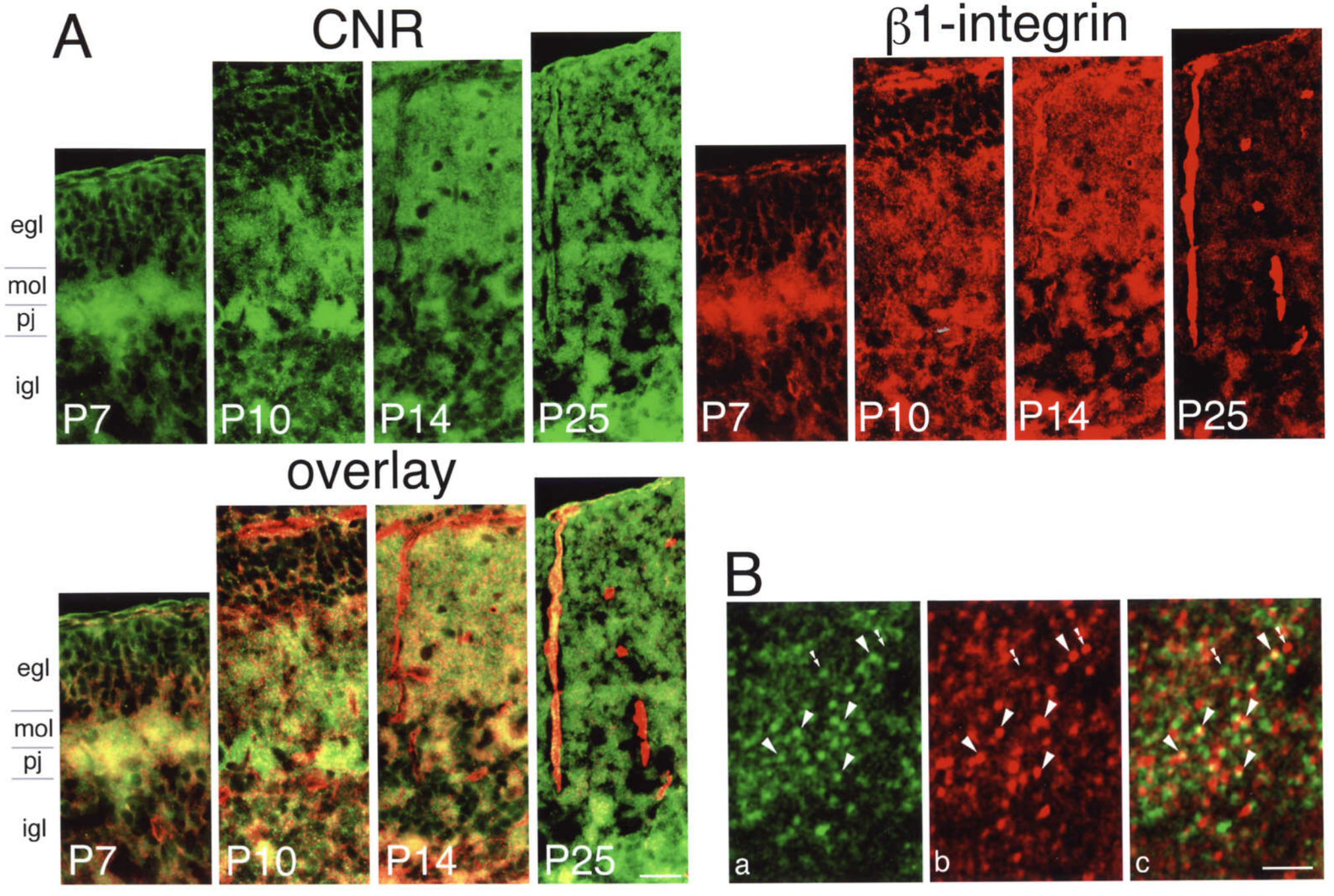

A
CNR
β1-integrin
egl
mol
pj
igl
P7
P10
P14
P25
P7
P10
P14
P25
overlay
egl
mol
pj
igl
P7
P10
P14
P25
B
a
b
c

Figure 10. Immunohistochemistry of postnatal mouse cerebellar cortex.

(A) CNR (Green) and β1 integrin (Red) expression in coronal sections of the mouse cerebellar cortex at P7–P25. Cerebellar layers (egl: extra granule layer, mol: molecular layer, pj: Purkinje layer, igl: inner granule layer) are indicated at the left side of the series of panels. Scale bar, 20 μm.

(B) Confocal microscope image showing the double labeling for CNR [a] and integrin [b] and the overlay image [c] in the molecular layer, in 5-μm sections from the p14 mouse cerebellar cortex. Double-labeled dots are indicated in yellow (single arrow). Red or green dominant dots were also observed (double arrow). Scale bar, 5 μm.

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