BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **250,** 143–149 (1998)
ARTICLE NO. RC989179

Structural and Functional Analysis of the Putative Inositol 1,3,4,5-Tetrakisphosphate Receptors GAP1^{IP4BP} and GAP1^{m}

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Received June 8, 1998

Previously we have purified and cloned a high affinity isomerically specific inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4)-binding protein which, because it is clearly a member of the GAP1 family of Ras GTPase-activating proteins (GAP), we have termed GAP1^{IP4BP}. Here we show that expressed full-length GAP1^{IP4BP} binds Ins(1,3,4,5)P_4 with an affinity and specificity similar to that of the originally purified protein, a binding activity which is dependent on a functional PH/Btk domain. Furthermore, we highlight a fundamental distinction between GAP1^{IP4BP} and its homologue GAP1^{m}, namely that both proteins function as Ras GAPs but only GAP1^{IP4BP} displays Rap GAP activity.

Key Words: **Ras; Rap; GAPs; Ins(1,3,4,5)P_4; GAP1^{IP4BP}; GAP1^{m}.**

In agonist stimulated cells inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) is produced directly by phosphorylation of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) via an Ins(1,4,5)P_3 3-kinase [1]. The kinetics of Ins(1,3,4,5)P_4 production and degradation provided the initial evidence that this ubiquitious inositol phosphate may function as a second-messenger. The exact second-messenger role of Ins(1,3,4,5)P_4 remains a matter of some controversy, both as to whether it has a role at all, and if so, what that role might be (see [2] for a review of this literature). In recent years we have taken the view that if Ins(1,3,4,5)P_4 does indeed constitute a novel second messenger then one criteria that must be fulfilled is the presence within cells of a protein(s) which specifically binds Ins(1,3,4,5)P_4 *i.e.* an Ins(1,3,4,5)P_4 receptor [3]. To this end we have described the purification [4,5] and cloning [6] of a highly isomerically specific Ins(1,3,4,5)P_4-binding protein, which is a GAP (a GTPase-activating protein), specifically a member of the GAP1 family, and this has led us to call it GAP1^{IP4BP}.

The GAP1 family can be broadly divided into two sub-families: those identical to human blood GAP1^{IP4BP}, namely bovine brain R-Ras GAP [7] and rat brain GAP-III [8], and homologues that are approximately 60% identical which include rat brain GAP1^{m} [9] and human brain GAP1^{m} [10] (see [2] for a more detailed discussion of this family). Recently we [11] and Kobayashi *et al* [12] have cloned the human peripheral GAP1^{m} from blood and ectocervical endothelial cells respectively. Both GAP1^{IP4BP} and GAP1^{m} appear to be ubiquitiously expressed within tissues, and generally cell lines contain both proteins (Reynolds *et al.*, unpublished). However, we have recently established that they have fundamentally distinct subcellular localisations, in that GAP1^{IP4BP} resides entirely at the plasma membrane [13,11], whereas GAP1^{m} has a perinuclear localisation with possibly a small cytosolic component [11].

Structurally both GAP1^{IP4BP} and GAP1^{m} possess a central Ras GAP domain that contains a highly conserved Ras-binding motif [14]; this is flanked by N-terminal C2A and C2B domains which by analogy with synaptotagmins may constitute Ca^{2+}-dependent and Ca^{2+}-independent phospholipid binding domains [15], and a C-terminal pleckstrin homology domain (PH) which is coupled to a Bruton's tyrosine kinase (Btk) motif [16]. *In vitro* both GAP1^{IP4BP} and GAP1^{m} function as GAPs on N-, H-, R- and K-Ras [6-10, 17,18], but interestingly when these assays are performed in the presence of membrane phospholipids, the GAP activity is completely dependent on the presence of Ins(1,3,4,5)P_4 [6,18]. These data suggest therefore, that *in vivo* GAP1^{IP4BP} and GAP1^{m} may function as Ins(1,3,4,5)P_4-dependent Ras GAPs.

Two issues regarding GAP1^{IP4BP} are unclear, and it is these we have addressed here. Firstly, GAP1^{IP4BP} purified from pig platelets, but not that isolated from bovine brain [7], will function not only as a Ras GAP but also

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0006-291X/98 $25.00

as a Rap GAP, although this activity would appear not to be regulated by Ins(1,3,4,5)P_4 [6]. Obviously it is not inconceivable that this Rap GAP activity may be due to a contaminating highly active Rap GAP present in the GAP1^{IP4BP} purified from pig platelets. Secondly, we have previously suggested [6] following the work of Fukuda *et al* [19], who studied Ins(1,3,4,5)P_4 and IP_6 binding to the C2B domain of synaptotagmin II, that Ins(1,3,4,5)P_4 would bind to the similar C2B domain of GAP1^{IP4BP}. However it has recently been reported that the Ins(1,3,4,5)P_4-binding to GAP1^m is not a function of its C2B domain but is in fact via the PH/Btk domain [18].

In this present study we have prokaryotically expressed the cDNA encoding for GAP1^{IP4BP} and analysed the resultant protein with respect to Ins(1,3,4,5)P_4-binding and GAP activity. We show that recombinant GAP1^{IP4BP} functions not only as an isomercially specific Ins(1,3,4,5)P_4-binding protein in which the PH/Btk domain constitutes the Ins(1,3,4,5)P_4-binding site, but also has the intrinsic ability to function as both a Ras and Rap GAP. Through the direct comparsion of this GAP activity with that of recombinant GAP1^m we have also highlighted, for the first time, a fundamental distinction in the GAP specificity of these two putative Ins(1,3,4,5)P_4 receptors.

MATERIALS AND METHODS

Cloning GAP1^{IP4BP} and GAP1^m into pGEX. The full length cDNA encoding human GAP1^{IP4BP} was cloned into the prokaryotic expression vector pGEX4T-2 using the polymerase chain reaction (PCR) and the primer combination of sense $^{5'}$-CCAGGTCGACCTCGGCGCGCGCTTGGGG-$^{3'}$ and antisense $^{5'}$-ATTAGCGGCCGCGTCTGCGGGACGCGCGCCC-$^{3'}$. After 35 cycles using Expand Hi-Fidelity *Taq* (Boehringer) and the pBK-CMV GAP1^{IP4BP} plasmid as template (each cycle consisting of denaturing at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 2 minutes) the product was isolated and digested with *Sal*1 and *Not*1. The digested DNA was gel purified and ligated into the corresponding sites within pGEX4T-2 prior to tranformation into the *E.coli* strain *BL21* which had been previously transformed with a plasmid expressing the GroES/EL chaperonins. pGEX-GAP1^{IP4BP} was fully sequenced before use.

Human GAP1^m was cloned into the *EcoR*1 / *Not*1 restriction sites of pGEX4T-2 after direct cloning of the entire GAP1^m ORF from the pBK-CMV GAP1^m plasmid [11]. pGEX-GAP1^m was transformed into *BL21*'s as above.

Expression of GAP1^{IP4BP} and GAP1^m. The GST-GAP1^{IP4BP} or GST-GAP1^m fusion proteins were isolated as follows. An overnight culture was diluted 100-fold into 5 litres (10 × 2.5L conical flasks) of fresh LB containing both ampicillin and kanamycin and grown at 25°C until an OD_{600} of 0.6 (approximately 5 hours). Expression was induced by addition of 0.1 mM IPTG for 2.5 hours at 25°C prior to harvesting the cells by centrifugation. All subsequent steps were performed at 4°C. Cells were lysed by sonication (3 × 15 secs with 1 minute on ice between sonications) followed by incubation for 1 hour in 1% (v/v) Triton X-100 and 2 mg/ml lysozyme. The suspension was centrifuged (36000g for 1 hour) and the resultant supernatant was incubated overnight with 2 ml of glutathione sepharose 4B resin (Pharmacia). Prior to elution the resin was thoroughly washed sequentially with 6 × 30 mls of PBS, 0.1% (v/v) Triton X-100 followed by a similar cycle with 75 mM Tris (pH 8.0), 300 mM NaCl, 0.1% (v/v) Triton X-100. At this point the bound GST-fusion protein was either liberated by incubation with 75 mM Tris (pH 8.0), 1M NaCl, 20 mM glutathione, 0.1% (v/v) Triton X-100 or by cleavage with thrombin (10 cleavage Units overnight at room temperature in PBS).

Construction of GAP1^{IP4BP} deletion mutants. This was achieved using PCR by the following primer combinations which contain either underlined *Sal*1 (sense) or *Not*1 (antisense) restriction sites (see Fig. 4a for regions of GAP1^{IP4BP} encoded by the various constructs); GST-2 (sense $^{5'}$-CCAGGTCGACCTCGGCGCGCGCTTGGGG-$^{3'}$ [primer A]; antisense $^{5'}$-ATTAGCGGCCGCCGGACGGAAGCGCTTTGGG-$^{3'}$ [primer B]). GST-3 (sense $^{5'}$-CCAGGTCGACACACTGGGGTCGTCTGCC-$^{3'}$ [primer C]; antisense $^{5'}$-ATTAGCGGCCGCCCTCCGGCAGTCCAGTCC-$^{3'}$). GST-4 (sense primer C; antisense primer B). GST-5 (sense primer C; antisense $^{5'}$-ATTAGCGGCCGCCCACCCGTCCGCCTACCTG-$^{3'}$ [primer D].). GST-6 (sense 5′-CCAGGTCGACCCTAAAGCCAGACGACC-3′ [primer E]; antisense 5′-ATTAGCGGCCGCGTCTGCGGGACGCGCGCCC-3′ [primer F]). GST-7 (sense 5′-CCAGGTCGACCAAGAGGGGCCAAGGACGG-3′ [primer G]; antisense primer F). GST-8 (sense primer G; antisense primer D). GST-9 (sense primer E; antisense primer B). PCR cycles consisted of 1 min at 94°C, 1 min at 50°C and 2 mins at 72°C for 35 cycles with a final 5 mins at 72°C. Using the same protocol as above the PCR products were isolated, digested with *Sal*1 and *Not*1 and ligated into corresponding sites within either pGEX4T-1 or 4T-2 depending on the required reading frame. All plasmid were verified by DNA sequencing. The deletion mutants were expressed and the resultant protein was isolated in a similar fashion to that described for full length GAP1^{IP4BP}.

ΔC2GAP1^m was construct by PCR using the primer combination sense $^{5'}$-TTTGTCGACCCAAAACTGATGACCTGGG-$^{3'}$ and antisense $^{5'}$-TTTTGCGGCCGCACTCTAAGATGCTTTCCC-$^{3'}$ containing the underlined *Sal*1 and *Not*1 site respectively. PCR and subsequent cloning was as described above.

Site-directed mutagenesis. Mutagenesis of GAP1^{IP4BP} was performed using the Transformer Kit from Clontech as previously described [11].

[^{32}P]Ins(1,3,4,5)P_4-binding assays. These were performed as described previously [4]. An individual binding assay contained 100 mM KCl, 20 mM NaCl, 10 mM Hepes/NaOH (pH 7.0), 1 mM EDTA, 30000 dpm [^{32}P]Ins(1,3,4,5)P_4 (prepared as in [13]), 0.5-1 μg of GST fusion protein and various concentrations of competing unlabelled inositol phosphates in a final volume of 0.5 ml. Equilibrium binding was reached by a 15 minutes incubation at 4°C after which the receptor-ligand complex was precipitated by the addition of 100 μl of 5 mg/ml γ-globulin and 1 ml of 25% (w/v) polyethylene glycol. The samples were spun for 10 minutes prior to removal of the supernatant with the resultant pellet being briefly washed prior to counting.

Ras and Rap GAP assays. These were performed under first order kinetics as described in [6]. Briefly, the particular GTP-binding protein was loaded with [γ-^{32}P]GTP (3000 Ci mmol^{-1}, Amersham) for 5 mins at 25°C. GTPase activity was assayed at 25°C by addition of the various GAP's to the loaded GTP-binding protein. At the required time points activity was stopped by addition of 5 mM silicotungstate, 1 mM H_2SO_4, with the liberated [^{32}P]P_i being extracted with isobutanol/toluene (1/1 v/v), 5% (w/v) ammonium molybdate, 2 M H_2SO_4. The upper phase was removed for scintillation counting.

Materials. Rap1a and Rap GAP were kind gifts from Professor Alfred Wittinghofer (Max-Planck Institut fur Molekulare Physiologie, Dortmund, Germany) and Dr Frank McCormack (ONYX Pharmaceuticals). H-Ras was purchased from Calbiochem (Cat. No. 553325). All inositol phosphates were kind gifts from Professor Robin Irvine (Department of Pharmacology, University of Cambridge, UK).

RESULTS

[^{32}P]Ins(1,3,4,5)P_4-Binding to Expressed Full Length GAP1^{IP4BP}

As can clearly be seen from Figure 1, Ins(1,3,4,5)P_4 bound to expressed GAP1^{IP4BP} with high affinity and

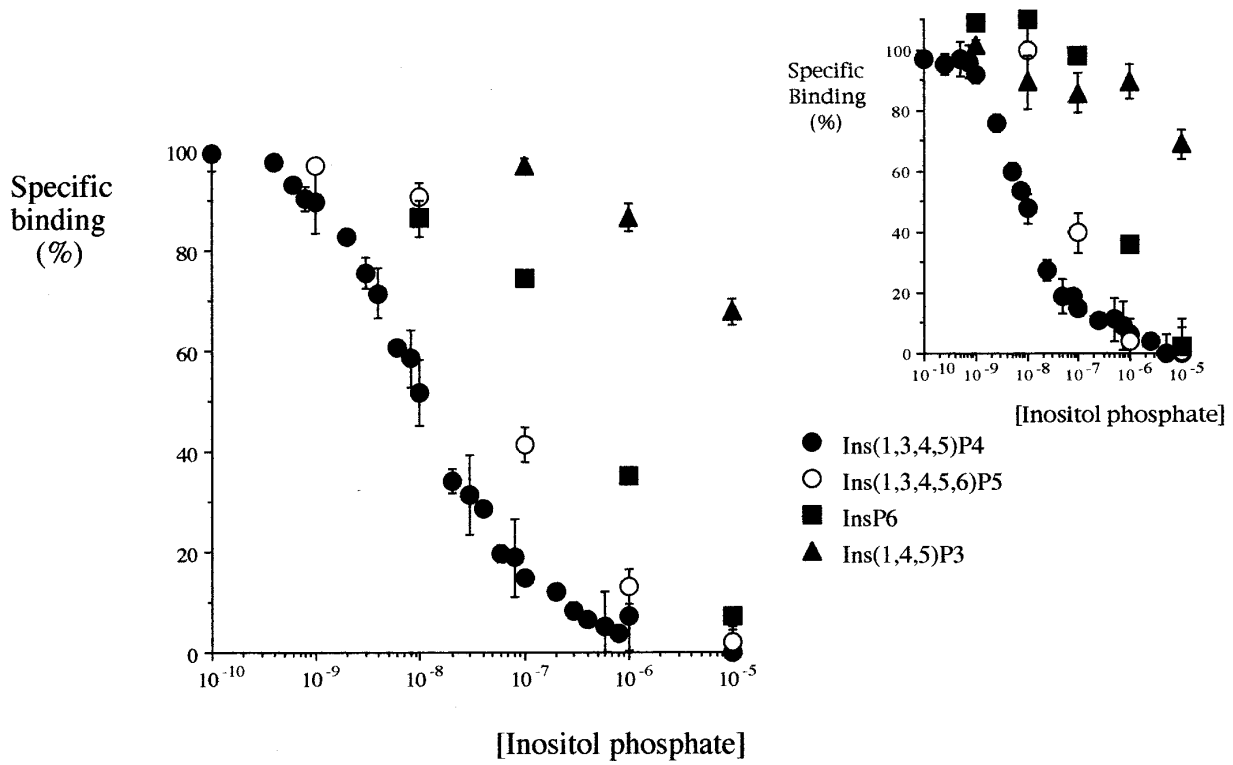

FIG. 1. Comparison of [^{32}]PIns(1,3,4,5)P_4-binding to prokaryotically expressed full length GAP1^{IP4BP} and endogenous GAP1^{IP4BP} purified from pig platelets. Data are from three individual determinations of the competition curve for [^{32}P]Ins(1,3,4,5)P_4. Binding was performed as described in Material and Methods on protein either purified from prokaryotic expression or as shown in the inset from pig platelets. Specificity of the binding site is shown using the various inositol phosphate isomers. Non-specific binding was defined as the binding remaining in the presence of 10 μM Ins(1,3,4,5)P_4. Data are means ± S.E.M.

specificity. The K_d value for Ins(1,3,4,5)P_4 {*i.e.* the concentration required to compete for 50% of bound [^{32}P]-Ins(1,3,4,5)P_4} was 11.3 ± 3.2 nM (n=3), with the other inositol phosphates tested, namely Ins(1,3,4,5,6)P_5, InsP_6 and Ins(1,4,5)P_3, having K_d's of 85.0 ± 4.5, 646.7 ± 28.7 and >10,000 nM respectively (n=3 for each inositol phosphate). Detailed Scatchard transformation of the Ins(1,3,4,5)P_4 competition curve (using 4-5 points per log unit) resolved a single class of binding site (data not shown). With respect to these parameters expressed GAP1^{IP4BP} closely resembles Ins(1,3,4,5)P_4-binding to GAP1^{IP4BP} purified from pig platelets which has K_d's for Ins(1,3,4,5)P_4, Ins(1,3,4,5,6)P_5, InsP_6 and Ins(1,4,5)P_3 of 6.3 ± 0.4, 85.0 ± 5.9, 800.0 ± 20.1 and >10,000 nM respectively (see inset to Fig. 1 and ref. [4,5]).

GAP Activity of Expressed Full-Length GAP1^{IP4BP}

As mentioned in the Introduction, an unusual feature of purified GAP1^{IP4BP} is that not only does it function as a Ras GAP but it also stimulates the GTPase activity of Rap1a [6]. In order to address whether this Rap GAP activity is an intrinsic function of GAP1^{IP4BP} we have analysed the GAP specificity of expressed GAP1^{IP4BP} (Fig. 2). Clearly expressed GAP1^{IP4BP} stimulates the GTPase activity of both H-Ras (Fig. 2a) and Rap1a (Fig. 2b) thus demonstrating that the ability of GAP1^{IP4BP} to enhance the GTPase activity of Rap1a is an integral property of the protein. Our recent cloning of the full length cDNA encoding for human blood GAP1^{m} [11] has allowed us to directly compare the GAP activity of expressed GAP1^{m} with that of GAP1^{IP4BP} under identical experimental conditions. Using the same Ras and Rap isoforms as those described for GAP1^{IP4BP}, GAP1^{m} only appears to function as a Ras GAP, there is no detectable Rap GAP activity (Fig. 2a and 2b).

Identification of the Ins(1,3,4,5)P_4-Binding Site within GAP1^{IP4BP}

To map the Ins(1,3,4,5)P_4-binding site we produced eight GST deletion mutants which traversed the entire ORF of GAP1^{IP4BP} (Fig. 3a). Although we had previously predicted that the C2B domain may constitute the Ins(1,3,4,5)P_4-binding site based on analogy with the non-specific binding of Ins(1,3,4,5)P_4 to the C2B domain of synaptotagmin II [6], the Ins(1,3,4,5)P_4-binding activity actually mapped to the PH/Btk domain (Fig. 3b). However, the PH/Btk domain alone (GST-8) does not show a high degree of activity but requires both N- and

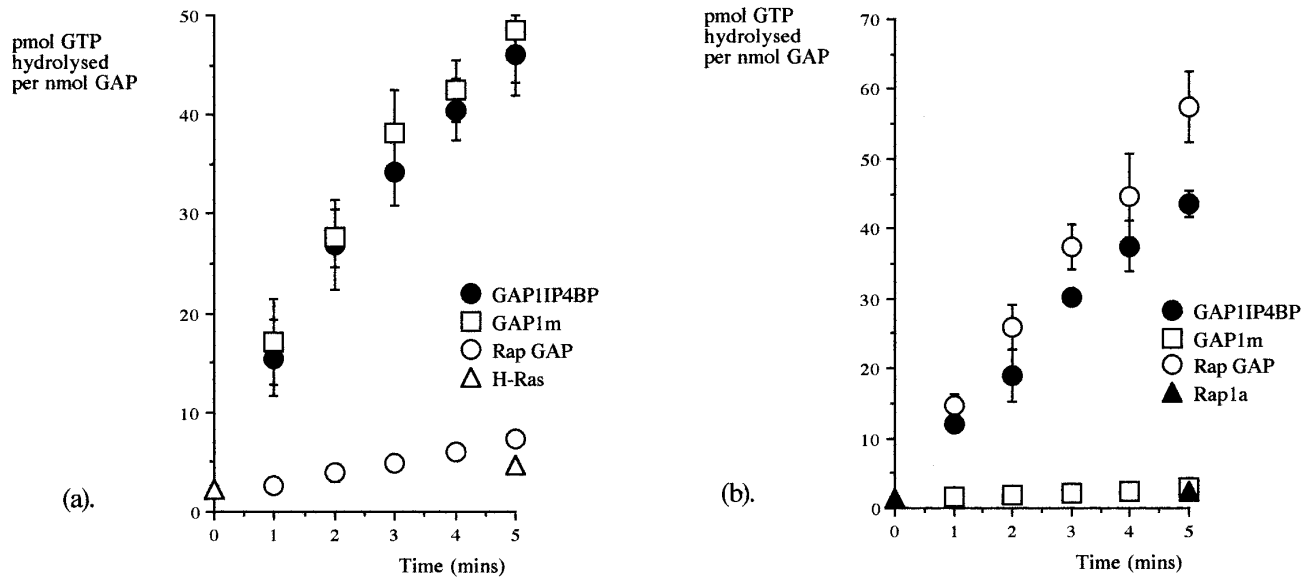

FIG. 2. GAP activities of expressed full length $GAP1^{IP4BP}$ and $GAP1^{m}$. Assays were performed through the analysis of liberated $[^{32}P]P_i$ as described in Material and Methods. GAP activity of both full length $GAP1^{IP4BP}$ or $GAP1^{m}$ was assayed using either H-Ras (*a*) or Rap1a (*b*) as the small GTP-binding protein. The endogenous GTPase activity of H-Ras and Rap1a was assayed by addition of buffer in which the various GAPs were diluted. Note that Rap GAP only functions on Rap1a and not H-Ras. Data are the means ± S.E.M. from three individual determinations using Rap1a obtained from two distinct sources.

C-terminal flanking regions in order to bind with a comparable activity to full length $GAP1^{IP4BP}$ (see GST-6). $Ins(1,3,4,5)P_4$ bound to this C2A and C2B deletion mutant (ΔC2-$GAP1^{IP4BP}$) with a similar affinity and specificity to that of full length $GAP1^{IP4BP}$ [11], having K_d's for $Ins(1,3,4,5)P_4$ of 30.8 ± 1.5 nM and for $Ins(1,3,4,5,6)P_5$, $InsP_6$ and $Ins(1,4,5)P_3$ of 450.7 ± 56.2, 946.7 ± 22.5 and >10,000 nM respectively. This data suggests that for high affinity isomerically specific $Ins(1,3,4,5)P_4$-binding, $GAP1^{IP4BP}$ requires at least the GRD, PH/Btk domain and carboxyl terminus.

Site-Directed Mutagenesis of the PH/Btk Domain within $GAP1^{IP4BP}$

The N-terminal region of the PH/Btk domain contains a cluster of positively charged amino acids that are likely to be involved in $Ins(1,3,4,5)P_4$-binding based on the $Ins(1,3,4,5)P_4$-binding site located in $GAP1^{m}$ [18] and Btk [20] (Fig. 4a). We have therefore introduced mutations into this region and analysed the effect on $Ins(1,3,4,5)P_4$-binding on the mutated full length $GAP1^{IP4BP}$. $GAP1^{IP4BP}$ (R601C) possesses a conversion of arginine into cystine at position 601, which corresponds to the mutation in Btk in the *Xid* mice [21], whereas $GAP1^{IP4BP}$ (K599Q,K600Q,R601Q) is a triple mutant neutralising the positive charge. $Ins(1,3,4,5)P_4$-binding activity was reduced to approximately 10% of control values in $GAP1^{IP4BP}$ (R601C) and is completely ablated in $GAP1^{IP4BP}$ (K599Q,K600Q,R601Q) (Fig. 4b). It should be stressed that the GAP activity of these mutants was indistinguishable from wild type $GAP1^{IP4BP}$ (data not shown).

DISCUSSION

In this study we have described the expression of the full length cDNA which previously had been predicted to encode for a specific $Ins(1,3,4,5)P_4$-binding protein [6]. Indeed as expected the expressed protein binds $Ins(1,3,4,5)P_4$ with an affinity and inositol phosphate specificity similar to $GAP1^{IP4BP}$ purified from pig platelets and hence demonstrates that the cloned cDNA does indeed encode for a protein which has the characteristics predicted of a putative $Ins(1,3,4,5)P_4$ receptor. Furthermore using both deletion and site-directed mutagenesis we have identified that the $Ins(1,3,4,5)P_4$-binding site does not appear to reside in the C2B domain [6], but rather is a function of the PH/Btk domain. However, for high affinity isomerically specific $Ins(1,3,4,5)P_4$-binding, $GAP1^{IP4BP}$ requires at least the GRD, PH/Btk domain and the carboxyl terminus.

Previously we [11] and others [18] have shown that $GAP1^{m}$ also constitutes an isomercially specific $Ins(1,3,4,5)P_4$-binding protein as demonstrated by the binding activity of ΔC2-$GAP1^{m}$. In this present study however, we were unable to isolate a sufficient quantity of full length $GAP1^{m}$ to analyse in detail the $Ins(1,3,4,5)P_4$-binding activity and hence the exact affinity and specificity of $GAP1^{m}$ will only be documented when this is achieved.

In general PH domains are protein modules of approximately 100 amino acids which have been implicated in binding $\beta\gamma$ subunits of heterotrimeric G proteins [22], phosphatidylinositol 4,5-bisphosphate [23] and in the case of the PH domain from phospholipase C-

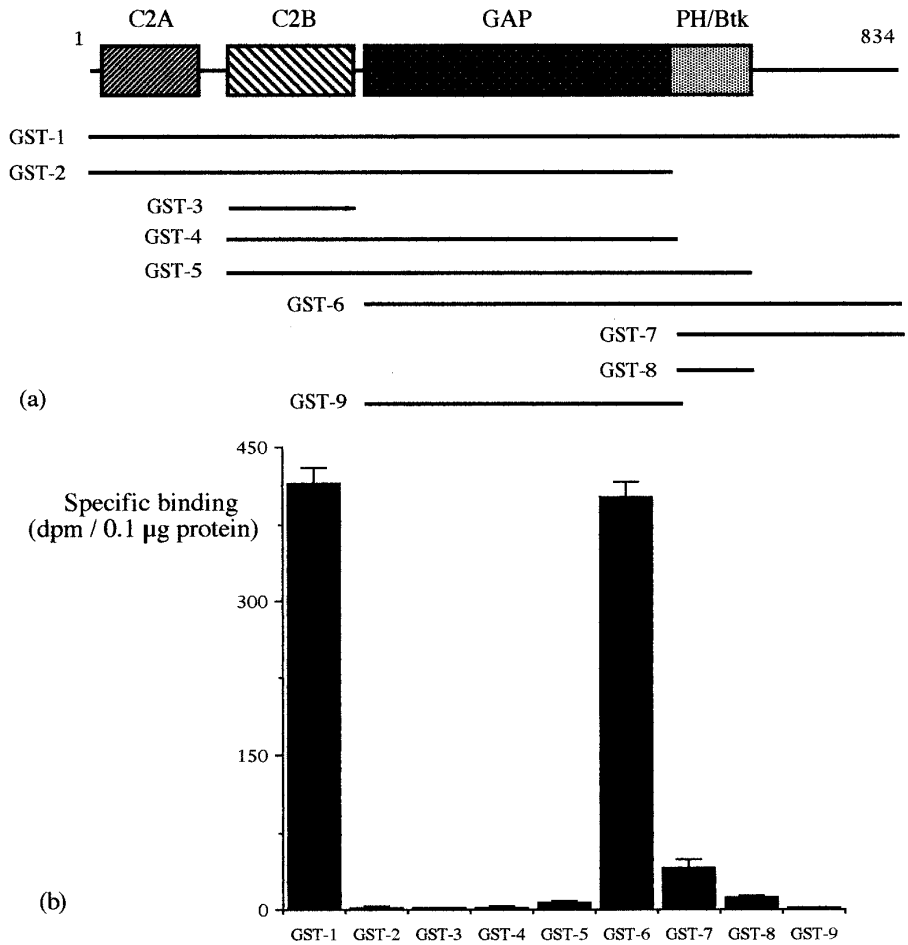

FIG. 3. Mapping of the [^{32}P]Ins(1,3,4,5)P_4-binding site to the PH/Btk domain of GAP1^{IP4BP}. The schematic representation of the various domains which constitute GAP1^{IP4BP} are shown in *a* where C2A and C2B, GRD and PH/Btk depict the Ca^{2+}-dependent and -independent phospholipid binding domains, the Ras GAP related domain and the PH domain containing the Btk motif respectively. The fragments which where expressed as GST deletions mutants are shown by the solid lines. *b*, analysis of the ability of the various deletion mutants to bind [^{32}P]Ins(1,3,4,5)P_4. Assays were performed on protein isolated as described in Material and Methods with significant binding only being observed in those constructs expressing the PH/Btk domain. Data are means ± S.E.M. (n=3).

δ1, Ins(1,4,5)P_3 [24]. The PH/Btk domains of GAP1^{IP4BP} and GAP1^{m} are members of a distinct class of PH domains which shows a high degree of homology to the PH domain from Bruton's tyrosine kinase (Btk); a homology that is perhaps not surprising since Btk itself has been shown to function as a high affinity Ins(1,3,4,5)P_4-binding protein [20]. Mutational analysis within this PH domain has implicated various residues in Ins(1,3,4,5)P_4-binding [20], a conclusion which has been confirmed by the recent proposed crystal structure of the Ins(1,3,4,5)P_4-binding site from Btk which highlights that these residues, and in particular the highly conserved KKR motif, do indeed constitute the Ins(1,3,4,5)P_4-binding pocket [16]. The point mutants we describe here are centred around the corresponding KKR sequence within GAP1^{IP4BP} and confirms that this PH/Btk domain does indeed constitute the Ins(1,3,4,5)P_4-binding site (see also [18]). Furthermore our data demonstrating that this particular GAP-1^{IP4BP} mutant no longer resides at the plasma membrane but is instead cytosolic [11] implicates a dual role for the PH/Btk domain in both Ins(1,3,4,5)P_4-binding and plasma membrane localisation possibly via an interaction with phospholipids. It should not be overlooked that the Ins(1,3,4,5)P_4-binding site within GAP-1^{IP4BP} may structurally be similar to that described for Btk [16] and hence this implicates other residues which may be important for Ins(1,3,4,5)P_4-binding.

Significantly, we have shown that expressed GAP1^{IP4BP} is able to function both as a Ras and Rap GAP; an unique activity that is an intrinsic property of the protein and not due to a minor but highly active contamination present in the originally purified GAP1^{IP4BP} [6]. Intriguingly we have also demonstrated that under identical experimental condition to those used to assay the GAP activity of GAP1^{IP4BP}, full length GAP1^{m} only

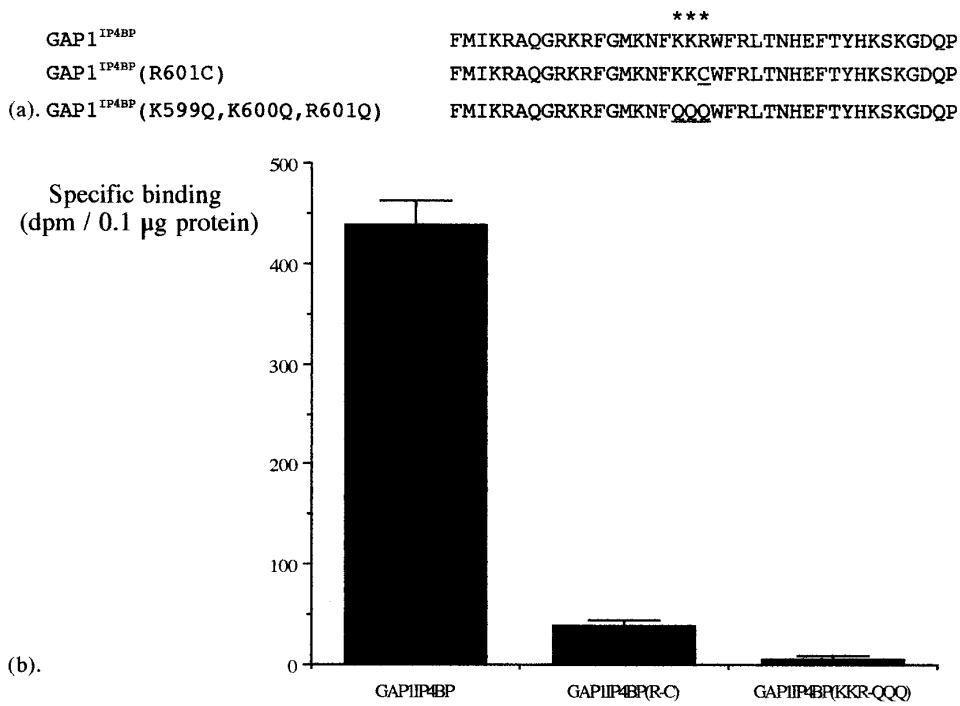

FIG. 4. Mutational analysis of the Ins(1,3,4,5)P_4-binding domain of GAP1^{IP4BP}. *a*, sequence alignment of the region within the PH/Btk of GAP1^{IP4BP} detailing the amino acids mutated in order to construct the various full length GAP1^{IP4BP} site-directed mutants. The ability of these GAP1^{IP4BP} mutants to bind [^{32}P]Ins(1,3,4,5)P_4 is shown in *b* were GAP1IP4BP(R-C) and GAP1IP4BP(KKR-QQQ) refer to GAP1^{IP4BP}(R601C) and GAP1^{IP4BP}(K599Q,K600Q,R601Q) respectively. Binding was performed as detailed in Fig. 1. Data are the means ± S.E.M. from three independent deteminations.

appears to function as a specific Ras GAP, there is no detectable Rap GAP activity. These data therefore suggests that not only do GAP1^{IP4BP} and GAP1^{m} have distinct subcellular localisations [11], but *in vitro* they have distinct specificities with regard to their target small GTP-binding protein, namely that they both function as Ins(1,3,4,5)P_4-regulated Ras GAPs [6,18] but only GAP1^{IP4BP} functions as a Rap GAP.

In summary, we have shown that the human circulating blood GAP1^{IP4BP} cDNA encodes for an isomerically specific Ins(1,3,4,5)P_4-binding protein. Furthermore, with the data described here and that of Fukuda and Mikoshiba [18], the PH/Btk domain of both GAP1^{IP4BP} and GAP1^{m} appears to constitute the binding site for Ins(1,3,4,5)P_4. Moreover we have confirmed that the Rap GAP activity of GAP1^{IP4BP} [6] is an intrinsic property of the protein and hence highlighted a fundamental distinction in the GAP specificity of these two proteins.

ACKNOWLEDGMENTS

We thank Dr. Kanamarlapudi Venkateswarlu, Professor Robin Irvine and the members of Inositide Lab in the Department of Pharmacology, University of Cambridge for helpful discussion. This work is funded by Grants from the MRC and the BBSRC Integration of Cellular Responses Initiative. J.S.R. is in receipt of a BBSRC Studentship.

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