Analyzing the Role of the Putative Inositol 1,3,4,5-
Tetrakisphosphate Receptor GAP1IP4BP in Intracellular Ca\(^{2+}\) Homeostasis*  

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Inositol 1,3,4,5-tetrakisphosphate (IP\(_4\)) has been linked to a potential role in the regulation of intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) following cellular stimulation with agonists that activate phosphoinositide-specific phospholipase C. However, despite many studies, the function of IP\(_4\) remains unclear and indeed there is still some debate whether it has a function at all. Here we have used various molecular approaches to address whether manipulation of the potential IP\(_4\) receptor, GAP1IP4BP, affects [Ca\(^{2+}\)]\(_i\) following cellular stimulation. Using single cell imaging, we show that the overexpression of a constitutively active and a potential dominant negative form of GAP1IP4BP appear to have no effect on Ca\(^{2+}\) mobilization or Ca\(^{2+}\) entry following stimulation of HeLa cells with histamine. In addition, through the use of small interfering RNA duplexes, we have examined the effect of suppressing endogenous GAP1IP4BP production on [Ca\(^{2+}\)]\(_i\). In HeLa cells in which the endogenous level of GAP1IP4BP has been suppressed by ~95%, we failed to observe any effect on Ca\(^{2+}\) mobilization or Ca\(^{2+}\) entry following histamine stimulation. Thus, using various approaches to manipulate the function of endogenous GAP1IP4BP, we have been unable to observe any detectable effect of GAP1IP4BP on [Ca\(^{2+}\)]\(_i\).  

Despite many studies on the possible role of inositol 1,3,4,5-tetrakisphosphate (IP\(_4\)) in cellular physiology, its function remains unclear and indeed there is still some debate over whether it has a function at all (1, 2). Formed by direct phosphorylation of inositol 1,4,5-trisphosphate (IP\(_3\)), a reaction catalyzed by a family of Ca\(^{2+}\)-regulated IP\(_3\) 3-kinases (1, 3), IP\(_4\) has been linked to a potential role in the regulation of intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) following cellular stimulation with agonists that activate phosphoinositide-specific phospholipase C (4–7). Evidence for this finding has come from a number of sources. For example, in endothelial cells, there is direct evidence (8) and, in neurons, there is direct (9) and indirect evidence (10) that IP\(_4\) can activate Ca\(^{2+}\) influx channels in the plasma membrane. Furthermore, one of the first effects of IP\(_4\) to be reported highlighted an ability of this compound to synergize with IP\(_3\) to mobilize Ca\(^{2+}\) and regulate subsequent store-operated Ca\(^{2+}\) influx (11–13). However, the marked sensitivity of this particular system to experimental protocols (11, 14–16) has also raised a significant degree of controversy over the role of IP\(_4\) in intracellular Ca\(^{2+}\) homeostasis. In recent years, we have taken the view that if IP\(_4\) does indeed constitute a novel second messenger, one criterion that must be fulfilled is the presence within cells of protein(s) that specifically bind IP\(_4\), i.e. an IP\(_4\) receptor. To this end, we have described the purification (17, 18) and cloning (19) of a highly specific IP\(_4\)-binding protein termed GAP1IP4BP. This protein, which functions as a GTPase-activating protein for members of the Ras-like family of small GTPases, at present constitutes the most promising candidate IP\(_4\) receptor.  

The Ras-like family includes H-Ras, N-Ras, and K-Ras4A and 4B, the R-Ras proteins, the Raf proteins, and the Rap proteins 1A, 1B, 2A, and 2B (20–22). These are ubiquitously expressed, evolutionarily conserved proteins that couple extracellular signals to various cellular responses (20–22). All of these proteins have the inherent ability to undergo conformational changes in response to the alternate binding of GDP and GTP. The GDP-bound “off” state and the GTP-bound “on” state recognize distinct effector proteins, thereby allowing these proteins to function as two-state molecular “switches.” Importantly, cycling between the two forms does not occur spontaneously. Activation requires guanine nucleotide exchange factors to induce the dissociation of GDP to allow association of the more abundant GTP, and deactivation requires GTPase-activating proteins (GAPs) to bind to the GTP-bound form to enhance the rate of intrinsic GTPase activity (20–22).  

GAP1IP4BP along with the related proteins GAP1m, RASAL, and CAPRI (23–30) is composed of tandem N-terminal C\(_\_\_\_\_\_\_\_) domains, a C-terminal pleckstrin homology (PH) domain adjacent to a Bruton’s tyrosine kinase (Btk) motif, and a central catalytic Ras GAP-related domain. Associated with the plasma membrane through a complex interaction between its PH/Btk domain and the inner plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P\(_2\)) (31, 32), GAP1IP4BP functions as a dual Ras and Rap1 GAP (19, 33). Importantly, at least in vitro, the Ras GAP activity of GAP1IP4BP is regulated by IP\(_4\) (19). The data that have raised the key issue of whether the effects of IP\(_4\) on the regulation of [Ca\(^{2+}\)]\(_i\), are mediated by GAP1IP4BP. At present, the only direct evidence in favor of this finding has emerged from l-1210 cells (34, 35). In these perme-
GAP1IP4BP and the Regulation of Ca2+ Signaling

abitized the cells, the addition of exogenous GAP1IP4BP enhances the well documented ability of IP3 to potentiate IP3-stimulated Ca2+ release (36). However, it is not yet clear as to the role of GAP1IP4BP in vivo. In this study, we have addressed this issue by using various molecular approaches including overexpression of potential constitutive and dominant negative forms of GAP1IP4BP and siRNA to examine a potential in vivo role for GAP1IP4BP in the regulation of [Ca2+]i in HeLa cells.

EXPERIMENTAL PROCEDURES

Expression and Purification of GST Fusion Proteins in E. coli—The pGEX plasmids containing the coding sequences for the GAP1IP4BP mutants, isolated using the Transformer kit from Clontech as previously described (31, 32), and ΔC2GAP1IP4BP were transformed individually into the E. coli strain BL21(DE3) to express and purify as GST fusion proteins using the procedure described previously (33). A single colony of the transformed strain was inoculated into 5 ml of LB containing ampicillin (100 μg/ml) and grown overnight at 37 °C with shaking at 250 rpm. The overnight culture was diluted 1:100 with fresh LB containing ampicillin and grown at 37 °C with shaking until the cell density reached an A600 of 0.5. Protein expression was induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C after which cells were collected by centrifugation and resuspended in 25 ml of ice-cold buffer A (PBS containing 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfon fluoride, and 1 mM β-mercaptoethanol). Cells were lysed by sonication (3 × 20-s pulses with 1 min at 4 °C between each sonication) and incubated at 4 °C with 1% (v/v) Triton X-100 for 1 h with gentle mixing prior to the removal of cell debris by centrifugation. 1 ml of a 50% slurry of glutathione-Sepharose 4B resin (Amersham Biosciences) prewashed with buffer A was added to the resultant supernatant and incubated overnight at 4 °C with constant shaking. The resin was washed with 3 × 10 ml of ice-cold buffer A prior to elution of bound GST fusion protein with 3 × 1 ml of 50 mM Tris-HCl containing 10 mM glutathione, pH 8.0, by incubating with constant mixing for 10 min at room temperature. The eluates were pooled, and protein concentration was estimated by the Bradford method using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was used to check the purity of the eluted protein.

[P2IP5P-binding Assays—These were performed as described previously. An individual binding assay contained 100 mM KCl, 20 mM NaCl, 10 mM Hepes/NaOH, pH 7.0, 1 mM EDTA, 30,000 dpm of [32P]IP4 (see Ref. 17). 0.5–1 μg of GST fusion protein, and various concentrations of competing unlabeled inositol phosphates in a final volume of 0.5 ml. Equilibrium binding was reached by a 15-min incubation at 4 °C after which the receptor-ligand complex was precipitated by the addition of 100 μl of 5 mg/ml γ-globulin and 1 ml of 25% (v/v) polyethylene glycol. This sample was incubated at 4 °C for 1 h, and the slurry was washed twice with 2 ml of extracellular medium. Ca2+ release (36). However, it is not yet clear as to the role of GAP1IP4BP in vivo. In this study, we have addressed this issue by using various molecular approaches including overexpression of potential constitutive and dominant negative forms of GAP1IP4BP and siRNA to examine a potential in vivo role for GAP1IP4BP in the regulation of [Ca2+]i in HeLa cells.

results

Generation of a Constitutively Active and a Potential Dominant Negative Form of GAP1IP4BP—Our initial approach to examining the role of GAP1IP4BP in the regulation of [Ca2+]i, was to design a series of site-directed mutants with the aim of isolating constitutively active and dominant negative forms that could potentially be used to interfere with the function of endogenous GAP1IP4BP. As shown in Fig. 1, the conversion of arginine into cysteine at position 601 within the PH/BD domain of GAP1IP4BP resulted in a mutant protein that, although

thus 1 day later, the pre-kil boost (250 μg in 150 μl NaCl, 10 μM K2PO4, pH 7.4) was administered intravenously. Three days later, test serum and the spleen were removed. Fusion was undertaken by mixing spleen cells with myeloma cells in a ratio of 5:1. Mixed cells were plated in 4 × 56-well plates containing MRC-5 feeder cells and 100 μl of 20% fetal calf serum in Dulbecco’s modified Eagle’s medium. These cells were incubated in a CO2 incubator at 37 °C. Subsequent culture of harvested and cloned by limited dilution were followed as standard. Antibodies were used in experiments in the form of untreated culture supernatants from cells grown to stationary phase.

Immunoprecipitation of Endogenous GAP1IP4BP Using the GP-3 Monoclonal Antibody—Immunoprecipitations were carried out using antibodies immobilized on protein G-Sepharose beads (Amersham Biosciences). Protein G beads were washed thoroughly in PBS and incubated with the GP-3 hybridoma supernatant for 4 h at 4 °C with constant mixing. The beads were washed in PBS and then twice in 0.2 M sodium borate. Dimethyl primelidiminate was added to a final concentration of 20 mM, and the slurry was mixed at room temperature for 30 min. After one wash in 0.2 M ethanolamine, the beads were incubated in 0.2 M ethan-olamine for an additional 2 h to complete the covalent coupling of the antibody to the beads. The beads were washed thoroughly in PBS and then stored at 4 °C in PBS containing 0.01% azide. For immunoprecipitations, cell pellets were resuspended in ice-cold lysis buffer (PBS, 1% Triton X-100, 1 mM EGTA, 0.01% azide), and then cell debris was pelleted by high speed centrifugation. The GP-3-coupled Sepharose beads were washed thoroughly in lysis buffer, and then the lysates were added to the beads and incubated, mixing end-over-end at 4 °C for between 2 and 4 h. Beads were washed several times with lysis buffer, and the final wash aspirated tight to the beads. Samples were separated by SDS-PAGE prior to visualization.

Isolation of Stable, Inducible HeLa Cells—pTet-Off-transfected HeLa cells (Clontech) were cultured in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal-calf serum (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml G418, and 2 μM doxycycline. Cells were co-transfected with GAP1IP4BP pTRE and pTRE-Hyg (Clontech) using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Note that pTRE does not contain a mammalian-se-quence marker, but pTRE-Hyg contains a neomycin (G418) resistant cell lines were selected using 500 μg/ml hygromycin B (Roche Molecular Biologicals) and maintained in 250 μg/ml hygromycin B. When the expression of GAP1IP4BP was desired, the medium was replaced with doxycycline-free medium 24–48 h prior to using the cells.

RNA Interference of Endogenous GAP1IP4BP Expression—GAP1IP4BP(+/−), cells were seeded into the wells of a six-well plate, and 24 h later at a confluency of 30%, they were transiently transfected using OligoFECTAMINE (Invitrogen) with 200 pmol of GAP1IP4BP-specific siRNA according to the manufacturer’s instructions (siRNA sequence 5′-AACUGACUCUGCCGUUUAC-3′). Cells were cultured in the presence of doxycycline for the required period prior to Ca2+ imaging and the analysis of GAP1IP4BP level by immunoblotting using the GP-3 monoclonal antibody.

Single-cell Ca2+ Measurements—Cells were grown on 22-mm cover-slips and then washed with 2 ml of extracellular medium (121 mM NaCl, 5.4 mM KCl, 1.6 mM MgCl2, 6 mM NaHCO3, 9 mM glucose, 25 mM Hepes, 1.8 mM CaCl2, pH 7.4) and incubated in 1 ml of extracellular medium containing 2 μM Pura-2/AM for 30 min at room temperature. Cells were then washed twice with 2 ml of extracellular medium. Ca2+ imaging was performed using the Merlin ratiometric Ca2+ imaging system (PerkinElmer Life Sciences), an UltraPlix FKL300 digital camera, and an Olympus IX50 inverted microscope fitted with a ×40 oil-immersion objective lens. The data were processed using Merlin software. All images was performed at 37 °C with constant mixing. Calibrations were calculated with Merlin software using 340/380 ratio maxima and minima obtained by the treatment of cells with 2 μM ionomycin followed by 20 mM EGTA.

Results

Generation of a Constitutively Active and a Potential Dominant Negative Form of GAP1IP4BP—Our initial approach to examining the role of GAP1IP4BP in the regulation of [Ca2+]i, was to design a series of site-directed mutants with the aim of isolating constitutively active and dominant negative forms that could potentially be used to interfere with the function of endogenous GAP1IP4BP. As shown in Fig. 1, the conversion of arginine into cysteine at position 601 within the PH/BD domain of GAP1IP4BP resulted in a mutant protein that, although
displaying a greatly reduced IP4 binding activity, ~10% of that seen with wild-type protein (33) (Fig. 1A) nevertheless retained full Ras GAP activity when assayed in vitro (Fig. 1B). In contrast, the conversion of leucine into alanine at position 484, a residue located within the predicted Ras-binding site present in the Ras GAP-related domain (37), resulted in a GAP1IP4BP mutant that, although displaying no detectable in vitro Ras GAP activity (Fig. 1B), retained the ability to bind IP4, with an affinity and isomeric specificity identical to that of wild type (Fig. 1A and data not shown).

To assay the Ras GAP activity of these mutants in vivo, we transiently transfected HeLa cells with H-Ras and the relevant GAP1IP4BP and assayed the level of Ras-GTP using a GST fusion of the Ras-GTP-binding domain from Raf. In serum-starved cells, a substantial quantity of overexpressed H-Ras was in the GTP-bound state (Fig. 2). Under these conditions, wild-type GAP1IP4BP caused a significant decrease in the amount of Ras-GTP that was recovered compared with controls. This basal Ras GAP activity was dependent on the Ras GAP-related domain because the expression of GAP1IP4BP(L484A) had no effect on Ras-GTP levels, even when this mutant was expressed at significantly higher levels than wild-type GAP1IP4BP (Fig. 2). The expression of the cytosolic GAP1IP4BP(R601C) resulted in a significant decrease in the amount of recoverable Ras-GTP when compared with the expression of the same level of wild-type GAP1IP4BP (Fig. 2). Similar data were also obtained using green fluorescent protein-tagged versions of wild type and the various GAP1IP4BP mutants (data not shown).

**Expression of the GAP1IP4BP Mutants Has No Apparent Effect on Intracellular Ca2+ Homeostasis following Histamine Stimulation of HeLa Cells**—To examine the effect of overexpressing wild-type GAP1IP4BP and the various GAP1IP4BP mutants on intracellular Ca2+ homeostasis, we transiently transfected HeLa cells with the GAP1IP4BP constructs tagged at their N termini with yellow fluorescent protein (YFP). This spectral variant of green fluorescent protein allowed visualization of those cells expressing GAP1IP4BP and, when coupled with Fura-2 imaging, allowed the analysis of Ca2+ transients in single cells following stimulation with histamine (Fig. 3). We
performed experiments in the absence of extracellular Ca\(^{2+}\) to allow discrimination between the Ca\(^{2+}\) released from internal stores and that from Ca\(^{2+}\) entry. In these assays, the initial increase in [Ca\(^{2+}\)], that arose from IP\(_3\)-stimulated release of Ca\(^{2+}\) from internal stores (38) was not significantly altered in those cells expressing YFP-GAP1\(^{IP4BP}\)(R601C), YFP-GAP1\(^{IP4BP}\)(L484A), or YFP-GAP1\(^{IP4BP}\) (Fig. 3). Such data suggest that the overexpression of wild-type GAP1\(^{IP4BP}\) or the various GAP1\(^{IP4BP}\) mutants has no detectable effect on IP\(_3\)-stimulated Ca\(^{2+}\) release when assayed under these conditions.

In addition, after allowing the [Ca\(^{2+}\)] to return to the basal state, we analyzed store-operated Ca\(^{2+}\) entry by adding extracellular Ca\(^{2+}\). This induced an elevation of [Ca\(^{2+}\)] that was not significantly altered by the expression of various GAP1\(^{IP4BP}\) constructs (Fig. 3). As a positive control, we also analyzed the histamine-stimulated Ca\(^{2+}\) transient that was present in cells overexpressing the store-operated Ca\(^{2+}\) influx channel Trp3. Here we observed a clear enhancement of Ca\(^{2+}\) influx, consistent with that reported previously (39). Thus, under these particular assay conditions, GAP1\(^{IP4BP}\) has no detectable effect on store-operated Ca\(^{2+}\) influx.

**Isolation of a Monoclonal Antibody Capable of Detecting Endogenous GAP1\(^{IP4BP}\) —** Although the data presented in Fig. 3 suggest that when assayed under these particular conditions GAP1\(^{IP4BP}\) appears to have no detectable effect on [Ca\(^{2+}\)], following histamine stimulation of HeLa cells, we extended our experimental approach by examining the effect of manipulating the level of endogenous GAP1\(^{IP4BP}\). To undertake this approach, we first generated a monoclonal antibody against GAP1\(^{IP4BP}\) that was capable of detecting endogenous protein (our previously characterized GAP1\(^{IP4BP}\) polyclonal antibody, H113 (19), was unable to detect endogenous levels of GAP1\(^{IP4BP}\) in HeLa cells (data not shown)). As described under “Experimental Procedures,” a mouse monoclonal antibody (GP-3) was raised against a GST fusion of a GAP1\(^{IP4BP}\) mutant lacking the N-terminal tandem C\(_2\) domains (33).

In HeLa cells overexpressing each of the mammalian GAP1 family members, GP-3 was only capable of detecting a protein in those cells transiently transfected with an expression vector for GAP1\(^{IP4BP}\) (Fig. 4A). In non-transfected HeLa cells, GP-3 detected an endogenous protein of ~96 kDa on Western blots (Fig. 4B). Furthermore, GP-3 was capable of immunoprecipitating a 96-kDa protein that was detected by the GAP1\(^{IP4BP}\) specific polyclonal antibody H113 (Fig. 4C). The GP-3 immunoprecipitate also displayed Ras GAP activity and specific IP\(_3\) binding activity (data not shown). Taken together, these data suggest that the 96-kDa protein detected by GP-3 in HeLa cell lysates is endogenous GAP1\(^{IP4BP}\). Therefore, this antibody was employed in the subsequent assays designed to silence the expression of endogenous GAP1\(^{IP4BP}\).

**Using Double-stranded RNA Interference to Deplete Endogenous GAP1\(^{IP4BP}\) —** To deplete endogenous GAP1\(^{IP4BP}\), we used RNA interference. RNA interference is the process of sequence-specific post-transcriptional gene silencing in animals and plants initiated by double-stranded RNA that is homologous in sequence to the silenced gene (40). Recently, 21-nucleotide siRNA duplexes have been shown to specifically suppress the expression of endogenous genes in mammalian cell lines (41–43). Therefore, we designed and used a specific siRNA to sup-
express the expression of endogenous GAP1IP4BP.

To achieve an experimental system amenable to manipulation, we initially isolated a stably transfected HeLa cells line expressing full-length GAP1IP4BP under the control of a tetracycline-inducible promoter (see “Experimental Procedures”). To examine the leakiness and inducibility of GAP1IP4BP, the expression in this cell line named GAP1IP4BP(o/e3) was visualized by immunoblotting using the GP-3 monoclonal antibody (1:1000).

press the expression of endogenous GAP1IP4BP.

To obtain a functional system, we isolated a stably transfected HeLa cells line expressing full-length human GAP1IP4BP under the control of a tetracycline-regulated promoter (see “Experimental Procedures”). To examine the leakiness and inducibility of GAP1IP4BP, the expression in this cell line named GAP1IP4BP(o/e3), we compared whole cell lysates derived from the parental pTet-Off HeLa cell line (Parental Line) and a Tet-Off HeLa cell line stably transfected with full-length GAP1IP4BP cultured for 24 h either in the presence (GAP1IP4BP(o/e3), 0.2 mg/ml doxycycline) or absence (GAP1IP4BP(o/e3), 2 mg/ml doxycycline) of 2 mg/ml doxycycline. By comparing the expression of GAP1IP4BP(o/e3) cells cultured for 24 h in the presence of varying concentrations of doxycycline, GAP1IP4BP(o/e3) cells were transiently transfected with siRNA specific for GAP1IP4BP or a control siRNA according to “Experimental Procedures.” Cells were cultured for either 24 or 48 h in the continued presence of 2 mg/ml doxycycline. In all experiments, GAP1IP4BP was significantly overexpressed. Third, culturing in the absence of doxycycline generated cells in which GAP1IP4BP was significantly overexpressed. Third, culturing in the absence of doxycycline generated cells in which GAP1IP4BP was significantly overexpressed. Third, culturing in the absence of doxycycline generated cells in which GAP1IP4BP was significantly overexpressed. Third, culturing in the absence of doxycycline generated cells in which GAP1IP4BP was significantly overexpressed. Third, culturing in the absence of doxycycline generated cells in which GAP1IP4BP was significantly overexpressed. Third, culturing in the absence of doxycycline generated cells in which GAP1IP4BP was significantly overexpressed. Third, culturing in the absence of doxycycline generated cells in which GAP1IP4BP was significantly overexpressed. Third, culturing in the absence of doxycycline generated cells in which GAP1IP4BP was significantly overexpressed.
Here, Ras-GTP levels were similar to control cells. Thus, manipulating GAP1IP4BP levels within this cell line resulted in the alteration of the levels of Ras-GTP.

To analyze the intracellular free Ca$^{2+}$ homeostasis within GAP1IP4BP(o/e) cells cultured under these conditions, we performed Ca$^{2+}$-imaging experiments in the absence of extracellular Ca$^{2+}$. However, using Fura-2-loaded cells, we failed to observe any effect of overexpressing GAP1IP4BP or depleting endogenous GAP1IP4BP on the initial increase in [Ca$^{2+}$], following histamine stimulation (Fig. 7). In addition, after allowing the [Ca$^{2+}$], to return to the basal state, we analyzed store-operated Ca$^{2+}$ entry by adding extracellular Ca$^{2+}$. This induced an elevation of [Ca$^{2+}$], that was again not significantly altered by the overexpression of GAP1IP4BP or the depletion of endogenous GAP1IP4BP (Fig. 7).

**DISCUSSION**

In this report, we have described results from a series of experiments aimed at addressing whether the putative IP$_4$ receptor GAP1IP4BP may function to allow IP$_4$ to regulate Ca$^{2+}$ entry. Our initial approach was to design a series of site-directed mutants with the aim of isolating constitutively active and interfering forms of GAP1IP4BP that could be used to potentially block the function of endogenous GAP1IP4BP. First, the conversion of arginine into cysteine at position 601 within the PH/Btk domain of GAP1IP4BP resulted in a mutant protein that, although displaying a greatly reduced IP$_4$ binding activity (33), retained full Ras GAP activity, a suppression that can be alleviated following the binding of IP$_4$ to the PH/Btk domain (19). In the case of the GAP1IP4BP(R601C) mutant, the inability of the PH/Btk domain to bind IP$_4$ is mirrored by a loss in binding to PtdIns(4,5)P$_2$ (32). This results in GAP1IP4BP(R601C) residing in the cytosol rather than at the plasma membrane (31). Importantly, the induced activity of the GAP1IP4BP(R601C) mutant does not appear to simply result from a loss in plasma membrane association. Targeting this mutant back to the plasma membrane by the addition of a CAAX motif does not result in a suppression of the Ras GAP activity (data not shown). This finding suggests that the constitutive activity of the GAP1IP4BP(R601C) mutant may result from the removal of the negative influence of PtdIns(4,5)P$_2$ binding to the PH/Btk on the Ras GAP activity. Such a scenario may help explain how IP$_4$ can activate the Ras GAP activity of this protein in vitro (19). IP$_4$ may remove the negative control exerted by PtdIns(4,5)P$_2$ by competing for binding to the PH/Btk domain.

In contrast, the conversion of leucine into alanine at position 484 resulted in a GAP1IP4BP mutant that, although displaying no detectable in vitro or in vivo Ras GAP activity, retained the ability to bind IP$_4$ with an affinity and isomeric specificity identical to wild-type GAP1IP4BP. This mutant also retained the ability to associate with the plasma membrane (data not shown). Therefore, it is possible that upon overexpression, this particular mutant, GAP1IP4BP(L484A), may interfere with the function of endogenous GAP1IP4BP by binding to IP$_4$, but because of the loss of GAP activity, this mutant will not induce the subsequent regulation of Ras function. Indeed, the overexpression of a comparable mutant of the GAP1IP4BP-related protein CAPRI appears to interfere with endogenous CAPRI function (30).

Having generated and characterized the various GAP1IP4BP mutants, we analyzed their effect on the changes in [Ca$^{2+}$], that occur following histamine stimulation of HeLa cells. Through single cell analysis of HeLa cells overexpressing each of these mutants, we failed to observe any alteration in the release of internally stored Ca$^{2+}$ or store-operated Ca$^{2+}$ entry following histamine stimulation. Thus, using these particular mutants, we were unable to detect any apparent role for GAP1IP4BP in the regulation of [Ca$^{2+}$], observed following histamine stimulation of HeLa cells.

To complement these studies and negate potential problems especially with interpreting the ability of the GAP1IP4BP(L484A) mutant to function as a dominant negative, we observed the effect of depleting endogenous GAP1IP4BP on [Ca$^{2+}$]. Using siRNA technology, we were able to significantly suppress the expression of endogenous GAP1IP4BP in a HeLa cell line stably expressing GAP1IP4BP under a tetracycline-regulated promoter. However, under conditions in which we manipulated the level of endogenous GAP1IP4BP either by overexpression or siRNA suppression, we again failed to observe any affect on the release of internally store-operated Ca$^{2+}$ or store-operated Ca$^{2+}$ influx following stimulation with histamine.

As far as we are aware, this is the first study in which agonist-dependent changes in [Ca$^{2+}$], have been analyzed in cells in which the endogenous level of GAP1IP4BP has been so dramatically varied within the same cell line. The only other study that has reported the effect of reducing the expression of GAP1IP4BP on [Ca$^{2+}$], within mammalian cells is that of Lu et al. (44). In this study (44), the authors examined the role of GAP1IP4BP in store-operated Ca$^{2+}$ entry by comparing two clonally selected human erythroleukemia (HEL) cell lines, one...
expressing a constitutively active GAP1IP4BP antisense construct (AS-HEL cells) and the other a vector lacking the antisense construct (V-HEL cells) (44). In the AS-HEL cells in which endogenous GAP1IP4BP was reduced by between 85 and 96%, a substantial increase in Ca2+ entry was observed following thrombin addition (44). This enhanced entry was shown to result from the activation of intermediate conductance Ca2+-activated K+ channels that induced membrane hyperpolarization, thereby stimulating Ca2+ entry (44). However, partly because the expression of the GAP1IP4BP antisense was not inducible, the authors (44) were unable to firmly establish that GAP1IP4BP expression of exogenous GAP1IP4BP in the AS-HEL cells. Therefore, it remains unclear whether the observed affect of expression of GAP1IP4BP on intracellular Ca2+ homeostasis within HEL cells stems from the direct loss of GAP1IP4BP.

Currently, the only direct evidence for a role of GAP1IP4BP in Ca2+ homeostasis has emerged from L-1210 cells (34, 35). In these permeabilized cells, the addition of exogenous GAP1IP4BP enhances the well documented ability of IP3 to potentiate IP3-stimulated Ca2+ release (36). This is a reproducible observation that clearly suggests that in these cells under these particular conditions the binding of IP3 to GAP1IP4BP does appear to play a role in the regulation of IP3-stimulated Ca2+ release (36). However, our approach of manipulating endogenous GAP1IP4BP within intact HeLa cells suggests that a global role for GAP1IP4BP in the regulation of [Ca2+]i may not be a detectable phenomenon.

Acknowledgments—We thank Jim Putney for the provision of the cDNA encoding for Trp3. We also thank the Medical Research Council for providing Infrastructure Award G4500006 to establish the School of Medical Sciences Cell Imaging Facility and Mark Jeppson and Alan Leard for assistance.

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