Cytoskeletal restraints regulate homotypic ALCAM-mediated adhesion through PKCα independently of Rho-like GTPases

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Summary

The activated leukocyte cell adhesion molecule (ALCAM) is dynamically regulated by the actin cytoskeleton. In this study we explored the molecular mechanisms and signaling pathways underlying the cytoskeletal restraints of this homotypic adhesion molecule. We observed that ALCAM-mediated adhesion induced by cytoskeleton-disrupting agents is accompanied by activation of the small GTPases RhoA, Rac1 and Cdc42. Interestingly, unlike adhesion mediated by integrins or cadherins, ALCAM-mediated adhesion appears to be independent of Rho-like GTPase activity. By contrast, we demonstrated that protein kinase C (PKC) plays a major role in ALCAM-mediated adhesion. PKC inhibition by chelerythrine chloride and myristoylated PKC pseudosubstrate, as well as PKC downregulation by PMA strongly reduce cytoskeleton-dependent ALCAM-mediated adhesion. Since serine and threonine residues are dispensable for ALCAM-mediated adhesion and ALCAM is not phosphorylated, we can rule out that ALCAM itself is a direct PKC substrate. We conclude that PKCα plays a dominant role in cytoskeleton-dependent avidity modulation of ALCAM.

Key words: ALCAM, CD166, PKC, Cytoskeleton, Avidity, Rho-like GTPases

Introduction

Activated leukocyte cell adhesion molecule (ALCAM, CD166) is a novel member of the immunoglobulin superfamily of adhesion molecules. Unlike its name suggests, ALCAM is not only expressed by activated leukocytes (Bowen et al., 1995) and monocytes (Levesque et al., 1998), but also by hematopoietic progenitor cells (Uchida et al., 1997; Nelissen et al., 2000a), bone marrow stromal cells (Cortes et al., 1999) and hematopoiesis-supporting osteoblastic cells (Nelissen et al., 2000a). In addition to expression by hematopoietic cells and hematopoiesis-related tissues, ALCAM is expressed by metastasizing melanoma (Degen et al., 1998) and by prostate adenocarcinoma (Kristiansen et al., 2003). Neuronal cells express substantial levels of ALCAM (Tanaka et al., 1991) and multiple other tissues stain positive for ALCAM (Bowen et al., 1995; Degen et al., 1998; Nelissen et al., 1999).

ALCAM consists of five extracellular Ig-like domains and is a highly glycosylated type I transmembrane molecule with a short (32 AA) cytoplasmic tail and an observed molecular weight of 105 kDa (Bowen et al., 1995). Besides binding to CD6 (Bowen et al., 1995; Bowen et al., 1996), ALCAM mediates homotypic ALCAM-ALCAM interactions (Uchida et al., 1997; Nelissen et al., 2000b). Although the precise function of ALCAM is largely unknown, ALCAM-mediated interactions are important during neural development (Tanaka et al., 1991; Stephan et al., 1999), maturation of hematopoietic stem cells in blood forming tissues (Uchida et al., 1997; Cortes et al., 1999; Ohneda et al., 2001), immune responses (Bowen et al., 1995) and tumor progression (Degen et al., 1998). In view of the diversity of cellular processes in which ALCAM is implicated, tight regulation of ALCAM-mediated adhesion is required to prevent aberrant adhesive homotypic or heterotypic interactions. However, the signaling pathways that lead to modulation of ALCAM-mediated adhesion remain elusive. Identification of the molecular mechanism underlying ALCAM-mediated adhesion will further contribute to our understanding of the function of ALCAM in diverse tissues.

We have shown that homotypic ALCAM-mediated interactions are dynamically regulated through the actin cytoskeleton (Nelissen et al., 2000b). We demonstrated that stable, homotypic ALCAM-mediated adhesion requires a transient release of ALCAM from the actin cytoskeleton, as induced by partial disruption of the cytoskeleton with cytochalasin D (CytD). Upon CytD treatment of the cells, we observed a significant increase in lateral mobility of ALCAM molecules. This release from the actin cytoskeleton and enhanced mobility results in the formation of high avidity clusters of ALCAM molecules on the cell surface, which are required for strong adhesive ALCAM-ALCAM interactions. K562 cells expressing a GPI-anchored ALCAM mutant failed to adhere to immobilized ALCAM-Fc in response to CytD treatment, demonstrating the importance of the cytoplasmic domain of ALCAM for regulation by the actin cytoskeleton.

In the present study, we extend these findings by examining

two signaling pathways potentially involved in ALCAM-mediated adhesion. The first pathway of interest involves the small GTPases RhoA, Rac1 and Cdc42, which are key players in the organization of the actin cytoskeleton (Hall, 1998; Schoenwaelder and Burridge, 1999). RhoA promotes the formation and maintenance of stress fibers and focal adhesions, whereas Rac and Cdc42 regulate the formation of actin-rich protrusions such as lamellipodia and filopodia. RhoA, Rac1 and Cdc42 are highly involved in the regulation of adhesion mediated by integrins (Mackay and Hall, 1998; D'Souza-Schorey et al., 1998; Gimond et al., 1999) and cadherins (Braga, 2002; Yap and Kovacs, 2003). The Rho-like GTPases are also implicated in the clustering and adhesion of a number of Ig-CAMs: RhoA regulates the assembly of stable adhesions between endothelial cells and monocytes via clustering of the receptors ICAM-1, VCAM-1 and E-selectin, and their association with the actin cytoskeleton (Wojciak-Stothard et al., 1999). Also, clustering of VCAM-1 and possibly other Ig-CAMs initiates a signaling pathway that involves Rac1 (Van Wetering et al., 2003). Here, in sharp contrast to integrins and cadherins, we find no evidence for involvement of these GTPases in adhesion mediated by ALCAM.

Protein kinase C isoforms are another group of signaling molecules that may be relevant with respect to ALCAM-mediated adhesion. They were shown to significantly contribute to cytoskeletal rearrangements (Masson-Gadais et al., 1997; Keenan and Kelleher, 1998) and even bind filamentous actin (Slater et al., 2000). In locomotory T cells, PKCβ is involved in LFA-1-mediated signaling and cytoskeletal scaffolding (Altman and Villalba, 2002). PKCθ appears to be associated with the actin cytoskeleton in T cells and is essential for T-cell activation (reviewed in Volkov et al., 2001). Moreover, PMA stimulation of lymphocytes induces β2-integrin-mediated adhesion in a manner similar to CytD (Kucik et al., 1996). These findings underscore the possible involvement of PKC in cytoskeleton-dependent, homotypic ALCAM-mediated adhesion. Here we demonstrate that PKCα plays an essential role in cytoskeleton-dependent avidity modulation of ALCAM.

Materials and Methods

Chemicals and antibodies

All chemicals were purchased from Sigma (St Louis, MO) unless stated otherwise. Stock solutions of cytochalasin D (CytD), latrunculin A (LatA, Molecular Probes, Leiden, The Netherlands), phorbol-12-myristate-13-acetate (PMA) and chelerythrine chloride were prepared in dimethylsulfoxide (DMSO) and stored at –20°C. Stock solutions of myristoylated cell-permeable PKC inhibitor 19-27 (Calbiochem, La Jolla, CA) were prepared in Tris-buffer and stored at –20°C. [^{32}P]-Disodiumphosphate and [^{35}S]methionine/cysteine were from MP Biomedicals Inc. (Irvine, CA).

Anti-ALCAM monoclonal antibodies: J4-81 (IgG1 isotype) and FITC-conjugated J4-81 were purchased from Antigenix America (Huntington Station, NY). AZN-L50 (IgG2A isotype) and AZN-L51 (IgG1 isotype) were generated in our laboratory by immunizing BALB/C mice with K562-ALCAM. Goat-anti-human Fc-(Fab′)$_2$ fragments were purchased from Jackson ImmunoResearch (Westgrove, PA), FITC-conjugated goat-anti-mouse (Fab′)$_2$ fragments were from Zymed Laboratories (San Francisco, CA) and FITC-conjugated goat-anti-human Fc-(Fab′)$_2$ fragments were from Cappel Inc. (West Chester, PA). Alexa 488-conjugated goat-anti-mouse IgG (H+L) and Texas Red-X Phalloidin were obtained from Molecular Probes (Eugene, OR). Anti-PKC isotype-specific antibodies and corresponding blocking peptides are from Gibco (Paisley, Scotland). Recombinant ALCAM-Fc was produced and purified as described elsewhere (Nelissen et al., 2000b), and recombinant CD6-Fc was purchased from R&D Systems (Minneapolis, MN).

Cells, expression constructs, transfections and retroviral transductions

All culture media, serum and antibiotics were purchased from Life Technologies (Breda, The Netherlands). All culture media were supplemented with 1% antibiotics/antimycotics. Myelomonocytic KG1 cells were cultured in Iscove's Modified Dulbecco's Medium containing 10% FCS. Erythroleukemic K562 cells were cultured in RPMI 1640 containing 10% FCS. K562-ALCAM were generated and maintained as described elsewhere (Nelissen et al., 2000b). To induce downregulation of PKC, cells were cultured for 16 hours in the presence of 50 nM PMA.

For mutagenesis, the full length cDNA of ALCAM was cloned in pALTER (Promega) and according to the manufacturer's protocol, serine-to-alanine and threonine-to-alanine mutations were introduced. For mutation of the serines in the cytoplasmic tail (at positions 554 and 558), the oligonucleotide 5′-CATGAAGAAGGGCAAAGACTG-CAGCAAAACATGTA-3′ was used and for mutation of the threonines at positions 556 and 581, the oligonucleotides 5′-GA-AGTCAAAGGCTGCATCAAAAC-3′ and 5′-CAATCACAAAGCT-GAAGCCTAAC-3′ were used, respectively. Sequence analysis was performed to confirm the mutations and the cDNA was recloned in pRc/CMV (Invitrogen). These constructs were used for transfection of K562 cells by electroporation with a Gene Pulser (BioRad, Hercules, CA) at 960 μF and 230 V resulting in K562-ALCAMΔSer and K562-ALCAMΔThr, respectively. The mutant transfectants were cultured like K562-ALCAM. Myc-tagged V14-RhoA, N19-RhoA, V12-Rac1, N17-Rac1, V12-Cdc42, N17-Cdc42, and L61-Rac1 and HA-tagged C1199-Tiam1, cloned into the retroviral vector LZRS-IRES-Neo, were introduced into KG1 cells and K562-ALCAM cells by retroviral transduction and selected on G418 (125 μg/ml). The expression levels of Rho-like GTPases and C1199-Tiam1 were analyzed by conventional western blotting procedures using the antibodies 9E10 (anti-Myc) and 3F10 (anti-HA).

Flow cytometry

Cells were washed with PBA [PBS containing 1% (w/v) BSA and 0.05% (w/v) NaN_3] and stained for 30 minutes at 4°C with primary antibody (2-5 μg/ml in PBA). Cells were washed with PBA and incubated with FITC-conjugated goat-anti-mouse (Fab′)$_2$ secondary antibody. After washing, cells were analyzed on a FACScan analyzer (Becton Dickinson, Oxnard, CA). The gates were set to exclude dead cells and 5000 gated cells were analyzed. Data are displayed as histograms of fluorescence intensity versus cell count.

Plate adhesion assay

Plate adhesion assays were performed as described earlier (Nelissen et al., 2000b). Briefly, flatbottom 96-well plates (Maxisorp, NUNC, Roskilde, Denmark) were pre-coated with 4 μg/ml goat-anti-human-Fc-(Fab′)$_2$ in TSM (20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 8.0). The plates were blocked with 1% (w/v) BSA and subsequently coated with 250 ng/ml ALCAM-Fc or CD6-Fc. Cells (2×10^4 per well) were labeled with Calcein-AM (Molecular Probes, Eugene, OR) and where applicable, cells were pre-treated with CytD (2.5 μg/ml) or LatA (5 μg/ml) for 30 minutes at 37°C in culture medium. Chelerythrine chloride (5 μM) and cell-permeable myristoylated PKC inhibitor (100 μM) or equivalent amounts of solvent were pre-incubated for 10 minutes at 37°C. Antibody AZN-L50 (10 μg/ml) was pre-incubated for 5-10 minutes at room temperature (RT).

Cells were allowed to adhere in triplicate wells to the coated plates for 45 minutes in culture medium at 37°C in the presence or absence of the blocking mAb. Non-adherent cells were removed by repeated washing with TSM/0.5% (w/v) BSA at 37°C. Cells were lysed with lysis buffer [50 mM Tris, 0.1% (w/v) SDS] and fluorescence was quantified in a cytofluorometer (Perseptive Biosystems). Adhesion was expressed as the mean percentage (±s.d.) of bound cells from triplicate wells. ALCAM-specific adhesion is calculated by subtracting adhesion in the presence of both blocking antibody and stimulus from adhesion in the presence of the stimulus alone.

Fluorescent bead adhesion assay

Carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 μM; Molecular Probes, Eugene, OR) were coated with ALCAM-Fc as described previously for ICAM3-Fc (Geijtenbeek et al., 1999), and the adhesion assay was performed as described (Geijtenbeek et al., 1999). In brief, cells were resuspended in adhesion buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.5% BSA) at a final concentration of 5×10^6 cells/ml. Fifty-thousand cells were pre-incubated with mAb AZN-L50 (10 μg/ml) for 10 minutes at RT. Ligand-coated fluorescent beads (20 beads/cell) were added, and the suspension was incubated for 30 minutes at 37°C. Adhesion was determined by measuring the percentage of cells that have bound fluorescent beads using flow cytometry.

Soluble ligand-binding assay

ALCAM-expressing cells (5×10^4 per well) were resuspended in 50 μl of the indicated dilutions of ALCAM-Fc in medium and allowed to bind ligand for 30 minutes at 37°C. Where indicated, cells were pre-incubated with chelerythrine chloride (5 μM) or cell-permeable myristoylated PKC inhibitor (100 μM) for 10 minutes at 37°C, or for 30 minutes at 37°C with CytD (2.5 μg/ml) prior to addition of ALCAM-Fc. Cells were washed with pre-warmed medium and subsequently incubated with FITC-conjugated goat-anti-human-Fc antibodies for 15 minutes at 37°C. After washing, the cells were analyzed by flow cytometry and the percentage of cells that have bound ligand was quantified.

GTPase activity assays

GTPase activity assays were performed as described elsewhere (Sander et al., 1999). Briefly, GST-PAK-CD and GST-C21 fusion proteins, containing the Rac1- and Cdc42-binding region from PAK1B and the RhoA-binding domain from Rhotekin, respectively, were produced in *Escherichia coli* BL21 cells. The fusion proteins were purified and bound to glutathione-Sepharose 4B beads (Amersham-Pharmacia) as described (Sander et al., 1999). Serum-starved KG1, K562, K562-ALCAM cells were treated with or without CytD (2.5 μg/ml) for 30 minutes at 37°C in suspension and cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 1% NP-40, 10% glycerol, 100 mM NaCl, 1 mM PMSF, 1 μg/ml leupeptin and aprotinin). Alternatively, serum-starved K562-ALCAM cells were treated with or without CytD, and subsequently allowed to adhere for 45 minutes to an ALCAM-Fc- (250 ng/ml) or BSA- (0.5 mg/ml) coated cell culture dish, in the presence or absence of the blocking antibody AZN-L50, before lysing the cells. The cell lysates were incubated with GST-PAK-CD or GST-C21 fusion proteins bound to glutathione-Sepharose beads. The beads and the proteins bound to the beads were washed three times in excess lysis buffer, eluted in Laemmli sample buffer, and analyzed for bound Cdc42, Rac1 or RhoA by western blotting. Aliquots of total lysates served as a control for total amounts of proteins. GTPases were detected using antibodies against Cdc42 (rabbit polyclonal antibody from Santa Cruz Biotechnology), Rac1 (mAb from Upstate Biotechnology) or RhoA (mAb from Santa Cruz Biotechnology).

Detection of PKC isotypes by western blotting

Cells were cultured in the presence or absence of PMA (50 nM) and washed once in PBS. Cell lysates were subjected to SDS-PAGE. Proteins were transferred to polyvinylidine difluoride membranes (Immobilon P, Millipore) by western blotting. Membranes were blocked for 1 hour with PBS containing 0.2% (w/v) I-Block reagent and 0.1% (v/v) Tween-20 and incubated overnight with PKC-isotype-specific antibodies diluted 1:500 in PBS containing 0.1% (w/v) I-Block reagent and 0.2% (v/v) Tween-20. To demonstrate the specificity of the reaction, control membranes were incubated with PKC-isotype-specific antibodies in the presence of the corresponding PKC-isotype-specific blocking peptide diluted 1:1000. Membranes were washed with PBS containing 0.3% (v/v) Tween-20 and incubated for 1 hour with goat-anti-rabbit IgG antibodies conjugated to alkaline phosphatase diluted 1:1000 in PBS containing 0.1% (w/v) I-Block reagent and 0.2% (v/v) Tween-20. Membranes were washed as before and stained with 0.1 M diethanolamine, 0.34 mg/ml Nitroblue tetrazolium, 0.18 mg/ml 5-bromo-4-chloro-3-indolylphosphate and 50 mM $MgCl_2$.

Confocal laser scan microscopy

Cells were mounted on poly-L-lysine coated glass slides and, where indicated, treated with CytD (2.5 μg/ml) and/or chelerythrine chloride (5 μM) for 30 minutes and 10 minutes, respectively. Cells were fixed with 3.7% formaldehyde, stained for 30 minutes at RT with mAb AZN-L50 and subsequently incubated with Alexa 488-conjugated goat-anti-mouse IgG and Texas Red-X phalloidin for 30 minutes at RT. ALCAM and actin distribution were analyzed by CLSM at 488 nm with a krypton/argon laser on a MRC1024 confocal microscope (BioRad). The instrument settings were: lens 60×, magnification 2.5×, iris 2.3 nm, gain 1355 (red) or 1440 (green), laser 26% (red) or 34% (green).

Radioactive cell labeling and immunoprecipitation

Cells were cultured for 16 hours in the presence or absence of PMA (50 nM) and were pre-incubated for 1 hour in serum- and phosphate-free RPMI 1640 medium prior to labeling with [^{32}P]-disodiumphosphate, 400 μCi per 5×10^6 cells for 3 hours at 37°C. Subsequently, cells were treated with or without 50 nM PMA for 15 minutes at 37°C. Alternatively, cells were labeled for 16 hours with a mixture of [^{35}S]methionine/cysteine (500 μCi per 10×10^6 cells) in serum- and methionine/cysteine-free RPMI 1640 medium. Cell lysates were subjected to immunoprecipitation with 1 μg of AZN-L51 antibody coupled to Protein G Sepharose 4 Fast Flow beads (Amersham Biosciences). Bound proteins were eluted by boiling in Laemli sample buffer and were analyzed on a 10% SDS-PAGE gel under reducing conditions.

Results

Induction of cytoskeleton-dependent ALCAM-mediated adhesion is not caused by enhanced ligand-binding affinity

KG1 myelomonocytic cells and ALCAM-transfected K562 cells (K562-ALCAM) both express similar levels of ALCAM at the cell surface (Fig. 1A). We have shown that ALCAM-mediated adhesion of K562-ALCAM cells is dynamically regulated through the actin cytoskeleton by treating the cells with agents that specifically disrupt the cortical actin cytoskeleton (Nelissen et al., 2000b). Likewise, treatment of KG1 cells with either CytD or LatA strongly induces ALCAM-specific adhesion to immobilized ALCAM-Fc (Fig. 1B). Kinetics and ALCAM specificity of KG1 ALCAM-mediated

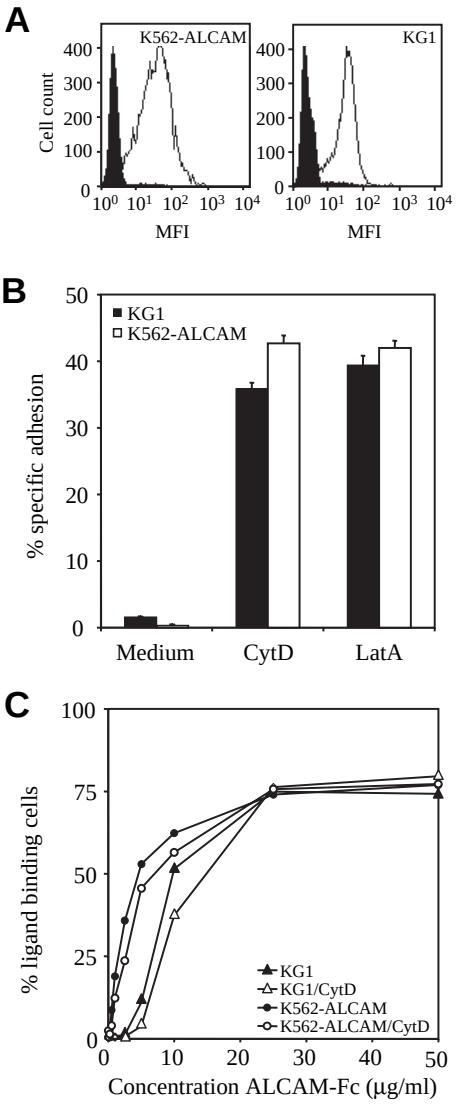

Fig. 1. (A) Flow-cytometric analysis of ALCAM expression by KG1 and K562-ALCAM cells. The shaded histograms represent the isotype control, whereas the white histograms represent ALCAM staining with AZN-L50. (B) ALCAM-mediated adhesion of both K562-ALCAM (white bars) and KG1 cells (black bars) to immobilized ALCAM-Fc requires disruption of the actin cytoskeleton. The cells were pre-incubated for 30 minutes at 37°C with or without CytD (2.5 μg/ml) or LatA (5 μg/ml) and subsequently allowed to adhere to an ALCAM-Fc coated plate (250 ng/ml ALCAM-Fc) for 45 minutes at 37°C in the presence or absence of the ALCAM-blocking mAb AZN-L50. Non-ALCAM antibodies did not affect binding, showing that the observed adhesion is ALCAM-mediated (data not shown). Specific adhesion is expressed as the mean percentage (±s.d.) of adherent cells from triplicate wells after subtraction of the adhesion in the presence of the blocking mAb AZN-L50. Data are representative of three experiments. (C) CytD treatment does not alter the affinity of K562-ALCAM (circles) and KG1 cells (triangles) for ALCAM-Fc. Cells were treated with (open symbols) or without (closed symbols) CytD (as in B) and subsequently incubated with the indicated dilutions of ALCAM-Fc. The percentage of cells that have bound ALCAM-Fc is detected by flow cytometry using a FITC-conjugated secondary goat-anti-human-Fc antibody.

adhesion are similar to what has been published for K562-ALCAM (Nelissen et al., 2000b) (Fig. 1B).

To investigate whether CytD-induced adhesion is regulated only by the release from cytoskeletal constraints (avidity regulation) or also affects the relative affinity of ALCAM-expressing K562 and KG1 cells, we compared binding to immobilized ALCAM with that of soluble ALCAM-Fc fusion proteins. CytD treatment does not increase the affinity of ALCAM-expressing cells for ALCAM-Fc (Fig. 1C). Similar results were obtained with LatA (data not shown). These findings demonstrate that release of ALCAM from the actin cytoskeleton leads to an increase in avidity (i.e. molecule clustering) rather than in affinity.

CytD, but not ALCAM-mediated adhesion, causes activation of RhoA, Rac1 and Cdc42

Disruption of the actin cytoskeleton by CytD treatment results in increased lateral mobility and cluster formation of integrins (Elemer and Edgington, 1994; Lub et al., 1995), cadherins (Baumgartner et al., 2003), and other cell adhesion molecules, such as Ep-CAM (Balzar et al., 1998), leading to increased adhesion. In addition to disrupting the actin cytoskeleton, Ren and co-workers (Ren et al., 1999) showed that in quiescent Swiss 3T3 fibroblasts CytD treatment activates RhoA. This, together with our previous observations that ALCAM-mediated adhesion is sensitive to cytoskeletal restraints (Nelissen et al., 2000b), raises the possibility that effects of CytD or LatA on ALCAM-mediated adhesion could at least in part be due to the activation of Rho-like GTPases. We analyzed the effect of CytD or LatA treatment on the activation of the small GTPases RhoA, Rac1 and Cdc42 in K562-ALCAM and KG1 cells in suspension. Upon treatment of serum-starved K562-ALCAM cells in suspension with 2.5 μg/ml CytD [the optimal concentration for inducing ALCAM-mediated adhesion (Nelissen et al., 2000b)], we observed a time-dependent and transient activation of RhoA. Interestingly, not only RhoA, but also Cdc42 and to a lesser extent Rac1 are activated upon CytD treatment (Fig. 2A). Similar observations were made with parental K562 cells, and when LatA instead of CytD was used (data not shown). In KG1 cells, a comparable time-dependent activation of RhoA and Rac1 was observed (Fig. 2B), though there are differences in kinetics. In K562-ALCAM cells, RhoA activation is maximal after 30-60 minutes, while Rac GTP levels are high already in untreated cells and are only slightly upregulated by CytD. In contrast, KG1 cells show highest RhoA activity already after 5-15 minutes of CytD treatment while maximum Rac1 activity is reached after 30-60 minutes.

Conversely, the activation state of the RhoA, Rac1 and Cdc42 was not altered by adhesion of cells to immobilized ALCAM or by cross-linking of ALCAM at the cell surface (data not shown), demonstrating that ALCAM-mediated adhesion itself does not induce activation of the Rho-like GTPases.

Dominant negative or constitutively active Rho-like GTPase mutants do not affect cytoskeleton-dependent ALCAM-mediated adhesion

To further investigate involvement of Rho-like GTPases in the

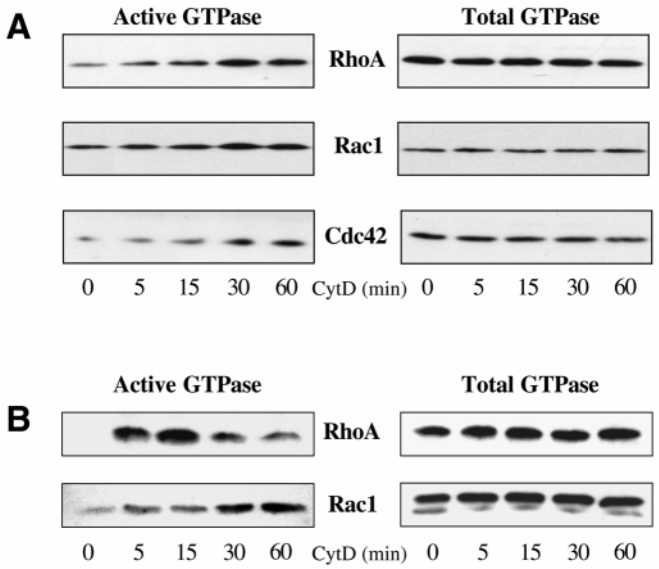

Fig. 2. CytD and LatA transiently activate RhoA, Rac1 and Cdc42. K562-ALCAM cells (A) and KG1 cells (B) in suspension were treated with 2.5 μg/ml CytD for the indicated time. Subsequently, cell lysates were incubated with GST-PAK-CD or GST-C21 and the bound RhoA, Rac1 and Cdc42 molecules were detected by western blot with the respective antibodies. Total lysates served as a control for analyzing total amounts of RhoA, Rac1 or Cdc42 on western blot using the respective antibodies. Similar observations were made using parental K562 cells (data not shown).

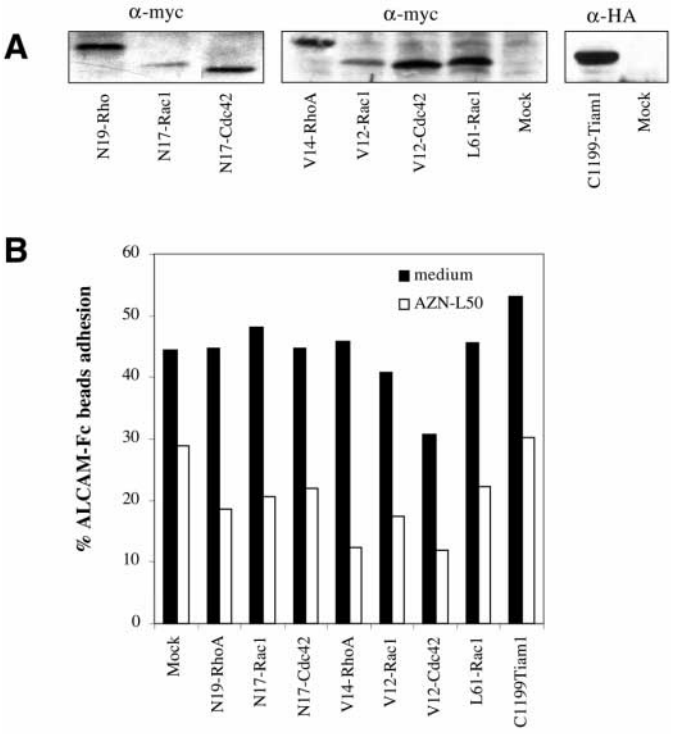

Fig. 3. (A) Expression of Myc-tagged Rho-like GTPase-mutants and HA-tagged C1199-Tiam1 in KG1. KG1 cells were retrovirally transduced with dominant negative (N19-RhoA, N17-Rac1, N17-Cdc42) or constitutively active (V14-RhoA, V12-Rac1, V12-Cdc42, L61-Rac1) GTPase mutants, or with C1199-Tiam1. Cells were lysed and expression of the mutants was detected by western blotting using anti-Myc and anti-HA antibodies, respectively. C1199-Tiam1 and the Rho-like GTPase mutants migrated according to the expected heights (RhoA > Rac1=Cdc42). (B) Rho-like GTPase mutants and C1199-Tiam1 do not alter adhesion of KG1 cells. Cells expressing either dominant negative GTPase (N19-RhoA, N17-Rac1, N17-Cdc42), constitutively active GTPase (V14-RhoA, V12-Rac1, V12-Cdc42, L61-Rac1) or C1199-Tiam1 were allowed to adhere to ALCAM-Fc-coated beads after pre-incubation with (white bars) or without (black bars) mAb AZN-L50, for 45 minutes at 37°C. Adhesion is expressed as the percentage of bound cells. Data are representative of three independent experiments.

induction of ALCAM-mediated adhesion, KG1 cells were retrovirally transduced with dominant negative (N19-RhoA, N17-Rac1 and N17-Cdc42) or constitutively active (V14-RhoA, V12-Rac1, L61-Rac1 and V12-Cdc42) GTPase mutants. A constitutively active variant of Tiam-1 (C1199-Tiam-1), an activator of the small GTPase Rac1 (Michiels et al., 1995), was used as an additional control to investigate the involvement of Rac1 in ALCAM-mediated adhesion. Expression of all GTPase variants and C1199-Tiam-1 was confirmed by western blotting (Fig. 3A). ALCAM surface expression, determined by flow cytometry, was not affected by stable expression of either of the Rho-like GTPase mutants or C1199-Tiam1 (data not shown).

Spontaneous adhesion to ALCAM-Fc coated beads was neither significantly altered upon expression of any of the Rho-like GTPase mutants or C1199-Tiam1 in KG1 cells (Fig. 3B), nor did we observe induction of spontaneous adhesion or alteration of CytD-induced adhesion to immobilized ALCAM-Fc (data not shown). Furthermore, treatment of KG1 and K562 ALCAM cells with the Rho-kinase inhibitor Y-27632 did not affect adhesion to both soluble and immobilized ALCAM (data not shown). Together, these data strongly indicate that Rho GTPase-activity does not affect ALCAM-mediated adhesion.

Cytoskeleton-dependent, homotypic ALCAM-mediated adhesion requires active PKC

PKC isoforms play a pivotal role in immune cell signaling and the regulation of cell adhesion, and have been implicated in cytoskeletal rearrangements (Masson-Gadais et al., 1997; Keenan and Kelleher, 1998; Volkov et al., 2001). Therefore, we investigated the role of PKC in the molecular mechanisms underlying cytoskeleton-dependent, homotypic ALCAM-mediated adhesion. KG1 and K562-ALCAM cells were pre-treated with CytD in combination with increasing amounts of either the PKC inhibitor chelerythrine chloride or with an inhibitory myristoylated PKC pseudosubstrate, specific for PKCα and PKCβ (Fig. 4A). A significant, concentration-dependent reduction of CytD-induced ALCAM-mediated adhesion was observed for both ALCAM-expressing cell lines.

To investigate whether the necessity for PKC is restricted to CytD or whether this applies to induction of ALCAM-mediated adhesion upon disruption of the actin cytoskeleton in general, the effect of chelerythrine chloride (5 μM) and myristoylated PKC pseudosubstrate (100 μM) on LatA-induced ALCAM-mediated adhesion was assessed. Inhibition of PKC not only reduced CytD-induced ALCAM-mediated adhesion, but also significantly inhibited LatA-induced

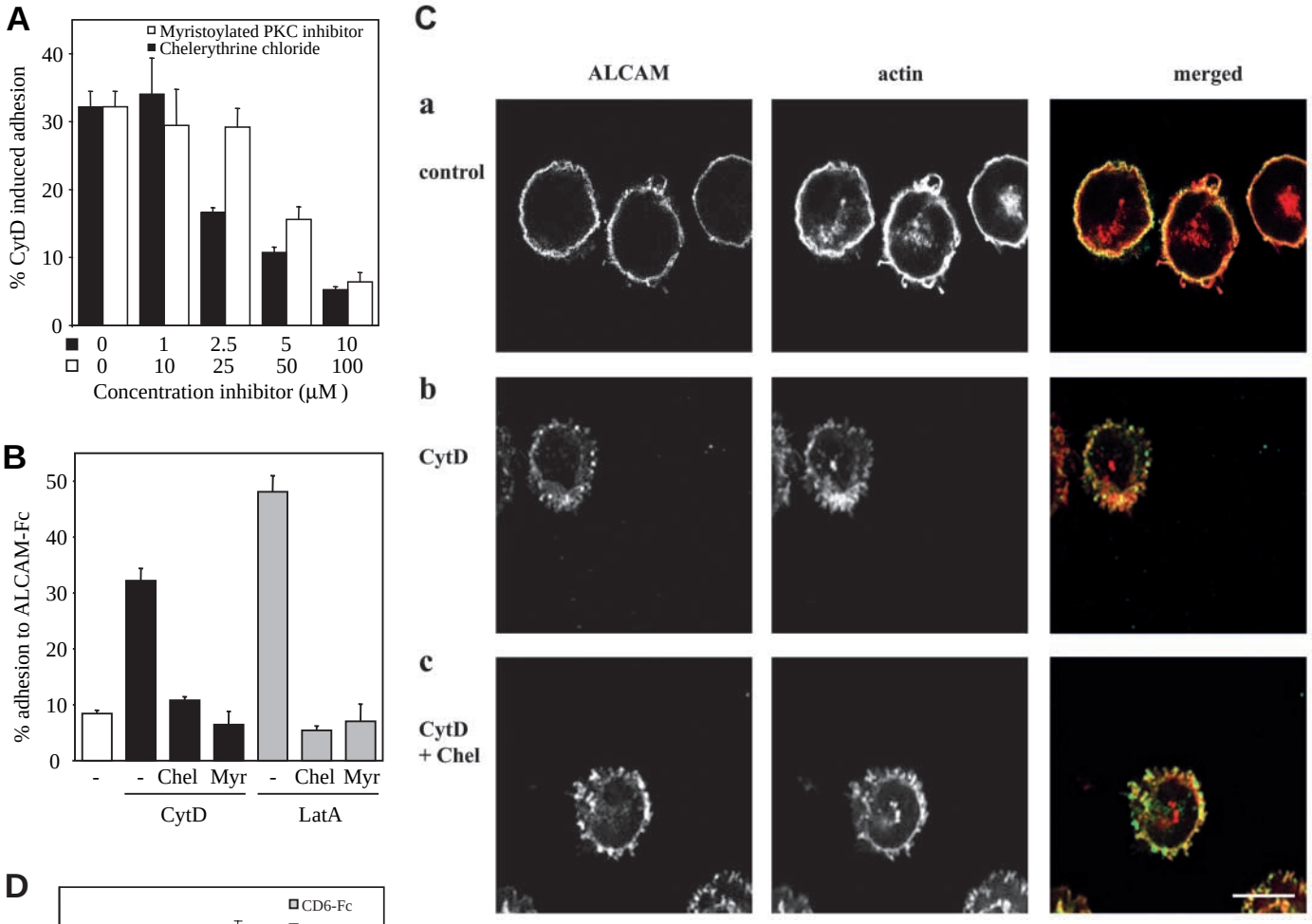

Fig. 4. (A) Inhibition of PKC dose-dependently reduced CytD-induced adhesion. KG1 cells were pre-treated for 10 minutes at 37°C with increasing concentrations of either chelerythrine chloride (black bars) or myristoylated PKC inhibitor (white bars) in combination with CytD (2.5 μg/ml). Both inhibitors of PKC significantly reduce CytD-induced adhesion to immobilized ALCAM. (B) PKC requirement is not restricted to CytD but applies to LatA as well. KG1 cells were pre-treated with chelerythrine chloride (Chel, 5 μM) or myristoylated PKC inhibitor (Myr, 100 μM) for 10 minutes at 37°C in combination with either CytD (2.5 μg/ml, black bars) or LatA (5 μg/ml, gray bars). Induction of adhesion by both cytoskeleton-disrupting drugs is equally inhibited by both chelerythrine chloride and myristoylated PKC inhibitor, demonstrating that the requirement for PKC is not restricted to the mode of action of CytD in itself. Similar observations were made for K562-ALCAM (not shown). (C) Analysis of the distribution of ALCAM and actin by CLSM. KG1 cells were pre-treated without (a) or with CytD (2.5 μg/ml, b), or with a combination of CytD and chelerythine chloride (Chel, 5 μM, c). Cells were stained with mAb AZN-L50 (green) and Texas Red-X phalloidin (red). For each preparation the same instrument settings were used. The scale bar represents 10 μm. Similar results were obtained with K562-ALCAM cells (not shown). (D) PKC requirement is not restricted to homotypic ALCAM-mediated adhesion induced by cytoskeleton-disrupting agents, but also applies to heterotypic ALCAM-CD6 adhesion. KG1 cells were pre-treated with PKC inhibitors as described in B, in the presence or absence of CytD (2.5 μg/ml). Cells were allowed to adhere to a plate coated with 250 ng/ml of CD6-Fc (white bars) or ALCAM-Fc (black bars) for 45 minutes at 37°C. Both spontaneous and CytD-induced ALCAM-CD6 adhesion are inhibited by chelerithrine chloride and myristoylated PKC inhibitor. Similar observations were made for K562-ALCAM cells (not shown).

adhesion (Fig. 4B), suggesting a contribution of PKC to clustering and subsequent stable adhesion. The effect of PKC inhibition on ALCAM-mediated adhesion was not due to a direct effect of CytD or LatA on PKC, since PKC activity, as determined by Bosch et al. (Bosch et al., 1998), was not altered in the presence of CytD or LatA (data not shown). Activation of PKC by the addition of PMA (50 nM) did not induce adhesion (data not shown), indicating that PKC alone is not sufficient to overcome the cytoskeletal restraints, but that disruption of the actin cytoskeleton has to coincide with PKC action.

To investigate whether the inhibition of PKC affects the cell surface distribution of ALCAM, CLSM analysis was performed. In non-treated KG1 cells, ALCAM is randomly distributed at the plasma membrane and co-localizes with cortical actin (Fig. 4Ca). Upon treatment with CytD we

Table 1. Expression of PKC isotypes in K562-ALCAM and KG1 cells, as detected by western blot analysis

PKC isotype	K562-ALCAM	KG1	Control*
α	++	+	++
β	–	–	+
γ	–	–	+
ε	–	–	++
η	+/–	+/–	+
ζ	+	+	+
θ	+	+	++

*Rabbit-brain homogenate.
–, no expression; +/–, weak expression; +, expression; ++, high expression.

observed a marked clustering of ALCAM at the cell surface, and these clusters partially co-localize with the local actin accumulations induced by CytD (Fig. 4Cb). Addition of the PKC inhibitor chelerythrine chloride did not affect the CytD-induced ALCAM clusters (Fig. 4Cc). Similar findings were obtained with myristoylated PKC inhibitor. Furthermore, neither chelerythrine chloride nor myristoylated PKC inhibitor had an effect on cells that were not incubated with CytD (data not shown). These results indicate that inhibition of PKC does not lead to changes in the cell surface distribution of ALCAM, suggesting that the involvement of PKC in ALCAM-mediated adhesion does not take place at the level of cluster formation.

PKC is involved in both homotypic and heterotypic ALCAM-mediated adhesion

To investigate whether PKC involvement applies only to homotypic ALCAM-ALCAM adhesion or also regulates heterotypic adhesion of ALCAM to CD6 (the other physiological ligand of ALCAM), we also examined the interaction between ALCAM and immobilized CD6-Fc. In contrast to ALCAM-ALCAM binding, we observed a spontaneous interaction between ALCAM and CD6 that did not require disruption of the actin cytoskeleton by CytD (Fig. 4D). However, adhesion was further increased after pre-treatment with CytD. Inhibition of PKC by specific inhibitors resulted in a markedly reduced ALCAM-CD6 binding, similar to what we observed for the CytD-induced, homotypic ALCAM-ALCAM interaction. These data point to an important role of PKC in the regulation of both homotypic and heterotypic adhesion mediated by ALCAM.

PKCα is the PKC isoform involved in ALCAM-mediated adhesion

To further explore the involvement of PKC in ALCAM-mediated adhesion, cells were cultured for 24 hours with PMA (50 nM) to induce downregulation of PKC. Western blot analysis revealed the expression of a number of PKC isotypes in KG1 and K562-ALCAM cells (Table 1). We observed that long-term PMA treatment dramatically lowered the amounts of PKCα and PKCθ (Fig. 5A), rendering homotypic ALCAM-mediated adhesion unresponsive to CytD and LatA (Fig. 5B), and reducing spontaneous K562-ALCAM adhesion to CD6-Fc (not shown). Of note, this treatment did not affect the levels of PKCζ and PKCη (data not shown). Importantly, the overall surface expression of ALCAM is not altered after long-term

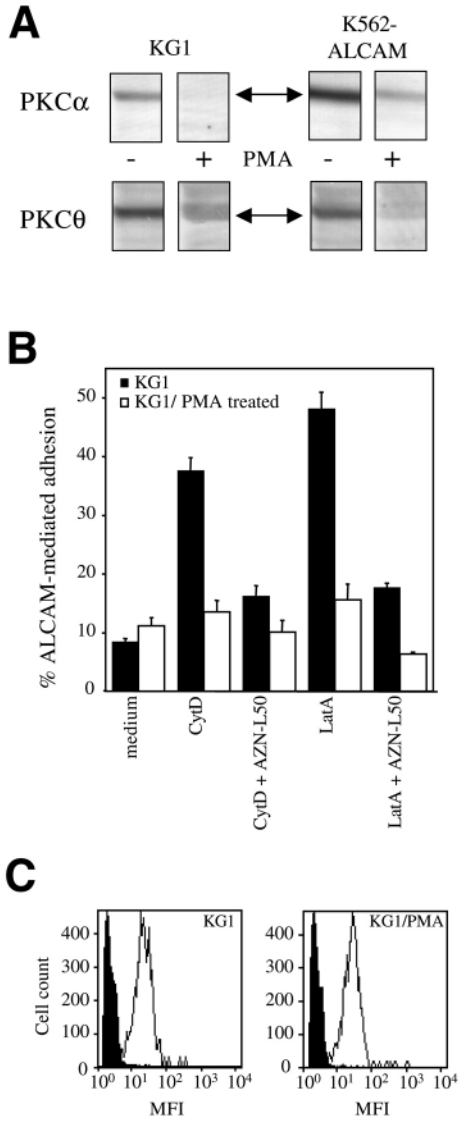

Fig. 5. (A) Long-term PMA treatment leads to downregulation of PKCα and PKCθ. KG1 and K562-ALCAM cells were cultured for 24 hours in presence or absence of PMA (50 nM) to induce downregulation of PKC. PKC expression was analyzed by western blotting. PKC isotypes α and θ are both downregulated upon culturing in the presence of PMA in both cell lines. Expression of PKC isotypes ζ and η was not affected (data not shown). (B) PMA culturing inhibits cytoskeleton-dependent ALCAM-mediated adhesion. KG1 cells were cultured for 16 hours in the presence (white bars) or absence (black bars) of 50 nM PMA. Subsequently, induction of adhesion by pre-incubation of the cells for 30 minutes at 37°C with CytD (2.5 μg/ml) or LatA (5 μg/ml) was determined, in the presence or absence of the blocking antibody AZN-L50 (10 μg/ml). Downregulation of PKC upon culturing with PMA inhibits CytD- and LatA-induced adhesion to background levels. Similar observations were made for K562-ALCAM (not shown). (C) PMA culturing does not affect the overall ALCAM expression. ALCAM expression on KG1 cultured in the presence or absence of PMA (50 nM) was determined by flow cytometry. Shaded histograms represent isotype control staining and white histograms represent ALCAM staining with mAb AZN-L50.

exposure to PMA (Fig. 5C). These data demonstrate a requirement for PKC in the regulation of ALCAM-mediated adhesion. PKCα is the key player in this process, since the inhibitor myristoylated PKC pseudosubstrate is specific for PKCα and PKCβ (but PKCβ is not expressed), and because strong downregulation of PKCα and PKCθ was observed (Fig. 5A).

PKCα modulates the avidity rather than the affinity of ALCAM

To investigate whether the block in CytD-induced ALCAM-activation by inhibition or downregulation of PKCα reflects changes in avidity or affinity of ALCAM, we performed a soluble ALCAM-Fc-binding assay. Downregulation of PKCα decreased the ligand-binding capacity of ALCAM expressed by KG1 to a limited extent, while the affinity of K562-ALCAM for ALCAM-Fc was not affected. Inhibition of PKC by chelerythrine chloride or myristoylated PKC pseudosubstrate did not significantly affect the affinity of either ALCAM-expressing cells for ALCAM-Fc (Fig. 6A,B). These data indicate that PKCα is involved in avidity- rather than affinity-modulation of ALCAM.

Serine and threonine residues in the cytoplasmic domain of ALCAM are not required for ALCAM-mediated adhesion

We have previously demonstrated the importance of the ALCAM cytoplasmic domain for cytoskeleton-dependent adhesion using a GPI-linked mutant of ALCAM (Nelissen et al., 2000b). To investigate whether the serine/threonine residues in the cytoplasmic tail are direct PKC targets and important in CytD-induced adhesion, we exchanged either the serine or threonine residues of the cytoplasmic domain for alanine residues. Both mutant ALCAM molecules were transfected in K562 cells (yielding K562-ALCAMΔSer and K562-ALCAMΔThr, respectively) and selected for expression levels comparable with those of wild-type ALCAM (Fig. 7A). Subsequently, the effect of the mutations on ALCAM-mediated adhesion was analyzed. Neither substitution of the serine-residues, nor of the threonine-residues had any effect on spontaneous or CytD-induced ALCAM-mediated adhesion (Fig. 7B).

We further investigated putative ALCAM phosphorylation by immunoprecipitation of ALCAM from [^{32}P]-phosphate labeled K562-ALCAM and KG1 cells. No phosphorylated ALCAM was detected in cells labeled with [^{32}P]-phosphate, not even after PKC stimulation with PMA (Fig. 7Cb). Long-term PMA treatment resulted in an overall decrease of protein phosphorylation due to downregulation of PKC activity. Similar results were obtained with KG1 cells. As a control, the presence of ALCAM in K562-ALCAM cells (but not in parental K562 cells) was confirmed by immunoprecipitation from [^{35}S]-Met/Cys labeled cell lysates (Fig. 7Ca). In addition, western blot analysis using phospho-serine- and phospho-threonine-specific antibodies showed no serine- or threonine-phosphorylation of wild-type ALCAM after immunoprecipitation (data not shown). Together, our findings demonstrate that the cytoplasmic tail is not a direct target of PKC or other serine/threonine kinases, but that PKC probably phosphorylates other (as yet unknown) molecules involved in the control of ALCAM-mediated adhesion.

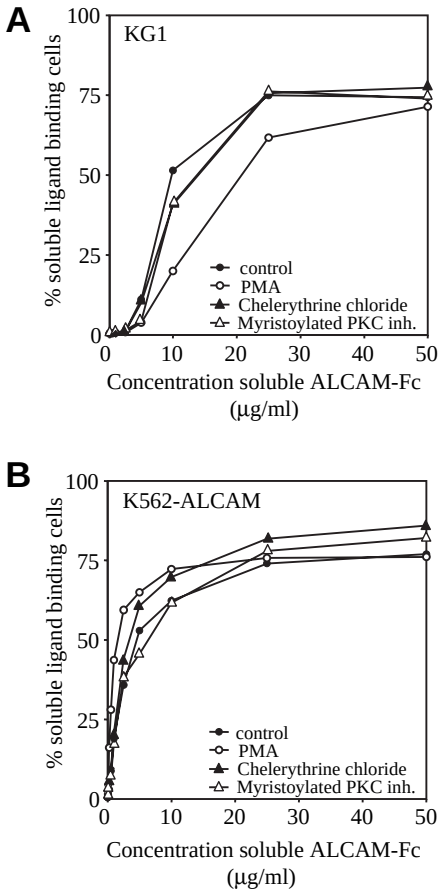

Fig. 6. Effect of PKC downregulation or PKC inhibition on ALCAM-affinity. Soluble ALCAM-Fc-binding assay with KG1 cells (A) and K562-ALCAM (B) either cultured for 16 hours with (○) or without (●) 50 nM PMA or pre-incubated for 10 minutes at 37°C with chelerythrine chloride (5 μM, ▲) or myristoylated PKC pseudosubstrate (100 μM, △). Cells are incubated with increasing concentrations of soluble ALCAM-Fc and the percentage of cells that have bound ligand is determined by flow cytometry. Data are representative of three independent experiments.

Discussion

Homotypic ALCAM-mediated adhesion is dynamically regulated through the actin cytoskeleton. In the present study we examined two signaling pathways potentially involved in the regulation of ALCAM-mediated adhesion.

Since Rho-like GTPases are key players in regulating cytoskeleton dynamics (Hall, 1998) and cellular adhesion (D'Souza-Schorey et al., 1998; Gimond et al., 1999; Braga, 2002), we analyzed the involvement of RhoA, Rac1 and Cdc42 in ALCAM-mediated, cytoskeleton-dependent adhesion. We observed activation of RhoA by CytD or LatA treatment in two hematopoietic cell lines, in accordance with the findings of Ren and co-workers (Ren et al., 1999), who demonstrated that CytD treatment activated RhoA in fibroblasts. Interestingly, we found that, in addition to RhoA, Rac1 and Cdc42 are activated in response to CytD. The observed differences in GTPase

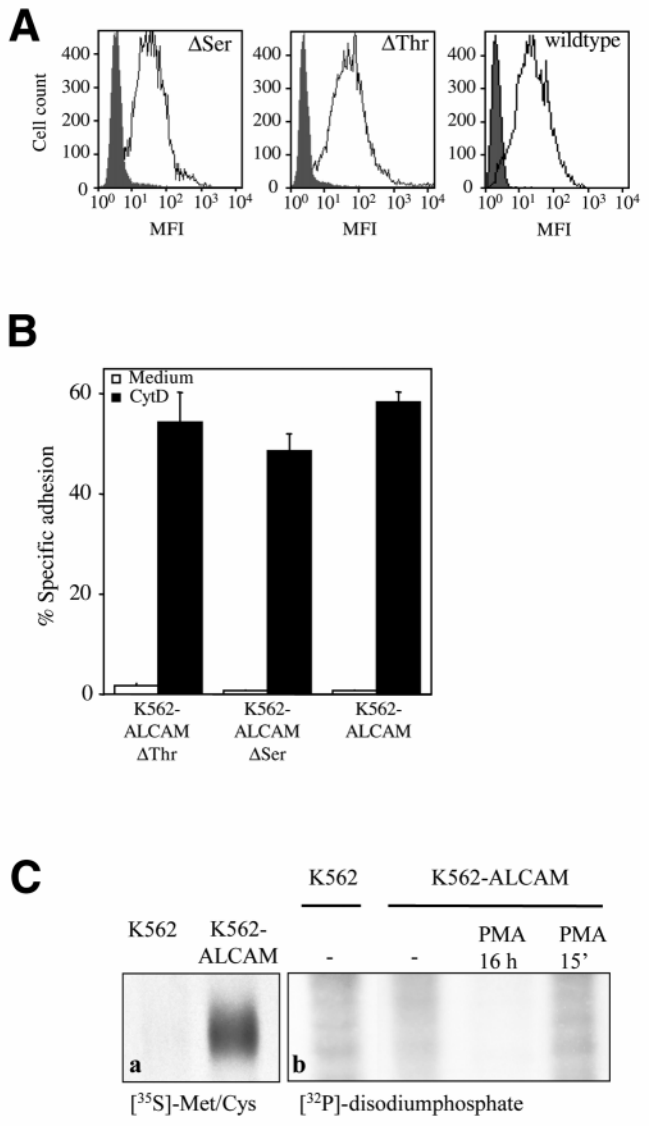

Fig. 7. (A) Surface expression of ALCAM on K562-ALCAMΔSer, K562-ALCAMΔThr and wild-type K562-ALCAM. K562 cells were transfected with expression constructs coding for mutant ALCAM proteins and were sorted to obtain homogeneous cell populations expressing similar levels of mutant ALCAM. Expression was analyzed by flow cytometry. The shaded histograms represent isotype control staining and the white histograms represent staining with ALCAM-antibody AZN-L50. (B) Replacement of serine- or threonine residues in the cytoplasmic domain of ALCAM does not affect spontaneous or CytD-induced adhesion. Adhesion of K562-ALCAMΔSer, K562-ALCAMΔThr and K562-ALCAM was analyzed after treatment of cells with or without CytD (2.5 μg/ml) as in Fig. 1B. Wild-type and mutant ALCAM-expressing cells show similar patterns of adhesion. Data are representative of three experiments. (C) No evidence for phosphorylation of ALCAM after treatment with [^{32}P]-phosphate. K562 and K562-ALCAM cells were incubated overnight with [^{35}S]methionine/cysteine (a) or for 3 hours with [^{32}P]-disodiumphosphate (b). Cells were pre-incubated with 50 nM PMA for 16 hours prior to [^{32}P]-phosphate labeling (PKC downregulation), or 15 minutes after [^{32}P]-phosphate labeling (PKC activation), respectively. ALCAM was immunoprecipitated from labeled cell lysates with 1 μg of AZN-L51. ALCAM was detected in [^{35}S]methionine/cysteine labeled K562-ALCAM lysate, but no [^{32}P]phosphate-labeled ALCAM could be detected, not even after PMA stimulation. Long-term PMA treatment resulted in decreased overall protein phosphorylation. Similar observations were made with KG1 cells (not shown).

activation kinetics between K562 and KG1 cells may be due to distinct sensitivity to actin cytoskeleton-disrupting agents, or differences in the association of RhoGAPs and/or RhoGEFs. No significant differences with respect to activation of Rho-like GTPases were observed between ALCAM-expressing cells plated on immobilized ALCAM-Fc or on BSA as a control substrate. In fibroblasts, Rho activation is regulated by a negative feedback loop dependent on cell-matrix adhesion, resulting in downregulation of Rho in adherent cells, whereas active Rho levels remain elevated in suspension cells (Ren et al., 1999). Our data provide no evidence for the existence of such a negative feedback loop induced by ALCAM-ALCAM-mediated cell adhesion.

Expression of dominant negative or constitutively active mutants of the Rho-like GTPases neither affected spontaneous nor CytD-induced ALCAM-mediated adhesion. Furthermore, constitutive activation of Rac1 caused by the introduction of C1199-Tiam1, or treatment with the Rho-kinase inhibitor Y-27632 had no effect on ALCAM-mediated adhesion. In contrast, β1-integrin-mediated adhesion and spreading on fibronectin is enhanced by expression of constitutively active Rac1 and Cdc42 in Jurkat cells (Del Pozo et al., 2003). We conclude that, in sharp contrast to adhesion mediated by integrins and cadherins, RhoA, Rac1 or Cdc42 are neither activated in response to ALCAM activation, nor directly required for induction of ALCAM-mediated adhesion.

Instead, PKC appears to be an important player in the induction of cytoskeleton-dependent, homotypic ALCAM-mediated adhesion. Based on the specificity of the PKC inhibitors used, and on the pattern of downregulation of PKC upon long-term PMA treatment, PKCα is the dominant isoform driving adhesion by ALCAM. Although the involvement of PKCθ can never be completely ruled out, it is unlikely that PKCθ plays a significant role. PKCα has been shown to co-localize with a range of cytoskeletal proteins including intermediate filament proteins (vimentin), membrane-cytoskeletal cross-linking proteins (MARCKS, ankyrin), and components of the actin filaments (F-actin) and microtubules (tubulin) (Keenan and Kelleher, 1998; Slater et al., 2000), and is widely implicated in integrin-mediated adhesion (Altman and Villalba, 2002; Li et al., 1998; McDowall et al., 2003).

All PKC isoforms have been shown to associate with F-actin (Slater et al., 2000). The actin cytoskeleton and PKC probably act in a coordinated manner, since disruption of the actin cytoskeleton by CytD caused a dose-dependent increase in expression and activation of PKCα in mesenchymal cells (Lim et al., 2000). These findings may explain the requirement for both disruption of the actin cytoskeleton and the presence of active PKCα for homotypic ALCAM-mediated adhesion in K562-ALCAM and KG1 cells. The crucial role for PKCα in ALCAM-mediated adhesion is strengthened by the fact that heterotypic ALCAM-CD6 adhesion requires active PKCα. Both CytD and PMA treatment lead to an equivalent increase in lateral mobility and adhesion of β2-integrins (Kucik et al., 1996; McDowall et al., 1998). However, for ALCAM-mediated

adhesion, stimulation with phorbol ester alone is not sufficient to induce adhesion.

The macrophage-enriched, myristoylated, alanine-rich C kinase substrate (MacMARCKS), a direct PKC substrate, was shown to be required for releasing the cytoskeletal restraints on integrin molecules during PKC-mediated integrin activation (Zhou and Li, 2000). However, we observed that PKC activation (and hence the subsequent phosphorylation of MARCKS) by short-term treatment with PMA neither induced spontaneous adhesion nor altered CytD-induced adhesion. Thus, although we cannot rule out a function for MARCKS in ALCAM-mediated adhesion, MARCKS activation alone is not sufficient for the induction of adhesion.

The cytoplasmic domain of ALCAM is not a direct target for PKC, since it was not phosphorylated, not even after PKC activation (Fig. 7C). Although two serines and two threonines are present in the cytoplasmic domain of ALCAM, the cytoplasmic domain does not contain conserved PKC-phosphorylation motifs. Moreover, mutation of either the serine or threonine residues did not affect cytoskeleton-dependent adhesion. Therefore, induction of ALCAM-mediated adhesion in response to CytD and PKC probably depends on other cytoskeletal components subject to PKC-regulation.

An important group of proteins that constitute a link between extracellular receptors and the intracellular cytoskeleton is the ezrin/radixin/moesin (ERM) family. These proteins bind actin filaments through their C-terminal domain and the conserved globular N-terminal half associates with the cytoplasmic domains of several other transmembrane adhesion receptors such as CD43 (Serrador et al., 1998), CD44 (Legg and Isacke, 1998), ICAM-1, ICAM-2 (Heiska et al., 1998), ICAM-3 (Serrador et al., 1997) and L1-CAM (Dickson et al., 2002). ERM proteins, in cooperation with Rho-like GTPases, are involved in the formation of stress fibers and focal adhesions (Mackay and Hall, 1998) and in the clustering of adhesion receptors (Wojciak-Stothard et al., 1999). They bind positively charged amino acid clusters in the cytoplasmic domains of CD44, CD43 and ICAM-2 (Yonemura et al., 1998), which makes this family of proteins candidates to bind to the highly charged cytoplasmic domain of ALCAM as well. However, thus far we have not been successful in confirming this association by GST pulldown assays or immunoprecipitations (our unpublished results), again demonstrating that ALCAM-mediated adhesion is differently regulated compared with other adhesion molecules.

Two actin bundling proteins, fascin and filamin, are substrates for PKCα and are candidates in the cytoskeleton-dependent regulation of ALCAM-mediated adhesion. Filamin promotes actin filament branching and stabilizes microfilament networks (Gorlin et al., 1990). Some β-integrin tails bind tightly to filamin, thereby restricting integrin-dependent cell migration by inhibiting transient membrane protrusion and cell polarization (Calderwood et al., 2001). Fascin is localized mainly at filopodia and membrane ruffles, as well as in stress fibres (Yamashiro-Matsumura and Matsumura, 1986). The actin bundling activity of fascin modulates the actin cytoskeleton, and is regulated by phosphorylation by PKCα (Adams et al., 1999).

Tomita and co-workers reported that ALCAM is co-recruited to sites of cell-cell contact upon correct assembly of E-cadherin/α-catenin complexes in epithelial cells (Tomita et al., 2000). Catenins link cadherins and, for example, PECAM-1 to the actin cytoskeleton (Ilan et al., 1999). Moreover, catenins can be phosphorylated by PKC and association of γ-catenin to PECAM-1 depends on phosphorylation (Ilan et al., 2000). Thus, catenins constitute a link between various adhesion molecules and PKC-signaling pathways. These findings suggest that α-catenin is another potential candidate linking the ALCAM-cytoplasmic domain to the actin cytoskeleton.

Although the PKC-substrate responsible for mediating ALCAM-mediated adhesion remains to be identified, we show here that the release of ALCAM from cytoskeletal restraints is essential for the induction of adhesion and requires PKCα. This PKC involvement is restricted to avidity modulation, since ligand-binding affinity is not changed by activation or inhibition of PKC. However, PKCα is not directly involved in ALCAM cluster formation, since PKC inhibition does not affect the cell surface distribution of ALCAM. Interestingly, regulation of ALCAM by the cytoskeleton differs from other adhesion molecules, such as integrins, cadherins and other Ig-like cell adhesion molecules, in that small Rho-like GTPases do not appear to be directly involved. Future research will unravel the physiological stimuli that generate ‘inside-out’ signals that induce cytoskeleton- and PKC-dependent ALCAM-mediated adhesion, either through avidity or affinity modulation.

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