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CRTAM: A Molecule Involved in Epithelial Cell Adhesion

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ABSTRACT

*C*lass I-*r*estricted *T* cell *a*ssociated *m*olecule (CRTAM) is a member of the immunoglobulin superfamily that complies with the structural characteristics of the JAM family of proteins and is phylogenetically more closely related to nectin-like proteins. Here we demonstrate for the first time, that CRTAM is expressed in epithelial cells along the lateral membrane and is important for early cell–cell contacts and cell–substrate interactions. CRTAM is sensitive to intermediate filament disruption and treatment of monolayers with soluble CRTAM enhances cell–cell dissociation and lowers transepithelial electrical resistance. Incubation of newly plated cells with anti-CRTAM antibody decreases the formation of cell aggregates and promotes cell detachment. Co-cultures of epithelial cells and fibroblasts that lack CRTAM expression and in vitro binding assays, demonstrate the participation of CRTAM in homotypic and heterotypic *trans*-interactions. Hence we conclude that CRTAM is a molecule involved in epithelial cell adhesion. J. Cell. Biochem. 111: 111–122, 2010.

KEY WORDS: CRTAM; JAM; CELL ADHESION; TIGHT JUNCTION; EPITHELIA

The *c*lass I-*r*estricted *T* cell *a*ssociated *m*olecule (CRTAM) received its name for its restricted expression pattern in T cells [Kennedy et al., 2000]. This protein was originally found to be highly expressed in activated $CD8^+$ T and NKT cells which are class I major histocompatibility complex (MHC) restricted [Kennedy et al., 2000].

More recently however, CRTAM expression has been detected in natural killer (NK) cells and in a minor population of activated CD4 T cells [Arase et al., 2005; Yeh et al., 2008].

In mouse, CRTAM mRNA is detectable in spleen, brain and testis, and in humans is present in spleen, thymus, intestine, lymph nodes, lung, testis, ovary and colon [Kennedy et al., 2000]. In human CNS, CRTAM mRNA is strongly expressed in cerebellum and immunocytochemistry studies have revealed that the protein is mainly localized to Purkinje neurons and granule cells [Patino-Lopez et al., 2006].

CRTAM belongs to the immunoglobulin superfamily (Ig-SF) due to the presence of two Ig-like domains at the extracellular region [Kennedy et al., 2000]. CRTAM exhibits at its carboxyl terminal end the class I PDZ binding motif ESIV [Arase et al., 2005; Patino-Lopez et al., 2006].

Previously we and others suggested a relationship between CRTAM and the nectin-like (necl) family of proteins [Boles et al., 2005; Patino-Lopez et al., 2006], although CRTAM shows below 20% amino acid identity to necl proteins. Nectins and necls are Ca^{2+}-independent cell adhesion molecules with three extracellular Ig-like domains, a single transmembrane region, and a PDZ binding motif at their carboxyl terminal ends which allows nectins but not necl proteins to bind afadin [Ogita and Takai, 2006].

The fact that CRTAM has two and not three Ig-like domains, prompted us to revisit our previous analysis and to explore the relationship of CRTAM to the JAM protein family.

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JAMs are proteins of the Ig-SF that exhibit two Ig-like domains with intramolecular disulfide bonds and two or more *N*-glycosylation sites in their extracellular domain. JAMs A, B and C exhibit a short cytoplasmic tail of 45–50 residues that ends with a type II PDZ binding motif, while others like CAR, ESAM, JAM4, and CLMP have longer tails of 105–165 amino acids ending in type I PDZ binding motifs [Hirabayashi and Hata, 2006].

JAMs are present at the intercellular junctions and lateral membrane of epithelial and endothelial cells as well as on the surface of leukocytes [Cunningham et al., 2000; Ostermann et al., 2002] and platelets [Kornecki et al., 1990; Santoso et al., 2002]. JAMs appear to be involved in tethering specific proteins to the tight junction (TJ) [Bazzoni et al., 2000; Ebnet et al., 2001; Itoh et al., 2001; Martinez-Estrada et al., 2001]. Besides their role in TJ assembly, JAMs participate in leukocyte transmigration [Martin-Padura et al., 1998; Johnson-Leger et al., 2002; Zen et al., 2004], platelet activation [Sobocka et al., 2000; Santoso et al., 2002], angiogenesis [Naik et al., 2003] and virus binding [Barton et al., 2001; Forrest et al., 2003].

Here we have analyzed the relationship between CRTAM, JAMs, nectin and necl proteins, studied the expression of CRTAM in epithelial cells and tested its role in epithelial cell–cell adhesion.

MATERIALS AND METHODS

BIOINFORMATICS

Multiple sequence alignments were performed using the T-Coffee method based on ClustalW pairwise pre-alignment approach with progressive optimization of the alignments using Geneious Software. Amino acid identities of sequences were determined after their pairwise alignment using CrustalW with the Gonnet protein weight matrix and settings of a gap open penalty of 10, a gap extension penalty of 0.1 and no gap separation penalty. Phylogenetic analysis and their plot as cladogram-like unrooted trees were conducted with PHYML method [Guindon and Gascuel, 2003] based on multiple bootstrapped data sets using standard settings.

CELL CULTURE

Epithelial MDCK cells (Madin Darby canine kidney cells) between the 60th and 90th passage were grown as previously described [Islas et al., 2002].

Total peripheral blood mononuclear cells (PBMCs), obtained from healthy donors at the Hospital Central Norte de Pemex blood bank, were isolated from blood samples using Ficoll-Paque Plus according to the manufacturer's protocol (Amersham, Bioscience, AB Uppsala, Sweden). Human CD8 T cells were purified from PBMCs thorough negative selection using magnetic beads (Miltenyi Biotec, Auburn, CA, Cat. No. 130-091-154). PBMCs or $CD8^+$ T cells were activated with PMA (2 ng/ml) and Ionomycin (200 ng/ml) as previously described [Patino-Lopez et al., 2006].

Chinese hamster ovary fibroblasts (CHO) obtained from the American Type Culture Collection, were cultured in a mixture of F12/DMEM. Cells were harvested with trypsin–EDTA and plated on dishes with or without coverslips. For the co-culture, CHO cells were pre-labeled with 10 μM 5-(and 6-)[(4-chloromethyl)benzoyl] amino-tetramethylrhodamine (C-2927, CellTracker Orange CMTMR; Molecular Probes, Eugene, OR) as described by the manufacturer.

For the co-culture, instead of doing a mixture of CHO and MDCK cell suspensions, CHO cells were first plated at sparse density on glass coverslips and 1 h later the MDCK suspension was plated at sparse density on the same coverslips. This procedure was followed in order to guarantee the presence of both types of cells in the culture, since MDCK cells adhere much faster to the glasscoverslips than CHO cells. Two days after plating the cultures were processed for immunofluoresence.

RT-PCR

Total RNA was isolated from MDCK cells using the Trizol method (Invitrogen. Cat. No. 15596). To measure CRTAM mRNA a semiquantitative RT-PCR was performed using The One Step System (Invitrogen). One microgram of total RNA was used in each analysis. RT-PCR amplification conditions for were as follows: 60 min at 55°C (RT), 5 min at 94°C, 30 cycles of amplification 30 s at 94°C, 30 s at 60°C, finally 30 s at 72°C for a final extension. Primers used for amplification were the following: CRTAM forward 5′-CCAAATACCAGCTTCTTCACCA-3′ and CRTAM reverse 5′-CTTCAAATCGGAAAGGTGCT-3′.

CELLULAR LYSATES AND PROTEIN BLOTTING

MDCK and CHO cells as well as pellets from resting or PMA/Ionomycin activated $CD8^+$ T cells were lysed with RIPA buffer as previously described [Hernandez et al., 2007]. Blotting was performed with the following antibodies—(1) Monoclonals: mouse IgG anti-human CRTAM (Catalog No. MAB16951, R&D Systems, Minneapolis, MN; dilution 1:200); rat IgG anti-E-cadherin (DECMA-1; Sigma U-3254, dilution 1:500); rat IgG against ZO-1 (R26.4C, Developmental Studies Hybridoma bank, University of Iowa, IA, USA, dilution 1:500); mouse IgG anti-actin (generously provided by Dr. Jose Manuel Hernandez from the Department of Cell Biology, at Cinvestav, Mexico, dilution 1:500). (2) Polyclonals: rabbit IgG against claudin-1 (Invitrogen 51-9000, dilution 1:1500); rabbit IgG anti-occludin (Invitrogen 71-1500; dilution 1:1,000); and goat IgG anti-JAM-A (R&D Systems AF1077, dilution 1:200).

As secondary antibodies the following peroxidase-conjugated antibodies were employed: goat IgG against mouse (Catalog No. 62-6520; dilution 1:3,000; Zymed Laboratories, San Francisco, CA); rabbit IgG anti-goat (Invitrogen 61-1620, dilution 1:3,000); goat IgG against rabbit (Sigma-Aldrich A9169, dilution 1:10,000). Then the ECL+Plus chemiluminescence detection Kit (GE Healthcare, Buckinghamshire, UK) was employed.

IMMUNOFLUORESCENCE

Monolayers grown on glass coverslips were fixed and processed as previously described [Hernandez et al., 2007]. The monolayers were incubated for 2-h with a rabbit polyclonal antiserum against the human and mouse CRTAM antigenic peptide $_{257}$DKEEKE$_{262}$ (dilution 1:10 in 0.5% of BSA), or else incubated with the anti-CRTAM polyclonal anti-serum pre-absorbed for 30 min with 5 μg/ml of antigenic peptide, or incubated with any of the following antibodies: (1) a rat monoclonal anti-E-cadherin (dilution 1:50 in 0.5% of BSA); (2) a mouse monoclonal anti-Desmoplakin (dilution

1:50 in 0.5% of BSA); (3) a mouse monoclonal anti-occludin (Zymed 33-1500, dilution 1:50); (4) a rat monoclonal against ZO-1 (dilution 1:50); (5) a goat polyclonal anti-JAM-A (dilution 1:20), (6) a rabbit polyclonal anti-occludin (dilution 1:50); and (7) a mouse monoclonal anti-cytokeratin-8 (1238-817; Boehringer, Mannheim, Germany; dilution 1:10).

As secondary antibodies we employed FITC-conjugated goat anti-rabbit IgG (Zymed 62-6111; dilution 1:100) and goat anti-rat IgG (Zymed 62-9511; dilution 1:100), a TRITC conjugated goat anti-mouse IgG (Zymed T2762; dilution 1:100) and a goat anti-rabbit IgG (Zymed 81-6114; dilution 1:100); CY5 conjugated goat anti-rat (Zymed 629516; dilution 1:75) and goat anti-mouse (Zymed 81-6516; dilution 1:75), an Alexa Fluor 488 donkey anti-goat (Molecular Probes A-11055; dilution 1:200), and Alexa Fluor 568 donkey anti-rabbit (Molecular Probes A-11042; dilution 1:200).

The fluorescence of the monolayers was examined using a confocal microscope (Leica SP2) with argon and helium-neon lasers and employing the Leica confocal software.

Immunofluorescence of rat kidney frozen sections was done as previously described [Gonzalez-Mariscal et al., 2000]. Rabbit pre-immune serum was employed for a negative control.

ACTIN AND INTERMEDIATE FILAMENTS DISRUPTION

Confluent monolayers grown on glass coverslips were washed twice with PBS and incubated: (A) for 2-h at 37°C with culture medium containing 5 mM acrylamide, previously prepared as a 100 mM stock in H_2O; or (B) for 1-h at 37°C with cytochalasin B (Sigma, Cat. No. C6762) in Hepes buffer saline with glucose (HBSG): 10 mM Hepes, pH 7.4, 5.4 mM KCl, 137 mM NaCl, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5.6 mM glucose. Cytochalasin B was prepared as a 5 μg/μl stock in DMSO and added to HBSG buffer to a final concentration of 5 μg/ml. DMSO final concentration in medium (0.1%) had no effect in control experiments. Monolayers were then fixed and processed for immunofluorescence.

IMMUNOELECTRON MICROSCOPY

For the localization of CRTAM on confluent MDCK cells, monolayers were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde and dehydrated in graded ethanols. The monolayers were detached from the culture dish by cutting the border with a knife, and then carefully lifting the monolayer from the Transwell insert by hand with the help of a razor blade. The samples were next embedded in LR White resin (Polysciences Inc., Warrington, PA; Cat. No. 17411) as indicated by the manufacturer. Thin sections of 60 nm were cut with a diamond knife and picked upon 200-mesh nickel grids coated with formvar films. Blockade of unspecific sites was done by first floating these grids for 1 h in 6% pig serum. Next the grids were incubated overnight in drops of mouse monoclonal anti-human CRTAM antibody (dilution 1:2). Grids were then washed 5 times in PBS, blocked again with 6% pig serum and incubated for 1 h in 20 nm gold conjugate coupled to goat anti-mouse IgG (Ted Pella Redding, CA; Cat. No. 15753; dilution 1:20 in 3% pig serum). Thin sections were contrasted with uranyl acetate and lead citrate and observed in a Jeol JEM-1011 electron microscope.

CALCIUM SWITCH ASSAY

This protocol was done as previously described by us [Gonzalez-Mariscal et al., 1985]. Briefly, trypsinized cells are plated at confluent density (5×10^5 cells/cm^2) in the presence of Ca^{2+} and with trypsin inhibitor. One hour later, when the cells have adhered to the substrate, the culture is gently washed 3 times with PBS without Ca^{2+} and left for 20 hr in low calcium media (1–5 μM Ca^{2+}). Then the monolayers are transferred to normal calcium medium (1.8 mM Ca^{2+}) for different periods of time in the absence (Supplemental Fig. 5) or presence of: 500 μg/ml of CRTAM-Fc and BSA (Figs. 6B and 7), or 2 mM of E-cadherin CAR peptide SHAVAS and scramble peptide AAHSSV (Supplemental Fig. 6).

PEPTIDE SYNTHESIS AND PURIFICATION

Peptides Ac-SHAVAS-NH_2 and Ac-AAHSSV-NH_2 with acetylated amine termini were synthesized employing standard Fmoc/tBu amino acid chemistry, using 0.1 mmol Rink Amide MBHA resin (100–200 mesh, substitution: 0.56 mmol/g). Protected amino acids were incorporated using *N*′,*N*-diisopropylcabodiimide/1-hydroxybenzotriazole as coupling agent. Fmoc group removal was performed using 37% piperidine in *N*,*N*-dimethylformamide with 0.07% triton® X-100. Amine termini was acetylated by treatment with 15% acetic anhydride, and *N*,*N*-diisopropylethylamine in *N*,*N*-dimethylformamide(2:1:16, v/v/v). Subsequent side chain protecting group removal and cleavage was performed by slow addition of 3 ml of a solution of trifluoroacetic acid, water and triisopropylsilane (94:4:2, v/v/v) for 1 h. The crude peptide was then precipitated with diethyl ether. The crude lyophilized peptides were purified by reverse phase HPLC using a Waters SymmetryPrep®, C18, 7 μm, 7.8 mm × 300 mm column, with a gradient of solvent system A (0.1% trifluoroacetic acid in H_2O) and solvent B (0.1% trifluoroacetic acid in acetonitrile). The final products were characterized by HPLC and Electrospray mass spectrometry. Mass spectra were obtained in a Agilent Accurate-Mass Time of Flight (TOF) MS spectrometer and the chromatograms were acquired using a Waters Symmetry300® column (C18, 5 μm, 4.6 mm × 150 mm column) with a gradient (0–100% B, 15 min, flow: 1 ml/min) of solvent A (0.05% trifluoroacetic acid in H_2O) and solvent B (0.01% trifluoroacetic acid in acetonitrile).

CELL AGGREGATION ASSAY

MDCK cells were trypsinized and sparsely plated (6.8×10^4 cells/cm^2) on glass coverslips. After 1-h incubation in CDMEM, non-adherent cells were removed by rinsing with fresh medium. Next the monolayers were incubated at 37°C with CDMEM alone or containing antibodies against CRTAM (dilution 1:10), or E-cadherin (dilution 1:50). The medium was removed 5-h later and the monolayers were fixed for 30 min with 2% *p*-formaldehyde. Monolayers were observed with a 20× objective in a light microscope (Nikon Eclipse E600, Japan) and photographed with a digital camera (Nikon Coolpix, Japan). The number of isolated cells and of cells forming aggregates was counted in photographs taken from seven different fields of the coverslip of each experimental condition. Two independent experiments were done.

PREPARATION OF RECOMBINANT PROTEINS

The CRTAM-glutathione transferase (GST) construct was previously described by us [Patino-Lopez et al., 2006]. This construct was used to transform competent *Escherichia coli* (Cat. No. 230196, Arctic Express RP competent cells; Stratagene, La Jolla, CA). Protein expression was induced for 24 h at 10°C with 0.5 mM isopropyl β-D-tiogalactoside. Fusion proteins were purified by standard methods.

Soluble recombinant proteins were generated by fusing the extracellular domain of hCRTAM or Nectin-like2 protein to the Fc domain of human IgG_1 through linkage by the thrombin recognition site, employing methods recently described by us [Medina-Contreras et al., 2010]. Ig fusion constructs were transfected by electroporation in Jurkat cells. Stable transfected cells were selected with G-418 (Invitrogen). CRTAM-Fc and Fc-Nectin-like2 proteins were purified from culture supernatants by protein G sepharose (Invitrogen).

INHIBITION OF TIGHT JUNCTION FORMATION WITH SOLUBLE CRTAM

Recombinant protein CRTAM-Fc was used to compete CRTAM mediated cell–cell adhesion in the following assays: In the first, done as previously described [Cereijido et al., 1981], confluent monolayers cultured on Transwell inserts were treated for 19 min at 37°C with 2.4 mM EGTA. Next the monolayers were transferred to normal calcium containing medium (NC; 1.8 mM $CaCl_2$) or NC medium with 500 μg/ml of CRTAM-Fc. TER was measured before and after EGTA addition and at different time points after the transfer to NC medium. In the second named Ca^{2+} switch assay [Gonzalez-Mariscal et al., 1985], after 20-h of incubation in LC medium, the monolayers were next cultured for 6-h in NC medium alone or containing 500 μg/ml of soluble CRTAM. TER was measured at different time points, with the EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL).

DISPASE-BASED DISSOCIATION ASSAY

This assay previously reported [Huen et al., 2002], was done on MDCK cells undergoing a Ca^{2+}-switch experiment. Briefly, after 20-h of incubation in LC medium, the monolayers were transferred for 6-h to normal calcium medium in the presence of 500 μg/ml of CRTAM-Fc or BSA. Next 2.4 U/ml of dispase II (Roche 11276921001) were employed for 1 h and 50 min at 37°C to release the monolayers from the Transwell inserts. The liberated monolayers were then carefully transferred with a P100 pipette tip cut at the tip to a 12-well dish containing PBS with 1 mM $CaCl_2$. The monolayers were then subjected to agitation at 300 rpm for 30 min in an orbital shaker (LABNET Orbit 1000). The strength of cell–cell adhesion was determined by counting under a dissecting microscope (Nikon SMZ1500) at 0.63× magnification, the number of fragments dissociated/well from the dispase-released monolayers.

RECOMBINANT PROTEIN BINDING ASSAY

Soluble proteins CRTAM (CRTAM-Fc) and Nectin-like-2 (Necl2-Fc) (2.5 μg/ml of each one) were immobilized overnight at 4°C in 96-well microtiter plates (Costar 3590, Corning Incorporated, New York). The plates were then blocked with 1% BSA for 2 h at room temperature. GST-CRTAM (5 μg/ml) or GST (negative control), were next added to the wells and incubated for 1 h at 37°C. After three washes with 0.05% Tween 20 in PBS, binding of GST or GST-CRTAM was detected by addition of a monoclonal antibody against GST (Zymed 13-6700; dilution 1:500) followed by a HRP conjugated goat anti-mouse IgG (Zymed 62-6520; dilution 1:3,000), and by the addition of the substrate *o*-phenylenediamine (OPD). Assessment of color development at 490 nm was evaluated in a microtiter plate reader (Bio-Rad Microplate Reader Benchmark, CA).

RESULTS

CRTAM HAS A STRUCTURAL ORGANIZATION SIMILAR TO THAT OF JAM PROTEINS ALTHOUGH ITS SEQUENCE IS MORE CLOSELY RELATED TO NECTIN-LIKE PROTEINS

First we analyzed with a cladogram-like unrooted tree the relationship of CRTAM with diverse members of the Ig-SF. CRTAM has in common with JAM proteins the presence of two Ig-like repeats and a PDZ binding motif at the carboxyl terminal end. Therefore in this analysis we included six proteins that have been identified as members of the JAM family: JAM-A, JAM-B, JAM-C, CAR, CLMP, and BT-IgSF [Hirabayashi and Hata, 2006]. We also included others proteins like A33 and CTH that belong to the *c*ortical *t*hymocyte *X*enopus (CTX) family of proteins characterized for having two Ig-like repeats with intramolecular disulfide bonds, but whose cytoplasmic region is devoid of a PDZ binding motif [Weber et al., 2007]. We have also included the nectin and nectin-like (necl) proteins in our analysis since a previous bioinformatic study suggested a relationship with CRTAM [Patino-Lopez et al., 2006]. The cladogram-like tree shown in Figure S1A indicates that hCRTAM is more closely related to the necl proteins 1, 2, 3, and 4 than to JAM proteins A, B and C, and is phylogenetically more distant to the group of JAM proteins with long cytoplasmic tails than to nectins 1–4 and necl 5. Since the overall percentage of identity between CRTAM and nectins, necl proteins [Boles et al., 2005] and JAMs is below 20%, we next decided to do the bioinformatics analysis only with the V type distal Ig-like repeat which is present in all the analyzed proteins. Figure S1B cladogram-like unrooted tree reveals that this segment of hCRTAM is more closely related to that of necl proteins 1–4, than to the other members of the Ig-SF analyzed.

CRTAM IS PRESENT IN THE LATERAL MEMBRANE OF EPITHELIAL CELLS

Next we asked if CRTAM is expressed in the MDCK epithelial cell line. Figure 1A shows that CRTAM mRNA is present in MDCK cells and as previously described in peripheral blood mononuclear cells (PBMCs) activated with phorbol myristate acetate (PMA) and ionomycin [Kennedy et al., 2000]. The Western blot (Fig. 1B), done with a monoclonal antibody against CRTAM, reveals a 93 kDa band in MDCK cells and in activated $CD8^+$ T cells. This band is also found in non-activated $CD8^+$ T cells, but at a lower level.

We next analyzed by confocal microscopy the localization of CRTAM in MDCK cells. For these experiments we employed a rabbit polyclonal antiserum generated by us against the human and mouse

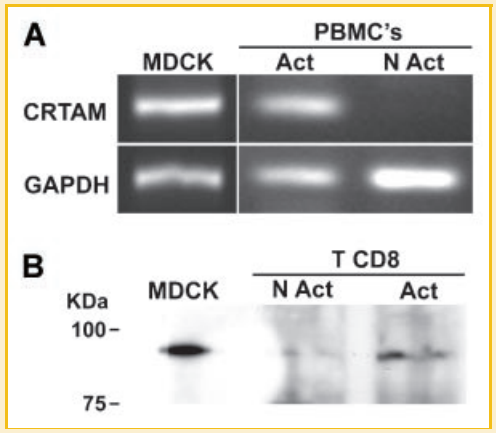

Fig. 1. CRTAM mRNA and protein is expressed in epithelial MDCK cells. A: RT-PCR with specific oligos for CRTAM amplifies a 500 bp segment in MDCK cells and in human peripheral blood mononuclear cells (PBMCs) activated (Act) with 2 ng/ml phorbol myristate acetate (PMA) and 200 ng/ml ionomycin. Non-activated (NAct) PBMCs do not express CRTAM mRNA. Two independent experiments with similar results were performed. B: Western blot analysis done with a monoclonal antibody against human CRTAM, reveals a 93 kDa band present in MDCK cells and activated $CD8^+$ T cells. This band is also found in non-activated $CD8^+$ T cells, but at a lower level. The results shown are representative of two independent experiments.

CRTAM antigenic peptide DKEEKE. As a control of the specificity of the antibody we incubated MDCK monolayers with CRTAM antibody and the antibody preabsorbed to the antigenic peptide. Supplemental Figure 2 shows how the cell border staining of CRTAM disappears when the antibody is pre-absorbed with the antigenic peptide. Then we performed double labeling experiments in which we compared the localization of CRTAM to that of the TJ protein occludin (Fig. 2A) and the adherent junction specific protein E-cadherin (Fig. 2B). At the TJ region identified by the conspicuous presence of occludin at the cell borders, CRTAM gives a diffuse and barely detectable signal (Fig. 2A, upper line). Instead at the lateral membrane, below the TJ region, CRTAM co-localizes with E-cadherin (Fig. 2B, second line). YZ sections confirm the localization of CRTAM below the TJ region (Fig. 2A) and along the lateral membrane (Fig. 2B). The dotted expression of CRTAM at the lateral membrane (arrowheads in Fig. 2A and B) prompted us to compare its distribution with that of desmoplakin, a specific desmosomal marker. Figure 2C shows a partial co-localization of CRTAM and desmoplakin (DP) along the lateral membrane of epithelial cells. YZ sections reveal a lateral membrane beaded staining pattern similar to that observed for desmoplakin.

Since the MDCK cell line is derived from kidney epithelial cells, we next explored the expression of CRTAM in mammalian kidney. Supplemental Figure 3 illustrates how in rat kidney frozen sections CRTAM and occludin are present at the cellular borders of renal tubules. Incubation with rabbit pre-immune (PI) serum indicates that CRTAM staining is specific. Closer inspection (see insert in upper right panel) reveals that CRTAM staining is located below the sites stained with occludin antibody. These results hence reinforce the observation that CRTAM is present at the lateral membrane of epithelial cells below the TJ region.

PERTURBATION OF INTERMEDIATE FILAMENTS RESULTS IN DISRUPTION OF CRTAM

Next we explored if the distribution of CRTAM at the cellular border was affected by disruption of intermediate filaments and desmosomal structures using acrylamide [Shabana et al., 1994; Zen et al., 2004]. Figure 3A shows that while the TJ protein ZO-1 remains at the cellular borders upon treatment with acrylamide, CRTAM distribution pattern exhibits a disruption which is even more intense

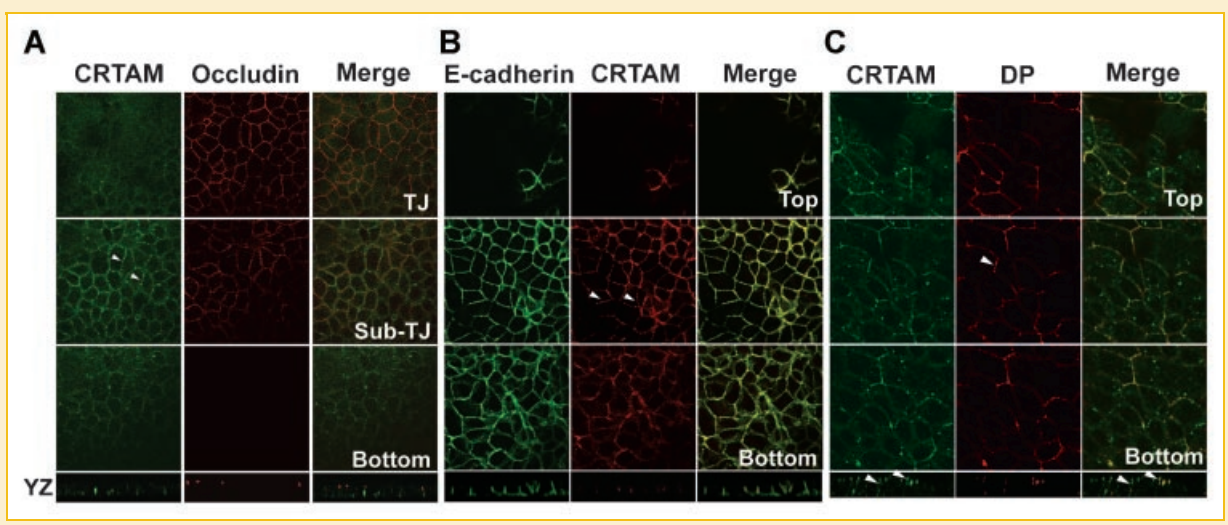

Fig. 2. In epithelial MDCK cells CRTAM distributes along the lateral membrane. Monolayers were treated with the polyclonal antibody against CRTAM together with the following specific antibodies: mouse anti-occludin (A), rat anti-E-cadherin (B) or mouse anti-desmoplakin (C). A goat anti-rabbit FITC-coupled secondary antibody and goat anti-mouse TRITC coupled antibody were employed in (A,C); and a goat anti-rat FITC-coupled secondary antibody and a goat anti-rabbit TRITC antibody were employed in (B). DP, desmoplakin; arrowheads indicate the beaded distribution pattern of CRTAM and desmoplakin. The results shown are representative of five independent experiments.

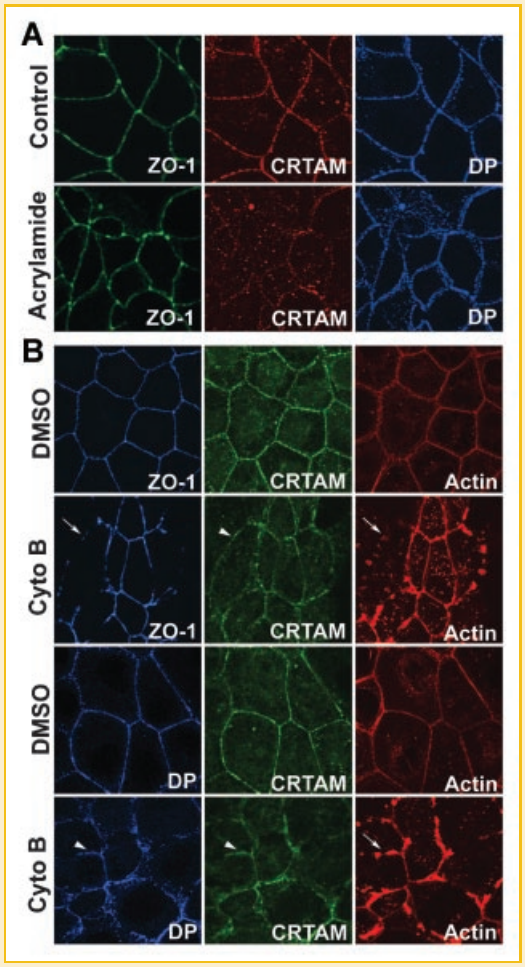

Fig. 3. CRTAM distribution is sensitive to intermediate filaments disruption. MDCK monolayers were treated with acrylamide (A) or cytochalasin B (B) before being processed for immunofluorescence with rhodaminated phalloidin and antibodies against ZO-1, CRTAM and desmoplakin (DP). As secondary antibodies the following were used: (A) goat anti-rat coupled to FITC, a goat anti-rabbit coupled to TRITC and a goat anti-mouse coupled to CY5; (B) goat anti-rabbit coupled to FITC and goat anti-rat (upper panel) or goat-mouse coupled to CY5 (lower panel). The results shown are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

than that observed for desmoplakin. To determine if perturbation of the actin cytoskeleton promotes changes in CRTAM localization we treated the cells with cytochalasin B, a drug that impairs actin polymerization [Theodoropoulos et al., 1994], inhibits the development of transepithelial electrical resistance (TER) [Meza et al., 1980, 1982; Gonzalez-Mariscal et al., 1985] and alters the distribution of TJ proteins [Nybom and Magnusson, 1996]. Figure 3B shows that this treatment that induces the appearance of ZO-1 and actin aggregates along the cell borders (arrows), does not alter in a significant manner CRTAM and desmoplakin distribution (arrowheads).

Since keratin intermediate filaments are a major constituent of the cytoskeleton of vertebrate epithelial cells, we next studied in MDCK cells the distribution of CRTAM and cytokeratin-8, a type I keratin characteristic of single layered epithelia [Moll et al., 1982]. Supplemental Figure 4 shows how cytokeratin-8 and CRTAM exhibit very different distribution patterns: CRTAM is present at the cell borders while cytokeratin-8 forms a complex network within the cytoplasm that extends from the cell periphery in a perpendicular manner to the outer nuclear membrane. The merged image in Figure S4 shows how cytokeratin 8 filaments from two neighboring cells (arrowheads) align at the cell border at sites where CRTAM is present (arrow).

Taken together these results suggest that CRTAM is more strongly associated to intermediate filaments than to the actin cytoskeleton.

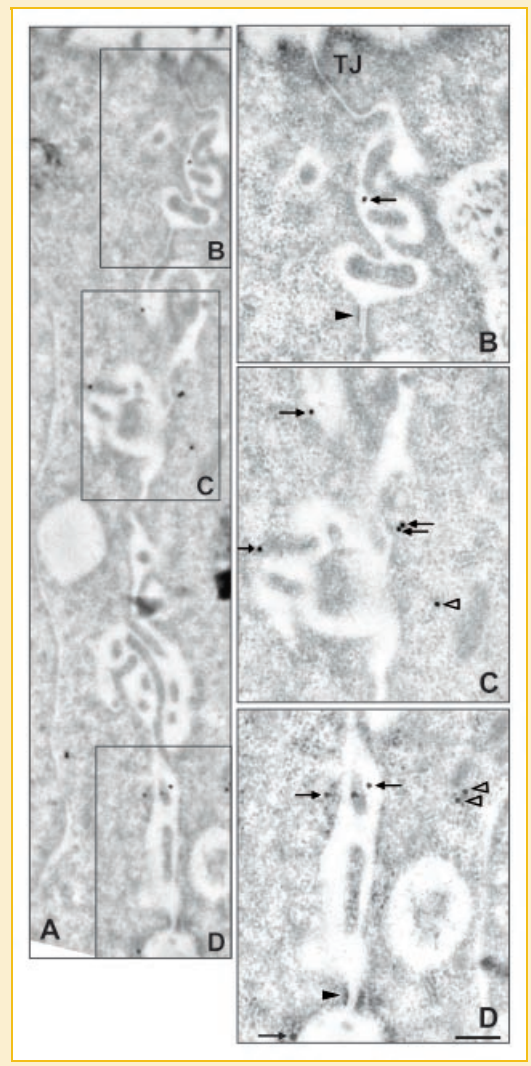

Fig. 4. Immunogold electron microscopy reveals the presence of CRTAM along the lateral membrane of epithelial cells. MDCK monolayers were stained with the anti-CRTAM monoclonal antibody followed by immunogold electron microscopy. B, C, and D, respectively, correspond to amplifications of the upper, middle, and lower rectangles delineated in (A). Bar 200 nm. TJ, tight junction; full arrowheads, desmosomes; arrows, gold particles at interdigitations along the lateral membrane; empty arrowheads, gold particles at the cytoplasm proximal to the lateral membrane. The results shown are representative of three independent experiments.

ELECTRON MICROSCOPY REVEALS THAT CRTAM IS PRESENT ALONG THE LATERAL MEMBRANE BUT IS NOT CONCENTRATED IN THE TIGHT JUNCTIONS OR THE DESMOSOMES

To determine with more precision the sub-cellular localization of CRTAM in epithelial cells, we performed a gold immuno-labeling experiment in MDCK monolayers embedded in LR white. Gold particles of 20 nm diameter present in thin sections, were observed by transmission electron microscopy to be distributed along the interdigitations of the lateral membrane facing the intercellular space and not to concentrate at either the TJ region or the desmosomes (Fig. 4). A few gold particles are also observed at the cytoplasm proximal to the lateral membrane, in agreement to that observed by immunofuorescence (Supplemental Fig. 2) and probably corresponding to CRTAM in transit to and from the plasma membrane.

CRTAM CONCENTRATES AT THE CELL BORDERS WHEN INTERCELLULAR JUNCTION FORMATION IS TRIGGERED BY CA^{2+} AND USAGE OF A BLOCKING PEPTIDE FOR E-CADHERIN, REDUCES CRTAM ARRIVAL TO THE CELL BORDERS

In order to determine if CRTAM concentrates at the plasma membrane, upon the novo intercellular junction formation, we performed a kinetic analysis on MDCK monolayers that after being cultured for 20-h in low calcium (LC) medium are switched to medium with normal calcium (NC). We observe (Fig. S5A) that 20 min after the Ca^{2+}-switch CRTAM can already be located at the cell borders (arrow). However its distribution pattern is less continuous and intense than that observed for E-cadherin and desmoplakin. Supplemental Figure 5B illustrates that a similar situation is found with CRTAM in comparison to ZO-1 and occludin. Supplemental Figure 5C shows how the intensity and distribution pattern of JAM-A at the cell borders is similar to that of occludin after the Ca^{2+}-switch.

Taken together these results indicate that CRTAM appears in early cell–cell contacts, although it concentrates at the cell borders after E-cadherin, ZO-1, occludin, JAM-A and desmoplakin.

In order to investigate the role of E-cadherin-mediated adhesion on CRTAM arrival to the cell borders, we next performed a Ca^{2+}-switch assay in the presence of the *c*ell *a*dhesion *r*ecognition (CAR) peptide SHAVAS, known to be specific for the blockade of E-cadherin function [Makagiansar et al., 2001]. As shown in Supplemental Figure 6, monolayers treated with SHAVAS peptide exhibit a less intense and discontinuous display of E-cadherin at the

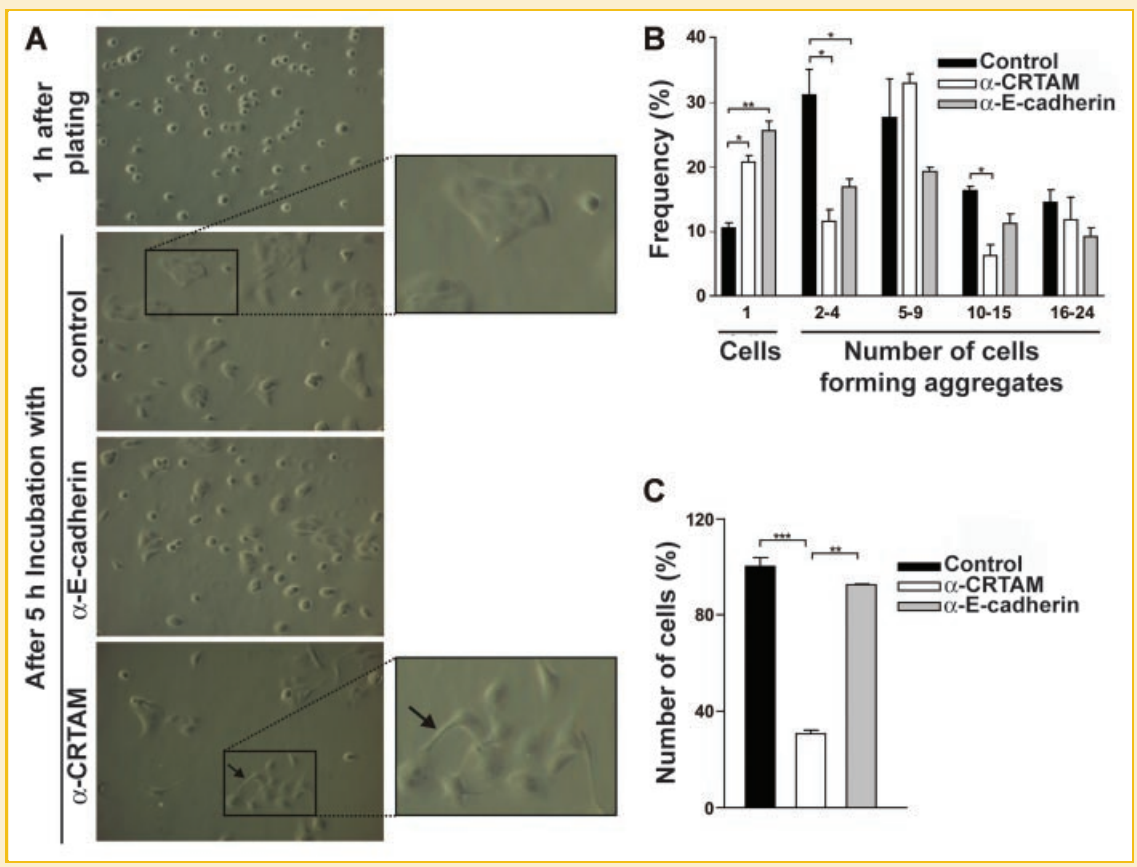

Fig. 5. Treatment with the antibody against CRTAM promotes cell detachment from the substrate and the formation of atypical cell aggregates. A: Representative light microscopy images of sparse cultures of MDCK cells 1-h after plating and after their subsequent transfer for 5-h to control medium or medium containing the monoclonal antibody against E-Cadherin or the polyclonal antibody anti-CRTAM. Inserts show at a higher amplification the morphology of the cells in the aggregates. Arrows indicates a cell in the aggregate with a spindle like morphology. B: Quantitative analysis of two independent experiments, counting the number of isolated cells and of those forming aggregates in seven different fields per experiment. C: Amount of cells present in the coverslips after the 5-h transfer to control medium or medium containing antibodies against E-Cadherin or CRTAM. The values found in cells transferred to control medium were taken as 100 for normalization. The results shown are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cell borders, than cultures treated with the scramble peptide AAHSSV, confirming the functionality of the CAR peptide. CRTAM staining is diminished or absent only at areas where E-cadherin expression has significantly lessen. These results hence indicate that CRTAM arrival at the cell borders is negatively affected upon E-cadherin-mediated cell adhesion disruption.

TREATMENT WITH CRTAM ANTIBODIES INHIBITS CELL AGGREGATION AND PROMOTES THE DETACHMENT OF CELLS FROM THE SUBSTRATE

We next sought to analyze if CRTAM participates in the establishment of intercellular adhesion. For this purpose we performed a cell aggregation competition assay in which live MDCK cells were treated with an antibody against CRTAM that recognizes the epitope DKEEKE present at the extracellular domain of the protein. As a positive control we included monolayers treated with DECMA-1 antibody against E-cadherin that recognizes the extracellular domain of E-cadherin and is thus capable of inhibiting the formation of cell–cell aggregates [Vestweber and Kemler, 1985; Vagin et al., 2006]. Figure 5A shows light microscopy images of two independent experiments and Figure 5B the quantitative results obtained counting the number of isolated cells and of those forming aggregates. As expected treatment with E-cadherin antibody augments the number of isolated cells in the culture and decreases the frequency of cell aggregates. In cultures treated with CRTAM we observe that the number of cells present in the coverslips suffered a threefold decrease (Fig. 5C), thus suggesting that CRTAM might be involved in the attachment of cells to the substrate. The cells that remain on the coverslips are frequently found forming cell aggregates, being those of five to nine cells the most abundant. However these cell aggregates are not as compact as in control cultures and are formed by cells with a fusiform or spindle-like morphology (Fig. 5A, arrow).

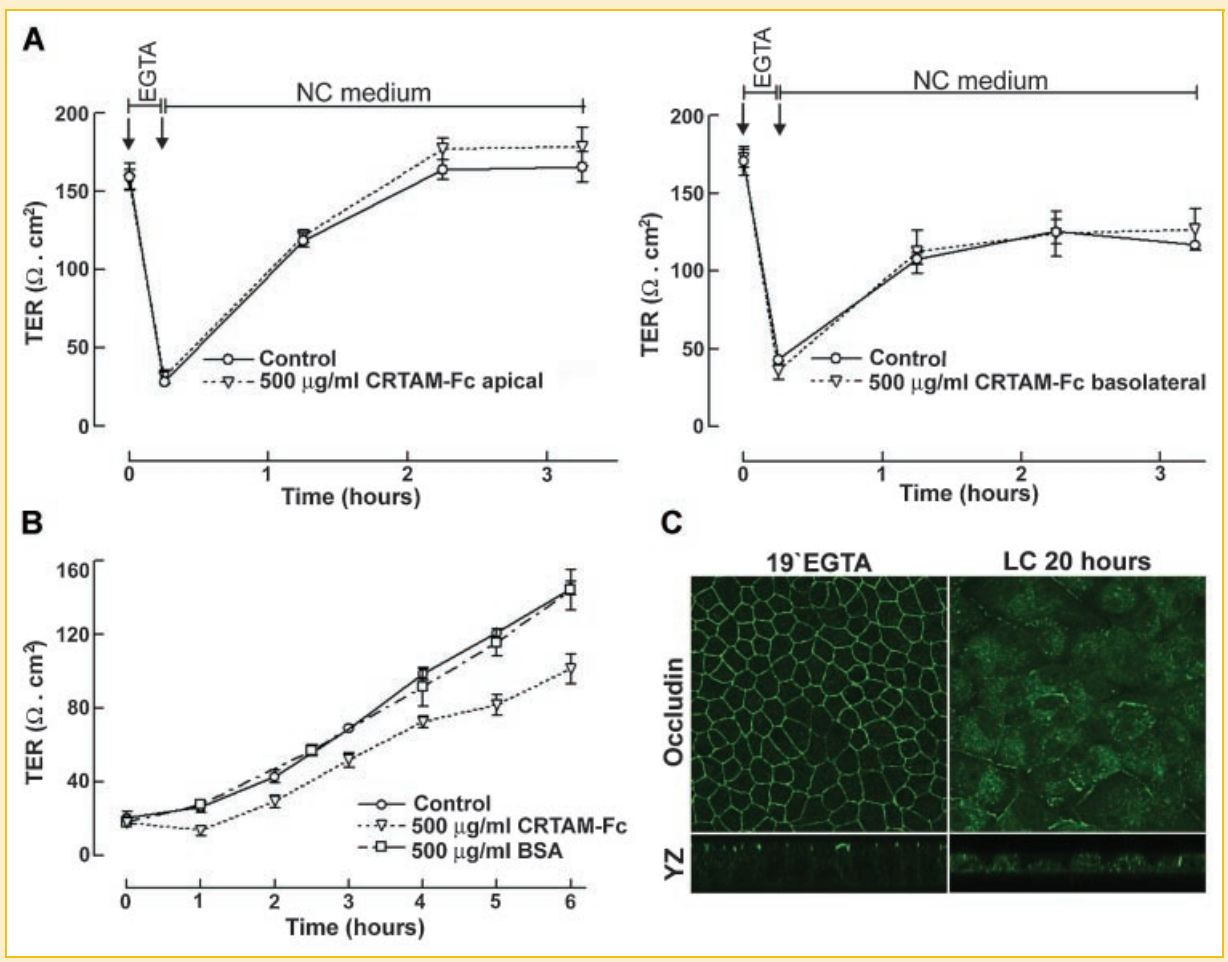

Fig. 6. Soluble CRTAM (CRTAM-Fc) inhibits the optimal development of TER. A: MDCK monolayers cultured on Transwell filters were treated for 19 min with 2.4 mM EGTA. Then the monolayers were bathed with NC medium alone or NC medium containing 500 μg/ml of CRTAM-Fc at either the apical (left graph) or basolateral (right graph) surfaces. B: Monolayers plated on Transwell filters were incubated for 20-h in LC medium and then switched for the indicated periods of time to NC medium. The apical NC medium of some monolayers contained 500 μg/ml of CRTAM-Fc or BSA. TER was measured at the indicated time points. C: Immunofluorescence localization of occludin with a rabbit polyclonal antibody, in MDCK monolayers incubated for 20-h in LC medium (right) or cultured in CDMEM and treated for the last 19 min with EGTA (left). The results shown are representative of at least two independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TREATMENT WITH SOLUBLE CRTAM ALTERS CELL–CELL ADHESION

To determine if CRTAM has a role in the development of TER, we competed CRTAM mediated cell–cell adhesion by the addition of soluble human CRTAM (CRTAM-Fc). Figure 6A shows that the addition of 500 μg/ml CRTAM-Fc either to the apical or the basolateral surface of MDCK monolayers cultured on transwell filters, has no effect on TJ sealing induced by calcium in monolayers previously treated with EGTA. In the other hand CRTAM-Fc added to the apical surface of monolayers transferred to NC medium after a 20-h incubation in low calcium (LC) medium, exhibit a significantly lower TER, compared with that obtained in control monolayers and in cultures incubated with 500 μg/ml of bovine serum albumin (BSA; Fig. 6B). These results suggest that CRTAM mediated adhesion is important for the optimal development of TER.

We hypothesize that CRTAM-Fc is effective in the Ca-switch but not in the monolayers treated with EGTA, because in monolayers incubated for 20-h in LC medium the intercellular space is wider and not sealed at the uppermost region, giving CRTAM-Fc a better access to its target. Observe in Fig. 6C (right panel), that after 20-h in LC medium, almost no staining of occludin is detected in the monolayers. In contrast, in cultures treated with a short pulse of EGTA, although TER has fallen, cell–cell adhesion is not completely dismantled as regions positive for the TJ protein occludin can be observed at the cell borders (Fig. 6C, left panel). This situation may hamper the access of CRTAM-Fc to its target along the lateral membrane.

Next we explored if treatment with CRTAM-Fc could enhance cell–cell dissociation. For this purpose we employed the dispase-based dissociation assay in monolayers that had been subjected for 6-h to a calcium switch assay in the presence of 500 μg/ml of CRTAM-Fc or BSA. In this experiment the ability of dispase to cleave fibronectin and type IV collagen [Stenn et al., 1989] is employed to release the monolayer from the extracellular matrix molecules that provide attachment to the Transwell inserts. The strength of cell–cell adhesion is next determined by counting the number of dissociated fragments liberated from the dispase-released monolayers after treatment with mild mechanical stress. Figure 7A illustrates how a significantly higher number of monolayer fragments are observed in the dispase-released monolayers treated with CRTAM-Fc, than in control monolayers and in those incubated with BSA. The number of fragments/well counted under a dissecting microscope in this assay is shown in Figure 7B.

This experiment hence indicates that the strength of intercellular junctions in the monolayer, decreases upon treatment with CRTAM-Fc and hence supports the idea that CRTAM is involved in cell–cell adhesion.

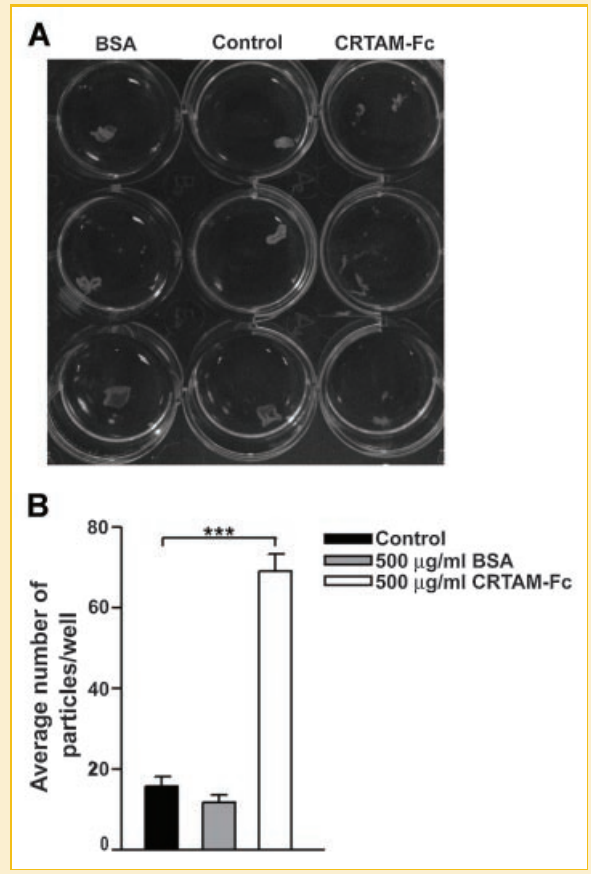

Fig. 7. Dispase-based dissociation assay reveals a lower degree of cell–cell adhesion in monolayers treated with CRTAM-Fc. A: Monolayers switched for 6 h to NC medium in the absence (control) or presence of CRTAM-Fc or BSA, were separated from the Transwell inserts via incubation with dispase II. Liberated monolayers were transferred to separate wells in a 12-well multiplate and agitated in an orbital shaker. Observe that the degree of fragmentation of the monolayers incubated with CRTAM-Fc is higher than that in incubated in BSA or left untreated (control). B: The dissociation assay was quantified by counting the number of total particles/well under a dissecting microscope. The results shown are representative of two independent experiments.

CRTAM MEDIATES CELL–CELL ADHESION IN TRANS

Our next aim was to determine if CRTAM mediates cell–cell adhesion in a *trans*-type manner, meaning the interaction of a CRTAM molecule located in one cell with another molecule located in the adjacent neighboring cell. For this purpose we needed to culture MDCK cells together with cells not expressing CRTAM. CHO fibroblast were chosen because our analysis by Western blot and immunofluorescence reveals that they lack several cell–cell adhesion proteins including claudin-1, E-cadherin, and JAM-A, and exhibit a barely detectable amount of occludin (Supplemental Fig. 7A). In agreement with observations done in L fibroblasts [Itoh et al., 1999], CHO cells express similar amounts of ZO-1 than those found in MDCK cells, however in CHO cells the protein is found scattered in the cytoplasm and not concentrated at the cell borders (Supplemental Fig. 7B). With regards to CRTAM, Western blot analysis reveals that CHO cells express a lower amount than MDCK cells, and by immunofluorescence a diffuse cytoplasmic signal is observed (Supplemental Fig. 7B). Figure 8A shows the MDCK/CHO co-culture in which the CHO cells were for identification purposes, labeled before hand with the CellTracker Orange CMTMR. We observe that at the cell borders between MDCK and MDCK cells, CRTAM is present (arrowhead), instead at the heterotypic borders between MDCK and CHO cells, no CRTAM staining is detected

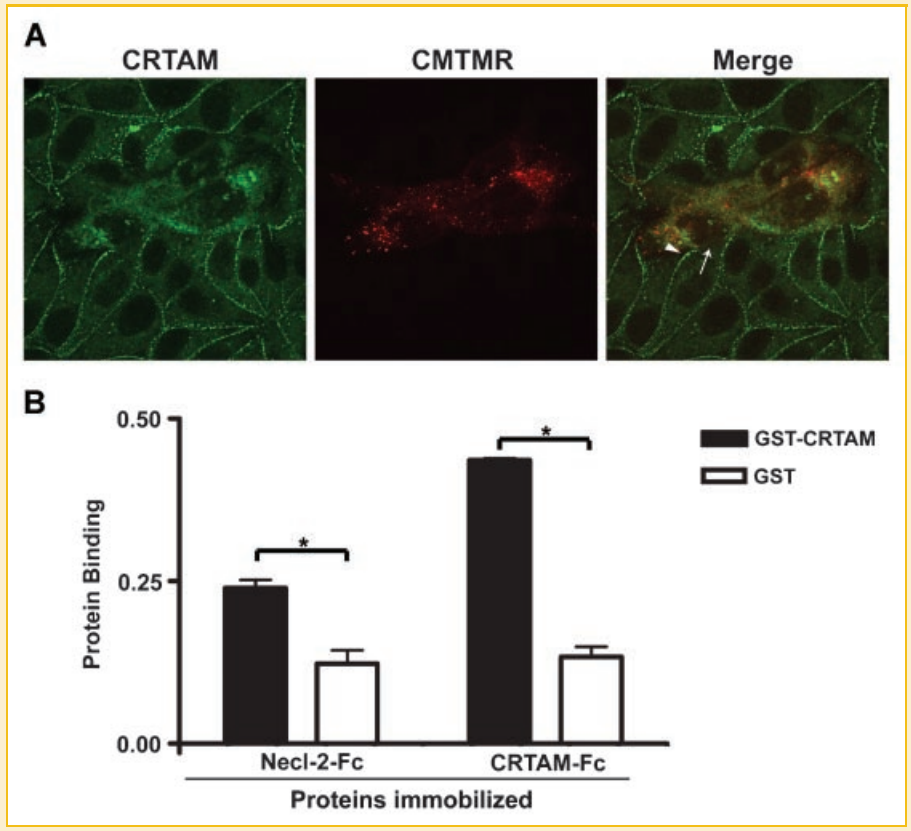

Fig. 8. CRTAM establishes homotypic and heterotypic trans-interactions. A: MDCK cells only express CRTAM in homotypic MDCK/MDCK borders, but not in those contacting CHO cells. MDCK cells were co-cultured with CHO fibroblasts, previously stained in red with CellTracker Orange CMTMR, and assayed with the polyclonal antibody against CRTAM (green). Arrow denotes a heterotypic border between MDCK and CHO cells. Arrowhead indicates a homotypic MDCK/MDCK border. B: Recombinant protein binding assay. Soluble CRTAM (CRTAM-Fc) and Necl-2 (Necl2-Fc) proteins immobilized in individual wells of 96-well microtiter plates interact with GST-CRTAM fusion protein. Bound protein was detected by the addition of a mouse monoclonal anti-GST, followed by HRP-conjugated goat anti-mouse IgG, and the substrate OPD for color development. The results shown are representative of at least two independent experiments.

(arrow). This result reveals that CRTAM can only be detected if it is expressed at the cell borders by the two neighboring cells, thus indicating that CRTAM establishes a *trans*-type of interaction.

However we cannot rule out the possibility that CRTAM establishes a heterotypic instead of a homotypic *trans*-interaction, and that in the co-culture of MDCK/CHO cells this interaction cannot be established due to the generalized absence of cell adhesion proteins in CHO cells.

To analyze the homotypic or heterotypic-*trans* interactions of CRTAM we next performed a recombinant protein binding assay in which we tested the interaction of the GST-CRTAM fusion protein to either CRTAM-Fc or Necl2-Fc, immobilized to microtiter wells. Necl2-Fc was included as a positive control since it was previously demonstrated to interact in a heterotypic *trans* manner with CRTAM in T cells hybridoma [Arase et al., 2005]. Figure 8B shows a significant binding activity between the fusion protein GST-CRTAM and CRTAM-Fc, that is even higher than that present between the fusion protein GST-CRTAM and Necl2-Fc. Specificity was confirmed by the poor binding of the GST control protein. These results thus indicate that CRTAM can establish trans homotypic interactions and confirms its heterotypic interaction with Necl-2.

DISCUSSION

CRTAM was initially described as a molecule of cells of the immune system [Kennedy et al., 2000]. Here we have demonstrated that CRTAM is present in epithelial cells at the lateral membrane.

The structural organization of CRTAM complies with all the requisites that a molecule has for belonging to the JAM protein family [Hirabayashi and Hata, 2006], and can therefore be considered as a new family member. However, the full length sequence of CRTAM as well as that of its first Ig-like domain exhibits a higher level of amino acid identity and phylogenetic closeness to necl proteins 1 to 4 than to JAM proteins A, B and C.

With regards to localization, CRTAM displays a distribution similar to that of certain JAM proteins. Thus while JAM-A, JAM-C, CAR, ESAM, JAM4, CLMP and BT-IgSF are located at the TJ [Hirabayashi and Hata, 2006], others such as A33 [Johnstone et al., 2000], and JAM-B [Palmeri et al., 2000], distribute like CRTAM to the lateral membrane below the TJ region.

It is noteworthy that CRTAM distribution pattern is intensively distorted upon treatment with the intermediate filament disruptor acrylamide. This observation together with CRTAM-colocalization with desmoplakin suggested the possibility of CRTAM being present

in desmosomes. However our immunogold experiments clearly show that CRTAM is not concentrated at desmosomes and is instead distributed along the whole lateral membrane. The observation of the cytokeratin-8 pattern in MDCK cells, further suggest that CRTAM could function as a cell–cell adhesion molecule to which intermediate filaments might attach.

CRTAM can be detected at the cell borders 20 min after intercellular junctional assembly is triggered by Ca^{2+} in monolayers previously incubated in LC medium. This observation suggests that CRTAM is involved in early cell–cell contact, although its concentration at the plasma membrane might not be as high as that of JAM-A, E-cadherin, ZO-1, desmoplakin, and occludin. The involvement of CRTAM in early cell adhesion is reinforced by the observation that treatment of epithelial monolayers with soluble CRTAM lowers the values of TER achieved in a TJ de novo formation experiment and promotes cell–cell dissociation in the dispase assay. Treatment with the E-cadherin CAR peptide, has no great impact on the de novo arrival of CRTAM to the cell borders, since a reduction in CRTAM expression is only observed in areas where E-cadherin is absent or greatly diminished.

An unexpected result from this study is the observation that treatment with the antibody against CRTAM promotes cell detachment from the substrate and the formation of atypical cell aggregates that may represent groups of cell unable to properly attach and spread in the culture dish. These results resemble the massive detachment of epithelial cells from the substrate provoked by ouabain, an inhibitor of the Na^{+},K^{+}-ATPase [Contreras et al., 1999], which has recently been recognized as a protein with cell–cell adhesion properties [Shoshani et al., 2005]. Alternatively, our results might suggest that CRTAM can regulate cell adhesion to the substrate by association to proteins that interact with molecules of the extracellular matrix. In this regard it is important to mention that several JAMs [Cunningham et al., 2000; Ostermann et al., 2002; Naik et al., 2003; Naik and Naik, 2006], nectins and necl proteins [Mueller and Wimmer, 2003; Ikeda et al., 2004; Sakamoto et al., 2006] are known to associate to integrins, and that here we were able to demonstrate by an vitro recombinant protein binding assay, the trans interaction of CRTAM with the extracellular domains of Necl-2.

We demonstrated in the CHO/MDCK co-culture that CRTAM is present at the cell borders only when the two neighboring cell express CRTAM. This *trans* CRTAM/CRTAM interaction was further confirmed in vitro with a fusion protein binding assay.

In summary we have been able to demonstrate that CRTAM is present along the lateral membrane of epithelial cells and is involved in cell–cell and cell–substrate interactions.

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