Phosphoinositide 3-OH Kinase Activates the β2 Integrin Adhesion Pathway and Induces Membrane Recruitment of Cytohesin-1*

(Received for publication, October 14, 1997, and in revised form, February 23, 1998)

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Signal transduction through phosphoinositide 3-OH kinase (PI 3-kinase) has been implicated in the regulation of lymphocyte adhesion mediated by integrin receptors. Cellular phosphorylation products of PI 3-kinases interact with a subset of pleckstrin homology (PH) domains, a module that has been shown to recruit proteins to cellular membranes. We have recently identified cytohesin-1, a cytoplasmic regulator of β2 integrin adhesion to intercellular adhesion molecule 1. We describe here that expression of a constitutively active PI 3-kinase is sufficient for the activation of Jurkat cell adhesion to intercellular adhesion molecule 1, and for enhanced membrane association of cytohesin-1. Up-regulation of cell adhesion by PI 3-kinase and membrane association of endogenous cytohesin-1 is abrogated by overexpression of the isolated cytohesin-1 PH domain, but not by a mutant of the PH domain which fails to associate with the plasma membrane. The PH domain of Bruton’s tyrosine kinase (Btk), although strongly associated with the plasma membrane, had no effect on either membrane recruitment of cytohesin-1 or on induction of adhesion by PI 3-kinase. Having delineated the critical steps of the β2 integrin activation pathway by biochemical and functional analyses, we conclude that PI 3-kinase activates inside-out signaling of β2 integrins at least partially through cytohesin-1.

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 either in development, or in somatic functions such as wound healing, and the regulation of complex cell-cell or cell-matrix interactions within the immune system (1–3). The avidity of integrins for their ligands is dependent on the activation state of the cell on which they are expressed (4). This type of regulation of cell adhesion has been termed inside-out signaling, because intracellular signaling pathways, triggered by, e.g., protein-tyrosine kinase or G-protein-coupled receptors, have been shown to contribute to integrin-mediated adhesiveness (5, 6). The mechanisms by which cytoplasmic signals are transmitted across the plasma membrane through integrin receptors remain unclear, but compelling evidence suggests that the intracellular domains of both α (7–11) and β chains participate in this process (12–17).

Previous studies have attempted to elucidate these signaling pathways. In T lymphocytes, a variety of cell surface receptors have been shown to regulate PI 3-kinase activity by recruiting the p85/110 isoform via SH2-phosphotyrosine interactions, including the T cell antigen receptor, CD2, and CD28 (reviewed in Ref. 18). All of these receptors are capable of inducing integrin activation, and PI 3-kinase has therefore been implicated in the up-regulation of cell adhesiveness (19–23). PI 3-kinase has also been strongly implicated in the activation of the α4β2 integrin in platelets and megakaryocytic cells, respectively (24, 25). However, the precise nature of the underlying mechanisms remained unknown because, first, the proximal regulatory elements of integrin affinity modulation were not characterized, and, second, the cellular mode of action of PI 3-kinase was not well understood.

Recently, candidate cytoplasmic regulatory factors of integrin activation have been identified, either by biochemical methods or with the help of the two-hybrid system (26–29). 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 α2β1 (CD11a/18, LFA-1) (29). Cytohesin-1 bears a short amino-terminal domain that may aid in oligomerization, an extended central homology region that is similar to the yeast Sec7 protein, and a carboxyl-terminal pleckstrin homology (PH) domain. Overexpression of cytohesin-1 or subdomain constructs in the Jurkat T cell line was shown to have pronounced in vitro effects on the binding of αβ2 to its ligand, the 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. Since the PH domain was not found to be mediating the interaction with the integrin cytoplasmic domain, it has been postulated that its unidentified cellular ligand may be an upstream component of the inside-out signaling pathway of αβ2. The finding that the overexpressed, isolated Sec7 domain acted only as a partial agonist pointed in the same direction (29).

PH domains are structural modules present in more than 100 proteins that play known or postulated roles in signal transduction. It is a commonly found thread that PH domains may aid in membrane recruitment of proteins through their interactions with phosphorylated ligands present at the inner leaflet of cellular membranes (30–32). Although a subgroup of PH domains is capable of interacting with tyrosine-phosphorylated

* This work was supported by the Deutsche Forschungsgemeinschaft and by the Bundesministerium für Forschung, Bildung, und Technologie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PI 3-kinase, phosphoinositide 3-kinase; SH, Src homology; PH, pleckstrin homology; βark, β-adrenergic receptor kinase; GST, glutathione S-transferase; PBS, phosphate-buffered saline; HBSS, Hanks’ buffered salt solution; ICAM-1, intercellular adhesion molecule 1; HS, hypotonic solution; MBSI, Mops/NatCl/IGEPAL CA-630; IP3, inositol (1,3,4,5)-tetrakisphosphate; IP6, phosphatidylinositol (3,4,5)-triphosphate; Mops, 4-morpholinepropanesulfonic acid; PMA, phorbol 12-myristate 13-acetate.
lated proteins (30), much reminiscent of the SH2 domain function, several isolated PH domains have been shown to bind to phosphoinositides such as phosphatidylinositol (4,5)-bisphosphate in vitro (33–37). Interestingly, certain PH domains show in vitro binding preference to lipid compounds, which are in vivo phosphorylation products of PI 3-kinase. The PH domains of Akt, Btk, and of GRP-1, a close homolog of cytohesin-1, have been demonstrated to belong to the latter group because they bind either phosphatidylinositol (3,4)-bisphosphate (Akt), or phosphatidylinositol (3,4,5)-trisphosphate (PIP3) with high affinity (38–41).

One of the important topics that remain to be addressed is the characterization of the lymphocyte inside-out signaling pathway proximal to the integrin cytoplasmic domains. In this study, we show for the first time that a constitutively active version of PI 3-kinase suffices to activate the αβ2 adhesion pathway in a T cell line. Functional, biochemical, and cell biological evidence is provided, which suggests that cytohesin-1 is located downstream of PI 3-kinase and that it is regulated by the recruitment of its PH domain to the plasma membrane.

MATERIALS AND METHODS

Constructs—Cytoplasmic immunoglobulin fusion proteins have been described previously (29). A cDNA fragment for the PH domain of murine Btk (corresponding to amino acids 8–170) has been subcloned into pcglTkg vaccinia expression vector (29). The cDNAs for Myc-P110α and Myc-P110α* (917–950) were subcloned into the pcglTkg vaccinia expression vector (70).

Adhesion Assay—Jurkat E6 cells were infected with recombinant vaccinia viruses as described (29). 6 h after infection, cells were labeled with 12 μg/ml bismiuzmide H33342 fluorochrome trihydrochloride (Calbiochem) for 30 min at 37 °C, collected by centrifugation, resuspended in Hanks’ buffer saline solution (HBSS), and delivered to 96-well plates (Nunc, Maxisorp) at 1.5 × 10⁶ cells/ml. Prior to 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) bovine serum albumin in PBS, incubated with culture supernatants from COS cells expressing ICAM-1-Rg fusion protein, and subsequently used in the assay. Where indicated in the figures, cells were incubated with 100 nM wortmannin (Sigma) 0.5 h prior to the adhesion assay. Cells were then allowed to adhere for 1 h at 37 °C, adherent cells were carefully washed off with 3 × 300 μl of HBSS. Bound cells were assayed in 100 μl of 2% (v/v) formamide in PBS using a fluorescence plate reader (Cytofluor II, PerSeptive). The signal of 1.5 × 10⁵ cells/well at 490 nm corresponds to 100% adhesion. Each value is the mean of two determinations carried out in triplicate.

Lactoperoxidase-Deposition Analysis—Displacement assay for [3H]IP3 binding activity was carried out according to Ref. 71 with modifications; 5 μM of purified, polyhistidine-tagged PH domains derived from expression in Escherichia coli BL21 (29) were rebound to 10 μl of nickel-agarose at 20 mCi imidazole in MBSD (50 mM Mops, pH 6.8, 100 mM NaCl, 0.1% Igepal CA-630) in a final volume of 40 μl. After incubation with 10 nM [3H]IP3 for 10 min on ice, increasing amounts of unlabeled inositol phosphate were added for 15 min. Then samples were transferred to ultraspun microfilters (Millipore, cut-off 10 kDa) and washed with 50 μl of MBSD by centrifugation at 1500 × g. Protein-bound radioactivity was eluted by MBSD, containing 200 mM imidazole. Samples were collected by centrifugation and counted by solid phase scintillation. Values were corrected for nonspecific binding of 10 nM [3H]IP3 to nickel-agarose. Each value represents the mean of two determinations carried out in duplicate.

Gel Filtration Assay—Purified polyhistidine-tagged full-length cytohesin-1 derived from expression in E. coli BL21 at 10 μM was mixed with 10 nM [3H]IP3 and [3H]Ins(1,4,5)P3, respectively, and with 20 μM of the appropriate unlabeled isomer in MBSD. The mixture was applied to an Amersham Pharmacia Biotech Fast Desalting Column HR. The elution position of the protein was monitored at 215 nm with the SMART system (Amersham Pharmacia Biotech). Scintillation counting was used to detect the elution position of the radioactively labeled inositol phosphates. Binding specificity was assessed with a mixture of 10 nM [3H]IP3 and purified GST.

Cellular Fractionation—Cells that had been infected with recombinant vaccinia viruses or uninfected cells were collected by centrifugation and resuspended on ice in 0.5 ml of ice-cold hypotonic solution (HS: 10 mM Hepes, pH 7.5, 10 mM KCl, 10 mM MgCl2, 0.5 mM dithiothreitol) containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Then cells were sheared, the nuclei were removed, and the supernatant cytosol was collected as described (72). The cytoclastic fraction was brought to a final concentration of 125 (w/v) CaCl2-60 and 150 mM NaCl and used directly for immunoprecipitation. The pellet was resuspended, washed with HS, and centrifuged at 15,000 × g for 15 min. The resulting pellet was resuspended in HS containing 1% (w/v) Igepal CA-630 and 150 mM NaCl, centrifuged, and the supernatant representing the particulate fraction was subjected to immunoprecipitation at Protein A-Sepharose employing an antigen-antibody directed against cytohesin-1 (29). Immunoprecipitates were analyzed by standard Western blot techniques. To assess that cytoplasmic contents were not trapped in the particulate fraction, lactate dehydrogenase activities were monitored as described (59).

Indirect Immunofluorescence—Six hours after infection of Jurkat E6 cells with recombinant vaccinia viruses, cells were placed on poly-L-lysine-covered microscope slides for 1 h in a humidified chamber at 37 °C. Non-adherent cells were then washed off with HBSS, and adherent cells were fixed and immobilized with freshly prepared 2% (w/v) formaldehyde in PBS overnight at 4 °C. Subsequently, cells were permeabilized for 15 min with 0.2% (w/v) Triton X-100 in PBS, blocked with 2% (v/v) glycerin in PBS, and incubated with a fluorescein isothiocyanate-labeled goat anti-human IgG (Fc-specific antibody) (Dianova) in PBS for 2 h at room temperature. In double-label experiments, tetramethylrhodamine isothiocyanate-coupled phalloidin (Sigma) at 1 μg/ml was included. 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 at 20 mg/ml as antifading reagent. Then samples were either examined on a Zeiss Axioshot microscope using a Zeiss Neofluor 40 × 1.3 oil immersion objective, or on a confocal laser scanning apparatus (Leica TCS-NT system, Leica) attached to a Leica DM IRB inverted microscope with a PLAPO 63 × 1.3 oil immersion objective. Conventional immunofluorescence images were recorded on Kodak T-MAX 400ASA film. Confocal images were collected as 512 × 512 pixel files and processed with the help of the Photoshop program (Adobe).

p110α Expression and Activity—Six hours after infection of Jurkat E6 cells with recombinant Myc-P110α and Ig control, cells were lysed in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1 mM Myc-IP3, 0.5 mM dithiothreitol, 0.1 mM sodium orthovanadate, containing 1% (v/v) Igepal CA-630, protease inhibitors leupeptin and aprotinin at 10 μg/ml, and 1 mM phenylmethylsulfonyl fluoride. After collection of the supernatant by centrifugation at 15,000 × g, the samples were subjected to immunoprecipitation with mouse anti-c-Myc antibody (monoclonal antibody 9E10; American Type Culture Collection) for 1 h at room temperature. For detection of p110α expression, precipitates were washed three times with lysin buffer and subjected to immunoblot analysis, using monoclonal antibody 9E10 as first antibody.

The assay for immunoprecipitated PI 3-kinase activity was essentially carried out according to Ref. 73 with modifications. Following washing with the lysis buffer, assay was performed with 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA three times and with kinase buffer containing 50 mM Hepes, pH 7.6, 5 mM MgCl2, 1 mM EDTA twice. Finally, the beads were resuspended in 100 μl of kinase buffer containing 20 μg of sonicated phosphatidylinositol (PI). The reaction was started by adding 1 μl of γ-[32P]ATP and 100 μM ATP. After incubation of the sample for 20 min at 25 °C with agitation, the reaction was stopped by adding 20 μl of 6 N HCl and the radiolabeled lipid was extracted with 160 μl of chloroform:methanol (1:1) by brief mixing. Thin layer chromatography was run with the chloroform extracts on a Silica Gel 60 plate (Merck) using a solvent system of chloroform:methanol:concentrated NH3:water (60:47:2:11). Radiolabeled phosphatidylinositol was visualized by autoradiography.

Phospholipid Binding to GST-Cytohesin-1 Measured by IAsys Bio-sensor Technology—Large unilamellar liposomes were generated by dialysis according to (74). Liposomes consisted 60% (w/v) β-palmitoyl-γ-oleoyl-t-o-phosphatidylycholine, 30% (w/v) dioleoyl-t-o-phosphatidylyglycerol, obtained from Sigma and 10% (w/v) PI(3,4,5)P3 (Mantrey, Inc.). Lipids were dried and resuspended in 10 mM Hepes, pH 7.4, 80 mM KCl, 15 mM NaCl, 0.7 mM NaH2PO4, 1 mM EDTA, containing 50 μM 1-o-octyl-γ-glucero-pyrophosphorylglycerol. To 1 μl of liposomes were added 1 μl of IAsys surfact (FCS-0301, not derivatized) at 0.1 mg of lipid/ml of PBS. Subsequently, remaining unspecific binding sites were blocked by GST and binding of either GST-cytohesin-1 or its mutant GST-cytohesin-1 (R281C) was analyzed at 2.5 μg/ml. Cuvette was regenerated by subsequent washing with 1 N NaOH/1% (w/v) SDS, water, 1 N HCl, water, and methanol.
RESULTS

Expression of a Constitutively Active PI 3-Kinase in Jurkat Cell Induces Binding of the αLβ2 Integrin to ICAM-1—Previous reports implicated a role of PI 3-kinase in the regulation of integrin-mediated adhesiveness (see Introduction and references therein). We therefore investigated whether a constitutively active PI 3-kinase can up-regulate lymphocyte adhesion directly. Overexpression of the catalytic subunit of mammalian PI 3-kinase is usually not sufficient for a constitutively active phenotype, but three groups have recently generated chimeric, mutant, or membrane-associated versions of PI 3-kinase, which were shown to result in an activation of cellular signaling pathways of fibroblast-like cells (42–45).

A chimera (p110*) that comprises the catalytic subunit (p110) of murine PI 3-kinase, fused at the amino terminus to a regulatory domain derived from the p85 subunit (42), was used in this study (Fig. 1A). A kinase deficient variant of p110*, bearing a short in-frame deletion (33 amino acids; Δ917–950) within the kinase domain, served as control (Fig. 1A).

Using recombinant vaccinia viruses, p110* or the kinase defective derivative thereof were expressed in Jurkat cells (Fig. 1E), and detected with a monoclonal antibody directed against the carboxyl-terminal Myc tag. Lipid kinase activity of the chimeric protein was assayed by its ability to specifically phosphorylate phosphatidylinositol in vitro (Fig. 1E). Subsequently the effect of p110* expression on αLβ2-integrin-mediated adhesion of Jurkat cells was analyzed. A 5-fold increase of adhesion to ICAM-1 was observed for cells that had been infected with vaccinia viruses expressing p110*, as compared with cells infected with control viruses (Fig. 1B). Stimulation of these cells with an anti-T cell receptor antibody, or overexpression of cytohesin-1 by recombinant vaccinia viruses, which had previously been shown to be sufficient for the activation of the αLβ2 integrin in Jurkat cells (29), were used as positive controls in this assay (Fig. 1B). Inhibitors of PI 3-kinase activity were then used to investigate the role of PI 3-kinase in Jurkat cell adhe...
sion to ICAM-1 in greater detail (Fig. 1C). We found that preincubation of Jurkat E6 cells for 0.5 h with known inhibitors of PI 3-kinase (46), either 50 nM wortmannin or 50 μM LY294002, inhibited OKT3-induced adhesion completely, whereas the responses to PMA or Mn2⁺ were not affected, the latter result being consistent with a previous report (23). These data suggest that PI 3-kinase acts downstream of the T cell antigen receptor in the LFA-1 adhesion pathway. p110* was partially inhibited, probably because the consequences of expression of p110* expression cannot fully be reversed when the indicated concentrations of inhibitors and short incubation times are used. Although prolonged incubation (i.e. several hours, data not shown) with Ly294002 or wortmannin reduced the adhesion levels induced by p110* dramatically, we found that all responses, including adhesion induced by phorbol ester and Mn2⁺ were strongly affected in this case, probably attributable to some rather nonspecific, global effect. LFA-1-dependent adhesion mediated by overexpression of cytohesin-1 was partially affected, suggesting that this phenotype is at least in part independent of PI 3-kinase, consistent with the idea that cytohesin-1 may be located downstream of PI 3-kinase. Taken together, these findings indicate that a constitutively PI 3-kinase is sufficient for the up-regulation of β₂ integrin-mediated cell adhesiveness.

The PH Domain of Cytohesin-1 Associates with Cellular Membranes—How is PI 3-kinase coupled to the regulation of integrin activity? We postulated that the PH domain of cytohesin-1 may provide a link between the activation of cell adhesion mediated by PI 3-kinase and the recruitment of cytohesin-1 to the plasma membrane. Therefore, membrane recruitment of cytohesin-1 in the Jurkat cell line was assessed biochemically by the separation of crude cell extracts. Localization of either overexpressed cytohesin-1 or the isolated PH domain in the cytosolic or the particulate fractions was detected by Western blot analysis. We found that a substantial fraction of both full-length cytohesin-1 and the isolated PH domain was associated with membranes (Fig. 2, A and B).

Membrane Association of the Cytohesin-1 PH Domain Is Required for Its Role in the Regulation of Cell Adhesion—Is the membrane recruitment mediated by the cytohesin-1 PH domain associated with its cellular function? To answer this question, we generated a point mutant (R281C) of the PH domain, which corresponds to a residue in the PH domain of Btk that had previously been shown to important for PIP₂ binding and cellular function (39, 47). Accordingly, we found that introduction of the R281C mutation abolished the membrane association of the cytohesin-1 PH domain (Fig. 2B). We then investigated the effect of this mutation on the ability of the cytohesin-1 PH domain to inhibit β₂ integrin-mediated adhesion of Jurkat cells. Fig. 2C shows that the R281C mutant had lost the dominant inhibitory potential, which corresponds directly to its inability to associate with membranes.

Cytohesin-1 Localizes to the Plasma Membrane—To which cellular membranes does cytohesin-1 bind? We have shown that cytohesin-1 can be co-precipitated with the α₅β₂ integrin from Jurkat cells (23) and therefore postulated that the PH domain of cytohesin-1 predominantly associates with the plasma membrane. Immunofluorescence studies were performed to test this hypothesis. To this end, a full-length cytohesin-1 fusion protein or the respective wild type or mutant PH domain constructs were expressed in the Jurkat line. The cells were subsequently immobilized on poly-L-lysine coated slides, fixed, permeabilized, and treated with an fluorescein-isothiocyanate conjugated antibody directed against the cIg portion. In addition to conventional immunofluorescence microscopy (Fig. 3, panels D, G, F, and M) subcellular distributions of the various cytohesin-1 fusion proteins were examined using the confocal laser scanning method. Measurement of the pixel intensity along a transect through a cell which was double stained for the respective Ig fusion protein and actin revealed colocalization of the full-length cytohesin-1 (panels D–F) or the PH-domain fusion construct (panels J–L) with the plasma membrane. By contrast, the cytosolic Ig control protein (panels
G–I) or the PH (R281C) mutant showed diffuse cytoplasmic expression. The observed plasma membrane association of cytohesin-1 through the PH domain is consistent with its role in the regulation of $\beta_2$ integrin activity. The R281C Mutation of the Cytohesin-1 PH Domain Abrogates Binding Either to Inositol (1,3,4,5)-Tetrakisphosphate or PIP3—Is the plasma membrane association of cytohesin-1 through its PH domain maintained by a ligand that may be generated by PI 3-kinase? One of the products of PI 3-kinase, phosphatidylinositol (3,4)-bisphosphate, has been shown to bind to the PH domain of the proto-oncogene Akt-1 and to regulate its kinase activity (38, 48, 49). Cellular effector functions of Akt-1 include cell survival (50). Recently, a murine member of the cytohesin family, GRP-1, was identified by its ability to bind a different product of PI 3-kinase, PIP3, when a cDNA expression library was screened with inositol phospholipid ligands (40). IP4 bears the same headgroup as PIP3 and has been postulated to play a role in detachment of the PH domain of cytohesin-1 and GRP-1 from the plasma membrane (40). We therefore investigated whether the R281C mutation of the cytohesin-1 PH domain interfered with its specific binding to inositol phosphate ligands. Using a gel filtration assay (51), we confirmed that IP4 co-migrated with purified, E. coli-derived full-length cytohesin-1 or with the isolated PH domain, whereas a control compound, inositol (1,4,5)-trisphosphate, did not co-elute with the purified proteins (Fig. 4A). By contrast, IP4 did not co-migrate with a control protein, GST (Fig. 4A).

More quantitative analyses, employing a ligand displacement assay revealed that the PH domain of cytohesin-1 binds to IP4, which is half-maximally displaced at a concentration of 2.5 $\mu$M (Fig. 4B). As expected, the R281C mutant of the cytohesin-1 PH domain does not bind to IP4 in vitro (Fig. 4B). No binding to IP4 was observed for the PH domain of the $\beta$-adrenergic receptor kinase ($\beta$ARK, Fig. 4B), which corresponds to its inability to block integrin adhesion in T cells (29).

Finally, we tested the effect of the R281C mutation on binding of cytohesin-1 to PIP3 in vitro. Liposomes containing PIP3 were immobilized onto IAsys biosensor surfaces, and GST fusion proteins of cytohesin-1 or cytohesin-1 R281C (Fig. 4D) were applied to the buffer phase above the liposomes. As shown in the diagram (Fig. 4C), GST-cytohesin-1 binds to PIP3-con-
taining liposomes with a fast on-rate and a very slow off-rate, indicating a high affinity interaction. In marked contrast, GST-cytohesin-1 (R281C) did not bind to PIP3-liposomes at all. Thus, the R281C mutation of the PH domain of cytohesin-1 interferes with plasma membrane association, function, and ligand binding.

Expression of p110* in Jurkat Cells Induces Membrane Association of Endogenous Cytohesin-1, but Overexpression of the PH Domain Abrogates Membrane Localization of Cytohesin-1—All data presented above suggested that PI 3-kinase regulates the activity of a β2 indirectly through membrane recruitment of cytohesin-1. We therefore overexpressed p110* in the Jurkat line, fractionated the cells, and assessed the subcellular distribution of endogenous cytohesin-1. We found that membrane association of cytohesin-1 was ~2.5-fold enhanced in the presence of P110*, as compared with cells in which the control constructs had been expressed (Fig. 5). Semiquantitative analysis of 12 independent experiments confirmed these results; the mean specific induction of cytohesin-1 in the membrane fraction following p110* expression was found to be 2–3-fold (Fig. 5 and data not shown). Moreover, overexpression of the PH domain construct interfered with membrane association of endogenous cytohesin-1 (Figs. 5 and 6E). This result is consistent with the observed dominant negative inhibition of Jurkat cell adhesion to ICAM-1 by the PH domain fusion protein.

A PH Domain Construct of Cytohesin-1 Blocks Up-regulation of aβ2 Integrin Adhesion Mediated by p110*, whereas the R281C Mutant or the PH Domain of Bruton’s Tyrosine Kinase Have No Effect—Adhesion assays as well as biochemical analyses were then performed to study the functional relationship of PI 3-kinase and cytohesin-1 in Jurkat cells directly. We had previously shown that dominant negative inhibition of LFA-1 adhesion to ICAM-1 by the cytohesin-1 PH domain was relatively specific (29), but it was not shown whether the PH domain controls used in this were capable of entering the same compartment. In order to examine this in greater detail, the PH...
Percent values represent the particulate portions of total immunoprecipitation of cytohesin-1, using the antibody bands as controls for loading normalization. Quantitation of cytohesin-1 was performed by densitometry reading of immunoprecipitated cytohesin-1, using the antibody bands as controls for loading normalization. Percent values represent the particulate portions of total immunoprecipitated cytohesin-1 within each sample, i.e. particulate and soluble fractions combined.

In this study, we show by both functional and biochemical analyses that PI 3-kinase is a candidate upstream regulator of cytohesin-1, an intracellular mediator of integrin activation, which has previously been shown to interact directly with the cytoplasmic domain of the β2 chain. Our experiments indicate that cytohesin-1 is recruited to the plasma membrane through its carboxyl-terminal PH domain following PI 3-kinase activation and that membrane association of cytohesin-1 through its PH domain appears to be a functional prerequisite for β2 integrin activation.

PH domains have been found in many signaling molecules. In an attempt to explain the finding that certain PH domains bind PIP2 in vitro, it was postulated that they may serve as membrane recruitment modules (30, 31). Broad evidence exists supporting this hypothesis (37, 53–55). However, some PH domains may have other functions, because in the case of the pleckstrin protein it was described that membrane localization is provided by the amino-terminal but not by the carboxy-terminal PH domain (59). Membrane recruitment of proteins mediated by PH domains is not necessarily constitutive, and it appears unlikely that PIP2, although it may be the physiological ligand for the PLC-δ PH domain (32), is a physiologically relevant interaction structure for most PH domains. Evidence points to the possibility that membrane association of PH domains is regulated by signal transduction events (37, 49, 60).

Since some PH domains show a binding preference for PIP2 or phosphatidylinositol (3,4)-bisphosphate in vitro, PI 3-kinase has been implicated in the regulation of membrane recruitment of PH domains. At least three types of PI 3-kinase isoform have been found in mammalian cells to date (61). One of them, PI 3-kinase α, is predominantly coupled to tyrosine kinase-activated pathways, whereas a different enzyme, PI 3-kinase γ, is induced by heterotrimERIC G-proteins. Binding specificity is conferred by the regulatory subunits, p85 and p101, respectively. Receptors that are involved in lymphocyte activation, such as the T cell receptor or CD28, couple to signal transduction through tyrosine kinase-activated pathways, and these are also known to induce PI 3-kinase function (46). Our findings presented here are consistent with the view that PI 3-kinase α is involved in the up-regulation of cell adhesion by hematopoietic receptors and we provide evidence for a mechanism. First, a constitutively active PI 3-kinase induces membrane association of cytohesin-1 as well as up-regulation of β2 integrin adhesion to ICAM-1; and second, overexpression of a PH domain construct of cytohesin-1 or treatment of the cells with PI 3-kinase inhibitors block cell adhesion stimulated by T cell receptor activation. Co-expression studies in Jurkat cells finally showed that expression of a cytohesin-1 PH domain construct but not the introduction of a mutant PH domain, incapable of membrane association, blocked p110α induced adhesion. Induction of cell adhesion to ICAM-1 was neither blocked by expression of the PH domain of Bruton’s tyrosine kinase, which was shown previously to bind PIP3 in vitro and to mediate membrane association in vivo. Therefore, cytohesin-1 appears to be coupling PI 3-kinase to the activation of cell adhesion by β2 integrin receptors. On the basis of our data, however, we still cannot fully exclude that other proteins, which are regulated by PI 3-kinase, may also play important roles in the regulation of β2 integrin-mediated cell adhesion. In fact we found that adhesion mediated by overexpressed cytohesin-1 was partially blocked by PI 3-kinase inhibitors, suggesting that PI 3-kinase may also contribute to a fully adhesive phenotype by other cellular functions. Incubating Jurkat cells with PI 3-kinase inhibitors for extended periods of time completely inhibited their ability to spread on ICAM-1-coated surfaces (data not shown), and this inability interfered with tight adhesion, irrespective of which stimulus was used.

Since integrin-mediated adhesiveness can also be triggered by chemokine receptors that signal through G-proteins (62, 63), an intriguing thought is that other members of the PI 3-kinase family may also be involved in the regulation of cell adhesion. This has in fact been suggested by a recent study (28).

PI 3-kinase appears to activate membrane recruitment of other signaling molecules, such as the Akt proto-oncogene, through their PH domains. How is signaling specificity regulated in vivo? One possible answer is that ligand specificity is not exclusively conferred by PI 3-kinase at the level of phosphoinositide phosphorylation. Akt, for example, preferentially binds to phosphatidylinositol-(3,4)-bisphosphate. It has therefore been postulated that PI 3-kinase as well as a phosphatidylinositol (5)-phosphatase are required to generate the Akt ligand in vivo (38). However, the PH domains of cytohesin-1, GRP-1, Bruton’s tyrosine kinase, and maybe more, all bind PIP2, with high affinity. A second layer of specificity may therefore be provided by additional PH domain ligands. The PH domain of Bruton’s tyrosine kinase can apparently bind protein...
Fig. 6. A, the PH domain of Btk partitions to both the cytoplasmic and the particulate fraction. An Ig fusion protein of Btk was expressed by recombinant vaccinia viruses and cellular fractionation analysis was subsequently performed as for the experiments shown in Fig. 2. The PH domain construct of cytohesin-1 and the cytosolic Ig control protein were used as controls. B, the Btk-PH domain is associated with the plasma membrane. Subcellular localization was detected as described in Fig. 3. C and D, adhesion assays. The PH domain of Btk had no effect on LFA-1 dependent adhesion to ICAM-1 (C). C, □, not stimulated; ■, OKT3. P110* inducedJurkat cell adhesion to ICAM-1 is blocked by overexpression of the PH domain of cytohesin-1. Double infections revealed that neither the PH (R281C) mutant of cytohesin-1 nor the PH domain of Btk exerts any dominant negative inhibition of β2 integrin adhesion. E, the PH domain of Btk does not interfere with membrane association of endogenous cytohesin-1. By contrast, overexpression of the PH domain of cytohesin-1 led to a competitive inhibition of endogenous cytohesin-1 from the particulate fraction (see also Fig. 5).
kinase C (64), and a carboxyl-terminal portion of the PH domain of the βark is required for the interaction of βark with β-γ subunits of heterotrimeric G-proteins (35). In our study, the PH domain of Bruton’s tyrosine kinase did not interfere with Jurkat cell adhesion nor did it compete with membrane association of endogenous cytohesin-1, although it was strongly associated with the plasma membrane. This points indeed to a highly selective ligand usage of these two PH domains with the plasma membrane. This is an intriguing thought.

Acknowledgments—We thank Anke Kippel, Lewis Williams, and Julian Downward for the kind donation of PI 3-kinase cDNAs; Pascale Sideras and Michael Reth for the Btk cDNA; and Ernst-Ludwig Winnacker for continuous support.

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