Mutation-deletion analysis of a Ca^{2+} -dependent phospholipid binding (CaLB) domain within p120 GAP, a GTPase-activating protein for p21 *ras*

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p120 GAP is a GTPase activating protein for p21 *ras*. It is a multidomain protein which exhibits sequence similarity with other GTPase-activating proteins, src, pleckstrin and a central portion of the protein kinase C conserved region 2 domain known as CaLB (Ca^{2+} -dependent phospholipid-binding). The presence of this CaLB motif has led to the speculation that p120 GAP may be a member of a family of structurally related proteins containing a Ca^{2+} -dependent membrane/lipid-binding domain. Here we have studied the *in vitro* Ca^{2+} -dependent phospholipid-binding properties of the isolated proposed CaLB sequence in human GAP and deduce that a phospholipid-binding sequence is indeed located between amino acids 606 and 648. Binding of phosphatidylserine and phosphatidylinositol,

but not phosphatidylcholine, within this sequence is Ca^{2+} -dependent, with an estimated EC_{50} for Ca^{2+} of approx. $1\ \mu\text{M}$. Using deletion-mutation analysis we have further defined the minimal boundaries for this *in vitro* phospholipid-binding activity. p120 GAP amino acids 612–643 exhibit full phospholipid-binding activity, but further deletion of either amino acids 612–617 or amino acids 633–648 significantly decreased or abolished phospholipid binding. These studies establish that amino acids 612–643 of p120 GAP indeed constitute a functional CaLB domain and thereby imply a role for Ca^{2+} in the regulation of p120 GAP association with cellular (membrane) phospholipids.

INTRODUCTION

Ras GTPase-activating protein (GAP) is a 120 kDa protein that specifically stimulates the GTPase activity of the p21 *ras* G-protein [1–3]. In common with a variety of diverse signalling proteins it comprises of a number of either proven or postulated discrete functional domains [1,4–6]. The most well studied of these domains are the src homology (SH2 and SH3) domains which play important roles in protein–protein interactions during cellular-signalling events triggered by receptor-activated tyrosine kinase signalling cascades [7].

The conserved region 2 (C2) domain [8,9] of protein kinase C (PKC) isoenzymes is approx. 114 amino acids long and contains within it a motif similar to a 43-amino-acid sequence found in p120 *ras* GAP [10]. This 43-amino-acid motif within human GAP has also been observed to have sequence similarity with motifs within proteins including cytosolic phospholipase A_2 (cPLA $_2$), phospholipase $\text{C}_{\gamma 1}$, raphilin-3A and synaptotagmin [10–13]. Since PKC isoforms containing C2 domains appear to require Ca^{2+} for kinase activation and lipid association, it has been suggested that this domain may therefore function as a Ca^{2+} sensor which may be important for phospholipid-binding activity [8]. However, because the CaLB motif, but not the full-length C2 domain, has been observed within both p120 GAP and cPLA $_2$, and cPLA $_2$ membrane association is also a Ca^{2+} -dependent process, it has been proposed, but is yet to be proved, that the CaLB motif itself may be sufficient for Ca^{2+} -dependent phospholipid-binding activity [10]. We have found that this motif is indeed sufficient to confer a Ca^{2+} -dependent cellular membrane-associated localization both *in vitro* and *in vivo* by p120 GAP [14], implying that this amino acid sequence may play a role in p120 GAP membrane association and subsequent regulation of *ras* signalling.

Since the proposed Ca^{2+} -dependent lipid-binding domain boundaries have to date only been assumed from amino-acid-sequence alignments between structurally related proteins, but have not as yet been either demonstrated in p120 GAP or structurally defined by mutational and functional analysis, we have undertaken to define and characterize *in vitro* this proposed domain in human p120 GAP.

MATERIALS AND METHODS

Construction of expression vectors and expression and purification of recombinant proteins

cDNA corresponding to amino acids 606–648 of human GAP and deletion and point mutations therein (as outlined in Figure 3 below) were synthesized and amplified by oligonucleotide-directed PCR and subcloned into the *Sma*I site of the pGEX-3X expression vector (Pharmacia). Fusion protein constructs were verified by DNA sequencing.

Glutathione S-transferase (GST) and GST–GAP fusion proteins were expressed in bacteria, purified by batch adsorption to, and released from, GST–agarose beads [15]. Binding experiments were performed with proteins dialysed against binding assay buffer in the absence of Ca^{2+} .

^{35}S metabolic labelling of GST fusion proteins

Bacterial cultures were grown in cysteine-and-methionine-free M9 media to which $13\ \mu\text{Ci/ml}$ [^{35}S]Met/[^{35}S]Cys ($1000\ \text{Ci/mmol}$; ICN) was added for the last 15 min of a 2 h protein induction period. Labelled GST fusion proteins were purified as above and

Abbreviations used: CaLB domain, Ca^{2+} -dependent phospholipid-binding domain; C2, conserved region 2; cPLA $_2$, cytosolic phospholipase A_2 ; PKC, protein kinase C; GST, glutathione S-transferase.

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Figure 1 GST and GST-GAP fusion proteins bind to phosphatidylserine-, phosphatidylinositol- and phosphatidylcholine-containing vesicles *in vitro*

^{35}S -labelled GST (open symbols), GST-GAP wild-type (closed symbols) binding in the presence of $1\ \mu\text{M}$ free Ca^{2+} and GST-GAP wild-type in the absence of Ca^{2+} (Δ) to (a) phosphatidylserine-, (b) phosphatidylinositol- or (c) phosphatidylcholine-containing vesicles. Results shown are means $\pm$ S.E.M. values for $n > 3$ independent experiments.

specific activities were determined by liquid-scintillation counting and SDS/PAGE [16].

Phospholipid-vesicle preparation

Portions ($400\ \mu\text{g}$) of phosphatidylserine, phosphatidylinositol or phosphatidylcholine (Sigma) were resuspended in chloroform, dried under nitrogen and then resuspended in assay buffer containing 20 mM HEPES, pH 7.5, containing 2.5 mM EGTA, 1 mM dithiothreitol, leupeptin and aprotinin ($10\ \mu\text{g}/\text{ml}$ each) and 1 mM phenylmethanesulphonyl fluoride. The mixture was sonicated five times for 1 s on ice. For fusion-protein-binding assessment, $25\ \mu\text{l}$ of vesicles were used in an assay volume of 1 ml. Vesicles were prepared freshly immediately prior to use.

^{35}S -labelled-fusion-protein binding assay

Labelled GST fusion proteins were incubated with phospholipid-vesicle preparations in assay buffer containing the indicated concentration of free Ca^{2+} for 30 min at 30°C . Samples were then centrifuged at $100000\ g$ for 15 min at 4°C , and pelleted vesicles were then analysed for radiolabelled fusion-protein association by liquid-scintillation counting. Radiolabelled fusion proteins did not associate with pellet fractions in the absence of vesicles, nor was their precipitation observed after centrifugation in the presence or absence of Ca^{2+} . Free Ca^{2+} concentrations in the Ca^{2+} -EGTA buffers were calculated by the Chelator program (distributed by BioTechniques BioTech Net BBS).

RESULTS

GST-GAP fusion protein binds to phospholipid vesicles

GST-GAP⁶⁰⁶⁻⁶⁴⁸ was observed to bind to vesicles containing phosphatidylserine (Figure 1a), or phosphatidylinositol (Figure 1b), but not significantly to phosphatidylcholine (Figure 1c). Binding was dependent on both fusion-protein and Ca^{2+} concentration. Binding did not appear to be saturable with fusion-protein concentrations within the range 10^{-9} – $10^{-6}\ \text{M}$, and significant binding (compared with the GST control) was not observed until the fusion-protein concentration was greater than $10^{-7}\ \text{M}$. Similar levels of protein binding were observed for phosphatidylserine and phosphatidylinositol vesicles. These results suggest

Figure 2 Ca^{2+} -dependence of phospholipid-vesicle binding

The Figure shows dose-response curves for ^{35}S -labelled GST-GAP wild-type ($3 \times 10^{-7}\ \text{M}$) binding to phosphatidylserine ($\circ$) or phosphatidylinositol ($\blacksquare$) vesicles in the presence of increasing concentrations of free Ca^{2+} . The estimated Ca^{2+} EC_{50} value for both phosphatidylserine and phosphatidylinositol binding is approx $1\ \mu\text{M}$. Results shown are means $\pm$ S.E.M. values for $n = 3$ independent experiments.

that the GAP amino acid sequence 606–648 is capable of directly binding to phospholipid vesicles *in vitro*. Ca^{2+} increased the efficiency of fusion-protein binding to phosphatidylserine and phosphatidylinositol, but not to phosphatidylcholine vesicles. However, since binding saturability was not observed, we cannot estimate EC_{50} values for this process.

Ca^{2+} -dependence of phospholipid-vesicle binding

In order to determine whether GST-GAP binding to phospholipid vesicles was a Ca^{2+} -concentration-dependent process, dose-response measurements for vesicle binding were made in the presence of increasing concentrations of free Ca^{2+} (Figure 2).

Figure 3 Fusion-protein constructs used in binding-analysis studies

(a) Amino acid sequence for wild-type human GAP CaLB and deleted and mutated constructs which were fused to GST are shown schematically. Amino acid sequences of individual fusion proteins are given on the left-hand side, and the efficiency of phosphatidylserine binding is indicated on the right-hand side. Alignment of the CaLB consensus sequence within related proteins (Clark et al. [14]) and conserved residues within GAP are highlighted in **bold** type. (b) Coomassie Blue-stained polyacrylamide gel of expressed fusion-protein constructs. Approx. 2–3 μ g of each protein was loaded per well.

Figure 4 Mutational analysis of GST-GAP binding to phosphatidylserine vesicles

Increasing concentrations of ³⁵S-labelled fusion proteins were incubated with phosphatidylserine vesicles in the presence of 1 μ M free Ca²⁺, and protein binding to vesicles was assessed by liquid-scintillation counting as described in the Materials and methods section. (a) N-terminally truncated mutant constructs as described in Figure 1: constructs 612-648 (○), 618-648 (◇) and 618-632 (□). (b) C-terminally truncated mutant constructs: 606-647 (●), 606-643 (◆) and 606-632 (■). (c) Single-amino-acid point mutants: C616V (△) and T628A (◇). Results shown are means $\pm$ S.E.M. for $n > 3$ independent experiments.

Binding to phosphatidylserine and phosphatidylinositol vesicles was indeed Ca²⁺-dependent, with an estimated EC₅₀ value of approx. 1 μ M.

Fusion-protein constructs used in binding-analysis studies

GST and GST-GAP fusion proteins corresponding to amino acids 606-648 (wild-type), N- or C-terminally truncated and point-mutated constructs as illustrated in Figure 3(a) were purified from *Escherichia coli* as described in the Materials and methods section and analysed by SDS/PAGE (Figure 3b).

GAP⁶⁰⁶⁻⁶⁴⁸ contains the entire CaLB amino acid sequence of human p120 GAP fused to GST, and GAP⁶¹²⁻⁶⁴⁸ has the N-terminal leucine residue and the following five amino acids deleted in order to establish whether this relatively variant N-terminal portion within CaLB is necessary for phospholipid-

binding activity. GAP⁶¹⁸⁻⁶⁴⁸ has an additional deletion of amino acids 612-617 which removes the consensus sequence motif DPYVK.

The C-terminal mutants are GAP⁶⁰⁶⁻⁶⁴⁷, which lacks the invariant C-terminal proline residue, and GAP⁶⁰⁶⁻⁶⁴³, which lacks both this proline residue and the amino acids enclosed by and including the invariant phenylalanine residue at position 644.

GAP⁶⁰⁶⁻⁶³² is missing residues 633-648 containing the conserved CaLB consensus motif NPVWNE. GAP⁶¹⁸⁻⁶³² is a fusion protein of the central region in the CaLB sequence. This region is widely variant between proteins containing the proposed domain.

Finally, the mutant proteins containing single-amino-acid-residue changes are GAP^{T628A}, in which the codon for threonine-628, an invariant residue in CaLB motifs, was mutated to encode

Figure 5 GST–GAP fusion proteins bind to phosphatidylserine vesicles in a Ca^{2+} -dependent manner

^{35}S -labelled fusion proteins were incubated with phosphatidylserine vesicles at a concentration of 10^{-6} M in the presence (■) or absence (□) of $1 \mu\text{M}$ free Ca^{2+} , and binding was assessed as described in the Materials and methods section. Results shown are means $\pm$ S.E.M. for $n = 3$ independent experiments.

alanine, and $\text{GAP}^{\text{C616V}}$, in which the codon for cysteine-616 was mutated to encode valine, the amino acid residue found at this position in all CaLB motifs other than p120 GAP.

Mutational analysis of the GAP CaLB domain and Ca^{2+} -dependence of binding to phosphatidylserine vesicles

GST–GAP constructs shown in Figure 3(a) were assayed for phospholipid-vesicle binding (Figure 4). N-terminal deletion of the GAP CaLB sequence, amino acids 606–611, had no effect on phosphatidylserine-vesicle binding in the presence of Ca^{2+} ($1 \mu\text{M}$). However, removal of six additional residues (amino acids 606–617) significantly reduced, but did not abolish, vesicle binding. Mutants lacking proline-648 or amino acids 644–648 at the C-terminal end of the GAP CaLB sequence did not show altered phosphatidylserine-vesicle binding. However, deletion of amino acids 633–648 abolished vesicle binding (Figure 4b). Similarly $\text{GAP}^{618-632}$ did not significantly bind phosphatidylserine vesicles (Figure 4a). The mutant domains $\text{GAP}^{\text{C616V}}$ and $\text{GAP}^{\text{T628A}}$ still bound to vesicles (Figure 4c).

To determine whether phospholipid binding by wild-type and mutant fusion proteins was a Ca^{2+} -dependent process, fusion proteins were assayed for Ca^{2+} -dependence of binding (Figure 5). All constructs which were observed to bind phosphatidylserine vesicles exhibited enhanced vesicle binding in the presence of Ca^{2+} ($1 \mu\text{M}$).

Binding of mutant GST–GAP constructs to phosphatidylinositol vesicles

Constructs were assayed for phosphatidylinositol-vesicle binding (Figure 6), and the binding profiles obtained were similar to those obtained for phosphatidylserine vesicle binding; that is, $\text{GAP}^{618-648}$, $\text{GAP}^{606-632}$ and $\text{GAP}^{618-632}$ showed reduced binding to phosphatidylinositol vesicles. However, subtle differences in binding were observed; the rank order of binding to phosphatidylserine vesicles was $\text{GAP}^{618-648} > \text{GAP}^{606-632} > \text{GAP}^{618-632}$, but for phosphatidylinositol binding it was $\text{GAP}^{618-632} \geq \text{GAP}^{606-632} > \text{GAP}^{618-648}$.

Figure 6 Analysis of mutant fusion-protein constructs binding to phosphatidylinositol vesicles

^{35}S -labelled fusion proteins (10^{-6} M) were incubated with phosphatidylinositol vesicles in the presence of $1 \mu\text{M}$ free Ca^{2+} , and binding was assessed as described in the Materials and methods section. Results shown are means $\pm$ S.E.M. for $n = 2$ independent experiments.

DISCUSSION

Here we have presented evidence that the CaLB motif sequence in p120 GAP is sufficient and able to bind phospholipid vesicles in a Ca^{2+} -dependent manner with an estimated EC_{50} for Ca^{2+} of approx. $1 \mu\text{M}$. The Ca^{2+} EC_{50} values determined for phospholipid–fusion protein associations is comparable with the estimates of 5 and $30 \mu\text{M}$ reported for synaptotagmin binding to phospholipid vesicles [17] and synaptic vesicle membranes [18] respectively. It is still unclear to what component(s) Ca^{2+} is directly binding; studies with synaptotagmin suggest that Ca^{2+} does not bind to protein sequence alone, but to a protein–lipid complex [19].

By using a range of GST–GAP fusion-protein constructs, we have analysed the boundaries of the p120 GAP CaLB domain. Our results indicate amino acids 612–643 are sufficient for Ca^{2+} -dependent phospholipid binding *in vitro*. We suggest these are the minimal boundaries of this domain, but cannot rule out the requirement for the flanking conserved sequences, which comprise the entire CaLB motif in the context of the full-length protein for *in vivo* activity. It is, however, surprising that deletion of the C-terminal proline-648 or amino acid sequence up to and including phenylalanine-644 had no effect on phospholipid-binding activity, since these proline and phenylalanine residues are highly conserved in the CaLB motif. Although these C-terminal residues are not required for *in vitro* lipid binding, we cannot rule out the possibility that they play a role in p120 GAP interactions with phospholipids *in vivo*.

We suggest that membrane lipid association would allow access of p120 GAP to plasma-membrane-associated *ras*. Also, because phospholipids have been shown to influence the catalytic activity of p120 GAP [20–22], it is possible that a Ca^{2+} -regulated lipid association with the GAP CaLB domain could affect the activity of the adjacent GTPase-activating domain and, in turn, control the signalling activity of *ras*. It is therefore feasible that this domain may play a role in the regulation of *ras* signalling indirectly through cellular localization or directly through regulation of catalytic activity.

Thus amino acids 612–643 (CaLB) *in vitro* constitutes a functional Ca²⁺-dependent phospholipid-binding domain in p120 GAP and therefore, possibly, in other structurally related proteins. However, it remains to be established if our boundaries of the domain are sufficient for p120 GAP–membrane lipid association *in vivo*. Studies with mutant proteins are now being undertaken in order to establish whether CaLB-mediated cellular membrane association is indeed due to membrane lipid binding. Finally, we do not rule out the possibility that the CaLB domain may also possess a protein-binding function analogous to that demonstrated for the C2 region in PKC [23,24], particularly since we have proposed that more than one Ca²⁺-dependent binding component is responsible for GAP membrane association *in vivo* [14].

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