Communication

The Ca\textsuperscript{2+}-dependent Lipid Binding Domain of P120\textsuperscript{GAP} Mediates Protein-Protein Interactions with Ca\textsuperscript{2+}-dependent Membrane-binding Proteins

**EVIDENCE FOR A DIRECT INTERACTION BETWEEN ANNEXIN VI AND P120\textsuperscript{GAP}**

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The CaLB domain is a 43-amino acid sequence motif found in a number of functionally diverse signaling proteins including three Ras-specific GTPase activating proteins (GAPs). In the Ras GTPase activating protein, P120\textsuperscript{GAP}, this domain has the ability to confer membrane association in response to intracellular Ca\textsuperscript{2+} elevation. Here we have isolated three proteins, p55, p70, and p120, which interact with the P120\textsuperscript{GAP} CaLB domain in vitro. We identify p70 as the Ca\textsuperscript{2+}-dependent phospholipid-binding protein annexin VI. Using co-immunoprecipitation studies, we have shown that the interaction between P120\textsuperscript{GAP} and annexin VI is also detectable in rat fibroblasts, suggesting that this interaction may have a physiological role in vivo. Thus, the CaLB domain in P120\textsuperscript{GAP} appears to have the ability to direct specific protein-protein interactions with Ca\textsuperscript{2+}-dependent membrane-associated proteins. In addition, annexin VI is known to have tumor suppressor activity. Therefore, it is possible that the interaction of annexin VI with P120\textsuperscript{GAP} may be important in the subsequent modulation of p21\textsuperscript{ras} activity.

p21\textsuperscript{ras} proteins play a critical role in cellular proliferation and differentiation pathways (1, 2). Mutations in p21\textsuperscript{ras} which result in constitutive activation are associated with certain forms of cancer (3, 4). GTPase activating proteins (GAPs)\textsuperscript{1} are important in inactivating p21\textsuperscript{ras} (5–7) and, in addition, may themselves be signal transducers (8, 9). Primary sequence analysis of Ras-specific GAPs indicates that P120\textsuperscript{GAP}, GAP1\textsuperscript{m}, and GAP1\textsuperscript{IP4BP} display 25–30% sequence identity with each other in a region called the “CaLB” (Ca\textsuperscript{2+}-dependent lipid binding) domain (10–13). The function of the CaLB domain is unknown. However, since these GAPs are all able to specifically regulate p21\textsuperscript{ras} activity, it is possible that the CaLB domain may play a common signaling role in these proteins which could be important in the Ras signaling pathway. Discrete modular domains found within proteins in the Ras signaling pathway have been shown to mediate important protein-protein interactions which are involved in signal transduction (14–16). We therefore sought to determine whether protein-protein interactions could be mediated via the CaLB domain in P120\textsuperscript{GAP}. We rationalized that if CaLB-binding proteins could be isolated and identified, then the role of the CaLB domain in Ras GAPs may become apparent. Here we demonstrate that the CaLB domain of P120\textsuperscript{GAP} interacts directly with annexin VI which is a major Ca\textsuperscript{2+}- and phospholipid-binding protein (17, 18). Thus, it is possible that the CaLB domain in P120\textsuperscript{GAP} may be a convergence point in Ca\textsuperscript{2+} and Ras signaling pathways. In addition, these protein-protein interactions could have important implications in the tumor suppressor activity associated with annexin VI expression (19).

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Membrane Preparation**—Cellular membranes were prepared from rat 2 fibroblasts which had been grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, and streptomycin and maintained in a 5% CO\textsubscript{2} atmosphere. Prior to harvesting, cells were washed twice with phosphate-buffered saline and then lysed on ice in 20 mM Hepes, 25 mM EGTA, free Ca\textsuperscript{2+} at a final concentration of 1 mM, pH 7.5. Cells were homogenized by 25 strokes in a dounce homogenizer at 4°C for 5 min at 1000 × g to remove nuclei. The postnuclear supernatant was centrifuged at 100,000 × g for 30 min at 4°C, and the pellet fractions containing cellular membranes (P1) were collected. Membranes were then washed by resuspension in 20 mM Hepes containing 25 mM EGTA, pH 7.5, in the presence or absence of 1 mM free Ca\textsuperscript{2+}, sonicated and incubated at 4°C for 30 min. Supernatant (S2) and pellet (P2) fractions were collected by centrifugation at 100,000 × g at 4°C for 30 min.

**Fusion Protein Binding Assay**—For binding assays, membranes (P2) were resuspended by sonication in 20 mM Hepes containing 25 mM EGTA and free Ca\textsuperscript{2+} at a final concentration of 1 mM, pH 7.5.

\textsuperscript{35}S]Methionine-radiolabeled GST fusion proteins were prepared and purified as described previously (21). Labeled GST fusion proteins were incubated with 200 μg of P2 membrane preparations in 20 mM Hepes containing 25 mM EGTA and free Ca\textsuperscript{2+} at a final concentration of 1 mM, pH 7.5, for 30 min at 30°C. Samples were then centrifuged at 100,000 × g for 30 min at 4°C to allow collection of membrane pellets. Pellets were analyzed for radiolabeled fusion protein association by liquid scintillation counting. Free Ca\textsuperscript{2+} concentrations in all Ca\textsuperscript{2+}-EGTA buffers were calculated by the Chelator program (distributed by BioTechniques Biotech Net BBS).

**Detection of Complexing Proteins in Rat Fibroblast Cell Extracts**—Rat fibroblast membranes (P1) were prepared, and EGTA was washed as described above. The resulting supernatant (S2) fraction which contained EGTA-extractable proteins was collected by centrifugation at 100,000 × g and incubated with glutathione-agarose immobilized GST fusion proteins in the presence of 1 mM free Ca\textsuperscript{2+} for 1 h at 4°C. GST fusion proteins and complexing proteins isolated from cell extracts were precipitated by centrifugation for 2 min at 6,500 × g and washed three times. The resulting pellets were resuspended in 20 mM Hepes, 25 mM EGTA, pH 7.5, and the amounts of GST fusion proteins were counted.

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times with ice-cold phosphate-buffered saline (PBS) containing 1% (v/v) Triton X-100, 10% (v/v) glycerol, and 1 mM phenylmethylsulfonyl fluoride. Protein complexes were resuspended in PBS containing 1 mM NHS-biotin solution (Bio-Rad) and incubated with constant agitation at 4 °C for 1 h. The fusion proteins and complexed proteins were washed three times with ice-cold PBS and resolved by SDS-PAGE, Western transferred and associated (biotin labeled) proteins were detected with avidin-HRP and ECL (Amersham).

For Western Analysis—Proteins separated on SDS-polyacrylamide gels were transferred onto nitrocellulose, blocked in 5% (w/v) bovine serum albumin for 1 h and incubated with 30 nM GST or GSTCaLB fusion protein for 1 h at 25 °C. Membranes were then washed three times with PBS, and bound fusion proteins were detected using anti-GST polyclonal antibodies followed by anti-rabbit-HRP and ECL detection.

**Purification of Ca²⁺-dependent Membrane-binding Proteins—Ca²⁺-dependent membrane-binding proteins were purified from sheep liver using the Ca²⁺ precipitation method described previously (23). Proteins were detected in gels by silver staining (Bio-Rad).**

**Immunoprecipitation Studies in Rat Fibroblasts—Rat fibroblasts were lysed on ice in 10 mM Hepes, pH 7.4, containing 1 mM CaCl₂, 1% (v/v) Triton X-100, 10% (v/v) glycerol, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were homogenized by 25 strokes in a dounce homogenizer at 4 °C and then centrifuged for 5 min at 1000 × g to remove nuclei. Rabbit anti-GAP antibodies (26) and rabbit anti-annexin VI antibodies (27) were conjugated to protein A-Sepharose by incubation at 4 °C for 1 h. The antibody-bead complexes were washed three times with ice-cold phosphate-buffered saline, and then cell lysates prepared as described above were added and incubated at 4 °C for 1 h. Antibody-protein complexes were then precipitated by centrifugation at 6,500 × g for 2 min and washed three times with ice-cold phosphate-buffered saline containing 1% Triton X-100 and 10% glycerol. Immunoprecipitated samples were analyzed by SDS-PAGE and Western blotting. Anti-GAP and anti-annexin VI antibodies were used at a 1:5,000 dilution and detected with anti-rabbit-HRP-conjugated antibodies followed by visualization using ECL and detection on film.

**GST-Annexin VI Fusion Protein Preparations—GST-annexin VI fusion protein was constructed by subcloning human annexin VI cDNA into the pGEX-2T bacterial expression system. Protein was prepared and purified as described previously (21) with the exception that bacterial cells expressing the fusion protein were lysed in the presence of 25 mM EGTA, in the absence of Ca²⁺ to increase soluble protein yield. GST-annexin VI fusion protein was cleaved for 2 h at 25 °C with thrombin at a w/w ratio of 1:10. 300 ng of total annexin VI protein was loaded per lane on an SDS-polyacrylamide gel and analyzed by Far Western binding studies as described above.**

**RESULTS AND DISCUSSION**

Our previous work has shown that the CaLB domain of P120GAP is important for the Ca²⁺-dependent association of this protein with cellular membranes (20). We have demonstrated that one mechanism involved in this process may be the binding of this domain to negatively charged phospholipids in a Ca²⁺-dependent manner (21). In addition, we have suggested that a high affinity Ca²⁺-dependent interaction of the domain with membranes may also occur (20). In order to investigate whether the CaLB domain of P120GAP could be interacting with Ca²⁺-dependent membrane-binding components, we extracted rat fibroblast membranes with a buffer containing a high EGTA concentration. After extraction, we undertook membrane-binding studies to establish if the association of the CaLB domain with cellular membranes was altered. Using 35S-labeled GSTCaLB fusion protein to quantify the association of the CaLB domain with membranes (Fig. 1), we observed that EGTA extraction caused a significant reduction in binding of the fusion protein to membranes. One explanation for this could be that a membrane component, to which the fusion protein binds, had been removed upon chelation of calcium. Therefore, we investigated whether the CaLB domain could be interacting with protein(s) which associate with membranes in a Ca²⁺-dependent manner. Such proteins were isolated by preparing membranes firstly in the presence of Ca²⁺ and then subsequently extracting them with a buffer containing a high concentration of EGTA. In this way, EGTA-extractable proteins were collected as an “S2” supernatant fraction after centrifugation. Proteins contained in this S2 fraction were then labeled with biotin and analyzed by Western blotting. We detected many proteins in this extractable fraction (data not shown) but, upon incubation of this S2 fraction with GST or GSTCaLB fusion proteins, specific proteins were found to complex with the GSTCaLB fusion protein but not with GST (Fig. 2A). In addition, subsequent Far Western analysis indicated that only one of these proteins bound directly to the CaLB domain (Fig. 2B). However, this finding may reflect either the loss of CaLB binding activity of some proteins upon SDS denaturation or may indicate that only one protein in the complex has the ability to directly bind to the CaLB domain.

Previously, several groups have characterized Ca²⁺-dependent membrane-binding proteins from various tissues (17, 18). Liver has been shown to be a particularly rich source of these proteins (22, 23). We therefore prepared a purified fraction of Ca²⁺-dependent membrane-binding proteins from sheep liver (Fig. 2C). We subsequently incubated GST and GSTCaLB fusion proteins with these purified proteins and again used biotin to label complexing proteins. We detected three proteins which bound specifically to GSTCaLB but not GST fusion proteins. We have called these proteins p55, p70, and p120 with respect to their apparent molecular weights as assessed by SDS-PAGE (Fig. 2D). The Ca²⁺-dependent association of p70 with cell membranes suggested that it could be the protein annexin VI. This protein has an apparent molecular mass of approximately 68 kDa and displays Ca²⁺-dependent membrane binding properties (17, 18, 24). To establish if p70 was annexin VI, we used anti-annexin VI antibodies to identify proteins which were complexing with GSTCaLB fusion protein. The 70-kDa protein was indeed recognized by our anti-annexin VI antibody (Fig. 3A). Since our in vitro studies were undertaken using only the isolated CaLB domain sequence, it was important to establish if full-length P120GAP protein could also be detected as interacting with annexin VI in cell extracts. We immunoprecipitated P120GAP from rat fibroblast cell lysates using anti-P120GAP antibodies and probed Western blots for the presence of annexin VI. Our results confirmed that annexin VI is indeed able to complex with P120GAP (Fig. 3A). We then labeled all proteins which co-precipitated with P120GAP with biotin in order to
evaluate the specificity of these co-immunoprecipitation studies. Using Western blot analysis, we detected co-precipitating proteins with molecular masses of 190 kDa, 120 kDa, ~90–100 kDa, and ~60–70 kDa (Fig. 3B). It is well established that P120GAP binds to tyrosine-phosphorylated proteins p190 and p62 (28). Additionally, the 120-kDa protein band detected may be P120GAP and also our 120-kDa CaLB domain interacting protein. Similarly, the protein band detected at ~90–100 kDa was not detected by the anti-annexin VI antibody, p55 is unlikely to be a proteolytic product of p70. Instead we believe that p55 is a distinct protein in the CaLB binding complex. We have also obtained similar results using GSTCaLB to isolate proteins from sheep liver extracts (data not shown). Therefore, these data strongly suggest that p70, which binds to the P120GAP CaLB domain in fibroblast and sheep liver extracts, is annexin VI.

Since it was conceivable that our anti-annexin VI antibody could be recognizing a protein with a similar molecular weight and related amino acid sequence to annexin VI, we used recombinant annexin VI to verify its interaction with P120GAP. We expressed recombinant human annexin VI in the form of a GST fusion protein, immobilized the fusion protein on glutathione-agarose beads, and incubated it with rat fibroblast cell lysates. Interaction of P120GAP with annexin VI was investigated by Western blot analysis (Fig. 4A). The results obtained confirmed that recombinant annexin VI can complex with P120GAP. Further verification that annexin VI can bind directly to the CaLB domain of P120GAP was obtained using Far Western analysis. Annexin VI was prepared from the GST-annexin VI fusion protein by proteolytic cleavage. Both intact annexin VI and proteolytic fragments of the protein were observed (Fig. 4B). The CaLB domain of a GSTCaLB fusion protein was able to bind to both the full-length annexin VI and proteolytic fragments of the protein (Fig. 4C). The GST protein alone did not bind to cleaved annexin VI or to its proteolytic fragments, and the anti-GST antibody only detected the cleaved GST fragment of the blot (Fig. 4C). These data are consistent with the CaLB domain recognizing a sequence motif within annexin VI because the protein-protein interaction is detected after SDS denaturation of annexin VI. It still remains to be established whether other proteins containing CaLB do-
Annexin VI binds directly to the CaLB domain of P120GAP. Similarly, P120GAP was immunoprecipitated from lysates of glutathione-agarose beads were incubated with lysates of rat fibroblasts. Proteins were separated by SDS-PAGE and transferred to nitrocellulose by Western blotting. Blots were then probed with anti-P120GAP antibodies to detect P120GAP presence in protein complexes. Lane 1, cut fusion protein; lane 2, uncut fusion protein; lane 3, GST-annexin VI but not GST

In summary, we conclude that annexin VI can directly interact with the CaLB domain of P120GAP. P120GAP relocates from a cytoplasmic location to the plasma membrane in response to calcium elevation. Similarly, P120GAP relocates from a cytoplasmic location to the plasma membrane to access Ras proteins. This process can be triggered by Ca$^{2+}$ elevation and is dependent upon the CaLB domain. However, the precise role which Ca$^{2+}$ plays in the annexin VI/P120GAP CaLB domain interaction still needs to be established. Also, the identity and function of the associating proteins p55 and p120 need to be elucidated. However, we have established that p120 is not P120GAP since it is not recognized by our anti-P120GAP antibodies.

Finally, our work suggests that the CaLB domain is probably another example of a "modular binding domain" (14–16). Modular binding domains appear to be of central importance in mediating protein-protein interactions and signal transduction processes.

Our data suggest that the CaLB domain could function as a regulatory or integration point between convergent signaling pathways. In particular, it may be directing protein-protein interactions between components of Ca$^{2+}$ and Ras signaling pathways. Future work should establish more fully the role of P120GAP and the function of this CaLB domain and annexins in cell signaling processes.

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