Identification of Targets for Calcium Signaling through the Copine Family of Proteins

CHARACTERIZATION OF A COILED-COIL COPINE-BINDING MOTIF*

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We provide evidence that copines, members of a ubiquitous family of calcium-dependent, membrane-binding proteins, may represent a universal transduction pathway for calcium signaling because we find copines are capable of interacting with a wide variety of “target” proteins including MEK1, protein phosphatase 5, and the CDC42-regulated kinase, that are themselves components of intracellular signaling pathways. The copine target proteins were identified by yeast two-hybrid screening and the interactions were verified *in vitro* using purified proteins. In the majority of cases the copine binds to a domain of the target protein that is predicted to form a characteristic coiled-coil. A consensus sequence for the coiled-coil copine-binding site was derived and found to have predictive value for identifying new copine targets. We also show that interaction with copines may result in recruitment of target proteins to membrane surfaces and regulation of the enzymatic activities of target proteins.

Calcium regulates a number of intracellular activities in stimulated cells by associating with specific motifs in calcium-binding proteins. In some cases the calcium-binding motif is embedded in an enzyme or structural protein that is directly regulated by calcium. However, in the case of the important signaling protein calmodulin, calcium first binds to calmodulin and then the calcium-calmodulin complex binds to and regulates a wide variety of “target” proteins. Copines, proteins of unknown function first described in Paramecium (1, 2), are expressed from multigene families in plants, animals, and protozoa. They bind phospholipid membranes through the action of two “C2 domains” in the N-terminal portion that are activated by calcium. The C-terminal half of the copine molecule is distantly related to the “A domain” (3) (or “I domain”) that enables the extracellular portion of integrins to bind extracellular matrix proteins. The binding of the integrin A domain to matrix proteins is dependent upon the presence of a bound magnesium or manganese ion through a mechanism that has been termed MIDAS for metal-induced adhesion site (3). Two lines of evidence have led to the hypothesis that the copine A domain may also be involved in targeted protein-protein interactions. First, critical residues involved in the chelation of Mg2+ are conserved in the copine molecule (1), and second, native copine I binds Mg2+ and Mn2+ (4). It is, however, unknown whether the copine A domain is in fact a protein-protein interaction motif. We report here, based on *in vivo* and *in vitro* evidence obtained by yeast two-hybrid screening and “pull-down” experiments using immobilized copine partners, that the copine A domain binds to a number of intracellular target proteins. We also demonstrate that full-length copine mediates the Ca2+-dependent association of target proteins with phospholipids, a phenomenon that could influence the activities and intracellular localization of the target proteins. The copines may thus comprise another pathway for calcium signaling to proteins involved in a wide range of biological activities including growth control, exocytosis, mitosis, apoptosis, gene transcription, and cytoskeletal organization.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—A mouse embryo cDNA library (5) in vector pVP16 was used in a yeast two-hybrid screen based on the Clontech GAL4 two-hybrid System 3. The A domains for human copines I, II, and IV (SER260 to the C terminus of copine I; the homologous portions of copines II and IV) were subcloned from EST clones obtained from the American Type Culture Collection into the bait vector pG-BKT7 (Clontech). Library and bait cotransformants of yeast strain AH109 (Clontech) were selected for growth on His−, Ade− medium and for development of blue color on 5-bromo-4-chloro-3-indolyl-b-p-galactopyranoside indicator plates. Approximately 8 × 10⁶ transformants were screened for interaction with copines I and II, and 1.6 × 10⁶ for copine IV. Library plasmids captured from positive clones were retransformed into AH109 transformed with the copine A domain bait vector or with the empty bait vector to identify false positives that were obtained at a frequency of about 25% of the true positives.

In Vitro Pull-down Assay—Interacting cDNAs from the two-hybrid screen were excised from pVP16 with NotI and subcloned into pGEX4T (Clontech). The GST fusion proteins were purified by binding to agarose-glutathione beads as previously described (4) omitting the solubilization in Sarkosyl. The interaction of copines with MEK1 was studied using a commercial preparation of murine MEK1-GST bound to agarose beads (Calbiochem 444952). CDNAs encoding the A domains of copines I and IV, as used in the two-hybrid screen, were subcloned into pET28a or pET30a (Novagen) and expressed in Escherichia coli as His-tagged proteins. Purification was carried out by metal chelation chromatography under denaturing conditions in 6 M urea using nickel-nitrilotriacetic acid columns (Novagen 70971-3) according to the manufacturer’s protocol. Fractions containing the purified A domains as assessed by SDS-PAGE (6) were combined and renatured by slowly removing the urea by dialysis against decreasing concentrations of urea in 50 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, and 1 mM dithiothreitol. Pull-down experiments were carried out by incubating the GST target domain fusion proteins bound to agarose-glutathione beads with the His-tagged copine A domains for 2 h on a shaker at room temperature in 130 μL of 140 mM NaCl, 10 mM Hepes-NaOH, pH 7.4, 2 mM MgCl₂, 0.1% Triton X-100. MgCl₂ was omitted from this buffer and from the dialysis buffer in experiments aimed to test the Mg2+ sensitivity of the binding. Because of the variable efficiency of binding of GST-tagged proteins to

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1 The abbreviations used are: GST, glutathione S-transferase; MIDAS, metal-induced adhesion site; MEK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.
agarose-glutathione beads, the amount of beads for each protein was adjusted between 20 and 130 μl to obtain similar amounts of protein, as judged by SDS-PAGE (roughly equivalent to a 10-μg band of bovine serum albumin). Blank beads were used when necessary to make up the volume of beads to 130 μl. The amount of copine A domain ranged from 3 to 9 μg (7). After incubation, the supernatant was withdrawn and the beads were washed three times with 1 ml of the same buffer containing no copine. Separation of beads from buffer was achieved by sedimentation at 100 × g for 1 min. Finally, proteins were eluted from the beads with electrophoresis sample buffer and loaded on SDS-PAGE gels. To reveal the presence of His-tagged proteins, gels were transferred to nitrocellulose membranes and probed with 1:1,000 commercial mouse monoclonal anti-His antibodies (Novagen 70976-3). Detection was carried out using 1:10,000 polyclonal goat anti-mouse, peroxidase-labeled antibodies (American Qualex), and a chemiluminescence kit (Pierce Supersignal). In addition to GST alone as a control (Fig. 1), two additional negative controls were used: GST fused to residues 28 to 24-kDa subunit of murine mitochondrial NADH-ubiquinone oxidoreductase (GenBankTM Q9D6J6), and GST fused to residues 28–171 of the murine protein of unknown function MGC19415 (AAH11286). These constructs generated fusion proteins that are of a size comparable with the interacting proteins but that do not bind copines.

**Protein Phosphatase 5 Activity**—Protein Phosphatase 5 was expressed and purified as a GST fusion protein as described (4), omitting solubilization in Sarkosyl, using the plasmid pET GST-PP5 (8) kindly provided by Dr. Sandra Rossie (Purdue University). The phosphatase was eluted from the agarose beads by incubation for 1 h with an equal volume of 50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione, 0.1% β-mercaptoethanol. Full-length human copine I was produced in yeast with the expression vector YepDB60 previously used for the production of recombinant annexins (9) and the protein was isolated by calcium-dependent binding to lipids (4). This recombinant protein was found to have calcium-dependent lipid binding properties indistinguishable from those of native bovine copine I (10). Phosphatase activity was measured colorimetrically by monitoring transformation of colorless p-nitrophenol phosphate into yellow p-nitrophenol (11). Assays were carried out in 200 μl of 50 mM Tris-HCl, 10 mM p-nitrophenol phosphate, 10 mM MgCl₂, 300 μM arachidonic acid, 0.1% β-mercaptoethanol, 200 μg of egg albumin, 0.5–1.8 μg of protein phosphatase 5, and 4.5–6.8 μg of copine, full-length or A domain. Reactions were started by the addition of the substrate, allowed to proceed for 15 min at room temperature, and stopped with 500 μl of 0.25 M NaOH. Mixtures were centrifuged at 20,000 × g for 1 min and the absorbance of the supernatants measured at 410 nm. Reactions were linear up to 30 min.

**Phospholipid Overlay Assay**—Overlay assays were carried out as described by Cheever et al. (12) with minor modifications. Briefly, pieces of nitrocellulose membrane were spotted with 1–μl (100 pmol) drops of phosphatidylserine dissolved in 1:2:0 chloroform:methanol:H₂O and allowed to dry for 1 h. Membranes were blocked for 1 h with 5% fatty acid-free bovine serum albumin (Sigma; A-8806) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20). All subsequent incubations and washes were carried out in TBST containing 0.5% fatty acid-free bovine serum albumin and 3 mM CaCl₂ or 5 mM EGTA. Blocked membranes were then sequentially incubated for 1 h each with: 0.5 μg/ml full-length recombinant human copine I produced in yeast, 0.01 μg/ml GST-copine target fusion protein or GST, 1:2000 commercial rabbit polyclonal anti-GST antibodies (Sigma G-7781) and 1:10,000 polyclonal goat anti-rabbit, peroxidase-labeled antibodies (American Qualex). For these experiments a full-length murine MEK1-GST fusion protein was generated in E. coli using an expression vector kindly provided by Dr. Thomas Sturgill of the University of Virginia. After each membrane was blocked for 1 h with 5% fatty acid-free bovine serum albumin (Sigma; A-8806) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20). All subsequent incubations and washes were carried out in TBST containing 0.5% fatty acid-free bovine serum albumin and 3 mM CaCl₂ or 5 mM EGTA and detection was carried out using a commercial chemiluminescence kit (Pierce Supersignal) supplemented with 3 mM CaCl₂ or 5 mM EGTA. All steps were carried out at room temperature. The GST and GST fusion proteins were prepared by the method described earlier (4). Protein phosphatase 5, and 4.5–6.8 μg of copine, full-length or A domain. Reactions were started by the addition of the substrate, allowed to proceed for 15 min at room temperature, and stopped with 500 μl of 0.25 M NaOH. Mixtures were centrifuged at 20,000 × g for 1 min and the absorbance of the supernatants measured at 410 nm. Reactions were linear up to 30 min. Overlay assays were carried out as described above.

**Derivation and Application of a Consensus Sequence for the Copine-Binding Coiled-coil Domain**—The amino acid sequences encoded by the inserts of the 11 clones obtained in the two-hybrid screen for the two-hybrid assay were aligned by the University of Wisconsin Genetics Computer Group PRETTY program (13): octamer-binding protein, radixin, bicaudal D, CDC42-binding kinase, and WTP. The Blosum62 scoring matrix was used with a gap creation penalty of 8 and a gap extension penalty of 2. The program was used to generate a consensus sequence based on a parameter of scoring of 3 of 5 similar (nonidentical) matches. The consensus sequence obtained (see “Results” and “Discussion”) was used to probe Release 72 of the Protein Information Resource of the National Biomedical Research Foundation with the FASTA program (14) with the Blosum50 scoring matrix, gap creation penalty of 12, and gap extension penalty of 2. The 20 closest matches included several of the proteins used to derive the consensus sequence (radixin, the CDC42-binding kinase, and octamer-binding protein), some known and unknown sequences homologous to these proteins, and three entries for the mitogen-activated protein kinase kinase, MEKI.

**General Methods**—SDS-polyacrylamide gels were run according to Laemmli (6). Protein concentrations were determined according to Bradford (7).

**RESULTS AND DISCUSSION**

**Identification of Copine Targets by Yeast Two-hybrid Screening**—To search for possible targets for copines we used the isolated A domains of three of the seven human copines (copines I, II, and IV) as baits in a yeast two-hybrid assay to screen a mouse embryo cDNA library. Unique classes of interacting cDNAs, summarized in Table I, were isolated for each of the three copines.

Characteristic of the library used for the screen, the isolated cDNAs are generally less than full-length (average size about 400 to 500 base pairs), of a size likely to encode only domains of the target proteins. Each copine was found to select from the library certain target cDNAs that dominated the screen. Screening with copine I repeatedly identified protein phosphatase 5 (8 of 10 isolates), copine II the NEDD8-conjugating enzyme UBC12 (4 of 10 isolates), and copine IV the stranded RNA-binding protein, octamer-binding protein (18 of 38 isolates). In addition, a complement of other specific targets for each copine was also observed as detailed in Table I. Interestingly, copine I was found to be a target of copine IV. Although it was previously demonstrated that copine I exhibits calcium-dependent self-association in vitro (4), interactions between different copines have not been previously recognized. This result may indicate that copines can function as oligomers, or that copines can regulate the activities of one another. When the cDNAs of targets interacting with each copine were tested in the two-hybrid assay using the other two copine A domains as bait, it was found that some targets would interact with only a single copine (e.g., UBC12, octamer-binding protein, and CDC42-binding kinase) whereas others were less specific (e.g., Mhc-binding protein, radixin, and E2-230K ubiquitin-conjugating enzyme).

**Binding of Copines to Target Proteins in Vitro**—The interactions between the copine A domains and their targets were further analyzed in an in vitro pull-down assay (Fig. 1). The cDNAs isolated in the two-hybrid screen were subcloned into a GST fusion expression vector to generate immobilized protein fragments to test for binding to recombinant A domains. The copine A domains were expressed in E. coli as His-tagged fusion proteins that facilitated isolation of the proteins and detection in the pull-down assay with anti-His tag antibodies. However, the copine II A domain could not be obtained in soluble form and so was not examined for in vitro binding of target proteins. Two yeast two-hybrid clones were excluded from this study (and from Table I) because one of them failed to produce a fusion protein in E. coli and the other one produced a protein that did not bind copine in vitro. All other clones obtained in the two-hybrid screen produced fusion proteins that bound to the copine I or IV A domains and are listed in Table I. Many of the target proteins bound to both copine A domains, although often with a differential affinity. Sometimes this reflected specificity that was also apparent in the yeast two-hybrid assay (Table I). However, in many cases the two-hybrid assay was more discriminating than the in vitro pull-down assay.
**Table I**

*Copine targets*

COP, copine “I”, “II”, or “IV” A domain used to identify the target cDNA in the initial two-hybrid screen. ID, GenBank™ accession number for the target cDNA. If the mouse sequence as obtained in the screen is not present in GenBank™, the accession number of the closest human homolog is given. NAME (SYNONYM), common name of the target protein (synonyms given in parentheses). ISOLATES, number of times overlapping clones were obtained for the same cDNA in the two-hybrid screen. RESIDUES, amino acid residues encoded by the cloned target cDNAs. If more than one clone was obtained, the minimum overlapping sequence is represented. DOMAIN, recognized structural domains encoded by the partial target cDNA. COILS, graphic output of the COILS program giving the probability that the target protein domain will form a coiled-coil. Probability from 0 to 1 is plotted as a function of residue number (as given in the column “RESIDUES”). Dashed horizontal line represents probability of 0.5. TWO-HYBRID INTERACTION, level of interaction seen in the two-hybrid assay with the A domains of copines I, II, or IV. Range from “−” to “+++” depending on growth rate and suppression of the pink color of ade2 mutants on adenine-deficient medium. IN VITRO INTERACTION, ability of target protein domain fused to GST to pull down the A domains of copines I or IV, ranging from “−” to “+++.”

| COP | ID       | NAME (SYNONYM)                  | ISOLATES | RESIDUES | DOMAIN | COILS                           | TWO HYBRID INTERACTION | IN VITRO INTERACTION |
|-----|----------|---------------------------------|----------|----------|--------|---------------------------------|------------------------|----------------------|
|     |          |                                 |          |          |        |                                 | I          | II       | IV       | I        | IV       |                      |
| 1   | AF018262 | Protein phosphatase 5            | 8        | 6-152    | Tetratricopeptide repeats (coiled coil) | ++         | ++       | −        | ++       | ++       |
| IV  | XP_127166| Cdc-42-binding kinase (MRCK beta)| 4        | 569-737  | Coiled coil | −         | −        | ++++     | ++       | ++       |
|     | NP_032953| MEK1 (Map kinase kinase)        | 1        | 1-393    |         | (predicted – see text)          | ++         | +        |                      |                      |
|     |          |                                 |          |          |        |                                 | I          | IV       |                      |                      |

**Regulators of protein phosphorylation**

| COP | ID       | NAME (SYNONYM)                  | ISOLATES | RESIDUES | DOMAIN | COILS                           | TWO HYBRID INTERACTION | IN VITRO INTERACTION |
|-----|----------|---------------------------------|----------|----------|--------|---------------------------------|------------------------|----------------------|
|     |          |                                 |          |          |        |                                 | I          | II       | IV       | I        | IV       |                      |
| 1   | AF075587 | Myc binding protein             | 1        | 705-888  | RCC1 repeats | +         | ++++     | ++++     | ++       | +        |
|     |          |                                 |          |          |        |                                 | I          | IV       |                      |                      |
| II  | NP_035516| SNO proto-encogene              | 1        | 51-183   | SKI/SNO domain | −         | ++       | ++       | +        | ++       |
| IV  | XP_135977| Octamer-binding protein (Paraspeckle protein; NonA (Drosophila)) | 18       | 238-360  | Coiled coil | −         | −        | ++++     | ++       | +        |
|     | AAG41429 | BeoR (HIC-1 co-repressor)       | 1        | 1432-1514| Ankyrin repeat | −         | ++       | ++++     | +        | ++       |
| IV  | CAC10188 | Human WTAP (Wilms’s tumor-1 associating protein) | 1        | 105-216  | Coiled coil | −         | −        | ++       | ++       | +        |
| COP  ID | NAME (SYNONYM) ISOLATES RESIDUES | DOMAIN | COILS | TWO HYBRID INTERACTION | IN VITRO INTERACTION |
|--------|---------------------------------|--------|-------|-----------------------|---------------------|
|        |                                 |        |       | I II IV                | I IV                |
|        |                                 |        |       |                      |                    |
| IV AAK56087 | Copine 1 | 3     | 5-172 | First C2 domain and intervening region | - - +++  ++ ++ |
|        |                                 |        |       |                      |                    |
| IV AAB38108 | ALG-2 | 1     | 71-191 |                      | - + +++  + +    |
|         | (Apoptosis linked gene 2)       |        |       |                      |                    |
|        |                                 |        |       |                      |                    |
|         | **Calcium-binding proteins**    |        |       |                      |                    |
|        |                                 |        |       |                      |                    |
|        | **Regulators of ubiquitination/NEDDylation** |        |       |                      |                    |
|        |                                 |        |       |                      |                    |
| II XM_133184 | UBC12 homolog | 4     | 24-141 |                      | - +++  -  ++ ++  |
|         | (NEDD8 conjugating enzyme)      |        |       |                      |                    |
|        |                                 |        |       |                      |                    |
| II XM_126729 | E2-230K | 1     | 842-1022 | Coiled coil          | + ++ ++  ++ +    |
|         | Ubiquitin conjugating Enzyme    |        |       |                      |                    |
|        |                                 |        |       |                      |                    |
|         | **Cytoskeleton/structural proteins and regulators** |        |       |                      |                    |
|        |                                 |        |       |                      |                    |
| I NM_009041 | Radixin | 1     | 353-428 | Coiled coil          | ++ ++ ++  + ++    |
|        |                                 |        |       |                      |                    |
| II NM_007393 | Beta actin | 3     | 241-366 |                      | + ++ ++  ++ +    |
|         | (Cytoplasmic actin)             |        |       |                      |                    |
|        |                                 |        |       |                      |                    |
| IV NP_033286 | Beta spectrin | 2     | 1873-2053 | Spectrin repeat (triple coiled coil) | - - +++  + +    |
|         | ( fodrin, brain spectrin)       |        |       |                      |                    |
|        |                                 |        |       |                      |                    |
| IV CAC51293 | Bipolar D protein | 1     | 107-249 | Coiled coil          | - - +  -  ++    |
|         | (BDCD2, dynamitin/ dynactin-binding protein) |        |       |                      |                    |
|        |                                 |        |       |                      |                    |
| IV BAA28786 | Collagen V alpha | 1     | 1223-1312 | Collagen triple helix | - +++  +++  ++ ++    |
|         |                                 |        |       |                      |                    |
To test the possibility that the interaction between the copine A domain and the target proteins may occur through the MIDAS mechanism, the sensitivity of the binding to the removal of Mg\(^{2+}\) was tested in the in vitro pull-down assay. The binding of all of the proteins listed in Table I to the copine I A domain (or copine IV A domain when this was the preferred partner) was tested in the absence of Mg\(^{2+}\) as described under "Experimental Procedures." In the case of collagen, interaction with the copine I A domain was completely abolished by the absence of Mg\(^{2+}\), whereas the rest of the target interactions were insensitive to Mg\(^{2+}\) removal. This suggests that the MIDAS mechanism may not be essential for most of the interactions we have observed. In the case of collagen the in vitro interaction may be mechanistically similar to that between the integrin A domain and collagen, for which there is a crystallographic model (15). Because collagen is the only extracellular copine target that was identified, the mechanism of binding in this case may be different from the interactions with intracellular proteins. Although the biological significance of such an interaction is unclear because copines are believed to be exclusively intracellular proteins, the interaction may be relevant during developmental or pathological events that involve cell lysis.

**Identification of a Coiled-coil Copine-binding Motif**—Examination of the sequences and inferred structural features of the target domains revealed that a majority (14 of 21) included sequences predicted to form \(\alpha\) helical coiled-coils (Table I). This is a much higher occurrence of coiled-coils than would be expected to occur randomly. Computer analyses of fully sequenced genomes indicates that 5–9% of the open reading frames are predicted to encode proteins that contain coiled-coils (16), and that only 2–3% of residues in all proteins are in regions that are predicted to form coiled-coils (17). In contrast, 34.7% of all the residues encoded by the interacting target cDNAs obtained in the two-hybrid screen described here are predicted to be in coiled-coils. Although the actual structures of most of the target proteins reported here are unknown, the N-terminal tetratricopeptide repeat domain of protein phosphatase 5, which is the binding site for copine I (see Table I), is known to form a series of antiparallel \(\alpha\) helices (18). Use of the COILS program (19) to analyze the other targets revealed that many are predicted to have regions of coiled-coil structure with

### Table 1—continued

| COP ID  | NAME (SYNONYM) ISOLATES RESIDUES | DOMAIN | COILS | TWO HYBRID INTERACTION | IN VITRO INTERACTION |
|---------|----------------------------------|--------|-------|------------------------|---------------------|
|         |                                  |        |       | I  | II  | IV  | I   | IV  |
| IV BAA6531 | KIAA1217 protein, Homolog of SNAP (SNAP-25 interacting protein) | 317-520 | Coiled coil | -  | -  | ++++ | ++ | ++ |
| IV AAF07196 | LEK1 (kinetochore binding Protein, mitosis homolog) | 1472-1602 | Coiled coil | -  | +  | ++++ | +  | +  |
| IV AAF6951 | DXl100b protein (IM1 protein) | 481-589 | Coiled coil | -  | -  | ++++ | ++ | ++ |
| IV XP_124619 | Nir3 (PYK2 N-terminal Domain-interacting receptor, Retinal degeneration protein B2) | 206-292 | Phosphatidylinositol transfer, and adjacent coiled coil | -  | -  | ++++ | ++ | ++ |
| IV BAA76853 | KIAA1009 protein (Quail homolog is expressed during neural differentiation) | 1020-1169 | Coiled coil | -  | -  | ++++ | -  | +  |
short interruptions that could represent hairpin turns (Table I). Therefore, the copines may favor interaction with antiparallel coiled-coils, a motif that is also the basis of the interaction of the small GTP-binding protein Rho with its target proteins (20). Although the coiled-coils in the copine targets may be intramolecular, it is also possible that the target proteins formed dimers in the two-hybrid assay and in the in vitro pull-down assay. However, the two-hybrid assay as employed here would not have been able to reveal potential targets that are formed by the association of different protein subunits to create coiled-coils, as occurs in G-protein βγ dimers and in soluble NSF attachment protein receptor (SNARE) complexes. More direct methods must be employed to determine whether copines may interact with such heterologous coiled-coil motifs.

Recognition of a motif that is bound by copine was found to have predictive value for identifying copine targets that were not identified in the two-hybrid screen. As detailed under “Experimental Procedures,” a template motif was determined by aligning the predicted coiled-coil motifs within the copine target fragments of octamer-binding protein, radixin, CDC42-binding kinase, bicaudal D, and WTAP and then using the PRETTY program (13) to generate a consensus sequence (E...R.R.L.E.E.Q.R.K.R.L.E.R.L.K.R...E.L.Q.L.D.E.E). Probing the National Biomedical Research Foundation Protein Information Resource data base with this sequence in the FASTA program (14) yielded among the 20 best matches several of the proteins used to generate the consensus sequence as well as MEK1. The portion of MEK1 identified by this alignment was residues 29–71 in the N-terminal domain. This portion of MEK1 is strongly predicted by the COILS program (19) to form a coiled-coil. A full-length GST fusion construct of MEK1 was bound by copine I and IV A domains in the pull-down assay (Fig. 1), verifying the predicted interaction.

Seven proteins that are not known to form coiled-coils also interacted with copine (Table I). Some of these, such as ALG2 and β-actin, do have significant α-helical content in the unidentified interaction region. Possibly the character of these helices may allow presentation of a structural motif similar to that involved in the binding of coiled-coils to copines. However, further structural and deletion analyses are needed to define these interaction sites more precisely.

Recruitment of Target Proteins to Lipids—The binding of a full-length copine to a target protein might have the important consequence that the target protein would be localized to a membrane surface in a calcium-regulated fashion by the action of the C2 domains of copine. To test this possibility the interaction of full-length copine I with several of the target proteins was examined in detail using GST fusion proteins of the target cDNAs as well as GST fusion proteins of full-length protein phosphatase 5 and full-length MEK1. The full-length copine I was found to recruit these proteins to immobilized phosphatidylinerine in a calcium-dependent manner (Fig. 2), suggesting

**Fig. 1. Binding of copine A domains to representative copine targets in vitro.** Copine target proteins fused to GST and bound to glutathione-agarose beads were incubated with His-tagged copine I and IV A domains. Beads were subsequently washed and eluted with SDS-PAGE sample buffer and the extent of interaction assessed by probing Western blots of the eluted material with anti-His tag antibodies. Panel A, Ponceau S staining of Western blot of extracts from beads with bound GST alone (GST), GST-UBC12 ubiquitin-conjugating enzyme (UBC), or GST-α collagen (COL) after incubation with the copine I A (I) or IV A domains (IV). In each case the major band is the GST target domain fusion construct. STD, molecular weight standards, masses given in kDa. Panel B, immunostaining of the membrane in panel A for copine A domains with an anti-His tag antibody detected by chemiluminescence. Copine A domains are bound to UBC12 and collagen, but not to GST alone. Panel C, immunostaining of MEK1-GST saturated beads for bound copine. The differential binding of copines I and IV to MEK1 is indicated in the header. As shown in row 1, all proteins were recruited to immobilized lipids. In the case of α-collagen (CO), recruitment required the addition of 2 mM Mg2+ to all incubations and washes (CO + Mg). Rows 2 and 3 show that no recruitment was observed with the GST portion of the molecule or in the absence of calcium. Rows 4 and 5 show that the recruitment depends on the presence of CO I PL and is not promoted by the copine I A domain (COIP A). Rows 6–8 show the phospholipid- and calcium-dependent binding of copine to nitrocellulose membranes. In these cases, incubation with copine-binding proteins was omitted and copine was detected using anti-copine I antibodies (4). Copine binding occurs only in the presence of phospholipid and calcium (row 8). Panel B shows that the recruitment of protein phosphatase 5 (PP5) by CO I PL and calcium is inhibited by the presence of increasing concentrations of CO I A domain. Labels on the right indicate the COP I A/COP I PL concentration ratio. For both panels A and B exposure time was the same for all spots located in each column.

**Table II: Activation of protein phosphatase 5 by copine.**

| Condition          | Activity (mean ± S.D., n = 3 as a % of control) |
|--------------------|-----------------------------------------------|
| No Copine          | 100 ± 1.8%                                    |
| Copine I           | 129 ± 5.7%                                    |
| Copine I A domain  | 144 ± 4.1%                                    |
| Copine IV A domain | 141 ± 3.5%                                    |

Basal phosphatase activity (100%) was 2.7 nmol/mg-min. Assay performed in the presence of 0.5 μg of phosphatase, 6.0 μg of copine I, 4.5 μg of copine I A domain, 8.8 μg of copine IV A domain. Copine preparations alone exhibited no phosphatase activity; boiling for 15 min eliminated the ability of the copine preparations to activate the phosphatase.
that copines may indeed be able to localize their targets to membrane surfaces in the cell in response to calcium fluxes. This activity was independent of whether or not the target protein contained a coiled-coil motif. The recruitment of collagen to the lipid substrate required the presence of Mg\textsuperscript{2+} (Fig. 2), reflecting the requirement for Mg\textsuperscript{2+} that was seen in the pull-down assay with the copine A domain. In general, copines bind to mixed lipid systems that contain an acidic phospholipid such as phosphatidyserine, phosphatidylinositol, or phosphatic acid (4). Copines may therefore recruit target proteins to the cytoplasmic side of the plasma membrane, as well as to a number of intracellular membrane systems that contain acidic lipids. However, recruitment to the plasma membrane could be of particular significance for protein phosphatase 5 because it has been suggested that one of its substrates may be the plasma membrane atrial natriuretic peptide receptor (21). In addition, the mitogen-activated protein kinase signaling pathway might be enhanced by recruitment of MEK1 to the complex of proteins involved in signaling from growth factor receptors at the plasma membrane.

The copine I A domain by itself was unable to promote the association of the target proteins with lipids (Fig. 2), demonstrating that this property of copine depends upon the calcium- and lipid-binding C2 domains. Interestingly, the copine A domain was able to prevent, in a dose-dependent manner, full-length copine I from recruiting target proteins to lipids (Fig. 2). It is likely that the A domain is acting in these experiments in a dominant-negative fashion by competing for the copine-binding site on the target protein and thus preventing the full-length copine from attaching to lipids through the C2 domains. The A domain construct might therefore prove to be a useful dominant-negative probe for testing the functional significance of target relocation because of copine in cellular systems.

Effects on Target Protein Enzymatic Activity—The binding of a copine to a target protein might have a direct effect on enzymatic or other functional properties of the target, independent of the effect on target localization to membranes. In the case of protein phosphatase 5, the tetracopeptide repeat domain, which is the binding site for copine (see Table 1), is known to regulate the activity of the catalytic domain (22). In addition, mutagenesis of the coiled-coil region in the N terminus of MEK1, where we speculate copine may bind, is also known to influence the catalytic activity of MEK1 (23). To determine whether copine may influence the activity of protein phosphatase 5, the activity of the phosphatase against the model substrate p-nitrophenyl phosphate was tested in the presence or absence of the copine I or IV A domains or full-length copine I. These protein constructs were all found to activate the phosphatase by 30–40% (Table II). Although this is a modest activation, it is possible that more significant activation would be seen with specific protein substrates (which are generally unknown for this phosphatase) or in the presence of different lipid activators. As these experiments were done in the absence of calcium, the result also demonstrates that the copine C2 domains do not have to be activated by calcium for copine to bind a target protein. The importance of the C2 domains might therefore be limited to their ability to attach the copine-target complex to membranes. The possibility that the activity of MEK1 is also regulated by copines will be important to examine.

CONCLUSION

Multiple copines are expressed in a given organism (1, 2), thus multiple independent and branching pathways for calcium signaling may exist within cells based on this family of proteins. Because of their ability to associate with membranes the copines may be particularly important for regulating signaling processes on membrane surfaces. With the exception of collagen, all of the copine target domains identified here are known or predicted to be on intracellular proteins or on the intracellular portions of membrane proteins. Therefore, these targets should be accessible to copines in vivo. In many cases the targets are signaling molecules themselves, including calcium-binding proteins, kinases, a phosphatase, ubiquitin (or NEDD8)-conjugating enzymes, and transcription modulators. Mutation of a copine in Arabidopsis has been shown to lead to alterations of growth patterns and apoptotic responses to stress (24, 25). Possibly this reflects alterations in calcium signaling networks based on copine-target interactions that underlie these processes.

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