Localization of a high-affinity inositol 1,4,5-trisphosphate/inositol 1,4,5,6-tetrakisphosphate binding domain to the pleckstrin homology module of a new 130 kDa protein: characterization of the determinants of structural specificity

Hiroshi TAKEUCHI*†**, Takashi KANEMATSU*,**, Yoshio MISUMI‡, Hassan Bin YAAKOB*††, Hitoshi YAGISAWA§, Yukio IKEHARA‡, Yutaka WATANABE‖, Zheng TAN¶, Stephen B. SHEARS¶ and Masato HIRATA*‡‡

Departments of *Biochemistry and †Maxillofacial Surgery, Faculty of Dentistry, Kyushu University, Fukuoka 812-82, Japan, ‡Department of Biochemistry, Faculty of Medicine, Fukuoka University, Fukuoka 814-80, Japan, §Department of Life Science, Faculty of Science, Himeji Institute of Technology, Hyogo 678-12, Japan, ‖Department of Applied Chemistry, Faculty of Engineering, Ehime University, Matsuyama 790, Japan, and ¶Inositol Lipid Section, Laboratory of Cellular and Molecular Pharmacology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, U.S.A.

We have previously identified a novel 130 kDa protein (p130) which binds Ins(1,4,5)P_3 and shares 38 % sequence identity with phospholipase C-δ_1 [Kanematsu, Misumi, Watanabe, Ozaki, Koga, Iwanaga, Ikehara and Hirata (1996) Biochem. J. **313**, 319–325]. We have now transfected COS-1 cells with genes encoding the entire length of the molecule or one of several truncated mutants, in order to locate the region for binding of Ins(1,4,5)P_3. Deletion of N-terminal residues 116–232, the region which corresponds to the pleckstrin homology (PH) domain of the molecule, completely abolished binding activity. This result was confirmed when the PH domain itself (residues 95–232), isolated from a bacterial expression system, was found to bind [^{3}H]Ins(1,4,5)P_3. We also found that Ins(1,4,5,6)P_4 was as efficacious as Ins(1,4,5)P_3 in displacing [^{3}H]Ins(1,4,5)P_3, suggesting that these two polyphosphates bind to p130 with similar affinity. This conclusion was confirmed by direct binding studies using [^{3}H]Ins(1,4,5,6)P_4 with high specific radioactivity which we prepared ourselves. Binding specificity was also examined with a variety of inositol phosphate derivatives. As is the case with other PH domains characterized to date, we found that the 4,5-*vicinal* phosphate pair was an essential determinant of ligand specificity. However, the PH domain of p130 exhibited some novel features. For example, the 3- and/or 6-phosphates could also contribute to overall binding; this contrasts with some other PH domains where these phosphate groups decrease ligand affinity by imposing a steric constraint. Secondly, a free monoester 1-phosphate substantially increased binding affinity, which is a situation so far unique to the PH domain of p130.

INTRODUCTION

It is now well recognized that many of the proteins that participate in cell signalling contain modular domains that help to regulate interactions between components of signal transduction cascades. One such module is the pleckstrin homology (PH) domain, a region of approximately 120 amino acids that can form an electrostatically polarized tertiary structure [1,2]. Some PH domains have been shown to bind to PtdIns(4,5)P_2 and/or Ins(1,4,5)P_3 [3–6], which themselves are well known to separately participate in intracellular signalling pathways [7,8]. Therefore current efforts to determine the significance of the ligand binding characteristics of PH domains lie at the heart of the search for their overall physiological significance.

Among the growing number of recognized PH domains, that which is present in phospholipase C-δ_1 (PLC-δ_1) is so far unique in the detail with which its ligand specificity and functional significance have been analysed. This particular PH domain binds Ins(1,4,5)P_3 with about 10-fold greater affinity than PtdIns(4,5)P_2 [5]. A consequence of PLC-δ_1 binding to Ins(1,4,5)P_3 is the inhibition of the enzyme's lipid-hydrolysing activity [9,10], so it is possible that competition between these two ligands *in vivo* may contribute to the overall regulation of PLC-δ_1 activity.

However, it has been pointed out (e.g. [6]) that the information obtained from PLC-δ_1 is likely not to be generally applicable; despite the tertiary structure of PH domains being well conserved between individual proteins, there is nevertheless considerable variation in their primary amino acid sequences. This undoubtedly contributes to the variation that has been observed in the relative ligand affinities of PH domains, and an accompanying diversity in the functional significance of this module is therefore anticipated. For example, the PH domain of pleckstrin has a somewhat specific affinity for PtdIns(4,5)P_2, and the soluble Ins(1,4,5)P_3 head-group is only an effective competitor when it is present in large excess [3,5]. In the case of the PH domain of spectrin, the affinities for both PtdIns(4,5)P_2 and Ins(1,4,5)P_3 are sufficiently weak (K_d 40 μM) that only the binding of the lipid is likely to be physiologically relevant [4]. Moreover, it appears that the PH domain of human dynamin does not bind either PtdIns(4,5)P_2 or Ins(1,4,5)P_3 [5,11].

There is clearly a need to examine other PH domains before we can develop any general rules concerning the relationship between primary sequence, tertiary structure and ligand binding proper-

Abbreviations used: PH domain, pleckstrin homology domain; Gro*P*Ins(4,5)P_2, glycerophospho-D-*myo*-inositol 4,5-bisphosphate; PLC-δ_1, phospholipase C-δ_1; PVDF, poly(vinylidene difluoride).

** These authors contributed equally to this work.

†† Present address: Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia.

‡‡ To whom all correspondence should be addressed.

ties. To achieve this aim, we have examined whether a recently cloned Ins(1,4,5)P_3 binding protein of 130 kDa (p130) [12] might be a useful tool because of its candidate PH domain. This protein shares 38% sequence identity with PLC-δ_1 (in regions corresponding to the so-called X- and Y-boxes, as well as the PH domain) [12,13]. This similarity is intriguing, although we do not yet know the function of p130, neither the native nor the expressed protein has any apparent catalytic activity towards inositol lipids. The reason for p130 not having PLC activity may result from a single amino acid deviation in each of two separate sequences of amino acids [12] that otherwise match well-conserved X-box subdomains that are a feature of all PLC enzymes [14]. However, this aspect of p130 also presented us with a complication. While these X-box motifs in PLC enzymes may determine catalytic activity, there is also the possibility of a non-catalytic PtdIns(4,5)P_2 binding function [15]. Perhaps in the case of p130 these particular domains have retained an ability to bind the polyphosphate moiety. This is difficult to predict from the primary sequence, because there is currently little information on inositol polyphosphate binding motifs. Thus our first goal was to determine the extent to which the PH domain of p130 is responsible for the protein's affinity for binding inositol polyphosphates. Fortunately, previous work with the purified protein [9] has established that p130 has only a single class of Ins(1,4,5)P_3 binding sites.

There was another important reason for characterizing the regions of p130 that are responsible for binding inositol polyphosphates. This arises from the observation that the addition to Ins(1,4,5)P_3 of either a 3-phosphate or a 6-phosphate sterically interferes with ligand binding to PLC-δ_1, reducing ligand affinity 10-fold or more [10,11,16]. The situation with regard to p130 is somewhat different; although Ins(1,3,4,5)P_4 is not quite as good a ligand as Ins(1,4,5)P_3, the difference is only about 3-fold [9]. Moreover, Ins(1,4,5,6)P_4 was as efficacious as Ins(1,4,5)P_3 itself at displacing [^{3}H]Ins(1,4,5)P_3 from full-length p130 [12,16]. Again, the reason that the ligand binding characteristics of p130 are so exceptional may reflect the participation of regions that lie outside the PH domain. In particular, it would be a finding of particular relevance to efforts to determine the three-dimensional structure of PH domains if we could establish a precedent for at least one of these modules directly binding Ins(1,4,5,6)P_4 and Ins(1,4,5)P_3 with equal affinity.

The approach we have taken has been to express and analyse the polyphosphate binding characteristics of several deletion mutants of p130 in COS-1 cells, and ultimately of the PH domain itself. As a result of these studies, we not only determined that it is indeed the PH domain of p130 that binds Ins(1,4,5)P_3 and Ins(1,4,5,6)P_4, but we went on to derive some new information on other structural determinants of ligand specificity, by performing ligand displacement studies with a range of inositol derivatives.

MATERIALS AND METHODS

Materials

[α-^{32}P]dCTP, [γ-^{32}P]ATP (both specific radioactivity $\sim$ 110 TBq/mmol) and deoxyadenosine 5′-[α[^{35}S]thio]triphosphate (specific radioactivity $>$ 37 TBq/mmol) were obtained from Amersham. [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 (both specific radioactivity 777 GBq/mmol) were from du Pont–New England Nuclear (Boston, MA, U.S.A.). cDNA synthesis and sequencing kits were obtained from Stratagene (La Jolla, CA, U.S.A.) and United States Biochemicals (Cleveland, OH, U.S.A.). DNA labelling kits and a site-directed mutagenesis kit were from TaKaRa (Kyoto, Japan). Various DNA-modifying enzymes and restriction endonucleases were purchased from Nippon Gene (Toyama, Japan) and TaKaRa. pT7BlueT-vector was from Novagen (Madison, WI, U.S.A.). Lipofectamine® reagent was from Gibco–BRL (Gaithersburg, MD, U.S.A.). Biotinylated goat anti-(mouse IgG) antibodies were from Amersham. An alkaline phosphatase staining kit was obtained from Pierce (Rockford, IL, U.S.A.) or Vector Laboratories Inc. (Burlingame, CA, U.S.A.). A prestained molecular mass marker set for SDS/PAGE and poly(vinylidene difluoride) (PVDF) sheets for Western blotting were obtained from Bio-Rad (Hercules, CA, U.S.A.). (Nitrilotriacetic acid)–agarose resin was obtained from Quiagen (Chatsworth, CA, U.S.A.). 3-Deoxy-Ins(1,4,5)P_3, 2,3-dideoxy-Ins(1,4,5)P_3, Ins(4,5)P_2, Ins(1,4)P_2, Ins(1,5,6)P_3 and glycerophospho-D-*myo*-inositol 4,5-bisphosphate [Gro*P*Ins(4,5)P_2] were purchased from Calbiochem (La Jolla, CA, U.S.A.), and L-Ins(1,4,5)P_3 was from Sigma (St. Louis, MO, U.S.A.); these were used without further purification. Other inositol phosphate positional isomers and Ins(1,4,5)P_3 analogues used in the present study were synthesized as described [17–21], except for [^{3}H]Ins(1,4,5,6)P_4. This was prepared by first phosphorylating [^{3}H]Ins(1,3,4,5)P_4 to [^{3}H]Ins(1,3,4,5,6)P_5 with a partially purified 6-kinase prepared from turkey erythrocytes [22], and then the [^{3}H]Ins(1,3,4,5,6)P_5 was dephosphorylated to [^{3}H]Ins(1,4,5,6)P_4 using a purified multiple inositol polyphosphate phosphatase [23].

Construction of expression plasmids and truncation derivatives

A single *Xho*I site in the coding region of pcMT3 was destroyed by a silent mutation (at Arg^{24}; CGA^{538} to CGT^{538}) directed with the synthetic oligonucleotide 5′-CGACCCTCGTGCCGGCG-CCGAT-3′. To generate a new *Xho*I site (C^{390},T^{391} to T^{390},C^{391}) in the 5′-non-coding region of pcMT3, the synthetic oligonucleotide 5′-CCTTCTGGTGCTCGAGCGCACCCTTA-3′ was used as a primer. The designed cDNA was digested with *Xho*I and inserted into the *Xho*I site of the mammalian expression vector *Xho*I/pSG5, which was modified by introducing *Eco*RV–*Xho*I–*Not*I (GATATCTCGAGAGCGGCCGC) sites between the *Eco*RI and *Bgl*II sites of pSG5 (Stratagene). The orientation was confirmed by restriction endonuclease mapping and the resultant plasmid was designated construct I (full).

Truncated mutants were constructed by site-directed mutagenesis as follows. Two subclones were prepared for constructs II and III. One was an *Afl*II–*Sma*I/construct I vector, in which an *Afl*II site was introduced at position 400 of construct I, and was treated with bacterial alkaline phosphatase after being digested with *Afl*II and *Sma*I. The other was pcMT3 (1–1356)/Δ*Xho*I^{534}, *Xho*I^{389}, *Afl*II^{400}, which was subcloned into a pUC118 vector using an *Sma*I site after an *Afl*II site was introduced at position 400. To form construct II, an *Nco*I site was introduced at position 810 and an *Nco*I site at position 1048 was destroyed on pcMT3 (1–1356)/Δ*Xho*I^{534}, *Xho*I^{389}, *Afl*II^{400}. This cDNA clone was digested with *Nco*I and self-ligated. Then the self-ligated cDNA was digested with *Afl*II and *Sma*I, and the desired DNA fragment was inserted into the pre-paired *Afl*II–*Sma*I/construct I vector. The resultant plasmid was designated construct II (Δ1–115). To make construct III, an *Nco*I site was introduced at position 1161 into pcMT3 (1–1356)/Δ*Xho*I^{534}, *Xho*I^{389}, *Afl*II^{400}, and this clone was digested with *Nco*I to self-ligate. The *Nco*I-self-ligated cDNA was digested with *Afl*II and *Sma*I, and the desired DNA fragment was inserted into the pre-paired *Afl*II–*Sma*I/construct I vector. The resultant plasmid was designated construct III (Δ1–232). For construct IV (Δ727–1096) and construct V (Δ851–1096), stop codons were introduced into construct I at positions 2645 and 3017 respectively. Construct VI

was prepared as follows. A deletion mutant clone of pcMT3 (1–1356)Δ*Xho*I[534], *Xho*I[389], *Afl*III[400] at nucleotides 470–748 was created by site-directed mutagenesis with the synthetic oligonucleotide 5′-TAGAACGTGCTTGGCCATG[469]G[749]TGTCTTTCAGCAGCATGCCA-3′. The truncated clone was digested with *Afl*II and *Sma*I, and the desired DNA fragment was inserted into the pre-paired *Afl*II–*Sma*I construct I vector. The structure of the resultant plasmid was confirmed by restriction mapping analysis, and the molecular mass of protein product was checked by Western blotting using a monoclonal antibody against p130. Each plasmid (20 μg) was transfected into 5×10^6 COS-1 cells using liposomes (Lipofectamine reagent) according to the manufacturer's protocol. The transfected cells were cultured in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal calf serum in 10 cm dishes for 2 days.

Preparation of recombinant PH domain of p130

A cDNA fragment encoding the PH domain of p130 was generated by PCR on clone pcMT3 using the synthetic oligonucleotides 5′-ATGGATCCG[749]TGTCTTTCAGCAGCATGCC[769]-3′ and 5′-ATAAGCTTA[1162]AAGTCAAGTGGTTGCTTACTG[1141]-3′ as primers. The resultant DNA fragment was ligated into pT7 vector and the nucleotide sequence was confirmed. The clone was digested with *Bam*HI and *Hin*dIII, and the desired DNA fragment was ligated to *Bam*HI–*Hin*dIII-digested pQE30 His-tag vector (Quiagen). A construct expressing residues 95–232 of p130 was produced and purified as follows. After induction with isopropyl β-D-thiogalactoside (5 h), cells were lysed by sonication in 0.3 M NaCl and 50 mM phosphate buffer (pH 8.0). Triton X-100 was added to the homogenate to give a final concentration of 1 % (w/w), followed by incubation at 4 °C for 30 min. The mixture was centrifuged at 15000 ***g*** for 20 min and the resultant supernatant was mixed with a 50 % slurry of nitrilotriacetic acid resin at 4 °C for 1 h. Procedures for washing the resin and elution of adsorbed proteins were as described by the manufacturer. The isolated PH domain of p130 thus prepared was at least 95 % pure on an overloaded Coomassie Blue-stained SDS gel. The protein concentration was determined from an A_{280} value at 1 mg/ml of 0.88 ($\epsilon = 14000\ M^{-1} \cdot cm^{-1}$.

Preparation of cytosol fraction from transfected cells, and assay of [^{3}H]Ins(1,4,5)P_3 binding

Transfected COS-1 cells from a dish were harvested in a buffer solution (250 μl) containing 50 mM NaCl, 10 mM Hepes buffer (pH 8.0), 1 mM EDTA, 1 mM NaN_3, 10 mM 2-mercaptoethanol and a cocktail of protease inhibitors (1.25 μg/ml pepstatin A, 10 μM leupeptin, 1 μg/ml aprotinin, 0.1 mM *p*-amidinophenylmethanesulphonyl fluoride) and lysed by passing cells through a 26G needle 15 times, followed by a centrifugation at 100000 ***g*** for 60 min to obtain the cytosol fraction. The cytosol fraction was assayed for binding of [^{3}H]Ins(1,4,5)P_3 using a concentration of the latter of 1.3 nM, as described previously [9].

PAGE, immunoblotting and quantification

The cytosolic fractions from each construct were separated in SDS/8 %-polyacrylamide gels and transferred to a PVDF sheet. The sheet was incubated for 1 h with monoclonal antibody (2F9) against p130 [16] after blocking with 5 % (w/v) dried milk solution, and then bound antibodies were visualized by the alkaline phosphatase reaction using biotinylated goat anti-(mouse IgG) and an avidin–biotin complex kit. The quantification of expressed protein on the developed sheet was done with an image analyser (IS-1000; Alpha Innotech Corp.). For quantification, one sample of cytosol of construct I, together with the samples to be examined, was subjected to electrophoresis and immunoblotting as a reference.

RESULTS

Localization of the Ins(1,4,5)P_3/Ins(1,4,5,6)P_4 binding site of p130

Our first goal was to locate the region in the p130 molecule that is responsible for binding of Ins(1,4,5)P_3, so we prepared several groups of COS-1 cells, each of which was transfected with cDNA encoding either full-length p130 (construct I) or one of its five different truncated versions (constructs II–VI). Figure 1(A) is a schematic representation of the various constructs. Figures 1(B) and 1(C) show Western blots of the constructs, which were obtained using two monoclonal antibodies against p130 (designated 4C1 and 2F9). In each case, the apparent molecular mass of the band recognized by the antibody coincided with the expected molecular sizes: 130 kDa (construct I), 105 kDa (construct II), 93 kDa (construct III), 80 kDa (construct IV), 93 kDa (construct V) and 108 kDa (construct VI). Note that the monoclonal antibody designated 4C1 failed to interact with constructs IV and V, indicating that it recognizes a region within residues 851–1096. Antibody 2F9, which interacted with all of the

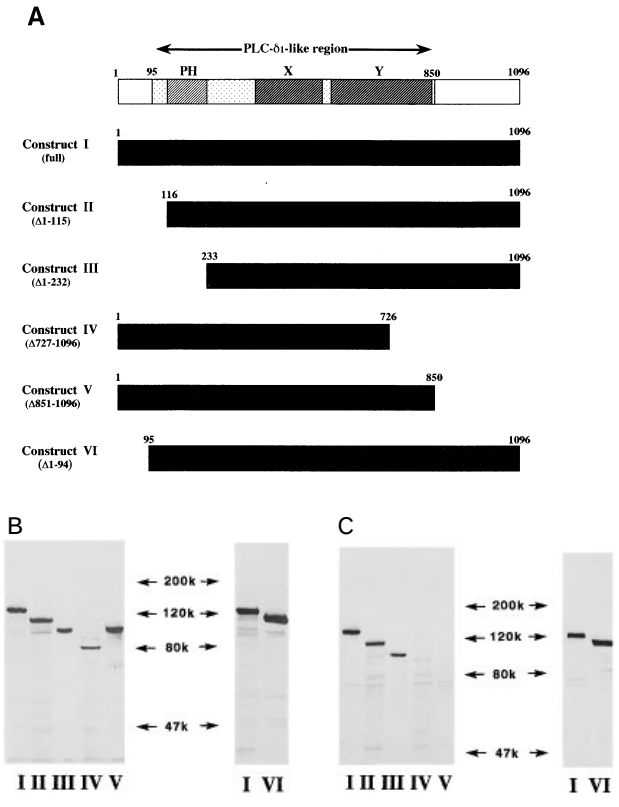

Figure 1 Schematic representation of constructs for full-length and deleted mutants of p130, and Western blotting analyses

(**A**) Schematic representation of p130 and its deleted mutants. X and Y indicate X- and Y-boxes respectively. (**B**) and (**C**) The cytosol fractions from COS-1 cells transfected with each construct were analysed by SDS/PAGE, followed by the electrical transfer to a PVDF membrane. The membrane was probed with monoclonal antibody 2F9 (**B**) or 4C1 (**C**) against p130 [16]. The amounts of cytosol applied to each lane were adjusted to provide similar densities. Positions of molecular mass markers in kDa (k) are indicated.

Table 1 Expression and [^{3}H]Ins(1,4,5)P_3 binding activity of the p130 constructs

Transfected cells grown in a dish of 10 cm diameter were homogenized and fractionated to obtain the cytosol fractions as described in the Materials and methods section. The cytosol fractions were analysed by SDS/PAGE, followed by Western blotting analysis by probing with antibody 2F9. The degree of expression of constructs II–VI was quantified relative to the value for one of seven samples of construct I. The standard sample was always subjected to electrophoresis together with the samples of interest. For measurement of [^{3}H]Ins(1,4,5)P_3 binding, 10 μl of each cytosol fraction was assayed for binding of 1.3 nM [^{3}H]Ins(1,4,5)P_3 as described [9,16]. Non-specific binding was measured in the presence of 1 μM Ins(1,4,5)P_3 and was subtracted from the binding data to give specific binding activity. For normalization (third column), the specific binding activities obtained in the second column were adjusted using the values in the first column to give pmol per unit amount of expressed protein. Values represents means $\pm$ S.E.M. ($n = 4$–7).

		[^{3}H]Ins(1,4,5)P_3 binding	
Construct	Relative expression (arbitrary units)	(pmol/mg of protein)	(pmol/unit amount of expressed protein)
I	0.81 $\pm$ 0.15	3.4 $\pm$ 0.8	4.2 $\pm$ 0.8
II	0.20 $\pm$ 0.07	0.10 $\pm$ 0.07	0.50 $\pm$ 0.03
III	11.5 $\pm$ 1.1	0	0
IV	0.25 $\pm$ 0.06	0.35 $\pm$ 0.13	1.4 $\pm$ 0.4
V	5.6 $\pm$ 1.7	7.9 $\pm$ 1.6	1.4 $\pm$ 0.3
VI	2.2 $\pm$ 0.6	3.5 $\pm$ 0.7	1.6 $\pm$ 0.3

constructs, must therefore recognize a region within residues 233–726. These epitopes had not previously been identified [16].

Cytosol was prepared from each group of COS-1 cells and assayed for its ability to bind [^{3}H]Ins(1,4,5)P_3. Control, non-transfected, cytosol did not show any [^{3}H]Ins(1,4,5)P_3 binding activity (results not shown; see also [12]). In these experiments there was no significant difference in the total protein concentration of the cytosol fractions obtained from each plate of confluent cells. However, a densitometric survey of Western-blotted sheets (Table 1, column 1) revealed some variation in the degree of expression of each construct. Therefore we normalized the binding activities of each construct to the amount of expressed protein (Table 1, column 3). Construct I (the full-length protein) exhibited the largest capacity for [^{3}H]Ins(1,4,5)P_3 binding (Table 1). Deletion of portions of the C-terminus, as in construct IV (Δ727–1096) and construct V (Δ851–1096), reduced binding capacity by approx. 65%. Removal of a portion of the N-terminus that included the PH domain (construct III; Δ1–232) completely eliminated [^{3}H]Ins(1,4,5)P_3 binding activity. Note that binding to construct III was not observed even though it was expressed at a 14-fold higher concentration than construct I. Even when the concentration of [^{3}H]Ins(1,4,5)P_3 was increased 10-fold to 13 nM, no binding to construct III was evident (results not shown). Importantly, [^{3}H]Ins(1,4,5)P_3 did bind to construct II, which also has some of the N-terminus deleted (Δ1–115), but in this particular case the region corresponding to the PH domain was intact. Our results (Table 1) indicate that residues 116–232 of p130 are essential for the binding of Ins(1,4,5)P_3. This region corresponds to the PH domain [12]. Nevertheless, some additional factors must have contributed to the differences between the ligand binding capacities of the constructs. We know that this variation was not due to the presence of multiple binding sites; p130 has only one such site [9,16], and indeed the affinity of this site for Ins(1,4,5)P_3 was similar in each construct where binding was detected [K_d values for Ins(1,4,5)P_3: I, 3.4 $\pm$ 0.3 nM; II, 3.1 $\pm$ 0.4 nM; IV, 3.9 $\pm$ 0.3 nM; V, 2.9 $\pm$ 0.4 nM]. It is possible that inappropriate folding of a variable proportion of each of the truncated constructs contributed to these differences in binding capacity.

Ins(1,4,5)P_3 binding to the isolated PH domain of p130

The data described above demonstrate that the PH domain of p130 is essential for the protein to bind inositol polyphosphates.

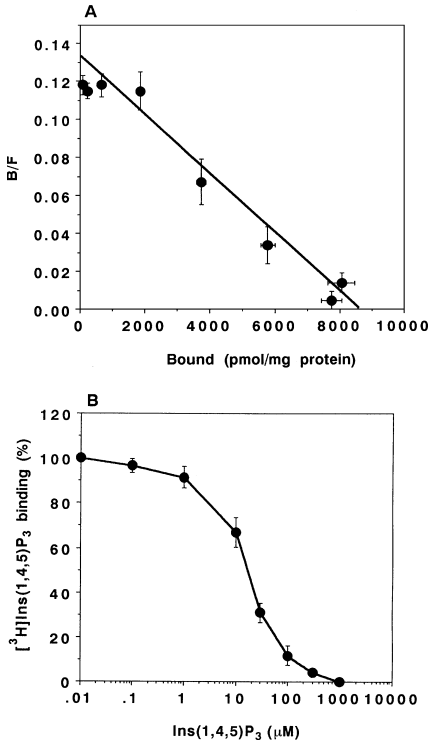

Figure 2 Binding of Ins(1,4,5)P_3 to the isolated PH domain of p130

(**A**) Scatchard analysis (B/F = bound/free); (**B**) displacement of [^{3}H]Ins(1,4,5)P_3 bound to the PH domain by Ins(1,4,5)P_3. Binding assays were performed essentially as described previously [9]. Results are means $\pm$ S.E.M. of four experiments.

The possibility was further explored by transfecting the cDNA for the PH domain itself (residues 95–232) into a bacterial expression system; the domain was then extracted and purified (see the Materials and methods section). Figure 2 shows the analysis of Ins(1,4,5)P_3 binding; Scatchard plots of three independent determinations revealed average K_d and B_{max} values of 12.2 μM and 8350 pmol/mg of protein respectively. These

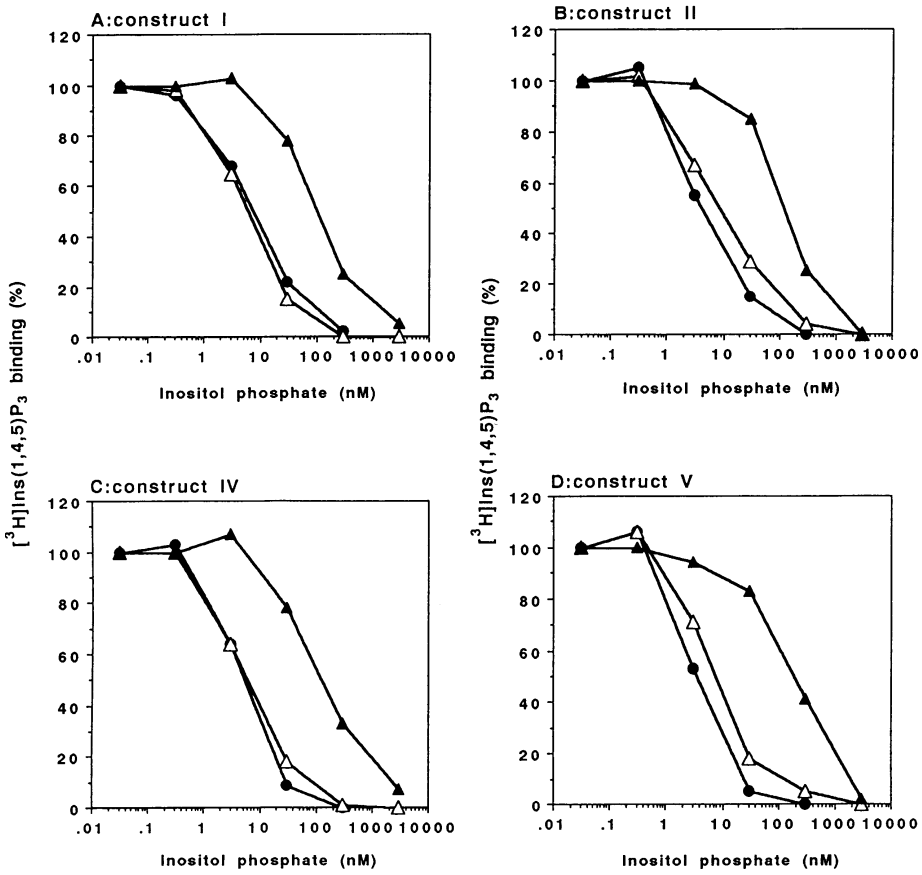

Figure 3 Displacement of [^{3}H]Ins(1,4,5)P_3 bound to each construct by Ins(1,4,5)P_3, Ins(1,4,5,6)P_4 and Ins(3,4,5,6)P_4

Each construct was assayed for the binding of [^{3}H]Ins(1,4,5)P_3 in the presence of various concentrations of Ins(1,4,5)P_3 (●), Ins(1,4,5,6)P_4 (△) or Ins(3,4,5,6)P_4 (▲). (**A**) construct I; (**B**) construct II; (**C**) construct IV; (**D**) construct V. Each point represents the mean of three determinations.

data directly demonstrate that the PH domain of p130 binds Ins(1,4,5)P_3. Nevertheless, the ligand affinity was about 3500-fold lower than that of the native protein (see Figures 3 and 4), indicating that regions outside the PH domain contribute significantly to the overall ligand binding characteristics.

Ins(1,4,5)P_3 and Ins(1,4,5,6)P_4 bind to the same site on p130

The PH domain of PLC-δ_1 binds Ins(1,4,5,6)P_4 about 10-fold more weakly than Ins(1,4,5)P_3 [10,16]. When this result is considered in relation to X-ray crystallography data [11], it appears that the the 6-phosphate of Ins(1,4,5,6)P_4 is a steric constraint on the ability of the molecule to be a ligand for the PH domain. In the case of p130, previous studies with the native protein [9,16] indicated that Ins(1,4,5,6)P_4 was as effective as Ins(1,4,5)P_3 itself at displacing [^{3}H]Ins(1,4,5)P_3. We have now confirmed and extended this result by showing that each of the p130 constructs that expressed this polyphosphate binding ability also had equal affinity for both Ins(1,4,5)P_3 and Ins(1,4,5,6)P_4 (Figure 3). Importantly, there was some stereoselectivity; Ins(3,4,5,6)P_4 was 10–30 times less effective than Ins(1,4,5,6)P_4 at displacing Ins(1,4,5)P_3 (Figure 3).

As well as studying the displacement of [^{3}H]Ins(1,4,5)P_3 from p130 by Ins(1,4,5,6)P_4, we consolidated the finding that Ins(1,4,5,6)P_4 is an important ligand by evaluating its binding characteristics directly, using [^{3}H]Ins(1,4,5,6)P_4 of high specific radioactivity that we synthesized ourselves (see the Materials and methods section). We found that the affinity and capacity towards [^{3}H]Ins(1,4,5,6)P_4 was not significantly different from those found for [^{3}H]Ins(1,4,5)P_3, both with regard to the native protein partially purified from rat brain {K_d and B_{max} values for [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,4,5,6)P_4 of 2.2 ± 0.3 nM and 1290 ± 153 pmol/mg of protein, compared with 2.1 ± 0.2 nM and 1210 ± 116 pmol/mg of protein respectively} (Figure 4), and with regard to the various constructs that we prepared (results not shown). Moreover, data showing the displacement of bound [^{3}H]Ins(1,4,5,6)P_4 by Ins(1,4,5,6)P_4, Ins(3,4,5,6)P_4 and Ins(1,4,5)P_3 confirmed that Ins(1,4,5,6)P_4 and Ins(1,4,5)P_3 bound to p130 about equally, whereas Ins(3,4,5,6)P_4 bound about 20-fold more weakly, both to native p130 (Figure 4) and to the constructs (results not shown).

Structural requirements of Ins(1,4,5)P_3 and Ins(1,4,5,6)P_4 for recognition by the PH domain of p130

The ligand specificity of p130 was studied by determining IC_{50} values for the displacement of [^{3}H]Ins(1,4,5)P_3 by a number of additional inositol phosphate derivatives (Table 2). COS-1 cytosol containing construct V was particularly useful for these studies because it afforded the highest binding activity per unit of cytosolic protein (see Table 1, column 2), while not exhibiting

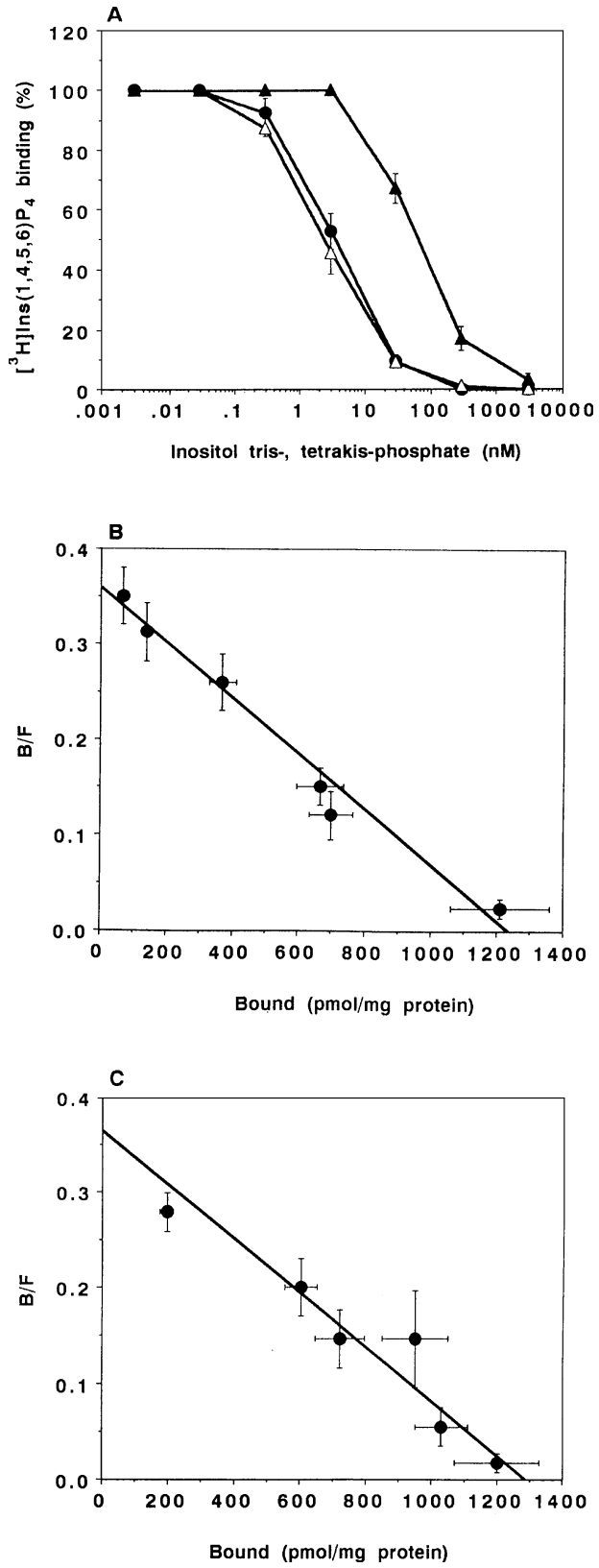

Figure 4 Direct binding of [^{3}H]Ins(1,4,5,6)P_4 to p130 isolated from rat brain

(**A**) Displacement of [^{3}H]Ins(1,4,5,6)P_4 bound to p130 partially purified from rat brain by Ins(1,4,5)P_3 (●), Ins(1,4,5,6)P_4 (△) and Ins(3,4,5,6)P_4 (▲). (**B**) Scatchard analysis of [^{3}H]Ins(1,4,5,6)P_4 binding. For comparison, Scatchard analysis of [^{3}H]Ins(1,4,5)P_3 binding to the same preparation was also performed (**C**). Values are means ± S.E.M. of four experiments.

Table 2 Binding specificity of construct V, purified p130 and the isolated PH domain

Binding specificity was examined by the displacement of bound [^{3}H]Ins(1,4,5)P_3 at 1.3 nM. Each value represents the mean of three to four determinations. Inositol phosphates used were D-isomers unless otherwise indicated. #209 is 2-*O*-(*p*-aminobenzoyl)-Ins(1,4,5)P_3 (for structure see [34,35]); #309 is 3-*O*-(*p*-aminobenzoyl)-Ins(1,4,5)P_3 (for structure see [36]). nd, not done.

Compound	IC_{50}		
	Construct V (nM)	Purified p130 (nM)	Isolated PH domain (μM)
Ins(1,4,5)P_3	4.3	4.5* (2.9†)	15.6
Ins(1,4)P_2	> 3000	nd	nd
Ins(4,5)P_2	2000	nd	nd
Gro*P*Ins(4,5)P_2	100	nd	125
DL-2-Deoxy-Ins(1,4,5)P_3	6.7	6.3	nd
3-Deoxy-Ins(1,4,5)P_3	21.6	nd	nd
2,3-Dideoxy-Ins(1,4,5)P_3	22.0	nd	nd
#209	3.9	4.3	nd
#309	20.7	nd	nd
L-Ins(1,4,5)P_3	> 3000	nd	nd
DL-Ins(2,4,5)P_3	92	42	137
Ins(1,4,6)P_3	1000	nd	200
Ins(1,3,6)P_3	600	nd	200
Ins(1,3,4)P_3	> 3000	> 1000	nd
Ins(1,5,6)P_3	2000	nd	nd
DL-Ins(1,2,4,5)P_4	7.3	nd	22.3
Ins(1,3,4,5)P_4	11.3	21 (9.7†)	nd
Ins(1,4,5,6)P_4	4.1	nd (3.1†)	16.7
Ins(3,4,5,6)P_4	110	nd (84†)	nd

* Cytosolic p130 taken from [9].
† Values in parentheses for membranous p130 taken from [16].

any significant differences in specificity compared with the native protein (Table 2). The isolated PH domain also expressed a similar rank order of affinities for the various ligands but, as discussed above, the absolute ligand affinities were much reduced.

We first examined the contributions made by the phosphate groups, beginning with the 5-phosphate. The importance of this group is revealed by two results. First, at concentrations of up to 3 μM, Ins(1,4)P_2 was unable to displace 1.3 nM [^{3}H]Ins(1,4,5)P_3 (Table 2). Secondly, Ins(1,4,6)P_3 was a 250-fold weaker ligand than Ins(1,4,5,6)P_4. The 4-phosphate is also an important determinant of specificity, as Ins(1,5,6)P_3 was nearly 500-fold more weakly bound than Ins(1,4,5,6)P_4. Ins(4,5)P_2 was about 500-fold less effective a ligand than Ins(1,4,5)P_3, indicating that the 1-phosphate also makes an important contribution. The fact that the affinity for DL-Ins(2,4,5)P_3 was intermediate between those of Ins(4,5)P_2 and Ins(1,4,5)P_3 indicates that a phosphate at the 2-position can only partly substitute for the 1-phosphate; the intermediate affinity of DL-Ins(2,4,5)P_3 has also been observed with the native protein (Table 2 in [9]). Gro*P*Ins(4,5)P_2 was about 25-fold less potent than Ins(1,4,5)P_3, indicating either that a fully ionized 1-phosphate is important and/or that the glycerol moiety provides some steric hindrance. This result is unprecedented; for all the other PH domains examined to date, a free monoester 1-phosphate is not critical, so they bind Ins(1,4,5)P_3 with virtually the same affinity as Gro*P*InsP_2 [2,4,5,24,25]. The fact that both Ins(1,4,5,6)P_4 and Ins(1,4,5)P_3 bound with equal affinity could be because neither the 6-phosphate nor the 6-hydroxy group has much impact on specificity. Alternatively, it is possible that these two groups each interact with different amino acids, perhaps by hydrogen bonding, so that the absence of one interaction is quantitatively compensated for by the

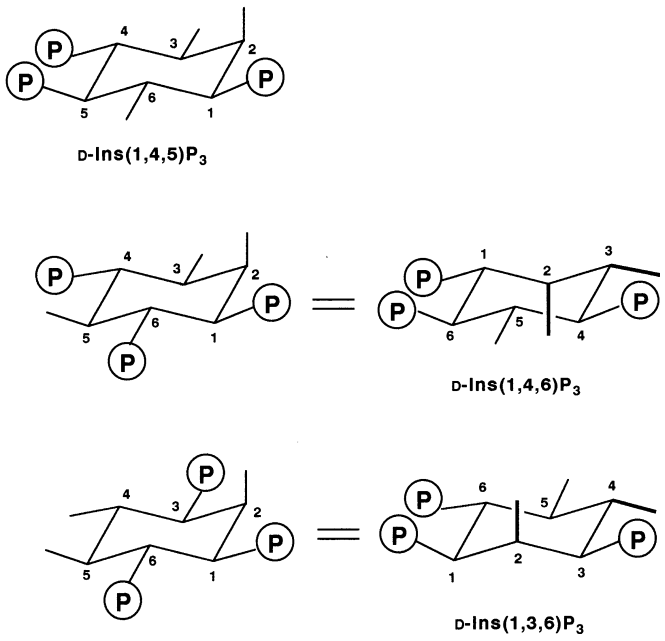

Figure 5 Structures of Ins(1,4,6)P_3 and Ins(1,3,6)P_3

The differences in the orientations of hydroxy groups of Ins(1,4,6)P_3 and Ins(1,3,6)P_3 compared with those of Ins(1,4,5)P_3 are shown by bold lines.

presence of the other. Further data indicate that this idea is feasible. For example, it appears that the 3-hydroxy group contributes to ligand specificity, presumably with a hydrogen-bonding interaction, since its substitution with a 3-deoxy group reduced affinity 5-fold (Table 2). This proposed contribution of the 3-hydroxy group is also supported by the 5-fold reduction in affinity brought about when it was substituted by a bulky aminobenzoyl group (as in analogue #309; Table 2). However, when the 3-OH was replaced by another relatively bulky substituent, a phosphate group, there was only a 2.5-fold reduction in affinity (Table 2). This result suggests that the 3-phosphate has its own interactions with p130 that can partly compensate for the loss of the 3-OH. Arguably the strongest evidence that the 3- and/or 6-phosphates do interact with amino acids in p130 comes from our new observation that the affinity for Ins(3,4,5,6)P_4 was 20-fold higher than the affinity for Ins(4,5)P_2 (Table 2).

The contribution of the 2-hydroxy group was also studied. An analogue (#209) with a bulky aminobenzoyl substituent at the 2-position was similar in potency to Ins(1,4,5)P_3 (Table 2). We also found that 2-deoxy-DL-Ins(1,4,5)P_3 and DL-Ins(1,2,4,5)P_4 both had less than a 2-fold lower affinity than Ins(1,4,5)P_3 (Table 2). There was unlikely to be a contribution from the L-enantiomers in these two racemic mixtures [since L-Ins(1,4,5)P_3 was not a significant ligand; Table 2]. Therefore it seems that the 2-hydroxy group makes no contribution to ligand specificity. The latter conclusion is also supported by the observation that 3-deoxy-Ins(1,4,5)P_3 had a similar affinity to 2,3-dideoxy-Ins(1,4,5)P_3 (Table 2). The poor affinity for both Ins(1,4,6)P_3 and Ins(1,3,6)P_3 (Table 2) was informative, in part because when these structures are inverted and rotated, they both satisfy the requirement for correctly oriented 1-, 4- and 5-phosphates of Ins(1,4,5)P_3 (Figure 5). However, in each case the orientation of the hydroxy groups fails to match that of Ins(1,4,5)P_3. Thus, although the hydroxy groups of Ins(1,4,5)P_3 do not greatly contribute to specificity *per se*, if their orientation is switched between the axial and equatorial configurations then a steric constraint is probably introduced that substantially reduces ligand affinity.

DISCUSSION

A consensus is emerging that the binding of PtdIns(4,5)P_2 to some PH domains can target a parent protein to the plasma membrane; more recently, some studies with PLC-δ_1 have raised the issue as to whether there might be PH domains where interactions with inositol phosphates could also be physiologically important [2,26]. To investigate this issue, we have used a novel 130 kDa protein as a model system. This protein was originally isolated by Ins(1,4,5)P_3 affinity chromatography [9,16,27]. We do not yet know the function of this protein and, although it shares approx. 38% sequence identity with PLC-δ_1, this similarity is insufficient to provide p130 with any lipid hydrolysing activity [9,12]. Although p130 does have a PH domain, there are additional regions of this protein that could theoretically serve to bind inositol polyphosphates (see the Introduction section). Therefore it was significant that we were able to confirm that the PH domain does contain the Ins(1,4,5)P_3 binding site. This conclusion arose from experiments using several truncated constructs of p130, which showed that loss of [^{3}H]Ins(1,4,5)P_3 binding was specific to deletion of the PH domain. Furthermore, the PH domain of p130 by itself (residues 95–232) was found to bind [^{3}H]Ins(1,4,5)P_3 (Figure 2). Thus we have validated p130 as a useful paradigm for studying the interaction of inositol derivatives with the PH module.

Our further studies on the structural determinants of ligand specificity indicated that critical contributions were made by the 4- and 5-phosphates of the inositol ring (Table 2). A similar conclusion has emerged from work with the PH domains of β-spectrin and PLC-δ_1 [4,6]. However, in two other respects the PH domain of p130 exhibited some unusual features. First, a free 1-phosphate was necessary for a strong interaction of Ins(1,4,5)P_3 with p130, since Gro*P*Ins(4,5)P_2 bound 25-fold more weakly (Table 2). All the other PH domains that have been analysed to date have not shown a significant difference in affinity between Ins(1,4,5)P_3 and Gro*P*Ins(4,5)P_2 [2,4,5,24,25]. Presumably the glycerol moiety provides a steric hindrance and/or blocks a negative charge of the 1-phosphate that is required for ligand binding to p130. Whatever the explanation, this observation strengthens the case for p130 providing the most credible example to date of an inositol phosphate being a physiologically important ligand for a PH domain. It will be interesting to discover how many other PH domains follow this particular prototype.

Our new data showing that the 3- and/or 6-phosphates can also provide sites of interaction with PH domains are also exceptional; neither has been identified as being important previously [4,6], and the effect of their presence was even suggested from X-ray crystallographic analysis to impose steric hindrance [4,6]. Indeed, our data indicate that Ins(1,4,5,6)P_4 should now be considered as potentially being a physiologically relevant ligand, not only for p130 but perhaps also for other PH domains. In relation to the search for a biological function for Ins(1,4,5,6)P_4, this information provides the most intriguing clue uncovered so far.

Of course, determination of which of a series of ligands is actually physiologically relevant depends not just on relative binding affinities, but also on relative intracellular abundance. Cellular levels of Ins(1,4,5)P_3 range from 1 μM (in 'resting' cells), to 10 μM (in receptor-activated cells) (see [8] for a review). Levels of Ins(1,4,5,6)P_4 are also about 1 μM [28]. Since these two polyphosphates have apparent binding affinities in the low

nanomolar region, they are both clearly capable of being physiologically relevant ligands. In the present study we have shown that p130 prefers a free monoester 1-phosphate (Table 2), indicating that Ins(1,4,5)P_3 and Ins(1,4,5,6)P_4 are much stronger ligands than PtdIns(4,5)P_2 However, we have not addressed the extent to which PtdIns(4,5)P_2 might compete *in vivo* because of uncertainty over the free concentration of this lipid; while total cellular levels have been estimated to be 30–160 μM [29], local concentrations are likely to be higher. On the other hand, the availability of cellular PtdIns(4,5)P_2 for binding to p130 depends upon the affinities of the lipid for a range of other cellular proteins, including some that do not contain PH domains [30–32].

The PH domain of p130 isolated from a bacterial expression system could bind [^{3}H]Ins(1,4,5)P_3, and showed a similar specificity to that of the native protein (Table 2). These data indicate that the PH domain itself forms a minimal structural module for accommodating Ins(1,4,5)P_3. However, the affinity for inositol polyphosphate binding was about 3500-fold lower than that seen with the parent protein. Therefore the tertiary structure of the isolated PH domain may not necessarily be the same as that in the whole molecule. For example, the orientations and/or bond lengths of hydrogen bonds and salt-bridges for the interaction between Ins(1,4,5)P_3 and the PH domain may be different from those formed with the whole protein. Alternatively, the isolated PH domain may not contain all the sites that are normally available for ligand interactions. This issue is relevant to the increasing use of isolated PH domains to study the properties of ligand binding [4,5]. Although the PH domain of PLC-δ_1 binds Ins(1,4,5)P_3 with similar affinity to that of the full-length protein [33], our data indicate that it may not generally be valid to extrapolate information obtained with the isolated PH domain to the native protein. The characterization of the relationship between primary sequence, tertiary structure and ligand binding characteristics, at least in the case of p130, will require a more complex analysis of the contributions of regions of the protein that lie outside the PH domain. Further studies with NMR spectrophotometric and X-ray crystallographic analyses are currently in progress in this laboratory to pursue this very issue.

We thank Dr. William Wetsel (LCMP, NIEHS) for help with densitometric analyses, and Professor K. Shirasuna (Department of Maxillofacial Surgery, Faculty of Dentistry, Kyushu University) for enabling H.T. to join this research project. This work was funded by a Grant-in-Aid for Scientific Research (International Scientific Research Program) from the Ministry of Education, Science, Sports and Culture of Japan (to M.H. and S.B.S), the Ciba–Geigy Foundation (Japan) for the Promotion of Science (to M.H.) and the Naito Foundation (to H.Y.). H.B.Y. was supported by The Hitachi Scholarship Foundation.

REFERENCES

1 Gibson, T. J., Hyvönen, M., Musacchio, A. and Saraste, M. (1994) Trends Biochem. Sci. **19**, 349–353
2 Ferguson, K. M., Lemmon, M. A., Sigler, P. B. and Schlessinger, J. (1995) Nature Struct. Biol. **2**, 715–718
3 Harlan, J. E., Hajduk, P. J., Yoon, H. S. and Fesik, S. W. (1994) Nature (London) **371**, 168–170
4 Hyvönen, M., Macias, M. J., Nilges, M., Oschkinat, H., Saraste, M and Wilmanns, M. (1995) EMBO J. **14**, 4676–4685
5 Lemmon, M. A., Ferguson, K. M., O'Brien, R., Sigler, P. B. and Schlessinger, J. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 10472–10476
6 Ferguson, K. M., Lemmon, M. A., Schlessinger, J. and Sigler, P. B. (1995) Cell **83**, 1037–1046
7 Berridge, M. J. and Irvine, R. F. (1984) Nature (London) **312**, 315–321
8 Shears, S. B. (1992) Adv. Second Messenger Phosphoprotein Res. **26**, 63–92
9 Kanematsu, T., Takeya, H., Watanabe, Y., Ozaki, S., Yoshida, M., Koga, T., Iwanaga, S. and Hirata, M. (1992) J. Biol. Chem. **267**, 6518–6525
10 Yagisawa, H., Hirata, M., Kanematsu, T., Watanabe, Y., Ozaki, S., Sakuma, K., Tanaka, H., Yabuta, N., Kamata, H., Hirata, H. and Nojima, H. (1994) J. Biol. Chem. **269**, 20179–20188
11 Ferguson, K. M., Lemmon, M. A., Schlessinger, J. and Sigler, P. B. (1994) Cell **79**, 199–209
12 Kanematsu, T., Misumi, Y., Watanabe, Y., Ozaki, S., Koga, T., Iwanaga, S., Ikehara, Y. and Hirata, M. (1996) Biochem. J. **313**, 319–325
13 Suh, P.-G., Ryu, S. H., Moon, K. H., Suh, H. W. and Rhee, S. G. (1988) Cell **54**, 161–169
14 Rhee, S. G., Suh, P.-G., Ryu, S.-H. and Lee, S. Y. (1989) Science **244**, 546–550
15 Simoes, A. P., Camps, M., Schnabel, P. and Gierschik, P. (1995) FEBS Lett. **365,** 155–158
16 Yoshida, M., Kanematsu, T., Watanabe, Y., Koga, T., Ozaki, S., Iwanaga, S. and Hirata, M. (1994) J. Biochem. (Tokyo) **115**, 973–980
17 Hirata, M., Narumoto, N., Watanabe, Y., Kanematsu, T., Koga, T. and Ozaki, S. (1994) Mol. Pharmacol. **45**, 271–276
18 Ozaki, S., Watanabe, Y., Ogasawara, T., Kondo, Y., Shintani, N., Nishii, H. and Matsuki, T. (1986) Tetrahedron Lett. **27**, 3157–3160
19 Ozaki, S. and Watanabe, Y. (1991) in Inositol Phosphates and Derivatives: Synthesis, Biochemistry, and Therapeutic Potential (Reitz, A. B., ed.), chapter 4, pp. 43–65, American Chemical Society, Washington, DC
20 Watanabe, Y., Hirofuji, H. and Ozaki, S. (1994) Tetrahedron Lett. **35**, 123–124
21 Watanabe, Y., Ogasawara, T., Ozaki, S. and Hirata, M. (1994) Carbohydr. Res. **258**, 87–92
22 Irvine, R. F., Letcher, A. J., Stephens, L. R. and Musgrave, A. (1992) Biochem. J. **281**, 261–266
23 Craxton, A., Ali, N. and Shears, S. B. (1994) J. Biol. Chem. **269**, 4337–4342
24 Harlan, J. E., Yoon, H. S., Hajduk, P. J. and Fesik, S. W. (1995) Biochemistry **34**, 9859–9864
25 Cifuentes, M. E., Delany, T. and Rebecchi, M. J. (1994) J. Biol. Chem. **269**, 1945–1948
26 Shaw, G. (1996) BioEssays **18**, 35–46
27 Hirata, M., Watanabe, Y., Ishimatsu, T., Yanaga, F., Koga, T. and Ozaki, S. (1990) Biochem. Biophys. Res. Commun. **168**, 379–386
28 Vajanaphanich, M., Schultz, C., Rudolf, M. T., Wasserman, M., Enyedi, P., Craxton, A., Shears, S. B., Tsien, R. Y., Barrett, K. E. and Traynor-Kaplan, A. (1994) Nature (London) **371**, 711–714
29 Bunce, C. M., French, P. J., Allen, P., Mountford, J. C., Moor, B., Greaves, M. F., Michell, R. H. and Brown, G. (1993) Biochem. J. **289**, 667–673
30 Zhou, M.-M., Ravichandran, K. S., Olejniczak, E. T., Petros, A. M., Meadows, R. P., Sattler, M., Harlan, J. E., Wade, W. S., Burakoff, S. J. and Fesik, S. W. (1995) Nature (London) **378**, 584–591
31 Lassing, I. and Lindberg, U. (1985) Nature (London) **314**, 472–474
32 Beck, K. A. and Keen, J. H. (1991) J. Biol. Chem. **266**, 4442–4447
33 Garcia, P., Gupta, R., Shah, S., Morris, A. J., Rudge, S. A., Scarlata, S., Petrova, V., McLaughlin, S. and Rebecchi, M. (1995) Biochemistry **34**, 16228–16234
34 Hirata, M., Watanabe, Y., Ishimatsu, T., Ikebe, T., Kimura, Y., Yamaguchi, K., Ozaki, S. and Koga, T. (1989) J. Biol. Chem. **264**, 20303–20308
35 Hirata, M., Yanaga, F., Koga, T., Ogasawara, T., Watanabe, Y. and Ozaki, S. (1990) J. Biol. Chem. **265**, 8404–8407
36 Hirata, M., Watanabe, Y., Kanematsu, T., Ozaki, S. and Koga, T. (1995) Biochim. Biophys. Acta **1244**, 404–410

Received 14 March 1996/26 April 1996; accepted 7 May 1996