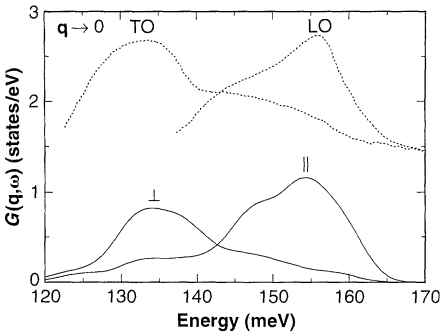

Fig. 3. Generalized density of states $G(\mathbf{q}, \omega)$ in the limit $\mathbf{q} \rightarrow 0$ for longitudinal and transverse excitations (solid lines). The LO and TO infrared absorption spectra (dashed lines) from (*3*) are shown for a qualitative comparison (in arbitrary units and on a semilogarithmic scale to enhance the structure in the shoulders).

modes parallel and orthogonal to the direction of **q**, a longitudinal and a transverse limit, respectively, were obtained (Fig. 3). The appearance of a well-defined splitting related to a LO-TO effect is now apparent. A qualitative comparison with the infrared absorption spectra measured by Kirk (*3*) shows good agreement: The position of the peaks as well as the structure in the wings are very similar in the theoretical and experimental spectra. Note, in particular, that the peak in the calculated longitudinal spectrum appears at higher frequencies than does the upper peak in the total density of states, in agreement with the position of the LO peak in infrared (*3*) and hyper-Raman (*28*) spectra as compared with the position of the upper peak in neutron spectra (*1*). The structure of the peaks can be further analyzed by considering the same long wavelength limit for the A_1 and T_2 projected modes. This reveals that the transverse spectrum originates almost exclusively from T_2 modes, with a vanishingly small contribution from A_1 modes. On the other hand, both modes contribute to the longitudinal spectrum; the A_1 mode contributes only to the principal peak, whereas the T_2 mode contributes both to the principal peak and to the wing at lower frequencies.

This first-principles study of the vibrational properties of a-SiO_2 provides a comprehensive understanding of the origin of the high-frequency doublet appearing in inelastic neutron scattering and its relation with infrared measurements. We expect that such detailed studies will play an important role in the assignment of other unidentified features in the vibrational spectra of amorphous systems.

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26. Compared to (*23*), we used improved basis sets with cutoffs of 24 and 200 rydbergs for the electronic wave functions and charge density, respectively.
27. We used the smallest finite **q** allowed by our periodic boundary conditions, avoiding the need for depolarizing fields (*9*).
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Signaling by Phosphoinositide-3,4,5-Trisphosphate Through Proteins Containing Pleckstrin and Sec7 Homology Domains

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Signal transmission by many cell surface receptors results in the activation of phosphoinositide (PI) 3-kinases that phosphorylate the 3′ position of polyphosphoinositides. From a screen for mouse proteins that bind phosphoinositides, the protein GRP1 was identified. GRP1 binds phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P_3] through a pleckstrin homology (PH) domain and displays a region of high sequence similarity to the yeast Sec7 protein. The PH domain of the closely related protein cytohesin-1, which, through its Sec7 homology domain, regulates integrin β2 and catalyzes guanine nucleotide exchange of the small guanine nucleotide–binding protein ARF1, was also found to specifically bind PtdIns(3,4,5)P_3. GRP1 and cytohesin-1 appear to connect receptor-activated PI 3-kinase signaling pathways with proteins that mediate biological responses such as cell adhesion and membrane trafficking.

Cellular 3-phosphoinositides are generated through the actions of a family of PI 3-kinases and appear to have regulatory roles in multiple cell functions. In yeast the Vps34 gene product, a PI 3-kinase with substrate specificity restricted to phosphatidylinositol (PtdIns), is required for correct sorting of carboxypeptidase Y to the vacuole (*1*). In mammalian cells three classes of PI 3-kinases have been identified in addition to a Vps34 homolog (*2*). These include p110 isoforms regulated by p85 subunits containing SRC homology 2 (SH2) domains (*3*), a p110γ PI 3-kinase regulated by heterotrimeric guanine nucleotide–binding proteins (*4*), and a PI 3-kinase containing a homology domain (C2 domain) thought to bind membrane lipids (*5*). The p85-p110 and p110γ type PI 3-kinases that are specifically activated by receptor signaling systems exhibit broad substrate specificities, and their activation leads to rapid phosphorylation of the inositol D-3 positions on PtdIns, PtdIns-4-phosphate [PtdIns(4)P], and

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PtdIns-4,5-bisphosphate [PtdIns(4,5)P_2] (6). Signaling by these 3-polyphosphoinositides appears to regulate such diverse cellular responses as membrane ruffling (7), chemotaxis (8), secretory responses (9), insulin-mediated membrane translocation of glucose transporters (10), membrane trafficking of growth factor receptors (11), and regulated cell adhesion (12).

Several protein targets of 3-phosphoinositides have been identified. These include certain protein kinase C isoforms (13) and the pleckstrin homology (PH) domain–containing protein kinases c-Akt and Btk (14). A series of sequentially activated protein kinases stimulated in response to 3-phosphoinositides causes activation of the p70 ribosomal protein S6 kinase (15). Additionally, 3-phosphoinositides appear to bind certain SH2 domains, including those within the p85 regulatory subunit of p110 PI 3-kinase (16). Thus, the phosphoinositides may serve as membrane localization elements that recruit target proteins to specific cellular organelles (17).

To identify general receptors for phosphoinositides (GRPs), we developed an expression library screening procedure using bovine brain phospholipids labeled with [γ-^{32}P]adenosine triphosphate (ATP) in the presence of the glutathione-S-transferase (GST) fusion protein GST–p110 PI 3-kinase (18). High-performance liquid chromatography (HPLC) analysis of the reaction products confirmed the presence of ^{32}P-labeled PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 in this probe mixture. We screened mouse 3T3-F442A adipocyte and brain cDNA expression libraries with the labeled brain phosphoinositides (Fig. 1). A single clone from each cDNA library screen reproducibly bound the ^{32}P label from the probe mixture upon subcloning and plaque purification (Fig. 1A). The proteins encoded by both brain and adipocyte cDNA clones bound [^{32}P]PtdIns(3,4,5)P_3 but not [^{32}P]PtdIns(3,4)P_2 or [^{32}P]PtdIns(3)P under the experimental conditions of the library screening procedure (Fig. 1B).

Both the brain and adipocyte cDNA clones encoded amino acid sequences of the same protein, GRP1. Standard hybridization techniques were used to obtain additional cDNA clones of this species that encode four more amino acids including a putative NH_2-terminal methionine, and a full-length sequence was deduced (Fig. 2). Database searches showed GRP1 to be highly similar to a protein encoded by the human cDNA B2-1, originally cloned from cytolytic natural killer T lymphocytes (19). Both GRP1 and the B2-1 protein contain PH domains (20) and Sec7 homology regions (21, 22) (Fig. 2A). The B2-1–encoded protein has been called

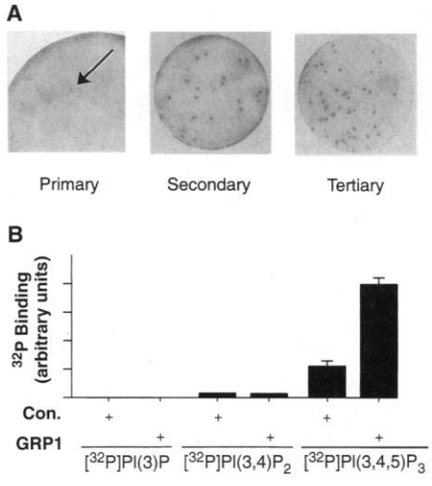

Fig. 1. Expression cloning of GRP1 cDNA. (**A**) Autoradiographs of nitrocellulose filters at different stages of purification of the cDNA clone identified in a mouse 3T3-F442A adipocyte cDNA expression library. Filters were incubated with mixed brain phosphoinositides labeled at the 3′ position with p110 PI 3-kinase and [γ-^{32}P]ATP, and then washed. The primary screen was performed in 15-cm dishes and subsequent screens in 10-cm dishes. (**B**) Binding specificity of the isolated cDNA clone. About 1000 pfu of the cDNA library (Con.) or the isolated cDNA clone (GRP1) were spotted on plates containing a layer of *Escherichia coli* and incubated with nitrocellulose filters as described. The filters were incubated with 0.5 × 10^6 cpm of either [^{32}P]PtdIns(3)P, [^{32}P]PtdIns(3,4)P_2, or [^{32}P]PtdIns(3,4,5)P_3 (PtdIns is abbreviated in labels as PI in all relevant figures), processed as described (18), and subjected to autoradiography and densitometry. The values are the means of four experiments, and the error bars represent the standard deviations. Similar results were obtained with a mouse brain cDNA expression library.

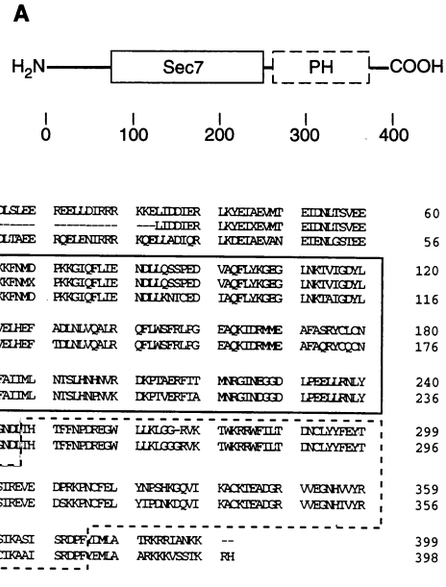

Fig. 2. Structure of GRP1. (**A**) Overall structure of GRP1 and cytohesin-1 (Ch-1). (**B**) Comparison of the deduced sequences of GRP1, B2-1/cytohesin-1, and EST 01394. The region corresponding to the Sec7 domain is boxed with a solid line, and the region corresponding to the PH domain is boxed with a dashed line. Sequence similarity between GRP1 and cytohesin-1 is 88%, and that between the Sec7 and PH domains of these two proteins is 93% and 94%, respectively. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

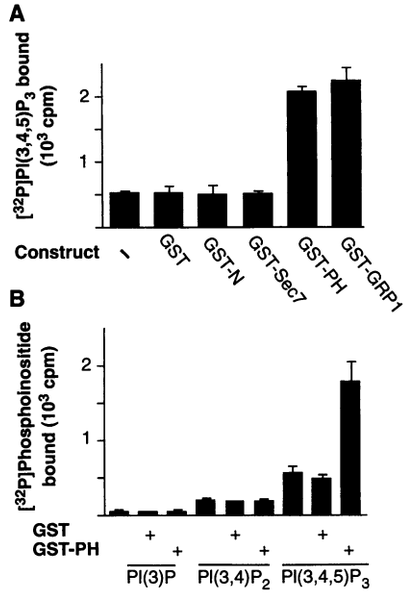

Fig. 3. Localization of PtdIns(3,4,5)P_3 binding to the PH domain of GRP1. (**A**) Identification of the binding domain for PtdIns(3,4,5)P_3 in GRP1. Proteins (150 pmol) were spotted on nitrocellulose filters and incubated with [32P]PtdIns(3,4,5)P_3 and the amount of bound lipid was determined as described (24). GST-N contains residues 5 to 71 of GRP1. The values are the means of quadruplicate determinations, and the error bars are the standard deviations. (**B**) Binding specificity of the PH domain of GRP1. Nitrocellulose filters with GST-PH were incubated with 25,000 cpm of each of the phospholipids, and the amount of bound lipid determined as described (24). The values are the means of quadruplicate determinations and the error bars are the standard deviations.

cytohesin-1 on the basis of its binding to the integrin β2 cytoplasmic domain through this Sec7 homology region (*23*), and the Sec7 domain of an isoform denoted ARNO (for ARF nucleotide binding site opener) has nucleotide exchange activity for the small guanine nucleotide–binding protein adenosine diphosphate–ribosylation factor 1 (ARF1) (*23*). A partial sequence of another probable isoform (Cts18) has been reported (*22*). The divergent amino acid sequences between GRP1, ARNO, cytohesin-1, and Cts18 appear not to result from species variation because a human expressed sequence tag (EST) found in the Institute for Genomic Research database shows a predicted amino acid sequence identical to that of mouse GRP1 between residues 34 and 120 (Fig. 2B). The sequence of the human EST differs from those of ARNO, cytohesin-1, and Cts18. Because there are additional EST sequences in the database, it is likely that more human isoforms of these proteins exist.

To determine whether PtdIns(3,4,5)P_3 binding could be localized to specific sequences within the GRP1 structure, we expressed GST fusion proteins containing either residues 239 to 399 (PH domain), residues 52 to 260 (Sec7 domain), residues 5 to 71, or residues 5 to 399 of GRP1 in bacteria and purified them on glutathione-conjugated beads (Fig. 3A). The nearly full-length GRP1 or its PH domain bound [^{32}P]PtdIns(3,4,5)P_3 (*24*), whereas the fusion proteins containing the Sec7 homology region or residues 5 to 71 did not (Fig. 3A). The GRP1 PH domain associated with [^{32}P]PtdIns(3,4,5)P_3 specifically and failed to bind [^{32}P]PtdIns(3,4)P_2 or [^{32}P]PtdIns(3)P (Fig. 3B). PH domains from PLC-δ (*25*) and various other proteins (*26*) bind PtdIns(4,5)P_2, but do not preferentially associate with a 3-phosphoinositide. In our [^{32}P]polyphosphoinositide binding assay, binding of [^{32}P]PtdIns(3,4,5)P_3 to GST fusion proteins containing the PH domains of the Son of Sevenless (SOS) protein and insulin receptor substrate (IRS)–1 was negligible (Fig. 4B).

The specificity of the GRP1 PH domain for binding [^{32}P]PtdIns(3,4,5)P_3 was also examined with respect to competition by the inositol phosphate head groups of the phosphoinositides. Unlabeled inositol-1,3,4,5-tetraphosphate [Ins(1,3,4,5)P_4] (100 μM) completely inhibited [^{32}P]PtdIns(3,4,5)P_3 binding to GST-PH$_{(GRP1)}$, whereas Ins(1,4,5)P_3, Ins(1,3,4,6)P_4, and Ins(1,2,5,6)P_4 had little or no effect at the same concentration (Fig. 4B) even though the latter two carry the same charge as the inhibitor Ins(1,3,4,5)P_4. The concentration of Ins(1,3,4,5)P_4 that inhibited binding of [^{32}P]PtdIns(3,4,5)P_3 half maximally was about 3 to 8 μM (Fig. 4A). Taken together, the data in Figs. 3 and 4 indicate extraordinary specificity of the GRP1 PH domain for PtdIns(3,4,5)P_3. A GST fusion protein of the cytohesin-1 PH domain also bound [^{32}P]PtdIns(3,4,5)P_3 (Fig. 4B), but not [^{32}P]-PtdIns3P or [^{32}P]PtdIns(3,4)P_2 (*27*). Analysis of the NH_2-terminal amino acid sequences of the GRP1 and cytohesin-1 PH domains in comparison with those of the IRS-1 and SOS PH domains (Fig. 4C) shows an additional lysine at position 273 and a Lys^{282}-Arg^{283}-Arg^{284} motif in the NH_2-terminal region, a region important for 4,5-polyphosphoinositide binding in other PH domains (*26*).

The selectivity of the GRP1 and cytohesin-1 PH domains for binding PtdIns(3,4,5)-P_3 indicates that the PH domain may function in the recruitment of these proteins to sites of PtdIns(3,4,5)P_3 synthesis in response to the action of receptor-regulated p110-type PI 3-kinases. Such recruitment to specific cell membrane regions would define their sites of action mediated presumably through their Sec7 domains. One function of the cytohesin-1 Sec7 domain is enhancement of cellular adhesion through direct association with the cytoplasmic region of integrin β2 (*22*). Transfection of Jurkat cells with the B2-1/cytohesin-1 cDNA or cDNA encoding this Sec7 homology domain alone enhanced their adhesion to ICAM-1, a ligand of integrins containing the β2 polypeptide. Our data thus suggest a molecular basis for regulation of integrin through PI 3-kinase (Fig. 5).

Another reported function of the Sec7 homology domain within cytohesin-1 and the similar protein ARNO is the catalysis of guanine nucleotide exchange on ARF1 (*23*). Regulation of ARF proteins by a PI 3-kinase–mediated pathway has been previously suggested on the basis of morphological data (*28*). Our demonstration that the cytohesin-1 PH domain is a target for PtdIns(3,4,5)P_3

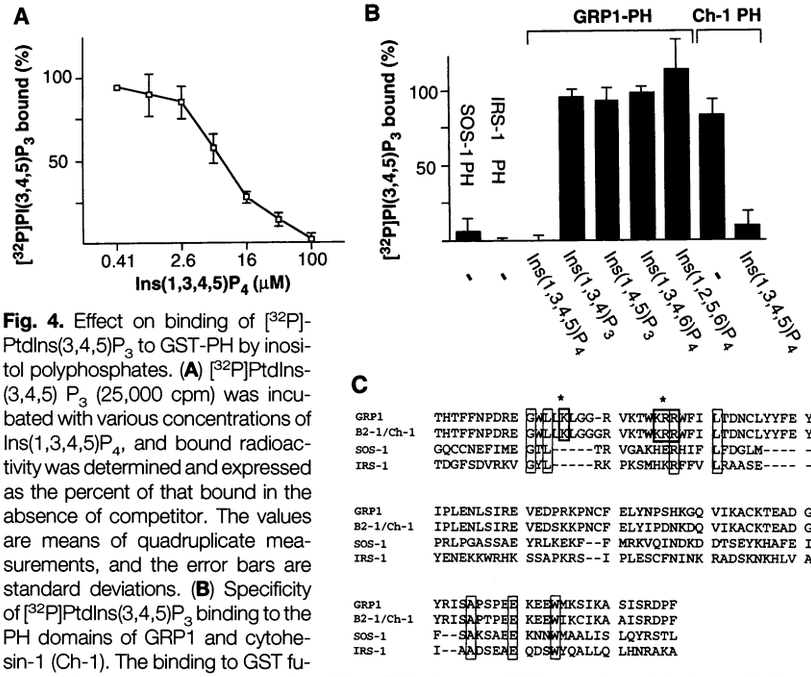

Fig. 4. Effect on binding of [^{32}P]-PtdIns(3,4,5)P_3 to GST-PH by inositol polyphosphates. (**A**) [^{32}P]PtdIns-(3,4,5) P_3 (25,000 cpm) was incubated with various concentrations of Ins(1,3,4,5)P_4, and bound radioactivity was determined and expressed as the percent of that bound in the absence of competitor. The values are means of quadruplicate measurements, and the error bars are standard deviations. (**B**) Specificity of [^{32}P]PtdIns(3,4,5)P_3 binding to the PH domains of GRP1 and cytohesin-1 (Ch-1). The binding to GST fusion proteins containing amino acids 462 to 569 of murine SOS or amino acid 13 to 115 of murine IRS-1 was assayed with 150 pmol of protein bound to the filters. Competition of binding to 7.5 pmol of GST-PH fusion proteins was measured in the presence of 100 μM of the competitors. The values were calculated as in (A) and are means of quadruplicate measurements and the error bars represent standard deviations. (**C**) Comparison of the amino acid sequences of the PH domains of murine GRP1, B2-1/cytohesin-1, SOS-1, and IRS-1. The basic motifs in the NH_2-terminal parts of the GRP1 and B2-1/cytohesin-1 molecules are outlined with heavy boxes and with asterisks. Light boxes delineate amino acid identities among the PH domains.

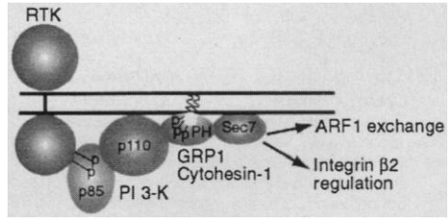

Fig. 5. Model of the mechanism by which GRP1 family proteins link receptor-activated PI 3-kinase signaling pathways to ARF1 and integrin β2 responses. Receptor tyrosine kinases (RTKs) recruit p85-p110–type PI 3-kinases to tyrosine phosphate sites, promoting the generation of 3,4,5-phosphoinositide, which binds the PH domain of GRP1 or cytohesin-1. Membrane-bound cytohesin-1 or GRP1 may interact through their Sec7 homology regions either with ARF1 to cause guanine nucleotide exchange, or with integrin β2 to modulate cell adhesion.

(Fig. 4B) suggests a model in which receptor-activated PI 3-kinase generates PtdIns(3,4,5)-P_3 to localize cytohesin-1, which in turn can regulate the guanine nucleotide exchange of ARF1 (Fig. 5). The PH domains of GRP1, cytohesin-1, and ARNO exhibit very high sequence similarity. Thus, the PH domain of ARNO may also bind PtdIns(3,4,5)P_3. This family of proteins appears to mediate the regulation of protein sorting and membrane trafficking by PtdIns(3,4,5)P_3.

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18. The 3-phosphatidylinositol probes labeled at the 3′-position were generated with GST–p110 PI 3-kinase purified from recombinant baculovirus-infected Sf9 cells and either purified PtdIns phosphates or unfractionated bovine brain lipid. Briefly, phospholipid (20 μg) in chloroform was dried under N_2, resuspended in 30 μl of buffer containing 20 mM tris-HCl (pH 7.4) and 1 mM EDTA and incubated in 200 μl of phosphorylation medium containing 20 mM tris-HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EGTA. 0.2 mM adenosine, GST-p110 PI 3-kinase, and 5 to 10 mCi of [γ-^{32}P]ATP (2.7 to 3.9 Ci/μmol). The reaction was incubated for 2 hours at room temperature, quenched by the addition of 0.2 ml of 1M HCl, followed by 0.5 ml of a chloroform:methanol (1:1) mixture. The organic phase was washed four times with 0.4 ml of methanol:HCl (1M) (1:1) and stored at −70°C. Just before use, the lipid was dried under a stream of nitrogen with phosphatidyl serine corresponding to a final concentration of 20 μg/ml. The identities of the PtdIns(3)P, PtdIns3,4)P_2, and PtdIns(3,4,5) P_3 were confirmed by thin-layer chromatography and HPLC analysis. Mouse 3T3-F442A adipocyte and mouse brain cDNA expression libraries were plated and protein expression induced by standard techniques. Briefly, 40,000 plaque-forming units (pfu) of the cDNA libraries were plated on each of 18 15-cm plates and incubated for 4 hours at 42°C. Nitrocellulose filters that had been soaked in 10 mM isopropylthio-β-D-galactoside and subsequently dried were placed on the plates and incubated for 14 to 16 hours at 37°C. The plates were cooled to 4°C and filters were removed and washed three to four times in 300 ml of assay buffer [25 mM tris (pH 7.4), 100 mM NaCl, 0.25% NP-40, 0.1% sodium cholate, 1 mM $MgCl_2$, and 0.5% dithiotreitol] under constant agitation. The filters were then incubated for 30 min in a crystallization bowl with the dissolved lipid in 30 ml of assay buffer at room temperature with labeled mixed brain lipid (2 μCi/ml) and shaken vigorously. The filters were washed with five changes of the same buffer, dried, and subjected to autoradiography.
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24. To generate the GST fusion proteins, we used for the polymerase chain reactions (PCRs) the following primers: GGAATTCCTTCGGCACGAGCGGTG and CCGCTCGAGCGGTGGCTATTTGCTTGTTCCTC for the GST-N (residues 5 to 71 of GRP1) construct; GGAATTCCGACAACCTGACTTCAGTGG and CCGCTCGAGCGGTGTGTGTCAGGTCATTTCC for the GST-Sec7 construct; GGAATTCCTATGAAAGTATCAAGAATGAGC and CCGCTCGAGCGGCTGGATCCTGACATTTACC for the GST-PH construct; and GGAATTCCTTCGGCACGAGCGGTG and CCGCTCGAGCGGCTGGATCCTGACATTTACC for the GST-GRP1 construct. The sequences of the PCR products were verified and cloned into pGEX-5X-3 in the Eco RI and Xba I sites. The cytohesin-1 PH domain corresponding to amino acids 286 to 398 was synthetically prepared by using a total of 16 oligonucleotides. In brief, three double-stranded oligonucleotides (219, 210, and 124 base pairs long) were prepared by annealing sets of either four or six oligonucleotides that contained 15 bases overlapping complementary sequences. Restriction sites (Eco RI at the 5′ ends and Sal I) were created in all the double-stranded oligonucleotides and used to subclone the oligonucleotides into the Puc 19 plasmid. The DNA inserts were excised and ligated in proper order at Stu I and Ban II sites. Finally, the completed PH domain of cytohesin-1 was subcloned into pGEX5X-3 by using Eco RI and Xho I sites. The bacteria expressing the pGEX constructs were lysed, and the fusion proteins were bound to glutathione immobilized on agarose according to standard procedures. The beads were incubated with one volume of 20 mM Hepes, 100 mM NaCl, 1 mM dithiothreitol (H buffer) supplemented with 10 mM glutathione and 1% sodium cholate, and the eluate was dialyzed extensively against H buffer. For binding assays, we bound protein to nitrocellulose with a Bio-Rad BIO-DOT apparatus using 150 pmol per well for binding assays and 7.5 pmol for competition assays. The nitrocellulose was washed in assay buffer, and 3-mm circles of the filters containing the protein were cut out and incubated in 40 μl of assay buffer with the relevant lipids and competitors for 2 hours under constant agitation. The filters were washed four times with 1 ml of assay buffer and counted in a scintillation counter.
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Nuclear Export of NF-ATc Enhanced by Glycogen Synthase Kinase–3

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The transcription factor NF-AT responds to Ca^{2+}-calcineurin signals by translocating to the nucleus, where it participates in the activation of early immune response genes. Calcineurin dephosphorylates conserved serine residues in the amino terminus of NF-AT, resulting in nuclear import. Purification of the NF-AT kinase revealed that it is composed of a priming kinase activity and glycogen synthase kinase–3 (GSK-3). GSK-3 phosphorylates conserved serines necessary for nuclear export, promotes nuclear exit, and thereby opposes Ca^{2+}-calcineurin signaling. Because GSK-3 responds to signals initiated by Wnt and other ligands, NF-AT family members could be effectors of these pathways.

In lymphoid cells, stimulation of the Ca^{2+}-calcineurin signaling pathway leads to the nuclear translocation of the NF-ATc family of transcription factors (*1*, *2*), which in turn activate immune response genes such as those encoding interleukin-2 (IL-2), IL-4, CD40 ligand, and Fas ligand (*3*). Inhibition of the nuclear translocation of NF-ATc is largely responsible for the immunosuppressive actions of cyclosporin and tacrolimus (FK506) (*4*), which specifically inhibit calcineurin (*5*). Calcineurin directly dephosphorylates NF-ATc on critical serines present in all family members, leading to