CUTL1 Is Phosphorylated by Protein Kinase A, Modulating Its Effects on Cell Proliferation and Motility*

Received for publication, January 30, 2006, and in revised form, March 29, 2006 Published, JBC Papers in Press, March 30, 2006, DOI 10.1074/jbc.M600908200

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CUTL1, also known as CDP (CCAAT Displacement Protein), Cut, or Cux-1, is a homeodomain transcription factor known to play an essential role in development and cell cycle progression. Previously, we identified CUTL1 as modulator of cell motility and invasiveness. Here we report that protein kinase A (PKA), known to inhibit tumor progression in various tumor types, directly phosphorylates CUTL1 at serine 1215 in NIH3T3 fibroblasts. The PKA-induced phosphorylation results in decreased DNA binding affinity of CUTL1 and diminished CUTL1-mediated cell cycle progression and cell motility. Furthermore, the expression of several CUTL1 target genes involved in proliferation and migration, such as DNA polymerase A and DKK2, was modulated by PKA-induced phosphorylation. These data identify CUTL1 as a novel target of PKA through which this protein kinase can modulate tumor cell motility and tumor progression.

Protein kinase A (PKA) belongs to the family of serine/threonine kinases whose activity is implicated in the regulation of a wide variety of cellular functions, such as cell metabolism, secretion, proliferation, differentiation, and neoplastic transformation (1). PKA consists of two separate subunits, the catalytic (C) and regulatory (R) subunits that interact to form an inactive holoenzyme complex (2). Activation of PKA is achieved by binding of the second messenger CAMP to the R subunit, which consequently induces a conformational change in the R subunit and leads to the dissociation of the holoenzyme into its constituent subunits (1, 2). The free active C subunit is then able to phosphorylate a variety of cytoplasmic and nuclear protein substrates, including transcription factors (1).

CAMP and protein kinase A have been shown to play a major role in the modulation of tumor progression. However, these effects appear to be dependent on the cellular context (3). CAMP is known to have antiproliferative effects by antagonizing ERK activation via PKA in a variety of cell systems including fibroblasts, glial cells, and lymphocytes (4). Furthermore, cAMP has been shown to inhibit cell motility and invasiveness of several epithelial cancers (5, 6). In several neuronal, endocrine, and other epithelial cells, however, cAMP is able to stimulate growth by activating ERKs (7–9).

CUTL1, also known as CDP (CCAAT displacement protein), belongs to a family of homeobox transcription factors involved in the regulation of cell growth and differentiation (10). It is evolutionarily conserved and contains four DNA binding domains, three of which are known as Cut repeats and one as a Cut homeodomain (11).

CUTL1 has been described as a transcriptional activator as well as a transcriptional repressor. Its activity has been associated with cellular proliferation and cell cycle progression (12–14) as well as modulation of genes involved in terminal differentiation (15, 16). Accordingly, knockout studies with the murine homologue Cux-1 revealed reduced growth, retarded differentiation of the lung epithelia, hair follicle defects, reduced male fertility, and deficient T and B cell function (17–19). In contrast, mice transgenic for Cux-1 showed organomegaly and multiorgan hyperplasia (20).

CUTL1 has been demonstrated to be a phosphorylation target of several kinases that modulate its DNA binding affinity: phosphorylations by cyclin A/cdk1 and by protein kinase C at various sites have been shown to inhibit DNA binding activity (21, 22). Studies in flies indicate that the Drosophila homologue of CUTL1, Cut, is a major determinant of cell type specification downstream of the notch pathway (23) and may also interact with the wingless signaling pathway (24).

Recent data indicate that CUTL1 plays a major role in tumorigenesis and tumor progression. Although initial data based on loss of heterozygosity studies suggested CUTL1 as a candidate tumor suppressor gene (25), a recent report suggests instead that CUTL1 could play a role in promoting breast cancer: Goulet et al. (26) describe a tissue-specific CUTL1 isoform that appears to be strongly expressed in some breast tumors and, when overexpressed, inhibited tubule formation of breast cancer cells in vitro. In addition, we have demonstrated that CUTL1 strongly promotes cell motility and invasiveness in vitro and in vivo in a variety of cell systems and that CUTL1 expression is associated with a less differentiated phenotype and worsened prognosis in breast cancer (27).

Based on our previous data, we sought to investigate whether the transcriptional activity of CUTL1 is modulated by upstream signaling pathways implicated in the regulation of cell proliferation and motility. We could demonstrate that CUTL1 is phosphorylated by protein kinase A, leading to a decrease in DNA binding affinity and transcriptional activity. Furthermore, we were able to show that CUTL1 phosphorylation by PKA affected expression of several of its downstream target genes and inhibited CUTL1-mediated proliferation and migration.

EXPERIMENTAL PROCEDURES

Plasmids and siRNA—Myc-tagged full-length human CUTL1 in pMX as well as CUTL1-(831-1336) in pX were kind gifts from A. Nepveu. CUTL1 S1215A mutants and S1237D/S1270D double mutants were generated by using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA).

The short hairpin RNA (shRNA) for mouse CUTL1 (5'-AAGAAGAAA-CACTCCAGAGGATTT-3') was cloned as hairpin oligonucleotide into pSuper.retro.puro (Oligoengine, Seattle, WA) according to the manufac-
turer’s instructions, and stable clones were obtained after selection with puromycin. DNA polymerase α-pGL3-basic-Luc (−1517/+45) was a kind gift from A. Nepveu (14). pcDNA3-HA-dominant negative PKA and pcDNA-PKtwt constructs were kind gifts from M. L. Edin (28).

Materials and Cell Lines—Dibutyryl-cAMP (working concentration, 1 mM), forskolin (50 μM), 3-isobutyl-1-methyloxanthine (IBMX, 100 μM), U0126 (20 μM), rapamycin (200 μM), LY294002 (50 μM), and puromycin (2.5 μg/ml) were obtained from Sigma. The PKA inhibitor H89 (1 μg/ml) was from Calbiochem. All cell lines were obtained from ATCC.

Flow Cytometry—Fluorescence-activated cell sorter analysis was performed as described previously (29).

Reverse Transcription PCR—Reverse transcription was performed using the Superscript first strand synthesis kit (Invitrogen). PCR was performed for 30 cycles. Primer sequences are available on request. Quantitative real-time PCR analyses using the comparative CT method were performed on an ABI PRISM 7700 sequence detector system using the SYBR Green PCR Master Mix kit (PerkinElmer, Applied Biosystems) according to the manufacturers’ instructions. Following initial incubation at 50 °C for 2 and 10 min at 95 °C, amplification was performed for 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Primer pairs were determined with the PrimerExpress® program (Applied Biosystems). The murine cyclophilin A gene was used as the internal standard. Primer sequences are available on request.

Immunoprecipitation and Western Blotting—5 × 10⁵ cells were lysed in Heps buffer containing 1% Triton X-100, sonicated, and immunoprecipitated with mouse monoclonal anti-Myc antibody (clone 9E10; Abcam, Cambridge, MA) or with an antibody directed against phosphorylated PKA substrates (Cell Signaling Technology, Beverly, MA). The immunoprecipitates were size fractionated by SDS-PAGE on precast gels (NuPAGE; Invitrogen) and blotted onto polyvinylidene difluoride membranes (Millipore, Watford, UK). The following antibodies were used for immunodetection: our own rabbit anti-CUTL1 (antigen, GST-hCUTL1(2551-3451) (27) and anti-phospho-CUTL1 peptide antibody (antigen, peptide containing CUTL1 amino acids 1209-1222 including phosphorylated serine at position 1215), goat anti-actin (Santa Cruz Biotechnology), anti-phospho-PKA substrate antibody, anti-phospho-PKB substrate antibody (anti-pSer/Thr), p42/44 MAPK (ERK) antibody and phospho-p42/44 MAPK (ERK) antibody (Cell Signaling Technology, Beverly, MA), mouse anti-Myc clone 9E10 (Abcam, Cambridge, MA). Following peroxidase-coupled secondary antiserum (Amersham Biosciences), anti-phospho-PKA substrate antibody, anti-phospho-PKB substrate antibody, anti-phospho-PKB substrate antibody (anti-pSer/Thr), p42/44 MAPK (ERK) antibody and phospho-p42/44 MAPK (ERK) antibody (Cell Signaling Technology, Beverly, MA), mouse anti-Myc clone 9E10 (Abcam, Cambridge, MA). Following peroxidase-coupled secondary antiserum (Amersham Biosciences), blots were detected by ECL chemiluminescence (Amersham Biosciences).

Luciferase Reporter Assays—Cells were transiently transfected using Genelucite (Merck Biosciences) according to the manufacturer’s instructions and harvested 24 h after transfection. Because the internal control plasmid is itself often repressed by CDP/Cux, as a control for transfection efficiency the purified β-galactosidase protein (Sigma) was included in the transfection mix as previously described (14, 30). The luciferase assays were performed using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s instructions. β-galactosidase activity was detected using 2-nitrophenyl β-D-galactopyranoside (ONPG; Sigma) in substrate buffer (1 mM MgCl₂, 1 mg/ml ONPG, 75 mM sodium phosphate buffer, pH 7.4, 60 mM β- mercapto- ethanol) at 420 nm.

Wound Healing Assays—An artificial “wound” was created using a 10-μl pipette tip on confluent cell monolayers in 6-well culture plates. Photographs were taken at 0 and 8 h. Quantitative analysis of the wound closure was calculated by counting the number of cells/10⁵ μm² wound area at 8 h.

Two-chamber Migration Assays—Cell migration was determined by using the modified two-chamber migration assay (8-μm pore size; BD Biosciences) according to the manufacturer’s instructions. 2.5 × 10⁴ cells were seeded in serum-free medium into the upper chamber and migrated toward 10% fetal calf serum as a chemoattractant in the lower chamber for 18 h. Cells in the upper chamber were carefully removed using cotton buds, and cells at the bottom of the membrane were fixed and stained with crystal violet. 0.2% methanol 20%. Quantification was performed by counting the stained cells.

Video Time-lapse Microscopy—Time-lapse imaging of migrating cells was performed on an inverted Zeiss Axiovert 135TV microscope (X-Y-Z motorized stage/piezo Z control; Zeiss, Jena, Germany) over 24 h at 37 °C/10% CO₂. Images were obtained with a Hamamatsu Orca ER firewire CCD camera every 7 and 20 min and analyzing using image analysis software (AQ M Advance6 and Tracker; Kinetic Imaging Ltd, Bromborough, UK). Migration of each cell was analyzed by measuring the distance traveled by a cell nucleus over the 24-h time period. Statistical analysis was performed using the Mathematica 4.2 software (Wolfram Research, LongHanborough, UK). The average migration speed in μm/min was calculated by analyzing at least 24 cells/group.

Electromobility Shift Assays—Gel shift assays were performed as described previously (31). The consensus and mutant CDP binding oligonucleotides were obtained from Santa Cruz Biotechnology. 4 μg of nuclear extract from cells transiently transfected with CUTL1-(831-1336) WT or S1215A mutant were incubated in binding buffer (125 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 25 mM EDTA, 25% glycerol, 5 mM dithiothreitol, 6 μg/ml of bovine serum albumin, 5 μg/ml of poly(dI-dC)). For supershift assays, 6 μg of rabbit anti-CUTL1/CDP antibody (Santa Cruz supershift reagent) was included. The end-labeled probe was then added to each reaction and subsequently loaded onto a 4% non-denaturing polyacrylamide gel. Dried gels were scanned in a PhosphorImager (Storm 860; Amersham Biosciences).

RESULTS

CUTL1 Is Phosphorylated by PKA—By screening CUTL1 for putative phosphorylation sites using Scan site (scan site.mit.edu), we found several putative phosphorylation sites for PKA and protein kinase B, also known as Akt. One of these sites (MKRRHSSVS at position 1215) is evolutionarily conserved across species and was predicted to be a target site for PKA (phosphorylation of serine 1215) and for protein kinase B (phosphorylation of serine 1216). Initially, we wanted to test whether CUTL1 is phosphorylated by protein kinase B at serine 1216. Therefore, we used an antibody that was designed to recognize phosphorylated serine/threonine motifs of protein kinase B substrates. We and others have found, however, that this antibody has broader specificity for several phospho-serine/threonine motifs containing upstream basic residues (32). We found that the antibody recognized CUTL1 phosphorylation induced by dibutyryl-cAMP or forskolin as PKA stimulators (Fig. 1a). Activation of PKB did not result in CUTL1 phosphorylation (data not shown). Stimulation with cAMP induced a rapid phosphorylation of CUTL1, detectable by Western blot after 15 min with a peak at 45 min (Fig. 1b).

Because PKA-independent effects of cAMP and forskolin have been described (1), we aimed to examine direct involvement of PKA in the cAMP/forskolin-induced phosphorylation. As a first step, we used the pharmacological PKA inhibitor H89. Coincubation with H89 decreased the cAMP-induced phosphorylation markedly (Fig. 2a). In addition, cotransfection with dominant negative PKA or with the inhibitory peptide PKI (28) inhibited cAMP-induced phosphorylation (Fig. 2b), indicating that the CUTL1 phosphorylation is mediated by PKA and is not...
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a PKA-independent phosphorylation event induced by cAMP and forskolin. Immunoprecipitation with an antibody designed to detect phosphorylated PKA substrates revealed a detectable CUTL1 immunoblot signal upon cAMP stimulation (Fig. 2c), showing that the endogenous CUTL1 protein is phosphorylated following activation of PKA. To further rule out possible involvement of other serine/threonine kinases that might be able to phosphorylate similar motifs, we used several drugs such as the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor U0126 acting upstream of p90RSK, the mTOR inhibitor rapamycin acting upstream of p70S6K, and the phosphatidylinositol 3-kinase inhibitor LY294002. None of these inhibitors was able to decrease cAMP-induced CUTL1 phosphorylation to a significant extent, indicating that involvement of the serine/threonine kinases p90RSK and p70S6K is unlikely and that protein kinases controlled by phosphatidylinositol 3-kinase are also not involved (Fig. 2d).

PKA Phosphorylates CUTL1 at Ser-1215—To investigate which residue serves as the actual phosphorylation site for PKA, we generated serine to alanine mutants by site-directed mutagenesis at codons 1215 and 1216 as well as threonine to alanine mutants at codons 45 and 1454, which were also predicted as putative PKA sites by Scansite. Using these constructs, we determined that cAMP stimulation is unable to induce any detectable phosphorylation of the S1215A mutant (Fig. 3a).

To further confirm the PKA-mediated phosphorylation of CUTL1 at Ser-1215, we generated a phospho-specific peptide antibody against the motif around the phosphorylated Ser-1215. Using this antibody, we saw cAMP-inducible phosphorylation that was only detectable in WT CUTL1 but was absent in CUTL1 S1215A (Fig. 3b). This antibody detected cAMP-induced phosphorylation both in Western blots using immunoprecipitated Myc-tagged CUTL1 and in cAMP-stimulated whole-cell lysates with endogenous CUTL1, as demonstrated in Fig. 3c.

PKA Decreases CUTL1-mediated Proliferation and Migration—To study the transcripational effects of CUTL1, we have previously suppressed CUTL1 expression by vector-based transfection of specific shRNA in NIH3T3 fibroblasts, which express high endogenous CUTL1 levels. Stable CUTL1 RNAi clones that showed no detectable CUTL1 expression by Western blot were used for further studies in comparison with empty vector-transfected control clones (27). Using this cell system as well as other cell lines, we previously showed that CUTL1 expression is strongly associated with increased cell migration and invasiveness in vitro and in vivo (27). Furthermore, CUTL1 has been shown to enhance proliferation by stimulating S-phase progression in a variety of cell lines (13).

To test the biological significance of the CUTL1 modulation by PKA, we first examined the effect of CUTL1 on proliferation in the presence or absence of PKA activation. Suppression of CUTL1 by shRNA significantly reduced proliferation as measured by cell counting (Fig. 4a), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, and bromodeoxyuridine enzyme-linked immunosorbent assay (data not shown). cAMP significantly decreased proliferation in the presence of endogenous CUTL1. However, it did not further affect the reduced proliferation in the absence of CUTL1 (Fig. 4a). The CUTL1...
shRNA-mediated decrease in proliferation was paralleled by a decrease in S-phase progression as demonstrated by fluorescence-activated cell sorter analysis (Fig. 4b). cAMP reduced S-phase progression in wild-type cells to a significant extent but had only minor effects on S-phase progression in the absence of CUTL1. Based on these data, PKA appears likely to exert at least a significant part of its anti-proliferative effects through CUTL1.

Besides its effect on CUTL1 phosphorylation, PKA is known to decrease ERK activity in several cell systems. In the NIH3T3 cells used, PKA activation reduced basal ERK activity (Fig. 4c). However, ERK activation by epidermal growth factor (EGF) did not affect cAMP-induced phosphorylation of CUTL1 at Ser-1215 as detected by the phospho-specific CUTL1 antibody (Fig. 4d). This suggests that activation of ERK does not affect the transcriptional activity of CUTL1 by decreasing PKA-induced phosphorylation of CUTL1 at Ser-1215.

Given our previous results on CUTL1 as an enhancer of cell migration (27), we examined as a next step the effect of PKA activation on CUTL1-induced cell migration. For that purpose, we employed three different assays examining different aspects of cell motility: modified Boyden chamber assays, wound healing assays, and time-lapse video microscopy, which measures cell migration independent of a proliferation bias. As demonstrated above for proliferation, addition of cAMP significantly decreased the migration of NIH3T3 control cells. In contrast, the effect of cAMP on the migration of CUTL1 RNAi cells was markedly less pronounced. These results were obtained by two-chamber migration assays with a chemotactic gradient from serum-free to serum-containing medium (mean migrated cells/field ± S.E.: control + cAMP: 52 ± 7 cells, control: 75 ± 9 cells, CUTL1 RNAi + cAMP: 23 ± 5 cells, CUTL1 RNAi: 25 ± 5 cells) (Fig. 5a), by time-lapse microscopy (Fig. 5b) and by wound healing assays (Fig. 5c) and were also confirmed in HT1080 cells (data not shown). These data suggest that PKA mediates its anti-migratory effects to a significant extent through CUTL1.

PKA Decreases CUTL1 Transcriptional Activity—To investigate the effect of PKA on CUTL1 transcriptional activity at a single-gene level, we examined the transcriptional regulation of the known CUTL1 target gene DNA polymerase A (14) and several other CUTL1 target genes, which we previously identified by microarray experiments (27), in the presence of cAMP.
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FIGURE 5. cAMP decreases CUTL1-induced cell migration. a, two-chamber migration assay of NIH3T3 control and CUTL1 RNAi cells ± dibutyryl-cAMP/IBMX. Cells migrated through an 8-μm pore membrane on the lower side of the membrane were stained with crystal violet, quantified (see "Results"), and photographed. b, video time-lapse microscopy of NIH3T3 control and CUTL1 RNAi cells ± dibutyryl-cAMP/IBMX. At least 48 cells/condition/experiment were tracked. Data are presented as bar and whisker graphs and are representative of three independent experiments. *, p < 0.05 compared with control cells. c, wound healing assay with NIH3T3 control and CUTL1 RNAi cells ± dibutyryl-cAMP/IBMX. Cells in four defined areas/group/experiment were quantified. Data are representative for three independent experiments and are shown as mean ± S.E. *, p < 0.05 compared with control cells.

DISCUSSION

In this study, we have demonstrated that the homeodomain transcription factor CUTL1 is phosphorylated at serine 1215 by protein kinase A. This phosphorylation results in reduced DNA binding affinity and decreased CUTL1-mediated proliferation and migration, which was paralleled by a reduced transcription of CUTL1 target genes.

Several other protein kinases have been shown to modulate CUTL1 transcriptional activity by phosphorylation at distinct residues. Based on the observation that Cut-specific DNA binding was increased following phosophatase treatment, Coqueret et al. (21) found that PKC efficiently phosphorylated Cut repeats 1–3 at residues Thr-415, Thr-804, and Ser-987, which inhibited DNA binding. In addition, cyclin A/cdk1 was shown to bind to CUTL1 and modulate its DNA binding activity in vitro and in vivo (22): phosphorylation of serines 1237 and 1270 caused inhibition of DNA binding. In cotransfection studies, cyclin A/cdk1 inhibited CUTL1 stable DNA binding and prevented repression of the p21WAF1 reporter. In contrast, mutant CUTL1 proteins in which serines 1237 and 1270 were replaced with alanines were not affected by cyclin A/cdk1. The fact that serine 1215, the target residue for PKA in this report, and serines 1237 and 1270, the target residues for cyclin A/cdk1, are in close proximity suggests that phosphorylations
in this region induce conformational changes that lead to reduced transcriptional activity. All three residues are located between the conserved C-terminal cut repeat 3 and the homeodomain, both of which have recently been reported as the major determinants of CUTL1 activity after proteolytic cleavage of the N-terminal region (34). Despite similar effects on the transcriptional activity of CUTL1 induced by phosphorylations at both Ser-1237/Ser-1270 and Ser-1215, our data indicate that the phosphorylation at Ser-1237/Ser-1270 by cyclin A/cdk1 does not abrogate the effect of PKA on Ser-1215, underlining the importance of the phosphorylation event induced by PKA.

Protein kinase A is known to play an important role in tumorigenesis and cancer progression. However, its effect on tumor cell proliferation and migration is dependent on the cellular context. Although in certain cell systems PKA activity induced by cAMP is able to stimulate cell growth by enhancing ERK activity, in many other cell types, including the cells used here, cAMP inhibits ERK activity (4, 36) and inhibits cell proliferation. A number of mechanisms for the growth-inhibitory effect of cAMP have been proposed, only some of which involve cross-talk with the ERK pathway. First, PKA blocks Raf-1 activation by direct serine phosphorylation (37–41). Second, PKA may activate the Rap1

FIGURE 6. cAMP decreases CUTL1 transcriptional activity. a, luciferase assay of DNA polymerase A promoter luciferase construct in NIH3T3 cells with or without transiently transfected CUTL1-(831-1336) ± dibutyryl-cAMP/IBMX for 6 h. Data are representative for three independent experiments and are shown as mean ± S.E. *, p < 0.05 compared with empty vector-transfected control cells; **, p < 0.05 compared with CUTL1-transfected cells without cAMP. b, quantitative real-time reverse transcription PCR of selected CUTL1 target genes identified by previous microarray experiments ± dibutyryl-cAMP/IBMX for 6 h in NIH3T3 control and CUTL1 RNAi cells, normalized to cyclophilin expression. c, electrophoretic mobility shift assay of NIH3T3 cells transfected with CUTL1-(831-1336) WT or S1215A mutant ± PKA inhibitor H89 in the presence of 10% donor calf serum. Data are representative for three independent experiments. d, video time-lapse microscopy of NIH3T3 cells transiently transfected with CUTL1 WT or CUTL1 S1215A mutant ± dibutyryl-cAMP/IBMX. Cells were cotransfected with pE GFP, and fluorescent cells were tracked. *, p < 0.05 compared with CUTL1 WT cells without PKA activation. e, luciferase assay of DNA polymerase A promoter luciferase construct in NIH3T3 cells with or without transiently transfected CUTL1-(831-1336) (CUTL) or CUTL1-(831-1336) S1237D/S1270D double mutant (S1237/1270D) ± forskolin for 6 h. Data are representative for three independent experiments and are shown as mean ± S.E. *, p < 0.05 compared with CUTL1-(831-1336)-transfected cells without forskolin; **, p < 0.05 compared with CUTL1-(831-1336)-S1237/1270D-transfected cells without forskolin.
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GTpase via Src, thereby inhibiting ERK in some cell contexts (4). Third, PKA may inhibit Akt activity and thereby activate the FoxO4 transcription factor and induce the CDK inhibitor p27Kip1 (42). Based on our data, we propose that in addition, PKA activity decreases proliferation and cell motility, and possibly tumor progression, at least in part by phosphorylating CUTL1 and thereby inhibiting its transcriptional activity.

Detailed study of cAMP effects on carcinoma cell motility by Mercurio and co-workers (43) and others has shown that α6β4 integrin-mediated invasion required suppression of cAMP levels by activation of a phosphodiesterase. This was required for RhoA translocation from the cytosol to membrane ruffles (44). Elevated cAMP levels have also been reported to block RhoA activation (45, 46). Although it has been suggested that these effects are due, at least in lymphocytes, to direct phosphorylation of RhoA by PKA at Ser-188 (47), our data suggest that these effects are due, at least in lymphocytes, to direct phosphorylation of RhoA by PKA at Ser-188 (47), our data suggest that these effects are due, at least in lymphocytes, to direct phosphorylation of RhoA by PKA at Ser-188 (47), our data suggest that these effects are due, at least in lymphocytes, to direct phosphorylation of RhoA by PKA at Ser-188 (47), our data suggest that these effects are due, at least in.