Actin Cytoskeletal Association of Cytohesin-1 Is Regulated by Specific Phosphorylation of Its Carboxyl-terminal Polybasic Domain*

Henning Dierks, Johanna Kolanus, and Waldemar Kolanus‡

From the Laboratorium für Molekulare Biologie, Genzentrum der Universität München, Feodor-Lynen-Strasse 25, D-81377 München, Germany

Cell adhesion mediated by integrin receptors is controlled by intracellular signal transduction cascades. Cytohesin-1 is an integrin-binding protein and guanine nucleotide exchange factor that activates binding of the leukocyte integrin leukocyte function antigen-1 to its ligand, intercellular adhesion molecule 1. Cytohesin-1 bears a carboxyl-terminal pleckstrin homology domain that aids in reversible membrane recruitment and functional regulation of the protein. Although phosphoinositide-dependent membrane attachment of cytohesin-1 is mediated primarily by the pleckstrin homology domain, this function is further strengthened by a short carboxyl-terminal polybasic amino acid sequence. We show here that a serine/threonine motif within the short polybasic stretch of cytohesin-1 is phosphorylated by purified protein kinase Cα in vitro. Furthermore, the respective residues are also found to be phosphorylated after phorbol ester stimulation in vivo. Biochemical and functional analyses show that phosphorylated cytohesin-1 is able to tightly associate with the actin cytoskeleton, and we further demonstrate that phosphorylation of the protein is required for maximal leukocyte function antigen-1-mediated adhesion of Jurkat cells to intercellular adhesion molecule 1. These data suggest that both phosphatidylinositol 3-kinase and protein kinase C-dependent intracellular pathways that stimulate β2α-integrin-mediated adhesion of T lymphocytes converge on cytohesin-1 as functional integrator.

Integrins are a diverse family of heterodimeric transmembrane adhesion receptors that are present on most vertebrate cell types. They are known to play important roles in either development or somatic functions such as wound healing and the regulation of complex cell-cell or cell-matrix interactions within the immune system (1).

The avidity of integrins for their ligands is dependent on the activation state of the cell they are expressed on (1–3). This type of regulation of cell adhesion has been termed inside-out signaling because intracellular signaling pathways triggered by protein tyrosine kinase or G-protein-coupled receptors have been shown to contribute to integrin-mediated adhesiveness. The mechanisms by which cytoplasmic signals are transmitted across the plasma membrane through integrin receptors are just emerging, but compelling evidence suggests that the intracellular domains of both α and β chains participate in this process (4–8).

Previous studies have attempted to elucidate these signaling pathways. Recently, candidate cytoplasmic regulatory factors of integrin activation have been identified, either by biochemical methods or with the help of the two-hybrid system (7). One of them, cytohesin-1, is a 47-kDa intracellular protein that interacts specifically in several systems with the cytoplasmic domain of the leukocyte integrin αβ2 (CD11a/18, LFA-1) (9). Cytohesin-1 bears a short amino-terminal domain that may aid in oligomerization, an extended central homology region that is similar to that of the yeast Sec7 protein, and a carboxyl-terminal pleckstrin homology (PH) domain as well as a short polybasic region. Overexpression of cytohesin-1 or subdomain constructs in the Jurkat T-cell line was shown to have pronounced effects in vivo on the binding of αβ2 to its ligand, intercellular adhesion molecule 1 (ICAM-1). Whereas the overexpression of full-length cytohesin-1 resulted in a constitutive adhesion of αβ2 expression of the PH domain construct specifically inhibited the activation of LFA-1 in a dominant negative fashion (9).

PH domains are structural modules present in more than a hundred proteins that play known or postulated roles in signal transduction. It is commonly found that PH domains aid in membrane recruitment of proteins through their interactions with phosphorylated ligands present at the inner leaflet of cellular membranes (10–14). A number of PH domains, including the PH domain of cytohesin-1, were shown to bind phosphatidylinositol 3,4,5-trisphosphate (PIP3) in vitro with high affinity (12, 15–18). These findings implicated the involvement of phosphatidylinositol 3-kinase in the LFA-1 activation pathway, and we have subsequently shown that a constitutively active version of phosphatidylinositol 3-kinase suffices to activate the αβ2 adhesion pathway in a T-cell line (17). Functional, biochemical, and cell biological evidence furthermore suggested that cytohesin-1 is located downstream of phosphatidylinositol 3-kinase and that it is regulated by the recruitment of its PH domain to the plasma membrane (17, 20, 21). The PH domain was supported in this function by the carboxyl-terminal polybasic region of the protein, which proved to be necessary for membrane association and stabilized PIP3 binding in vitro (22). These data supported a model in which membrane

1 The abbreviations used are: PH, pleckstrin homology; ICAM-1, intercellular adhesion molecule 1; LFA-1, leukocyte function antigen-1; PKC, protein kinase C; PIP3, phosphatidylinositol 3,4,5-trisphosphate; ARF, ADP ribosylation factor; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; clg, cytoplasmic Ig.
In Vitro Phosphorylation of Cytohesin-1—Cytohesin-1 protein was expressed in COS-7 cells by DEAE-dextran transfection. Cells that had been transfected with cDNA coding for cytohesin-1 were collected by centrifugation and resuspended on ice in 0.5 ml of ice-cold hypotonic solution (10 mM HEPEs, pH 7.5, 10 mM KC1, 10 mM MgCl2, and 0.5 mM EGTA) containing 10 mg/ml cytochalasin D, 200 mg/ml phosphatidylserine, 200 mg/ml PMA, 0.1 mg/ml leupeptin, 10 mg/ml aprotinin, 2 mM ATP, 0.05% Nonidet P-40, and 20 mg/ml PKCδ at pH 7.4 and 30 °C for 120 min.

MATERIALS AND METHODS

Constructs—Expression of amino-terminal cytoplasmic immunoglobulin fusion proteins has been described previously (9, 17, 22, 25, 26). For some of the constructs, a so-called FLAG tag was used, which replaced the Ig portion as the amino-terminal fusion sequence in the respective chimeras. The following sequence for the FLAG tag was employed: 5'-AAG-CTT-GCC-ACC-ATG-GAT-TAC-AAG-GAT-GAC-GAC-GAT-3'.

In Vivo Phosphorylation of Reconstituent GST Fusion Proteins—Reaction conditions were 10 mM HEPEs, 50 mM KC1, 10 mM MgCl2/4°C, 10 mg/ml PMA, 200 mg/ml phosphatidylserine, 200 mg/ml PMA, 0.05% Nonidet P-40, 100 mg/ml protein/ml respective protein substrate, and 1 mg/ml PKCδ at pH 7.5 and 30 °C for 60 min.

Cellular Fractionation—Jurkat E6 cells were pretreated with cytochalasin D (50 μg/ml) where indicated to disrupt actin filaments or with nocodazole (2.5 μg/ml, 60 min) to disrupt microtubules. These concentrations of cytochalasin D or nocodazole were maintained throughout the whole fractionation procedure. Cells were collected by centrifugation and resuspended on ice in 0.5 ml of ice-cold hypotonic solution (10 mM HEPEs, pH 7.5, 10 mM KC1, 10 mM MgCl2, and 0.5 mM dithiothreitol) containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mm phenylmethylsulfonyl fluoride. Fractionation of cells was performed as described previously (31). Briefly, cells were sheared, nuclei were removed by centrifugation (10,000 × g for 10 min) and the supernatant cytosol was collected. The cytosolic fraction was brought to a final concentration of 1% (v/v) Nonidet P-40 and 150 mM NaCl and centrifuged at 15,000 × g, and the resulting pellet was resuspended in hypertonic buffer. A typical sample, containing mainly cytoskeletal components from –2 × 107 cells was incubated with 50 ng of GST-cytohesin-1 for 10 min, as indicated. The samples were centrifuged at 15,000 × g for 15 min, and the pellet (insoluble fraction containing cytoskeletal components) and supernatant (soluble fraction) were subjected to gel electrophoresis.

Adhesion Assay—Jurkat E6 cells were infected with recombinant vaccinia viruses 6 h after infection, cells were labeled with 12 μg/ml bisbenzimide H33342 fluorochrome trihydrochloride (Calbiochem) for 30 min at 37 °C, collected by centrifugation, resuspended in Hank’s buffered saline solution, and delivered to 96-well plates (NUNC, Maxisorp) at 1.5 × 105 cells/well. Before adhesion, plates were coated with goat anti-human IgG (Fc-specific) antibody at 0.85 μg/ml for 90 min at 25 °C, blocked with 1% (w/v) BSA in PBS, incubated with culture supernatants from COS-7 cells expressing ICAM-1-Ig fusion protein, and subsequently used in the assay. Where indicated in the figures, cells were incubated with 40 ng/ml PMA or 10 μg/ml cytochalasin D for 0.5 h before the adhesion assay. Cells were then allowed to adhere for 1 h at 37 °C, and unbound cells were carefully washed off with 3 × 300 μl of Hank’s buffered saline solution. Bound cells were assayed in 100 μl of 2% (w/v) formaldehyde in PBS using a fluorescence plate reader (Cytofluor II; PerSeptive). The signal of 1.5 × 105 cells/well at 490 nm corresponds to 1% of adherent cells.

In Vitro Dephosphorylation of Cytohesin-1—COS-7 cells were treated as described under “PMA-induced in Vivo Phosphorylation.” 5 units of alkaline phosphatase were added to 50-μl aliquots of cell extract where indicated. Samples were incubated at 37 °C and subjected to nondeaturing PAGE.
for 1 h at 4°C. Subsequently, cells were permeabilized for 15 min with
0.2% (v/v) Triton X-100 in PBS, blocked with 2% (w/v) glycine in PBS,
and incubated with a fluorescein isothiocyanate-labeled goat anti-hu-
man IgG (Fcγ-specific) antibody (Dianova) in PBS for 2 h at room
temperature. For the actin stain, slides were incubated with an anti-
actin antibody for 1 h. Slides were subsequently washed with PBS
and incubated with Texas Red-labeled donkey anti-rabbit IgG antibody.

After the final wash with PBS, slides were mounted on a 9:1 mixture of
glycerol and 100 mM Tris-HCl, pH 9.0, containing n-propyl-gallate (20
mg/ml) as an anti-fading reagent. The samples were then examined on
a confocal laser scanning apparatus (Leica TCS-NT system; Leica)
attached to a Leica DM IRB inverted microscope with a PLAPO 63 ×
1.32 oil immersion objective.

Measurement of Phosphatidylinositol Binding of GST-PH Domain
Construc ts by IAsys Biosensor Technology—PH domains of cytohesin-1
were expressed as GST fusion proteins as described previously (17). An
optical evanescent resonant mirror cuvette system (IAsys; Affinity
Sensors) was used to measure interaction of GST fusion proteins with
PDI. A lipid monolayer containing 70% (w/w) β-palmitoyl-γ-oleoyl-l-a-
phosphatidylcholine and 30% (w/w) dioleoyl-l-a-phosphatidyl-α-glyc-
erol or a lipid mixture of 60% (w/w) β-palmitoyl-γ-oleoyl-l-a-phosphati-
dylcholine and 30% (w/w) and 10% (w/w) PIP3 (Matreya, Inc.) was
mounted on a hydrophobic sensor surface (FCH-0601) at 0.1 mg/ml
lipid. The cuvette was subsequently washed with 0.1 M HCl, PBS, and
10 mM NaOH. After the final wash with PBS, the cuvette was equili-
brated in PDI binding buffer (PBS, 2 mM dithiothreitol, and 0.001%
(v/v) Igepal CA-630), and affinity-purified GST fusion proteins dissolved
in binding buffer were added to a final concentration of 150 nM each.

The binding of the GST fusion proteins was monitored for 500 s. Dis-
sociation was initiated by adding PDI binding buffer to the cuvette.

Determination of the association equilibrium constant was done by
equilibrium titration. The interaction profiles for each protein were
analyzed using FASTfit kinetics analysis software supplied with the
instrument.

RESULTS

Treatment of Jurkat Cells with Phorbol Ester Induces Phos-
phorylation of Cytohesin-1 in Vivo—With the help of in vivo
labeling experiments, we discovered that a cytoplasmic Ig (cIg)
fusion protein of cytohesin-1 was strongly phosphorylated in
Jurkat cells after incubation of the cells with PMA. COS-7 cells
were transfected with a cDNA coding for cIg-cytohesin-1 fusion
protein or control protein (Fig. 1); the cells were then labeled
with 2 mCi/ml [32P]orthophosphate and subsequently activated
for 1 h with PMA. After this treatment, cells were lysed, and the fusion proteins from the samples were immunoprecipitated
on protein A and subjected to SDS-PAGE and autoradiography.

Fig. 1 shows that the [32P] content of the cIg-cytohesin-1 fusion protein increases dramatically and specifically after
PMA activation, whereas background labeling of the control
protein remained constant. Loading of the fusion proteins was identical, as detected by anti-cytohesin-1 or anti-Ig immuno-
 blotting (Fig. 1; data not shown).

We then discovered that the phosphorylated form of endog-
enous cytohesin-1 in Jurkat cells could be visualized as a fast-
migrating species in nondenaturing Tris-glycine gels. Because
no difference in the migratory behavior of endogenous or over-
expressed cytohesin-1 from unstimulated or PMA-induced cells
was observed when SDS-PAGE was employed (Fig. 1; data not
shown), we subsequently used nondenaturing Tris-glycine gels
as well as an adapted immunotransfer protocol. Using this
methodology, we found that PMA stimulation of the cells re-
sulted in a second fast-migrating band that also reacted spe-
cifically with an anti-cytohesin-1 monoclonal antibody (Fig. 2;
data not shown). This band was hypothesized to be the phos-
phorylated form of the protein because in nondenaturing gels,
modification of a protein with charged compounds should nor-
mally lead to an increase in its electrophoretic mobility (30).

This was confirmed by adding alkaline phosphatase to the
samples before electrophoresis, transfer, and detection: calf
intestine phosphatase treatment was found to remove the
lower band quantitatively from the samples. This was consist-
ent with the notion that the lower band corresponded to in vivo
phosphorylated cytohesin-1 and not to the PMA-dependent in-
duction of a different isoform of the cytohesin family or to an
unknown modification (Fig. 2).

FIG. 1. A cytohesin-1 fusion protein is phosphorylated in phor-
bol ester-stimulated COS cells. cIg fusion proteins were employed in
this experiment. The chimeras were expressed in [32P]orthophosphate-
 labeled COS-7 cells and subsequently precipitated on protein A. Cells
were pretreated with phorbol ester (PMA) as indicated (+). The anti-
human Ig immunoblot shows similar expression levels in PMA-treated
and untreated cells (A). Aliquots of respective samples were subjected to
PAGE, and the resulting immunoblot was exposed to x-ray film. Sig-
ificant incorporation of [32P]orthophosphate into cIg-cytohesin-1 pro-
duct was detected after PMA stimulation (B, top panel). Background
phosphorylation was monitored by expression of the isolated cIg
portion. The incorporation of [32P]orthophosphate into cIg protein was
not enhanced by PMA (B, bottom panel).

FIG. 2. PMA-induced phosphorylation of cytohesin-1 visual-
ized by nondenaturing PAGE. Cytohesin-1 was expressed in COS-7
cells and analyzed by nondenaturing PAGE as described under “Mate-
rials and Methods.” Left lane, control. Middle lane, a fast-migrating,
lower band appears in PMA-stimulated cells. Right lane, the lower
band disappears after incubation of the sample with alkaline phosphatase
(AP).
residues in cytohesin-1. Using the nondenaturing gel assay, we
mutagenesis was used to specifically map the phosphorylated
the carboxyl-terminal polybasic stretch (Fig. 4A), site-directed
sequence stretch at all.

contain a potential phosphorylation site in the homologous
was conserved in ARNO (serine 392), whereas GRP1 did not
contained two potential phosphorylation sites in this region,
phospholipid binding. A close look revealed that cytohesin-1
laborated with the PH domain in membrane recruitment and
重要作用 for the function of cytohesin-1 because it col-
ated under the conditions used. Previously, we had produced a
fast-migrating but normally somewhat weaker band was de-
ylated after1 hour of PMA treatment. In the ARNO sample, a
produced. Usually about 40%
phosphorylated after PMA treatment (data not shown).
It was therefore assumed that the modification occurred on
serine(s) or threonine(s). Because cytohesin-1 belongs to a
family of at least four extremely conserved proteins in mammals,
we tested whether other family members became phosphoryl-
ated under the conditions used. Previously, we had produced a
rat antibody that reacted exclusively with cytohesin-1 (25), but
we tested whether other family members became phosphoryl-
ated upon PMA stimulation. Because the phosphorylation sites were
their respective PKC sites of cytohesin-1 were disabled by the introduction of respective
point mutations and used as specificity controls. All of these mutants
found that mutagenesis of both serine 394 and threonine 395
affected the phosphorylation status of cytohesin-1 (Fig. 4B).
However, complete inhibition of phosphorylation was observed
when both residues were simultaneously replaced by alanines
or glycines, respectively. This result led us to conclude that
serine 394 and threonine 395 are the phosphorylated residues
in cytohesin-1. Because it was formally possible that by mu-
tageneis we had obtained an indirect effect, e.g. by targeting
regulatory sites that remained unphosphorylated themselves,
we mutated all consensus PKC phosphorylation sites in the
protein and consistently found that none of these residues
affected the phosphorylation of the protein at all (Fig. 4C).
These results are therefore interpreted as a strong indication
that cytohesin-1 is exclusively phosphorylated at residues 394
and 395 after PMA treatment of Jurkat cells.

Fig. 3. Cytohesin-1 and ARNO, but not GRP1, are phosphorylated upon PMA stimulation. Cell extracts of COS-7 cells transfected with either FLAG-cytohesin-1, FLAG-ARNO, or FLAG-GRP1 were subjected to nondenaturing PAGE. Cells had been stimulated with PMA where indicated. Anti-FLAG immunoblots are shown. In the case of cytohesin-1 and ARNO, a faster migrating species is found in PMA-treated cells, whereas the electrophoretic motility of GRP1 remained unchanged.

Mapping the Site of PMA-dependent Phosphorylation in Cytohesin-1—Using the anti-phosphotyrosine antibody 4G10 as a
detecting agent, we found that cytohesin-1 was likely not ty-
rosine-phosphorylated after PMA treatment (data not shown).
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Fig. 4. Identification of PMA-dependent phosphorylation sites in cytohesin-1. A, sequence comparison of the carboxyl-terminal poly-

basic regions of cytohesin-1, ARNO, and GRP1. B, a cytohesin-1 mutant
that lacks serine 394 and threonine 395 is not phosphorylated upon
PMA stimulation in COS-7 cells. FLAG-cytohesin-1 (wild-type or mu-
tant constructs) had been transfected into COS-7 cells and stimulated
with PMA as indicated. All constructs were subjected to nondenaturing
PAGE and subsequent immunoblot analysis. C, the remaining putative
PKC sites of cytohesin-1 were disabled by the introduction of respective
point mutations and used as specificity controls. All of these mutants
still responded to PMA stimulation.

An important clue to the mapping of the phosphorylation site
was therefore obtained by comparison of the peptide sequences
of cytohesin-1, ARNO, and GRP1. There are several PKC con-
ensus phosphorylation sites in these proteins, but most of these
are conserved among them. However, the carboxyl-ter-
inal polybasic stretch is an exception, inasmuch as this por-
tion has among the most divergent sequence elements in the
otherwise highly conserved primary peptide structures of the
protein family. Furthermore, the polybasic stretch was shown
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A

B

C

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A

B

C

important for membrane recruitment and function (22), we speculated that the addition of negative charge through phosphorylation events might affect the association of cytohesin-1 with phospholipids. We therefore generated another set of mutants in which serine 394 and threonine 395 were replaced by "constitutively active" residues, aspartate or glutamate, respectively. The rationale of this approach was based on the frequent observation that negatively charged amino acids can functionally substitute for phosphorylated serines or threonines, respectively.

The resultant cytohesin-1 mutants, S394D and T395E, or the double mutant ST394/95DE were expressed in Jurkat cells using recombinant vaccinia viruses, and subcellular localization was subsequently analyzed with the help of laser scanning confocal microscopy. The result of this experiment is shown in Fig. 5. We observed that the subcellular localization of cytohesin-1 or of the proposed loss of phosphorylation mutants S394A or T395A was very similar and corresponded to that published previously for a wild-type cytohesin-1 (22): the mutant cytohesin-1 fusion proteins were expressed in the cytoplasm, and a large proportion of the detectable material was constitutively associated with the cell cortex. To our surprise, gain of charge mutants S394D, T395E, and ST394/95DE showed an identical staining pattern. Furthermore, PMA treatment of an aliquot of the sample in which wild-type cytohesin-1 was expressed had no effect (Fig. 5).

This result was unexpected, and therefore it was possible that the in vivo effects of cytohesin-1 phosphorylation were somehow perturbed by the experimental system employed. We therefore analyzed whether specific phosphorylation of cytohesin-1 could be obtained by in vitro methods. To this end, COS-7 cell lysates that contained overexpressed FLAG-cytohesin-1 or control protein were incubated with purified PKC δ protein in the presence of ATP. As shown in Fig. 6, PKC δ induced a fast-migrating species of FLAG-cytohesin-1 in native gels, very similar to what we observed with PMA-treated cells. Consequently, the migration pattern of the FLAG-tagged mutant cytohesin-1 394/5AG was not altered by PKC in this assay (Fig. 6). Therefore, phosphorylation of cytohesin-1 could be reliably obtained by using PKC δ in vitro.

GST fusion proteins harboring either the intact PH domain of cytohesin-1 (GST-PH) or the PH domain and the polybasic region (GST-PHc) were then purified from an Escherichia coli overexpression system (Fig. 7A). The proteins were subsequently phosphorylated in vitro using purified PKC δ and [γ-³²P]ATP. The degree of ³²P incorporation into the protein was monitored using scintillation counting (data not shown). GST-PHc, either phosphorylated or unphosphorylated, was then employed in phospholipid binding assays, using IAsys biosensor technology (22). However, as shown in Fig. 7B, phosphorylation of the carboxyl-terminal segment of cytohesin-1
had no effect on binding to PIP$_3$ in vitro. The observed binding of the carboxyl-terminal protein modules of cytohesin-1 to the phospholipid appeared to be specific because GST control protein had no affinity for the PIP$_3$-derivatized interaction matrix, and deletion of the polybasic domain strongly reduced the affinity of the fusion protein for PIP$_3$ (GST-PH), as described previously (22). We therefore concluded that the phosphorylation of cytohesin-1 had no effect on its association with phospholipid membranes.

Cytoskeletal Association of Cytohesin-1 Is Regulated by Phosphorylation—Assessed by microscopy, the interaction of cytohesin-1 with phospholipids of the plasma membrane might be indistinguishable from its cortical association with the cytoskeleton. Interestingly, it had been described previously that expression of a dominant negative mutant of cytohesin-1 in peripheral blood mononuclear cells or Jurkat cells had resulted in abrogation of cell spreading (25). Furthermore, introduction of the same mutant in Jurkat cells led to characteristic alterations of the actin cytoskeleton in adherent cells. We therefore

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2. W. Nagel and W. Kolanus, unpublished observations.
speculated that the phosphorylation of cytohesin-1 might regulate its association with the actin cytoskeleton.

The association of cytohesin-1 with the actin cytoskeleton in native cell particulate fractions was assessed biochemically through crude separation of Jurkat cell extracts. Recombinant GST-cytohesin-1 was preincubated with PKC/ and ATP, as indicated. Note that the normally soluble fusion protein (far left lane) associates with the insoluble fraction in the presence of PKC and ATP (lane 4 from the left); however, this association is abrogated when cells were incubated with cytochalasin D (far right lane). C, an experiment similar to that shown in B; however, association of endogenous cytohesin-1 with the insoluble Jurkat cell fraction is shown. Note that PMA stimulation induces association of cytohesin-1 with the insoluble fraction (lane 4 from the left); this association is abrogated in the presence of cytochalasin D, D, the microtubule-disrupting agent nocodazole has no effect on the PKC-dependent association of GST-cytohesin-1 with the insoluble cell fraction. E, analysis of the association of cytohesin-1 phosphorylation site mutants with the insoluble cell fraction. GST fusion proteins were employed; PKC and ATP were present throughout. Note that the double mutation of serine/threonine 394/395 is required to completely abrogate the association of the fusion protein with the insoluble cell fraction. The charge of the substituted amino acid does not play a role.

**Fig. 8.** Phosphorylated cytohesin-1 associates with actin cytoskeleton. A, Coomassie Blue stain of purified GST fusion proteins resolved by 10% SDS-PAGE. B, phosphorylated cytohesin-1 binds to the cytoskeletal fraction of Jurkat E6 cells *in vitro*. GST-cytohesin-1 was incubated with cellular lysates from which nuclei and membranes had been depleted and with additional reagents, as indicated. Note that the normally soluble fusion protein (far left lane) associates with the insoluble fraction in the presence of PKC and ATP (lane 4 from the left); however, this association is abrogated when cells were incubated with cytochalasin D (far right lane). C, an experiment similar to that shown in B; however, association of endogenous cytohesin-1 with the insoluble Jurkat cell fraction is shown. Note that PMA stimulation induces association of cytohesin-1 with the insoluble fraction (lane 4 from the left); this association is abrogated in the presence of cytochalasin D. D, the microtubule-disrupting agent nocodazole has no effect on the PKC-dependent association of GST-cytohesin-1 with the insoluble cell fraction. E, analysis of the association of cytohesin-1 phosphorylation site mutants with the insoluble cell fraction. GST fusion proteins were employed; PKC and ATP were present throughout. Note that the double mutation of serine/threonine 394/395 is required to completely abrogate the association of the fusion protein with the insoluble cell fraction. The charge of the substituted amino acid does not play a role.
Actin Cytoskeletal Association of Cytohesin-1

Regulation of Cell Adhesion to ICAM-1 by Phosphorylation Site Mutants of Cytohesin-1—We subsequently compared the ability of the cytohesin-1 mutants to induce LFA-1-dependent adhesion to ICAM-1 in a T-cell overexpression system. To this end, all described mutants were expressed in Jurkat cells or in SKW3 cells by recombinant vaccinia viruses, and adhesion of the infected cells to an ICAM-1-Fc chimera was measured as described previously (9). The results of these experiments are shown in Fig. 9, and they corresponded well to all of the data above. Overexpression of cytohesin-1 induced Jurkat adhesion to ICAM-1 5–10 fold (Fig. 9A; data not shown), as described previously, whereas the expression of the PH domain construct or the E157K mutant suppressed basal adhesion significantly. The phosphorylation site mutants had no measurable effect on basal adhesion, but we detected a significant reduction of PMA-stimulated adhesion when the mutants were employed, as opposed to wild-type cytohesin-1. In accordance with this finding, pretreatment of cells with cytochalasin D at a concentration that strongly interferes with actin polymerization significantly inhibited the effect of cytohesin-1 on cell adhesion in this system (Fig. 9B). Although it is possible that the effects of cytochalasin D are of a complex nature in this assay, our results hint at a contribution of cytoskeletal association of cytohesin-1 to its regulatory role in β2 integrin activation. We also tested whether actin-cytoskeletal association and membrane localization of cytohesin-1 are interdependent, which would complicate interpretation. To this end, we determined the subcellular localization of cytohesin-1 or of the 394/5AG mutant in cytochalasin D-treated Jurkat cells. Fig. 10 shows that the actin-depolymerizing agent did not change the distribution of cytohesin-1 constructs in these cells, which is consistent with the notion that the cortical association of the protein is primarily phospholipid-mediated. Taken together, our results furthermore suggest that membrane recruitment of the molecule and cytoskeletal association are cooperative functions.

DISCUSSION

In this study, we show that cytohesin-1 is phosphorylated in vivo after stimulation of Jurkat cells with phorbol ester. We mapped the phosphorylation sites of the protein by use of nondenaturing gel electrophoresis. This technique allowed us to detect both the phosphorylated and the unphosphorylated species, yielding two respective bands of differential electrophoretic mobility that were visualized by immunoblot analysis. Under the nondenaturing electrophoresis conditions employed, the phosphorylated isomer bears an additional negative charge due to the phosphate group, which overcompensates for the gain of mass and therefore allows faster migration. In consequence, the upper band observed corresponds to the unphosphorylated protein, whereas the lower band corresponds to the phosphorylated isomer. This is unlike the sometimes observed slow migration of phosphorylated proteins in SDS gels, which is not due to the addition of negative charge but may rather be attributed to indirect effects (32).

We show that the phorbol ester PMA induces phosphorylation of cytohesin-1 in vivo at both serine 394 and threonine 395. We furthermore observed that cytohesin-1 may be phosphorylated in vitro by the PKCδ isofrom. Both the respective serine and threonine residues are embedded in a favorable consensus motif of PKC-dependent phosphorylation. These findings indicate that cytohesin-1 may be phosphorylated in vivo by a member of the PKC family because phorbol esters are known to stimulate PKC activity (33). However, we cannot exclude the possibility that a kinase located downstream of PKC may phosphorylate cytohesin-1 in vivo.

In the next series of experiments, we attempted to unravel the functional relevance of the observed phosphorylation events. Previous analyses had implicated the carboxyl terminus of cytohesin-1 in membrane recruitment and phospholipid binding (22). It therefore appeared likely that this function might be affected by phosphorylation events. Interestingly, however, extensive analyses in these directions, employing in vitro assays as well as cell-based experimental systems, yielded no clues. Previous studies (34, 35) had described an interesting mode of regulation of the homologous protein ARNO (cytohesin-2). Either phosphorylation of a carboxyl-terminal serine residue of ARNO or constitutive addition of negative charge to the ARNO carboxyl terminus by substituting the serine residue with an acidic amino acid (Glu) had resulted in an electrostatic repulsion effect and yielded membrane detachment and abrogation of phospholipid binding (35). It is quite surprising that this type of regulation apparently does not hold true for cytohesin-1, although it contains two phosphorylated residues at that site; that is, electrostatic repulsion should be even more efficient. Recently, a study has been published in which the consequences of ARNO phosphorylation were analyzed in greater detail (36). The authors concluded that although non-specific binding of ARNO to phosphatidylserine was diminished by the phosphorylation, there was no effect of the modi-
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been proposed for ARNO, appears not to play a role in this system. Intriguingly, an actin cytoskeletal remodeling activity had previously been attributed to ARNO (34).

What is the mechanistic, functional role of the regulated association of phosphorylated cytohesin-1 with the actin cytoskeleton? In previous studies, we have shown that cytohesin-1 interacts specifically with the cytoplasmic domain of CD18, the β-subunit of the leukocyte integrin LFA-1 (9, 25). Furthermore, phorbol ester is widely known to induce LFA-1-mediated cell adhesion to ICAM-1 (38). These results and the observation that cytohesin-1 recruitment to the actin cytoskeleton is enhanced by phorbol ester stimulation suggest that the phosphorylation may aid in targeting cytohesin-1 to a favorable position for its interaction with LFA-1. We employed adhesion assays to approach this question at the functional level. Significantly, we found that the ability of cytohesin-1 to induce adhesion was altered upon introduction of the phosphorylation site mutations. The reducing effect of the mutations was not observed when basal adhesion levels were analyzed. However, there was a clear reduction of maximal adhesion obtained by the synergistic actions of cytohesin-1 overexpression and phorbol ester co-stimulation. This appeared plausible because there is a very good correlation with cytohesin-1 phosphorylation in this situation. Consequently, the effect of the mutants was only observable when PMA was employed.

We do not currently know how association of cytohesin-1 with elements of the actin cytoskeleton regulates membrane transport and actin polymerization during cell migration (19, 37, 40). Thus, there is an emerging theme of functional cross-talk between ARF- and Rho-regulatory proteins in cytoskeletal regulation pertaining to cell adhesion and migra-

Fig. 10. Subcellular localization of cytohesin-1 or cytohesin-1 394/5 AG double mutant in cytochalasin D-treated cells. Constructs were expressed as cIg fusion proteins in Jurkat E6 cells, which were incubated with 40 ng/ml phorbol ester (PMA) or 10 μg/ml cytochalasin D as indicated. In the presence of cytochalasin D, the cortical F-actin is depolymerized, resulting in a diffuse localization of actin (red panels), whereas cytohesin-1 proteins are still localized to the membrane (green panels). The overlay shows partial colocalization of cytohesin-1 and cortical actin in cells that were not treated with cytochalasin D.

fication on ARNO binding to PIP₃. From these data and from our studies, it may be concluded that nonspecific membrane association of the proteins, but not PIP₃ binding, might be abrogated by the phosphorylation events.

We have recently shown that the GDP/GTP exchange function of cytohesin-1 is involved in cell spreading (25). Our data and data from several other studies implicated the actin cytoskeleton in the spreading phenomenon. We therefore analyzed whether cytohesin-1 was regulated in its ability to associate with F-actin in cells. In marked contrast to our attempts to relate the phosphorylation of cytohesin-1 to membrane and phospholipid binding, we found a very clear correlation of cytohesin-1 phosphorylation and its association with the nucleidepleted, detergent-insoluble cell fraction. Furthermore, pretreatment of the cells with an agent known to prevent the formation of F-actin completely abrogated this association. It therefore appears highly likely that the phosphorylation events mediate direct or indirect association of cytohesin-1 with cellular F-actin. Notably, the gain of negative charge mutants were not capable of mimicking the effects of phosphorylation in vitro and in cells. The electrostatic switch mechanism, which had been proposed for ARNO, appears not to play a role in this

We do not currently know how association of cytohesin-1 with elements of the actin cytoskeleton regulates β₂ integrin-dependent adhesion. It is also unclear which elements of the actin cytoskeleton interact with the phosphorylated protein. The polybasic domain might not necessarily be directly dependent for this interaction, but its phosphorylation could also induce conformational changes of the whole protein, which is then enabled to form novel associations. However, the importance of F-actin remodeling for adhesion regulation has been stressed by a number of recent studies (reviewed in Ref. 39). A model has been proposed that predicts a highly dynamic interaction of the cytoplasmic domains of LFA-1 with the actin cytoskeleton (4). According to this model, ligand binding by LFA-1 is initiated by detachment of the relatively immobile LFA-1 molecule from the cytoskeleton. This, in turn, might enable lateral movement of the protein in the plane of the membrane and might thus favor oligomerization or clustering of the adhesion molecules, which would result in ligand capture. After ligand binding, the association of the receptor with the cytoskeleton could be reinforced, and the molecular assembly could thus be stabilized over longer periods of time. Cytohesin-1 could certainly play a role in these events, either by regulating calpain proteases (likely mediating the initial detachment of the integrins from the F-actin network (41)) or by later stabilizing a clustered state through the association of the ligand-bound receptor with actin fibers. We have considered the possibility that cytohesin-1 might be a direct adaptor between the cytoskeleton and LFA-1 and that this function may be regulated by the observed phosphorylation. However, preliminary binding studies did not reveal phosphorylation-induced regulation of the cytohesin-1-LFA-1 interaction to date. On the other hand, it might well be that the phosphorylation of cytohesin-1 affects LFA-1 function indirectly through regulated association of the integrin with other cytoskeletal linker proteins, which in turn bind LFA-1, such as α-actinin (42), talin (43), or filamin (44). Furthermore, a number of recent studies point to a specific involvement of the ARF-GTPase-activating protein p95-APP1, a PKL family member, in the coordination of membrane transport and actin polymerization during cell migration (19, 37, 40). Thus, there is an emerging theme of functional cross-talk between ARF- and Rho-regulatory proteins in cytoskeletal regulation pertaining to cell adhesion and migra-
tion. Future biochemical and functional studies employing direct microinjection or transfection of phosphorylated or unphosphorylated protein into cells may aid in further elucidation of the specific role of the phosphorylation event.

Taken together, we provide compelling evidence for a specific and functional phosphorylation of cytohesin-1 at its carboxyl terminus. Surprisingly, we observe different consequences of the phosphorylation event in cytohesin-1 as compared with data shown previously for the homologous protein ARNO. However, these differences may contribute to the functional specificities of the highly similar proteins in physiological contexts.

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