# Inhibition of $Ca^{2+}$ signalling by p130, a phospholipase-C-related catalytically inactive protein: critical role of the p130 pleckstrin homology domain

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p130 was originally identified as an Ins(1,4,5)$P_3$-binding protein similar to phospholipase C-$\delta$ but lacking any phospholipase activity. In the present study we have further analysed the interactions of p130 with inositol compounds *in vitro*. To determine which of the potential ligands interacts with p130 in cells, we performed an analysis of the cellular localization of this protein, the isolation of a protein–ligand complex from cell lysates and studied the effects of p130 on Ins(1,4,5)$P_3$-mediated $Ca^{2+}$ signalling by using permeabilized and transiently or stably transfected COS-1 cells (COS-$1^{p130}$). *In vitro*, p130 bound Ins(1,4,5)$P_3$ with a higher affinity than that for phosphoinositides. When the protein was isolated from COS-$1^{p130}$ cells by immunoprecipitation, it was found to be associated with Ins(1,4,5)$P_3$. Localization studies demonstrated the presence of the full-length p130 in the cytoplasm of living cells, not at the plasma membrane. In cell-based assays, p130 had an inhibitory effect on $Ca^{2+}$ signalling. When fura-2-loaded COS-$1^{p130}$ cells were stimulated with bradykinin, epidermal growth factor or ATP, it was found that the agonist-induced increase in free $Ca^{2+}$ concentration, observed in control cells, was inhibited in COS-$1^{p130}$. This inhibition was not accompanied by the decreased production of Ins(1,4,5)$P_3$; the intact p130 pleckstrin homology domain, known to be the ligand-binding site *in vitro*, was required for this effect in cells. These results suggest that Ins(1,4,5)$P_3$ could be the main p130 ligand in cells and that this binding has the potential to inhibit Ins(1,4,5)$P_3$-mediated $Ca^{2+}$ signalling.

Key words: $Ca^{2+}$ release, Ins(1,4,5)$P_3$ receptor, phosphoinositides.

## INTRODUCTION

It is now well established that the metabolism of phosphoinositides is important in a wide variety of cellular functions. The hydrolysis of PtdIns(4,5)$P_2$ by phospholipase C (PLC) yields the water-soluble messenger Ins(1,4,5)$P_3$, which binds to specific $Ca^{2+}$-channel receptors and mobilizes intracellular $Ca^{2+}$, while the hydrophobic moiety, diacylglycerol, activates protein kinase C isoenzymes [1,2]. PtdIns(4,5)$P_2$ can also be phosphorylated by phosphoinositide 3-kinase (type 1) to form PtdIns(3,4,5)$P_3$, a lipid product found in stimulated cells [3–5]. There is growing evidence that PtdIns(3,4,5)$P_3$, and some other products of phosphoinositide 3-kinase activation, could bind directly to specific intracellular protein targets and thus act as second messengers [4,6].

Considerable effort, with a variety of experimental approaches, has been made to identify proteins that could bind phosphoinositides and inositol phosphates and thus participate in a signalling network via these second messengers [7]. One such potential protein target, designated p130, was isolated by using affinity chromatography with an Ins(1,4,5)$P_3$ analogue as a ligand [7,8]. Subsequent cloning of the cDNA encoding p130 revealed extensive similarity with phosphoinositide-specific PLC enzymes and, in particular, the PLC-$\delta$ family [9]. On the basis of the crystal structure of PLC-$\delta$1 [10], p130 protein is predicted to have the same domain organization, incorporating the pleckstrin homology (PH), EF-hand, catalytic and C2 domains [10a]. However, p130 has some distinct characteristics. It is a larger molecule than the PLC-$\delta$ isoenzymes and unique regions are present at both the N-terminus preceding the PH domain and at the C-terminus. More importantly, the residues within the catalytic domain critical for PLC activity (Glu-390 and His-356) are not conserved in p130 [9]. As expected from a mutagenesis study of PLC-$\delta$1 [11] it has been found that p130 does indeed lack PLC activity [7,9]. Other molecules with similarity to p130 have also been described and designated human PLC-L [12] and K10F12.3 gene product from *Caenorhabditis elegans* [13]. Otsuki et al. [14] recently isolated a novel cDNA from mouse brain library with a similarity of 70% to PLC-L; they therefore designated it mouse PLC-$L_2$, calling the original PLC-L human PLC-$L_1$. Furthermore, the gene for human PLC-$L_2$ has been reported by Kazusa DNA Research Institute [15]. All these proteins have characteristic N- and C-terminal extensions, and replacements of critical catalytic residues. Finding a similar molecule in a simple organism such as *C. elegans* suggests that

Abbreviations used: BK, bradykinin; COS-$1^{p130}$, COS-1 cells stably expressing p130; EGF, epidermal growth factor; GFP, green fluorescent protein; GFP–p130(F), GFP-fused full-length p130; GFP–p130(ΔPH), GFP-fused p130 lacking the PH domain; GFP–p130PH, GFP-fused PH domain of p130; IP3R, Ins(1,4,5)$P_3$ receptor; p130, a new Ins(1,4,5)$P_3$-binding protein with a molecular mass of 130 kDa; PH, pleckstrin homology; PLC, phospholipase C; SAX, strong anion exchange.

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this catalytically inactive branch evolved early and separated from other PLC families to form a new protein family.

Further studies of p130 demonstrated that the PH domain of this molecule is responsible for binding inositol phosphates [16], a property that could be important for the function of the entire molecule. The functions of PH domains, present in more than 100 proteins, have been extensively studied [6,17,18]. This stable structural entity incorporates variable regions that, in different proteins, can be adapted to perform a specific function through binding to phosphoinositides present in the membrane or specific proteins. Several recent examples illustrate that the function of some PH domains, which interact with phosphoinositides, is to regulate membrane attachment [19–23]. Interactions with inositol phosphates have also been reported for a number of PH domains [6,16,17,21,23]. However, inositol phosphates have often been regarded as a mimic of the cytoplasm-exposed headgroup of the membrane-bound phosphoinositides, rather than as physiological ligands for these PH domains. A possible exception could be the PH domain of GAP1$^{IP4BP}$ (GAP being GTPase-activating protein), which was, like p130, originally purified as inositol-phosphate-binding protein [with a $K_d$ for Ins(1,3,4,5)$P_4$ of approx. 6 nM] [24]. Subsequently, this protein was found to have Ras-GAP activity [25]. However, the function of Ins(1,3,4,5)$P_4$ binding to GAP1$^{IP4BP}$ remains unclear. It could be required for the enhancement of GAP activity towards Ras or its functions could be related to Ins(1,3,4,5)$P_4$-regulated $Ca^{2+}$ entry.

Here we describe studies aiming at the characterization of a potential physiological ligand for the p130PH domain and functional properties of p130. The results provide evidence for the involvement of p130, found exclusively in the cytoplasm, in the Ins(1,4,5)$P_3$-mediated $Ca^{2+}$ signalling. They also suggest that a consequence of the binding of Ins(1,4,5)$P_3$ to the p130PH domain could be to sequester Ins(1,4,5)$P_3$ and therefore to prevent its interaction with the Ins(1,4,5)$P_3$ receptors and metabolizing enzymes.

## EXPERIMENTAL

### Materials

[$^3$H]Ins(1,4,5)$P_3$ (specific radioactivity 777 GBq/mmol) and *myo*-[1,2-$^3$H]inositol (specific radioactivity 2105 GBq/mmol) were obtained from Du Pont–New England Nuclear (Boston, MA, U.S.A.). phGFP-S65T and pEGFP-C1 vectors (in which GFP stands for green fluorescent protein) were from Clontech (Palo Alto, CA, U.S.A.). LipofectAMINE™ reagent and Geneticin (G418) were from Gibco BRL (Gaithersburg, MD, U.S.A.). Biotinylated or fluorescein-labelled goat anti-mouse IgG antibodies were from Amersham (Little Chalfont, Bucks., U.K.). Protein G–Sepharose was from Amersham Pharmacia Biotech (Uppsala, Sweden). An alkaline phosphatase staining kit was obtained from Pierce (Rockford, IL, U.S.A.). Lipids [phosphatidylethanolamine, PtdIns and PtdIns(4,5)$P_2$] were from Sigma (St Louis, MO, U.S.A.). Short-chain PtdIns(4,5)$P_2$ [(diC$_4$)-PtdIns(4,5)$P_2$] and PtdIns(3,4,5)$P_3$ [(diC$_4$)PtdIns(3,4,5)$P_3$] were from Echelon Research Laboratory (Salt Lake City, UT, U.S.A.). Fura 2 (free acid), fura 2 acetoxymethyl ester and Ins(1,4,5)$P_3$ were from Dojindo Laboratory (Kumamoto, Japan). All other reagents were of the highest grade available.

### Construction of plasmids for stable expression and cell culture for establishing COS-1$^{p130}$ cells

p130 cDNA (pcMT3; see [9]) was subcloned into BCMGSneo. The BCMGSneo/p130 was transfected into COS-1 cells with the use of LipofectAMINE reagent in accordance with the manufacturer's instructions and cultured in the presence of 0.8 mg/ml G418. Stable transfectants were established by selection with G418 and cloning.

### Construction of plasmids for expression of GFP fusion proteins

GFP fusion proteins of the full-length p130 [GFP–p130(F)], the PH domain deletion mutant [GFP–p130(ΔPH)] and the p130 isolated PH domain [GFP–p130PH] were constructed with the phGFP105-C1 and phGFP105/Sse vectors, modified from phGFP-S65T vector by changing several amino acids of GFP to enhance the fluorescent intensity [26]. To construct GFP–p130(F), the phGFP105-C1 vector was digested with *Eco*RI, followed by treatment with Klenow fragment for end-filling; it was then digested with *Xho*I again. The p130 cDNA, pcMT3 [9], was digested with *Hin*dIII (end-filling) and *Xho*I; the purified insert was ligated into phGFP105-C1. GFP–p130(ΔPH) and GFP–p130PH was constructed as follows: the *Sma*I fragment of pcMT3 was introduced into the phGFP105/Sse vector digested with *Sma*I; the *Bam*HI (end-filling) and *Sma*I fragments of pcMT3 were inserted into phGFP105-C1 vector digested with *Xho*I (end-filling). To construct GFP–PLC-$\delta$1 E341A, the full-length PLC-$\delta$1 E341A as a glutathione S-transferase fusion protein [11] was digested with *Bam*HI and *Sma*I; the resultant fragment was subcloned into pEGFP-C1 vector digested with *Bgl*II and *Sma*I. For construction of the PLC-$\delta$1 PH domain as a GFP fusion protein, a pGEX-2T plasmid encoding the N-terminal part of PLC-$\delta$1 (amino acid residues 1–290) [19] was digested with *Bam*HI and *Eco*RI; the fragment was subcloned into pEGFP-C1 vector digested with *Bgl*II and *Eco*RI. GFP–PKB/Akt PH domain plasmid was generated by using a PCR fragment of bovine PKB/Akt encoding the N-terminus (residues 1–147) and subcloning into pEGFP-C1 vector.

### Transfection and microinjection of GFPs for cell localization studies

COS-1, NIH 3T3 and Madin–Darby canine kidney ('MDCK') cells, cultured in Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum, were transiently transfected with LipofectAMINE Plus reagent, in accordance with the manufacturer's protocol. Procedures for the microinjection of MDCK cells were essentially the same as described previously [19].

### Immunostaining with monoclonal antibody against p130

COS-1$^{p130}$ cells were grown on a glass coverslips for 24 h. The cells were rinsed in PBS and fixed for 30 min in 4% (w/v) paraformaldehyde. All steps were performed at room temperature; coverslips were rinsed in PBS between each of the steps. Cells were permeabilized in PBS containing 0.2% (v/v) Triton X-100 for 20 min; p130 was detected with monoclonal anti-p130 antibody (2F9) and either FITC-conjugated goat anti-mouse IgG or biotinylated monoclonal antibody (biotin-2F9) and FITC-conjugated avidin. The coverslips were examined for fluorescent images with a Zeiss axiophot microscope using Zeiss $40 \times 1.3$ and $63 \times 1.4$ oil immersion objectives. Untransfected COS-1 cells or mock-transfected COS-1 cells were used as a control.

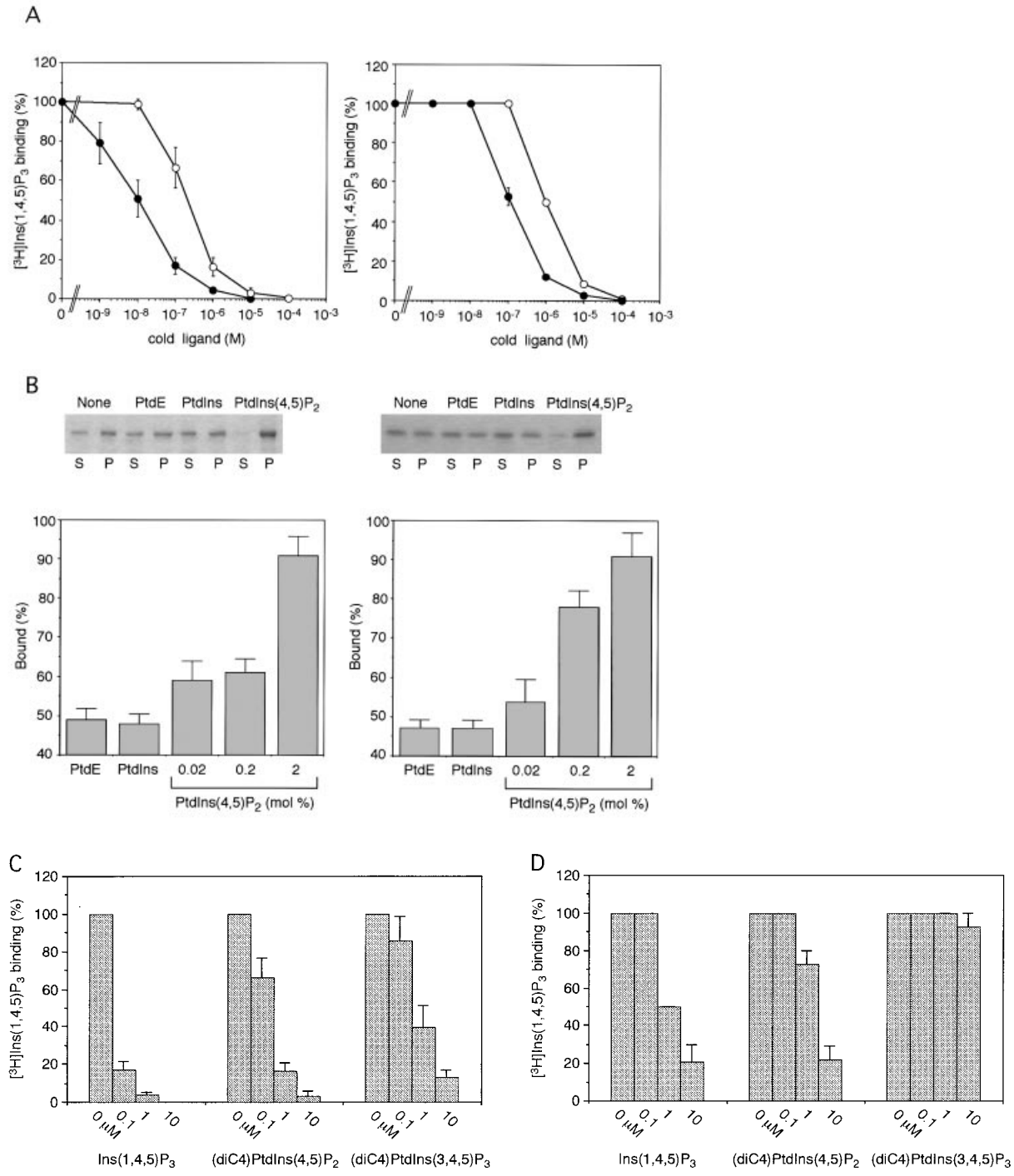


**Figure 1 Binding of inositol compounds**

(**A**) Displacement of [$^3$H]Ins(1,4,5)$P_3$ bound to p130 (left panel) and the p130PH domain (right panel) by Ins(1,4,5)$P_3$ (●) and water-soluble short-chain (diC$_4$)PtdIns(4,5)$P_2$ (○). (**B**) Binding of p130 (left panel) and p130PH domain (right panel) to liposomes containing naturally occurring PtdIns(4,5)$P_2$. Lanes marked S and P indicate supernatant and precipitate after centrifugation respectively. (**C**, **D**) Binding specificity of p130 (**C**) and PLC-$\delta$1 E341A (**D**). For displacing [$^3$H]Ins(1,4,5)$P_3$, unlabelled Ins(1,4,5)$P_3$, (diC$_4$)PtdIns(4,5)$P_2$ and (diC$_4$)PtdIns(3,4,5)$P_3$ were used. Each result is the mean ± S.E.M. for five to seven experiments.

## Protein expression and purification

Expression and purification of the full-length PLC-$\delta$1 E341A and PLC-$\delta$1 PH domain constructs were as described previously [11,19]. To prepare the full-length p130 (residues 1–1096), a baculovirus expression system was employed, with the use of standard procedures, and the fragment encoding p130 was subcloned into pAcHLT-B vector in frame with the $His_6$ tag. The recombinant PH domain of p130 was prepared as described previously [16].

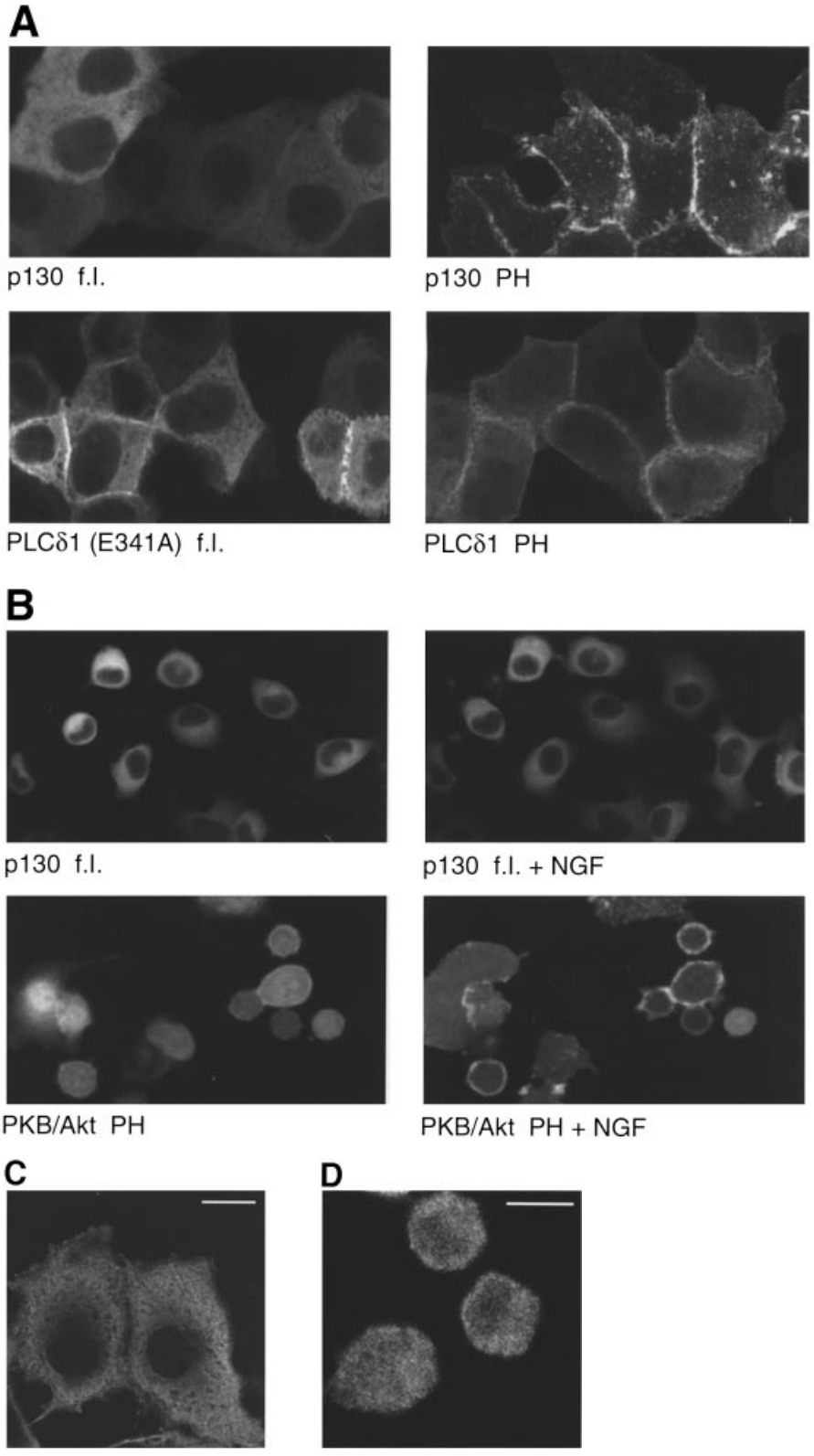


**Figure 2 Cellular localization studies of p130**

(**A**) The localization of the GFP fusion protein of the full-length p130 (p130 f.l.) and the isolated PH domain (p130PH) and corresponding constructs of PLC-$\delta$1 [PLC-$\delta$1 (E341A) f.l. and PLC-$\delta$1 PH domain respectively] was monitored after microinjection of the plasmids into MDCK cells. The images were recorded 2.5 h after microinjection. Experiments were repeated at least 10 times, giving essentially the same results. (**B**) Localization of the full-length p130 GFP fusion protein (p130 f.l.) in the PC12 cells was analysed 2.5 h after microinjection and images were recorded before

### Binding assay of recombinant proteins to [$^3$H] Ins(1,4,5)$P_3$ or to liposomes

The [$^3$H]Ins(1,4,5)$P_3$ binding assay was performed as described previously [27], except that the pH was 7.4. Binding to liposomes was assayed with the method described previously [28].

### Measurement of changes in free $Ca^{2+}$ concentration of the intact cells

Intracellular free $Ca^{2+}$ concentration was measured in COS-1 cells by using an Attofluor digital fluorescence microscopy system in combination with fura 2 (Atto Instruments, Rockville, MD, U.S.A.). Cells were loaded with fura 2 by incubation with fura 2 acetoxymethyl ester for 20 min at room temperature, then for a further 20 min at 37 °C. For measurement with control COS-1 or COS-1$^{p130}$, fura 2 was excited alternately at two wavelengths (340 and 380 nm) and the fura 2 fluorescence from a single cell was measured. For measurement with COS-1 cells transfected with the GFP-fused version of p130, successful transfection of the plasmid into the cell was confirmed by GFP fluorescence. Fura 2 fluorescence from a single cell in the same microscopic field was measured. Thus it was possible to measure $Ca^{2+}$ concentrations from only the vector-transfected, GFP-positive cells. All experiments were performed at room temperature (20–25 °C).

### Measurement of production of Ins(1,4,5)$P_3$

The cellular content of Ins(1,4,5)$P_3$ was measured by a radioreceptor competition assay by the method of Palmer et al. [29]. Cell labelling experiments were also performed with the method described by Shears [30], with a minor modification for the separation of labelled inositol phosphates by a strong anion exchange (SAX) column (Whatman) mounted on an HPLC system.

### Assay of $Ca^{2+}$ release from permeabilized cells by Ins(1,4,5)$P_3$

The procedures for obtaining and analysing permeabilized COS-1 cells were essentially the same as those previously described for other cell types [31]. The COS-1 cells were harvested 4–5 days after plating, then permeabilized with saponin. $Ca^{2+}$ uptake and release by saponin-permeabilized cells were assayed as described previously [31].

### Assay of Ins(1,4,5)$P_3$-metabolizing activity

Assays of Ins(1,4,5)$P_3$ metabolizing enzymes were performed as described previously, with recombinant enzymes [8].

### Binding of inositol phosphate in living cells to p130

COS-1$^{p130}$ cells were incubated for 48 h in inositol-free medium supplemented with *myo*-[$^3$H]inositol (50 $\mu$Ci/ml) and dialysed fetal bovine serum. The labelled cells were stimulated with bradykinin (BK) or epidermal growth factor (EGF) and then lysed with a buffer containing 1 % (v/v) Triton X-100/50 mM Hepes/NaOH (pH 7.2)/150 mM NaCl/5 mM EDTA. After centrifugation at 12000 ***g*** for 30 min at 4 °C, the resulting supernatant was incubated with 20 $\mu$g/ml anti-p130 monoclonal antibody (2F9), followed by the addition of 20 $\mu$l of a 50 % (w/v) slurry of Protein G–Sepharose for 2 h at 4 °C. Immunoprecipitate was treated with 5 % (w/v) trichloroacetic acid to release the radioactivity bound to p130. After neutralization of the sample, it was analysed by applying to a SAX column as described above.

## RESULTS

### Purified p130 and its isolated PH domain have a preference for Ins(1,4,5)$P_3$ over inositol lipids

The Ins(1,4,5)$P_3$-binding properties of p130 have previously been mapped to the N-terminus containing the PH domain [16]. Further characterization has also been performed with different inositol phosphates [32]. To compare the binding of p130 to both inositol phosphates and inositol lipids, short-chain water-soluble (diC$_4$)PtdIns(4,5)$P_2$ was directly compared with Ins(1,4,5)$P_3$ in competition experiments with radiolabelled [$^3$H]Ins(1,4,5)$P_3$ (Figure 1A). Both the full-length p130 and the N-terminal construct containing the PH domain had a higher binding affinity for Ins(1,4,5)$P_3$ than for PtdIns(4,5)$P_2$. {Previously we reported that the isolated PH domain of p130 showed approx. 3500-fold lower affinity for Ins(1,4,5)$P_3$ than did the full-length p130 [16]. We later noticed that this was simply because of the pH when assayed. At pH 7.4, which was used in the present study, only an approx. 10-fold difference in the apparent affinity was observed (Figure 1A). An alkaline pH (e.g. pH 8.3, used previously) significantly decreased the affinity of the isolated PH domain but not that of the full-length p130.} Calculated $K_d$ values for the full-length p130 were 3 nM for Ins(1,4,5)$P_3$ and 100 nM for PtdIns(4,5)$P_2$. Binding to PtdIns(4,5)$P_2$, isolated from natural sources, incorporated into liposomes was also observed for both the full-length p130 and the isolated PH domain. Figure 1(B) shows a typical SDS/PAGE pattern of the pellets and supernatants obtained with p130 and the PH domain and a summary of the liposome binding study. Although some protein was found in the pellet even in the absence of liposomes (lanes labelled 'none') [28] and associated with liposomes containing phosphatidylethanolamine or PtdIns, the inclusion of PtdIns(4,5)$P_2$ into liposomes resulted in a significant increase in the amount of bound protein. Binding of PLC-$\delta$1 to PtdIns(4,5)$P_2$ was also confirmed with the same assay (results not shown).

The binding properties of full-length p130 were directly compared with those of full-length PLC-$\delta$1 E341A by using Ins(1,4,5)$P_3$ and short-chain (diC$_4$)PtdIns(4,5)$P_2$ and (diC$_4$)-PtdIns(3,4,5)$P_3$ as ligands. The mutation E341A in PLC-$\delta$1 greatly decreases PLC activity from approx. 1000 units/mg, determined for the wild type, to 0.004 unit/mg [11]. This virtually catalytically inactive mutant was therefore suitable for comparison with p130, preventing the hydrolysis of PtdIns(4,5)$P_2$. As shown in Figures 1(C) and 1(D), p130 bound all three ligands more strongly than did PLC-$\delta$1. Furthermore, differences in relative specificities have been observed. A preference for Ins(1,4,5)$P_3$ over PtdIns(4,5)$P_2$ or PtdIns(3,4,5)$P_3$ was clearly demonstrated for p130 (30-fold or 130-fold respectively), whereas PLC-$\delta$1 demonstrated similar binding to both Ins(1,4,5)$P_3$ and PtdIns(4,5)$P_2$ (Figure 1D). However, PLC-$\delta$1 bound PtdIns(4,5)$P_2$ preferentially when compared with PtdIns(3,4,5)$P_3$ (30-fold difference), while p130 showed less selectivity.

and after the addition of nerve growth factor (NGF) for 10 min. The same conditions were used to analyse the localization of the GFP fusion protein of the PKB/Akt PH domain. (**C**) The localization of p130 in the stably transfected cell line COS-1$^{p130}$ was revealed with a monoclonal anti-p130 antibody. Experiments were repeated at least five times, giving essentially the same results. (**D**) Staining of PC12 cells with a monoclonal anti-p130 antibody. Experiments were repeated at least three times, giving essentially the same results.

### p130 is present in the cytoplasm

The ability of a number of PH domains to interact with phosphoinositides has been related to membrane localization [19,23]. To test the localization of p130, the microinjection or transfection of expression plasmids encoding GFP fusion proteins has been performed in several cell lines. Figure 2 shows a direct comparison of GFP–p130(F) and GFP–p130PH with the corresponding constructs of PLC-$\delta$1. Localization of all constructs was monitored after microinjection into MDCK cells. As assessed by GFP fluorescence, the preferential localization of GFP–p130PH to the plasma membrane was observed, whereas GFP–p130(F) was found throughout the cytoplasm (Figure 2A). In contrast, membrane localization could be detected for both the full-length E341A and the PH domain of PLC-$\delta$1 protein (Figure 2A). This observation was confirmed by using other cell types (COS-1 and NIH 3T3) in transient transfection experiments (results not shown).

Because p130 also binds PtdIns(3,4,5)$P_3$ with a high affinity (Figure 1C), we tested whether stimulation of cells, leading to the activation of type 1 phosphoinositide 3-kinase, could cause the translocation of p130 to the membrane. Using the PH domain of PKB/Akt as a positive control, we demonstrated that this did not occur (Figure 2B).

In addition to GFP fluorescent studies, we performed immunostaining of p130 with a monoclonal anti-p130 antibody. Because COS-1 cells contain no endogenous p130, COS-1 cells stably expressing p130 (COS-1$^{p130}$) were established. As shown in Figure 2(C), expressed p130 was abundant in the cytoplasm; no enrichment at the plasma membrane was observed. PC12 cells, the only cell line containing endogenous p130 among cell lines described in this study (PC12, MDCK, COS-1, NIH 3T3, rat basophilic leukaemic cells), also exhibited preferential localization in the cytoplasm as assessed by immunostaining (Figure 2D), indicating that the presence of p130 in the cytoplasm observed after transfection or microinjection was not caused by artificial or forced expression of p130. (We have found recently that GH3 cells also contain endogenous p130.)

### Addition of p130 to permeabilized cells inhibits Ins(1,4,5)$P_3$-triggered $Ca^{2+}$ release

Studies of cellular localization demonstrating the presence of p130 in cytoplasm, and the ability of p130 to bind strongly to Ins(1,4,5)$P_3$, suggested that it might be involved in the regulation of inositol signalling through binding to the soluble inositol phosphates rather than inositol lipids in the membrane. To test this possibility, $Ca^{2+}$ release in response to the addition of Ins(1,4,5)$P_3$ was analysed by using reconstitution experiments with permeabilized COS-1 cells (Figure 3). Permeabilized COS-1 cells were incubated in the presence of ATP and $NaN_3$, an inhibitor of mitochondrial $Ca^{2+}$ accumulation, but in the absence of EGTA and exogenously added $Ca^{2+}$. Thus the endoplasmic reticulum of permeabilized cells accumulated the water-contaminating $Ca^{2+}$ with the coupling of ATP hydrolysis. The amount of $Ca^{2+}$ outside the endoplasmic reticulum was monitored by the fluorescence changes of fura 2. The addition of ATP together with $Mg^{2+}$ caused a decrease in the fura 2 fluorescence due to the decrease in the $Ca^{2+}$ concentration, as shown in Figure 3(A). Neither ATP nor $Mg^{2+}$ alone could cause the observed decrease in fluorescence, demonstrating that the decrease in the fluorescence was most likely to have been due to the accumulation of $Ca^{2+}$ in the endoplasmic reticulum of permeabilized cells coupled to ATP hydrolysis. The sequential addition of Ins(1,4,5)$P_3$ caused a stepwise increase in the fluorescence, demonstrating that Ins(1,4,5)$P_3$ triggered a dose-dependent release of $Ca^{2+}$ accumulated in the endoplasmic reticulum (Figure 3A). When the recombinant PH domain of p130 at various concentrations (0.6–60 $\mu$M) was included in the assay mixture, the dose-dependent release of $Ca^{2+}$ by Ins(1,4,5)$P_3$ shifted to the

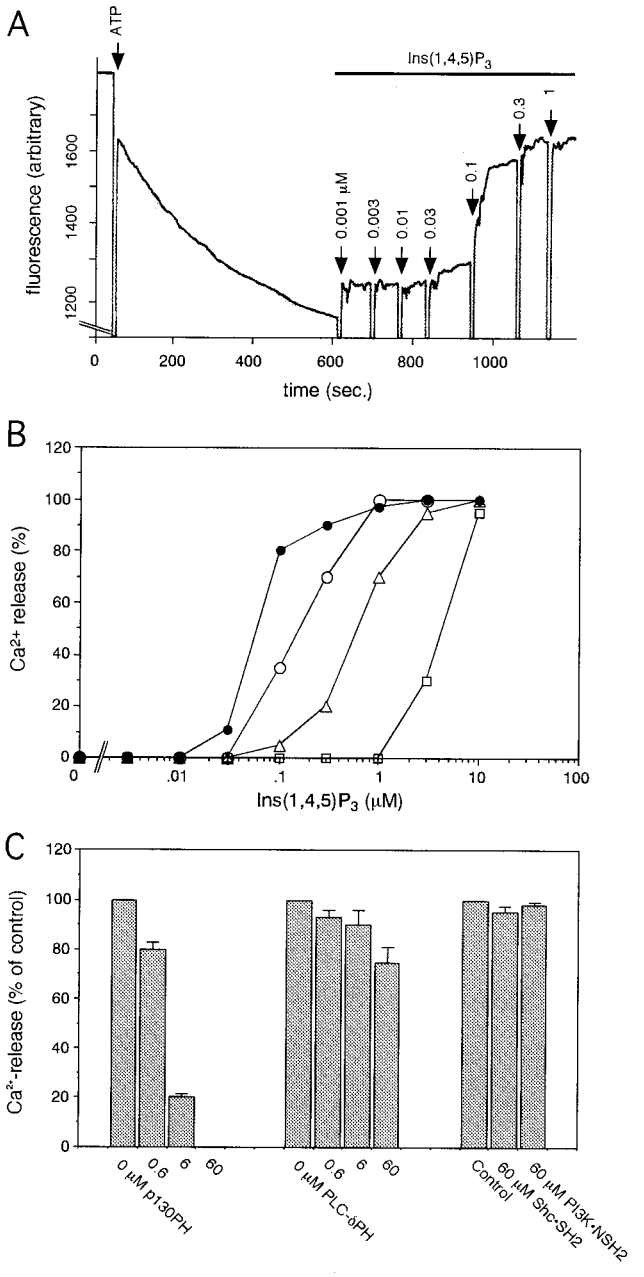


**Figure 3 Ins(1,4,5)$P_3$-mediated release of $Ca^{2+}$ from permeabilized COS-1 cells**

(**A**) $Ca^{2+}$ accumulation and release from permeabilized COS-1 cells were monitored with fura 2. At 7 min after the addition of ATP, increasing concentrations of Ins(1,4,5)$P_3$ were added sequentially to monitor the release of $Ca^{2+}$. Additions are indicated by arrows. (**B**) The $Ca^{2+}$ assay mixture included various concentrations of recombinant PH domain of p130. Each point represents the mean of two determinations with a different preparation of permeabilized cells; 100% $Ca^{2+}$ release was taken as that seen with 10 $\mu$M Ins(1,4,5)$P_3$. Symbols: ●, control; ○, 0.6 $\mu$M p130PH domain; △, 6 $\mu$M p130PH domain; □, 60 $\mu$M p130PH domain. (**C**) Inhibition of 0.3 $\mu$M Ins(1,4,5)$P_3$-mediated $Ca^{2+}$ release by PH domains from p130 and PLC-$\delta$1, or by SH2 domains. Each result is the mean for two determinations, relative to the $Ca^{2+}$ release caused by 0.3 $\mu$M Ins(1,4,5)$P_3$ (taken as 100%).

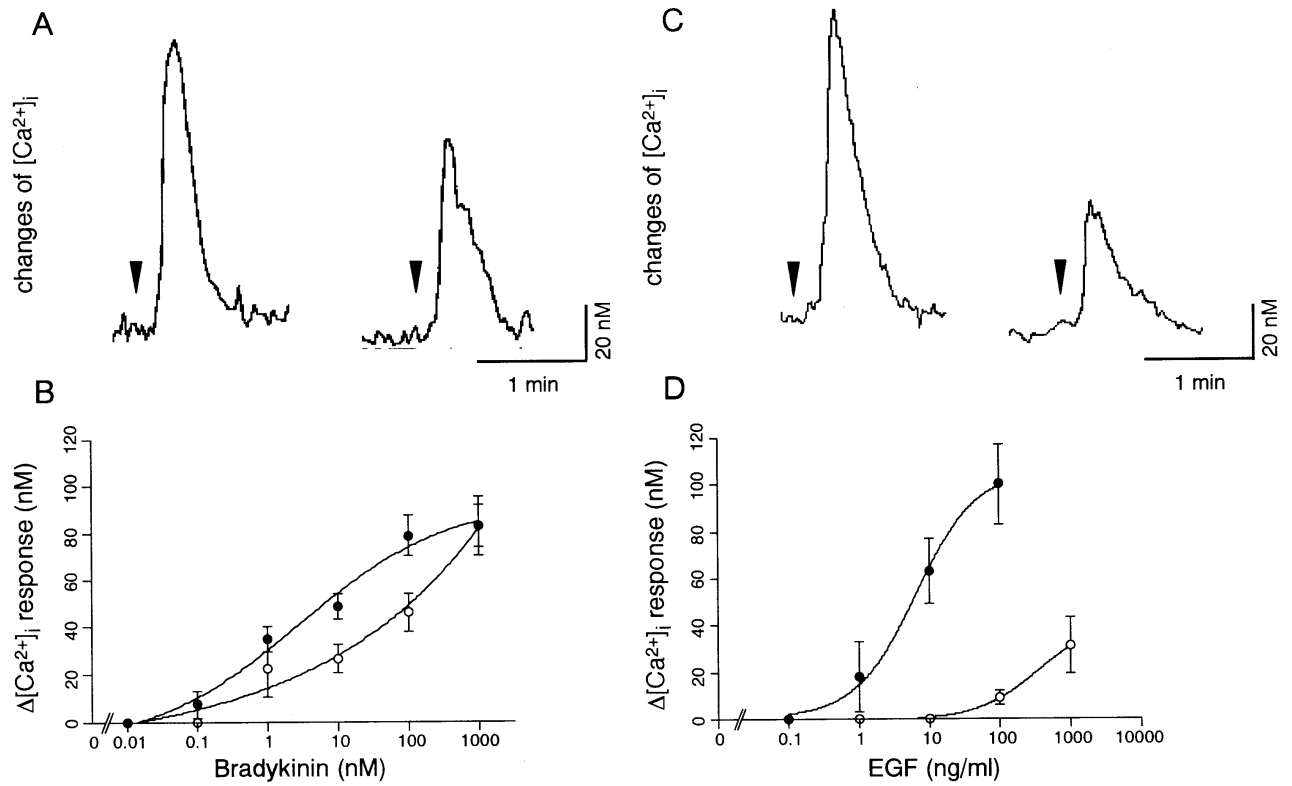


**Figure 4 Effects of extracellular stimulation with either BK (A, B) or EGF (C, D) on the change in free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) of COS-$1^{p130}$**

Control cells (left traces) or COS-$1^{p130}$ (right traces) were stimulated with BK (**A**) or EGF (**C**) after being loaded with fura 2. Arrowheads indicate the addition of agonist. The traces are representative of more than 300 cells examined. (**B**, **D**) The respective dose–response relationships. Symbols: ●, control cells; ○, COS-$1^{p130}$. Each point is the mean ± S.E.M. for at least five determinations.

right (Figure 3B). This shift was correlated with the amount of PH domain added, demonstrating that the PH domain of p130 was able to compete with the receptor involved in the $Ca^{2+}$ release for the Ins(1,4,5)$P_3$. Figure 3(C) also shows some inhibition of $Ca^{2+}$ release by the PH domain of PLC-$\delta$1 at high protein concentrations, presumably due to the binding of added Ins(1,4,5)$P_3$. This observation is consistent with the previous studies of PLC-$\delta$1 PH domain demonstrating that Ins(1,4,5)$P_3$ can compete for PtdIns(4,5)$P_2$ binding in permeabilized cells [33], and a recent study suggesting Ins(1,4,5)$P_3$-mediated membrane detachment of PLC-$\delta$1 PH domain after the microinjection of Ins(1,4,5)$P_3$ [23]. However, the potency of the PLC-$\delta$1 PH domain in inhibiting $Ca^{2+}$ release was much lower than that observed for the p130PH domain. This is in agreement with a higher binding affinity of p130 for Ins(1,4,5)$P_3$ and its preference for Ins(1,4,5)$P_3$ over PtdIns(4,5)$P_2$ observed in binding studies *in vitro* (Figure 1). In addition, recombinant SH2 domains of Shc and the 85 kDa subunit of type 1 phosphoinositide 3-kinase, proteins of similar molecular mass to the p130PH domain that are incapable of binding Ins(1,4,5)$P_3$, were also examined and had no effects on the $Ca^{2+}$ release (Figure 3C).

### p130 inhibits the agonist-induced rise in $Ca^{2+}$ concentrations when expressed in COS-1 cells

Further experiments were performed with COS-1 cells stably expressing p130 (COS-$1^{p130}$). To determine the concentration of p130 in this cell line, a direct comparison with PC12 cells containing endogenous p130 and known amounts of purified p130 was performed. The calculated concentration of p130 expressed in COS-$1^{p130}$ (40 ± 6 $\mu$M) was approx. 5-fold that of the endogenous p130 present in PC12 cells (8 ± 1 $\mu$M). As described previously, in control COS-1 cells we were unable to detect any p130 protein.

To analyse the involvement of p130 in $Ca^{2+}$ signalling, three extracellular agonists were chosen: BK and ATP as stimulators of PLC-$\beta$ isoforms, and EGF as a stimulator of the $\gamma$-isoforms of PLC. COS-1 cells lack the $\delta$ subtype of PLC as assessed by Western blotting with a specific polyclonal antibody (T. Kanematsu, H. Taneuchi and M. Hirata, unpublished work).

After the loading of cells with fura 2, COS-$1^{p130}$ and control COS-1 cells were stimulated with either BK or EGF and changes in free $Ca^{2+}$ concentration were monitored, in the absence of extracellular $Ca^{2+}$, indicating that the increase in free $Ca^{2+}$ concentration was a reflection of the release from intracellular store site(s), most probably the endoplasmic reticulum. The increase in free $Ca^{2+}$ concentration caused by either BK or EGF was lower in COS-$1^{p130}$ than in control cells (Figure 4). A trace from a single cell shown in Figures 4(A) and 4(C) is representative of more than 80 % of over 300 cells examined. The dose–response curve to increasing concentrations of BK and EGF was also shifted to the right in COS-$1^{p130}$ (Figures 4B and 4D). A higher concentration of BK (1 $\mu$M) restored the $Ca^{2+}$ response to the maximal level, whereas the decrease in $Ca^{2+}$ response caused by EGF was not restored to the control level even at 1000-fold higher agonist concentrations. The $Ca^{2+}$ response in the cells stimulated with ATP through the P2-purinergic receptor was

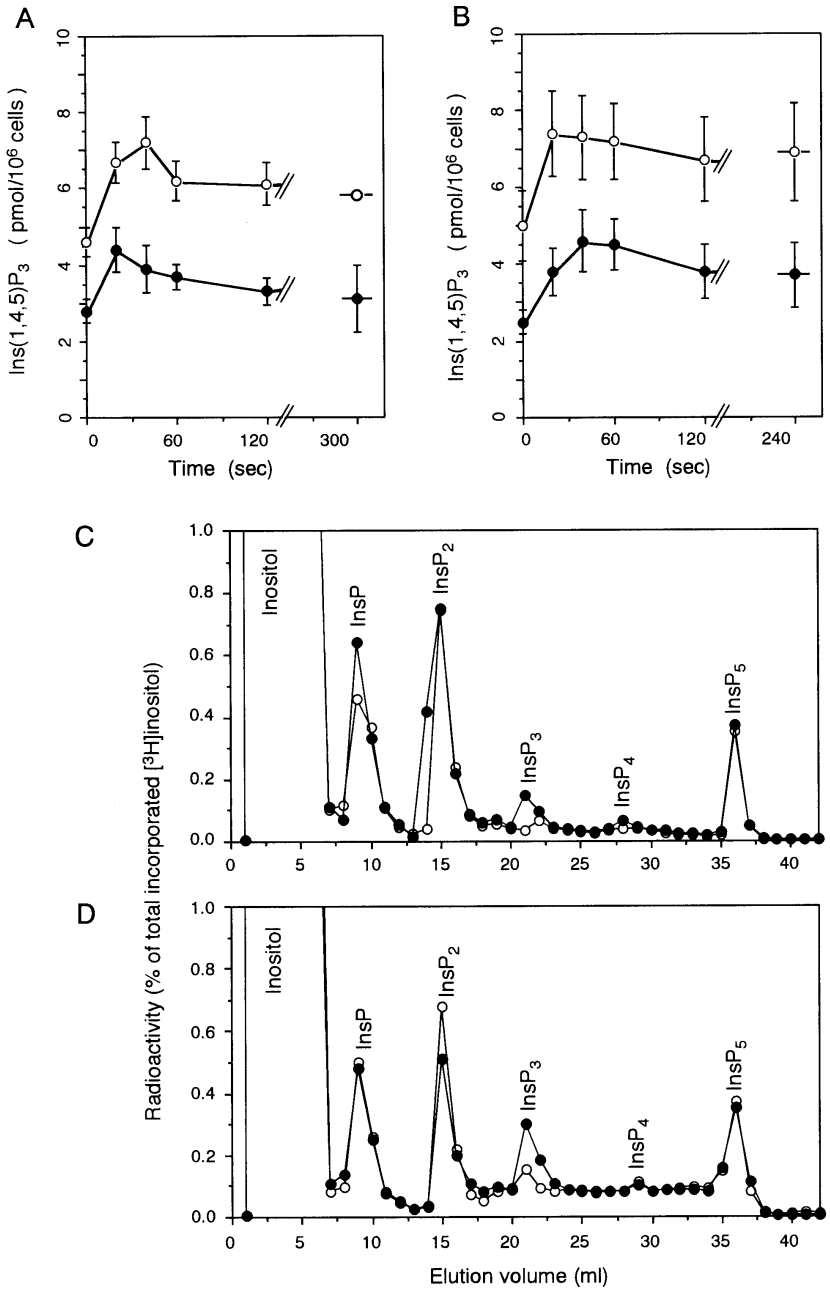


**Figure 5 Production of Ins(1,4,5)$P_3$**

The production of Ins(1,4,5)$P_3$ in control (●) or COS-1$^{p130}$ (○) cells stimulated with BK at 100 nM (**A**) or EGF at 100 ng/ml (**B**) was measured by a radio-receptor competition assay. Each point is the mean ± S.E.M. for at least five determinations. (**C**, **D**) The determination of Ins(1,4,5)$P_3$ after labelling of cells and the separation of [$^3$H]inositol phosphates by a SAX column of the cellular extract from control (**C**) or COS-1$^{p130}$ cells (**D**), either non-stimulated (○) or stimulated with 100 nM BK for 60 s (●). The results are expressed as percentages of total [$^3$H]inositol incorporated into inositol phosphates and phosphoinositides, and are representative of five experiments. Note that the radioactivities of Ins(1,4,5)$P_3$ in both non-stimulated and BK-stimulated control cells were lower than those in COS-1$^{p130}$ cells, consistent with the measurements described in (**A**).

also inhibited, as in cells with EGF stimulation (results not shown).

### Decrease in agonist-induced $Ca^{2+}$ concentration increase in COS-1$^{p130}$ is not caused by inhibition of PtdIns(4,5)$P_2$ hydrolysis

The possibility of an inhibitory interaction at the level of PtdIns(4,5)$P_2$ hydrolysis and Ins(1,4,5)$P_3$ production was analysed by measurements of total Ins(1,4,5)$P_3$ concentrations in the control and COS-1$^{p130}$ cells stimulated with BK or EGF. Consistent results were obtained with two different methods (Figure 5). The radio-receptor competition assay (Figures 5A and 5B) demonstrated that the production of Ins(1,4,5)$P_3$ was not inhibited (rather, a slight enhancement was observed) in COS-1$^{p130}$ cells stimulated with BK or EGF. The measurements made with the standard separation of radiolabelled inositol phosphates showed a similar accumulation of [$^3$H]Ins(1,4,5)$P_3$ in cells stimulated with BK (Figures 5C and 5D). Stimulation with EGF was

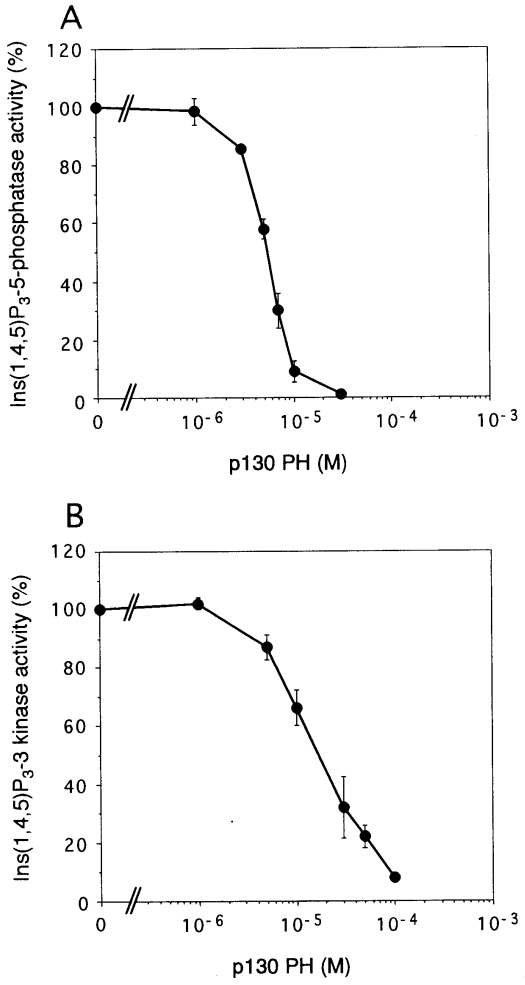


**Figure 6 Effect of p130 on Ins(1,4,5)$P_3$ metabolism by Ins(1,4,5)$P_3$-5-phosphatase (A) or Ins(1,4,5)$P_3$-3-kinase (B)**

Recombinant enzymes were incubated in the presence of increasing amounts of p130PH domain as described in the Materials and methods section. Each point is the mean ± S.E.M. for at least five determinations.

also performed and gave essentially the same results in the labelling experiments. The basal level of Ins(1,4,5)$P_3$ in COS-$1^{p130}$ was 1.5–2-fold that in the control cells, whereas the amount of Ins(1,4,5)$P_3$ produced after stimulation with BK or EGF was slightly higher than that in the control cells. In addition, a return to the basal level of Ins(1,4,5)$P_3$ produced by the stimulation was hardly observed, particularly after stimulation with BK. Although the basal level of Ins(1,4,5)$P_3$ in COS-$1^{p130}$ was maximally 2-fold that in the control cells, the basal concentration of free $Ca^{2+}$ in COS-$1^{p130}$ was the same as that seen in the control cells (results not shown). This was probably due to the competition for the binding of Ins(1,4,5)$P_3$, between p130 and the Ins(1,4,5)$P_3$ receptor on the endoplasmic reticulum. A similar explanation could apply to the inhibition of metabolism by p130 after agonist stimulation. The formation of a stable complex between Ins(1,4,5)$P_3$ and p130 might prevent not only the interaction with the receptor, as suggested by Figures 3 and 4, but also its further metabolism by either or both of Ins(1,4,5)$P_3$-5-phosphatase and Ins(1,4,5)$P_3$-3-kinase or other effects attributed to this inositol phosphate.

To test the possibility described above, we examined the effect of the p130PH domain on Ins(1,4,5)$P_3$ metabolism by Ins(1,4,5)$P_3$-5-phosphatase and Ins(1,4,5)$P_3$-3-kinase. Recombinant 5-phosphatase and 3-kinase enzymes were analysed *in vitro* with [$^3$H]Ins(1,4,5)$P_3$ as a substrate in the presence of various amounts of the p130PH domain. As shown in Figure 6, the p130PH domain inhibited both Ins(1,4,5)$P_3$-5-phosphatase (Figure 6A) and Ins(1,4,5)$P_3$-3-kinase (Figure 6B) activity in a dose-dependent manner.

### Ins(1,4,5)$P_3$ is bound to p130 in cells

*In vitro*, p130 is also able to bind to other inositol polyphosphates (Ins$P_5$ and Ins$P_6$), which are abundant in the resting cells [7,34], indicating the possibility that the binding capacities of cellular p130 are saturated by these inositol polyphosphates inside cells. However, the affinities for these inositol polyphosphates were approx. 100-fold lower than that for Ins(1,4,5)$P_3$ [7], indicating that above notion is unlikely. To obtain direct evidence that cellular p130 forms a complex with Ins(1,4,5)$P_3$ in living cells, COS-$1^{p130}$ cells were labelled with *myo*-[$^3$H]inositol. Cellular p130 was subsequently immunoprecipitated from the lysate of the labelled cells. Analysis of the protein content of the immunoprecipitates (Figure 7A) shows that p130 was a major protein. Labelled inositol phosphates bound to p130 were then analysed (Figure 7B). Under standard conditions of immunoprecipitation, Ins(1,4,5)$P_3$ was clearly identified as one of the peaks of radioactivity. Peaks of the abundunt free inositol and Ins$P$ could also be detected but were completely removed by stringent washing and therefore represented contaminants in p130 immunoprecipitates. The immunoprecipitation of p130 was not quantitative; five experiments performed under stringent conditions provided a minimum value for bound Ins(1,4,5)$P_3$. When cells were stimulated with BK, the radioactivity of only Ins(1,4,5)$P_3$ bound to p130 increased up to 2-fold, supporting a possibility that cellular p130 sequesters Ins(1,4,5)$P_3$ produced in response to cell stimulation.

### PH domain is required for the effect of p130 on $Ca^{2+}$ signalling

To examine whether the PH domain of p130 is involved in the decrease in the $Ca^{2+}$ response as shown in Figure 4, GFP–p130(F) and GFP–p130(ΔPH) were made. The use of GFP-fused proteins permitted the identification of living cells that expressed p130 in a transient expression system. After the transfection of COS-1 cells with the plasmid harbouring GFP–p130(F) or GFP–p130(ΔPH), cells were loaded with fura 2 for $Ca^{2+}$ measurement and then revealed by fluorescent microscopy. Almost all cells were fluorescent for fura 2, whereas approx. 30 % of cells were transfected and fluorescent for GFP. Stimulation of COS-1 cells was performed with BK, EGF and also with ATP through P2-purinergic receptors with essentially the same results; responses to stimulation of the P2-purinergic receptor are summarized in Figure 8. The increase in free $Ca^{2+}$ concentration in response to ATP was observed in COS-1 cells that were fluorescent only for fura 2 (Figure 8A) and was similar to $Ca^{2+}$ responses in untransfected COS-1 cells (results not shown). Figures 8(B)–8(D) show the ATP-induced $Ca^{2+}$ response in cells with GFP alone, with GFP–p130(F) and with GFP–p130(ΔPH) respectively. $Ca^{2+}$ responses were not altered by GFP alone, whereas GFP–p130(F) caused 70–80 % inhibition, as expected from results in COS-$1^{p130}$ cells. In contrast, GFP–p130(ΔPH) was not capable of mediating such a decrease in $Ca^{2+}$ responses. Figure 8(E) summarizes these results, showing clearly that the inhibition of the $Ca^{2+}$ response required the presence of the PH domain of p130.

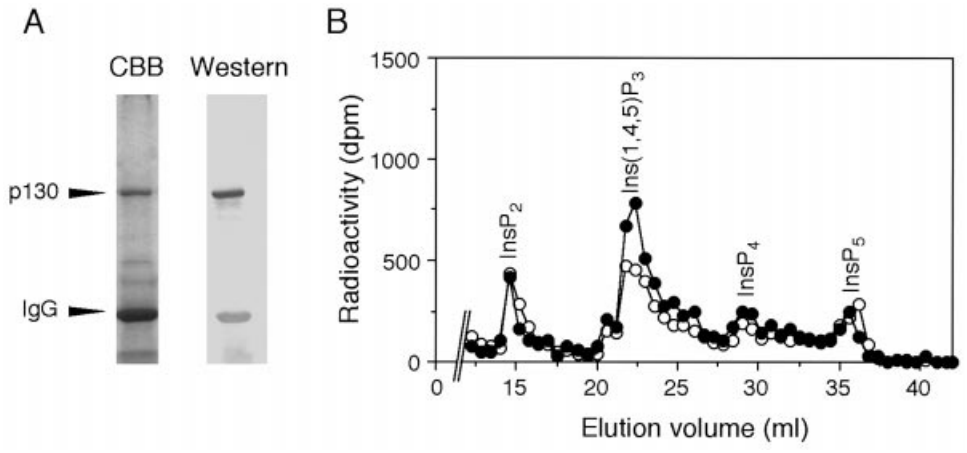


**Figure 7 Immunoprecipitation from the lysate of COS-1$^{p130}$ cells labelled with *myo*-[$^3$H]inositol by monoclonal anti-p130 antibody**

(**A**) Labelled COS-1$^{p130}$ cells were lysed and subjected to immunoprecipitation with monoclonal anti-p130 antibody (2F9) and Protein G–Sepharose. The immunoprecipitates were analysed by SDS/PAGE followed by CBB staining or Western blotting with monoclonal anti-p130 antibody. (**B**) The inositol phosphates bound to the immunoprecipitate were extracted by treatment with trichloroacetic acid and analysed by application to a SAX column. Results are representative of five experiments. Because there was much variation in labelling efficiency and immunoprecipitation, it was impossible to show statistical values. Symbols: ○, non-stimulated; ●, stimulated with 100 nM BK.

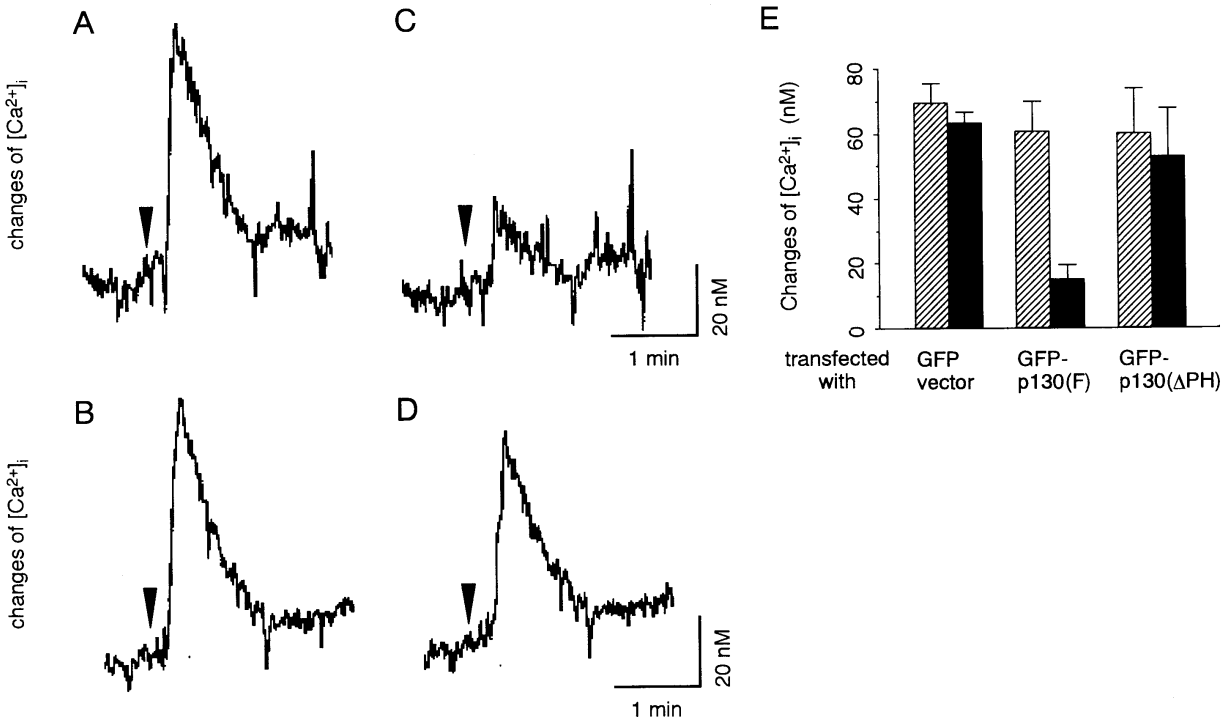


**Figure 8 $Ca^{2+}$ response by ATP stimulation in COS-1 cells transfected with GFP–p130(F) or GFP–p130(ΔPH)**

COS-1 cells were transfected with either plasmid encoding GFP–p130(F) or GFP–p130(ΔPH), followed by loading with fura 2. By using fluorescent microscopy, cells were selected for those with fura 2 but without GFP fluorescence (**A**) or those with fura 2, and GFP alone (**B**), GFP–p130(F) (**C**) or GFP–p130(ΔPH) (**D**). Cells were stimulated with 10 $\mu$M ATP as indicated by the arrowhead. Each trace is typical of at least 10 cells examined. The summary of the changes in $Ca^{2+}$ concentrations after ATP stimulation (**E**) shows mean values from cells without (hatched) or with (black) GFP fluorescence.

## DISCUSSION

In the present study we analysed the functional properties of p130 protein, which was originally isolated as a protein with high affinity for Ins(1,4,5)$P_3$ [7,35]. We have extended our analysis of ligand binding *in vitro*, used a combination of approaches to identify which of the potential ligands could interact with p130 in cells and further considered the implications of this binding for cellular function.

Studies of ligand binding *in vitro* have previously shown that the PH domain of p130 is responsible for the high-affinity

binding of Ins(1,4,5)$P_3$ by the full-length protein [16]. The binding *in vitro* is not restricted to this ligand: a few other inositol phosphates [Ins(1,4,5,6)$P_4$ and, to a smaller extent, Ins(1,3,4,5)$P_4$] can also bind to p130 through the PH domain [7,9,16,32,35]. Here we have also demonstrated that p130 can bind short-chain inositol lipids [($diC_4$)PtdIns(4,5)$P_2$ and ($diC_4$)PtdIns(3,4,5)$P_3$], although with lower affinity than for Ins(1,4,5)$P_3$; the affinity for PtdIns(4,5)$P_2$ isolated from natural sources is even lower (Figure 1). Nevertheless, affinities for short-chain versions of PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$ estimated in this study ($K_d$ values of 100 and 500 nM respectively) are comparable with those reported for proteins in which these phosphoinositides have been accepted as physiological ligands. For example, the $K_d$ for binding of PKB/Akt to ($diC_8$)PtdIns(3,4,5)$P_3$ was approx. 400 nM [36] and that for the binding of PLC-$\delta$1 to PtdIns(4,5)$P_2$ was approx. 1.7 $\mu$M [37]. Therefore, on the basis of the studies of ligand binding *in vitro*, several inositol compounds could potentially be physiological ligands for p130.

Factors that have to be considered when searching for a physiological ligand include the relative concentration of the free ligand and the cellular localization of both the ligand and the binding protein. Free concentrations of inositol lipids are difficult to measure; however, estimated concentrations in the inner leaflet of plasma membrane are within the millimolar range for PtdIns(4,5)$P_2$ and up to 200 $\mu$M for PtdIns(3,4,5)$P_3$ [38]. The cellular levels of Ins(1,4,5)$P_3$ are lower, ranging from 1 $\mu$M (in 'resting' cells) to 10 $\mu$M (in receptor-activated cells) [34]. Therefore, in spite of the higher affinity for Ins(1,4,5)$P_3$, the inositol lipids could possibly be bound to p130 in cells. This has been clearly shown for PLC$\delta$1 in non-stimulated cells, in which the full-length and isolated PH domains reside in the membrane [19–22] (Figure 2). Studies with several cell types have demonstrated that the isolated p130PH domain was associated with the membrane (Figure 2A). However, the full-length p130 was present only in the cytoplasm of cells endogenously expressing this protein or when introduced into a range of different cell types (Figures 2A, 2C and 2D). There are several possibilities for explaining why the PH domain within the entire p130 molecule and in the cellular context cannot act as a determinant of cellular localization through phosphoinositide binding. The possibility that other domains of p130 mask the PH domain is unlikely because the full-length p130 binds the ligands *in vitro* (Figure 1) and, as discussed below, also in cells. On the basis of the observation that the isolated PH domain can interact with the membrane (Figure 2A), it is also unlikely that the p130PH domain interacts with other cellular molecules that could block PtdIns(4,5)$P_2$ binding. One possible explanation could be that other region(s) of p130, outside the PH domain, make interactions that determine its presence in the cytoplasm. Although this remains to be clarified, the experiments presented here strongly support inositol phosphates rather than phosphoinositides as physiological ligands for the PH domain in p130. Evidence supporting this was provided by the isolation of a complex between p130 and Ins(1,4,5)$P_3$ after the immunoprecipitation of p130 from COS-1 cells that stably express this protein (Figure 7). As shown with some other proteins, this is not an ideal, quantitative, type of analysis. Nevertheless, we clearly demonstrated that the amounts of bound Ins(1,4,5)$P_3$ increase after stimulation with agonist.

To establish the identity of the ligand and the possible implications of the binding for cell function, the effects of p130 on $Ca^{2+}$ signals were monitored in permeabilized and transfected cells. We demonstrated that p130, through the function of the PH domain, was capable of attenuating the Ins(1,4,5)$P_3$-triggered and the agonist-induced increase in $Ca^{2+}$ concentrations (Figures 3, 4 and 8). Furthermore, experimental support has been provided for an interaction of p130 with Ins(1,4,5)$P_3$, rather than an inhibitory interaction at the level of PtdIns(4,5)$P_2$ hydrolysis as described for some related proteins [22,39]. Measurements of the production of Ins(1,4,5)$P_3$ in the control and COS-$1^{p130}$ cells stimulated with BK or EGF demonstrated that the production of Ins(1,4,5)$P_3$ was not inhibited (Figure 5). A possible role for p130, suggested by the results presented here, is to provide a level of control in $Ca^{2+}$ signalling through competition with Ins(1,4,5)$P_3$ receptor (IP3R) for Ins(1,4,5)$P_3$ binding. However, for this function to be fulfilled in cells one would expect that both affinities and concentrations of these proteins were comparable. The IP3Rs are a family of tetrameric ligand-gated $Ca^{2+}$ channels and the complete cDNA sequences encoding three distinct receptors (IP3R1, IP3R2 and IP3R3) have been determined. The affinities of three types of IP3R for the ligand are reported to be in the nanomolar range, with $K_d$ values of 1.5 nM (IP3R1), 2.5 nM (IP3R2) and 22 nM (IP3R3) [40]. These values are similar to those measured for p130 (2–5 nM) [7,16,35]. Therefore, on the basis of affinity, p130 could effectively compete for the Ins(1,4,5)$P_3$ ligand. Regarding their expression, both p130 and IP3Rs are expressed in a range of tissues, with high concentrations present in brain [41,42]. As described in the Results section, the concentrations of endogenous p130 measured in PC12 are approx. one-fifth of those in COS-$1^{p130}$ cells but are higher than the concentrations of protein that could inhibit $Ca^{2+}$ release in permeabilized cells triggered by physiological amounts of Ins(1,4,5)$P_3$ (Figure 3). These results therefore do not rule out the suggested function of p130. However, to obtain direct proof, further studies are clearly required.

The results described here can be summarized as follows. (1) p130 expressed in cells was mainly localized in the cytoplasm, although the PH domain of p130 is capable of binding PtdIns(4,5)$P_2$. (2) p130 did not inhibit the formation of Ins(1,4,5)$P_3$ from PtdIns(4,5)$P_2$ by PLC activation. (3) Most importantly, p130 attenuated the increase in free $Ca^{2+}$ concentration, possibly through binding to Ins(1,4,5)$P_3$ via the PH domain. p130 is therefore the first example of a PH-domain-containing molecule that might have a physiological role in Ins(1,4,5)$P_3$ binding and inhibition of the Ins(1,4,5)$P_3$-mediated signalling, suggesting that the amount of p130 present in cells could be one of the factors that determine the effectiveness of $Ca^{2+}$ signalling. Although these experiments argue that p130 could bind Ins(1,4,5)$P_3$ in cells, other experiments (currently in progress) focusing on endogenous p130 and the use of knock-out cells are needed to further our insights into the physiological role of this molecule.

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