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Review

Copines: a ubiquitous family of Ca^{2+}-dependent phospholipid-binding proteins

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Abstract. The copines are a novel family of ubiquitous Ca^{2+}-dependent, phospholipid-binding proteins. They contain two Ca^{2+}- and phospholipid-binding domains known as 'C2 domains' present in proteins such as protein kinase C, phospholipase C and synaptotagmin. Copines are thought to be involved in membrane-trafficking phenomena because of their phospholipid-binding properties. They may also be involved in protein-protein interactions since they contain a domain similar to the protein-binding 'A domain' of integrins. The biochemistry, gene structure, tissue distribution and possible biological roles of copines are discussed, including recent observations with *Arabidopsis* that indicate that copines may be involved in cell division and growth.

Key words. Copines; calcium; C2 domain; phospholipids; *Arabidopsis*; integrin A domain.

Discovery of copines

Copines are a family of proteins first identified in preparations of Ca^{2+}-dependent, phospholipid-binding proteins obtained from *Paramecium tetraurelia* [1]. These proteins are obtained by binding to phospholipid membranes in the presence of Ca^{2+}. Unlike similar preparations from other organisms, which consist mostly of annexins, the dominant species in *Paramecium* preparations is a single, 55-kDa band. Annexins are ubiquitous proteins thought to be involved in a host of Ca^{2+}-dependent cellular processes [2]. Early indications that the *Paramecium* protein was not an annexin or any other known protein were provided by the sequences of peptides produced by proteolytic digestion of the 55-kDa band. Further support for this observation was obtained by polymerase chain reaction (PCR) amplification of *P. tetraurelia* cDNA using primers designed according to the peptide sequences. Sequencing of the major amplification product – a 920-base pair product – showed that the 55-kDa protein contained two regions of strong similarity to the Ca^{2+}-dependent, phospholipid-binding domain known as C2, and a unique carboxy-terminal region or core domain that shows only a distant relationship to other proteins. This observation explained the biochemical properties of this protein and prompted the coinage of a name. Because of its behavior as a 'companion' of lipid membranes and the country where the initial observations were made (France), the feminine French noun 'copine' (pronounced 'ko-peen') was chosen. The feminine form was given because French nouns have genders and proteins are feminine.

Copines in nature

Proteins containing C2 domains exhibit Ca^{2+}-dependent binding to phospholipids. Some of them are enzymes such as protein kinase C (PKC) and phospholipase C (PLC), while others, such as synaptotagmin, rabphilin and Munc 13, are thought to play roles in exocytosis and membrane-trafficking phenomena [see refs 3 and 4 for reviews]. Data

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base searches with the *Paramecium* sequence showed the existence of uncharacterized sequences similar to that of copine in a host of organisms such as green plants, nematodes and humans. Analysis of human cDNA sequences indicated that there were five different human copine genes which were referred to using roman numerals as human copines I–V [1]. Since then, two additional human copines have been reported: copine VI or N-copine [5] and copine VII [6]. The degrees of identities in the known regions of overlap between copine I, the most abundant form in the expressed sequence tag (EST) databases, and the other copines are 60, 78, 53 and 56% for copine II through V, respectively. Five genes have also been characterized in *Caenorhabditis elegans*. Comparison of human copine with copines from other organisms in their region of overlap reveal 40, 40 and 33% identities for *C. elegans, Arabidopsis* and *Paramecium.* Further experimental studies of *Paramecium* cDNA showed the presence of two closely related genes for copine (CPN1 and CPN2) that are both expressed in wild-type *Paramecium.* Regarding expression of copines in humans, a quite comprehensive screen was recently carried out [7] using cDNA probes for copines I through VI to probe a 76 human tissue-type MTE array (Clontech). Copines I, II and III are expressed ubiquitously. Copine IV expression is restricted to brain, heart and prostate, whereas copine VI was brain specific as previously reported [5]. The actual content of copine proteins in human tissues has not been systematically investigated. We have carried out, however, a screening of different mouse and bovine tissues using anticopine I antibodies and found that copine I is ubiquitously expressed as detailed below. Figure 1 A shows the alignment of all seven human copine sequences obtained using data from current databases. A dendrogram illustrating the relationships between these sequences is presented in figure 1 B.

```
        1                                          *          * *   * 50
  copineI ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MAHCVT LVQLSISCDH LIDKDIGSKS
copineIII ~~~~~~~~~~ ~~~~~~~~~~ ~~~MAAQCVT KVALNVSCAN LLDKDIGSKS
 copineII ~~~~~~~~~~ ~~~~~~~~~~ ~~MGPQYCVC KVELSVSGQN LLDRDVTSKS
 copineIV MKKMSNIYE. SAANTLGIFN SP.....CLT KVELRVACKG ISDRDALSKP
copineVII ~~~MSAGSER GAAATPGGLP AP.....CAS KVELRLSCRH LLDRDPLTKS
 copineVI ~~~~~~~~~~ MSDPEMGWVP EPPTMTLGAS RVELRVSCHG LLDRDTLTKP
  copineV ~~~~~~~MEQ PEDMASLSEF DSLAGSIPAT KVEITVSCRN LLDKDMFSKS
Consensus ---------- ---------- -------C-T KVEL-VSC-- LLDRD-SKS
                                                      C
                                                  ____________________

       51 *  *          *     ***        *            **  * * 100
  copineI DPLCVLLQD. VGGGSWAELG RTERVRNCSS PEFSKTLQLE YRFETVQKLR
copineIII DPLCVLFLN. TSGQQWYEVE RTERIKNCLN PQFSKTFIID YYFEVVQKLK
 copineII DPFCVLFTE. NNG.RWIEYD RTETAINNLN PAFSKKFVLD YHFEEVQKLK
 copineIV DP.CVILKM. QSHGQWFEVD RTEVIRTCIN PVYSKLFTVD FYFEEVQRLR
copineVII DP.SVALLQ. QAQGQWVQVG RTEVVRSSLH PVFSKVFTVD YYFEEVQRLR
 copineVI HP.CVLLKL. YSDEQWVEVE RTEVLRSCSS PVFSRVLALE YFFEEKQPLQ
  copineV DPLCVMYTQG MENKQWREFG RTEVIDNTLN PDFVRKFIVD YFFEEKQNLR
Consensus DP-CVLL--- ----QW-EV- RTEV-RNCLN P-FSK-F--D Y-FEEVQ-LR
        C
                 ____________C2 DOMAIN____________________

      101*   *             ** *   * ** * *                   150
  copineI FGIYDID.NK TPELRDDDFL GGAECSLGQI VSS..QVLTL PLMLKPGKPA
copineIII FGVYDID.NK TIELSDDDFL GECECTLGQI VSS..KKLTR PLVMKTGRPA
 copineII FALFDQD.KS SMRLDEHDFL GQFSCSLGTI VSS..KKITR PLLLLNDKPA
 copineIV FEVHDISSNH N.GLKEADFL GGMECTLGQI VSQ..RKLSK S.LLKHGNTA
copineVII FEVYDTHGPS GFSCQEDDFL GGMECTLGQI VAQ..KKVTR PLLLKFGRNA
 copineVI FHVFDAE.DG ATSPRNDTFL GSTECTLGQI VSQ..TKVTK PLLLKNGKTA
  copineV FDLYDVDS.K SPDLSKHDFL GQAFCTLGEI VGSPGSRLEK PIVGIPGKKC
Consensus F-VYD-D--- ---L--DDFL G--ECTLGQI VSS---KLT- PLLLK-GK-A
          C C          C
          ____________________________________________________
```

Figure 1 A. Sequence alignment of the human copines. (*A*) The predicted amino acid sequences of the seven human copines were aligned using the PRETTY program of the Genetics Computer Group Wisconsin Package. The Consensus sequence represents residues that appear in the same position in at least four of the seven copines. Asterisks mark the positions of residues conserved in all seven copines. The positions of the C2 and A domains are marked by underlining. The residues in the C2 domains that are thought to coordinate calcium are marked with a *C*. The residues in the A domain that are thought to coordinate magnesium are marked with an *M*. The sequences for copines I, III, VI and VII were obtained from current GenBank entries for full-length proteins. The sequences for copines II, IV and V were inferred from current GenBank EST entries.

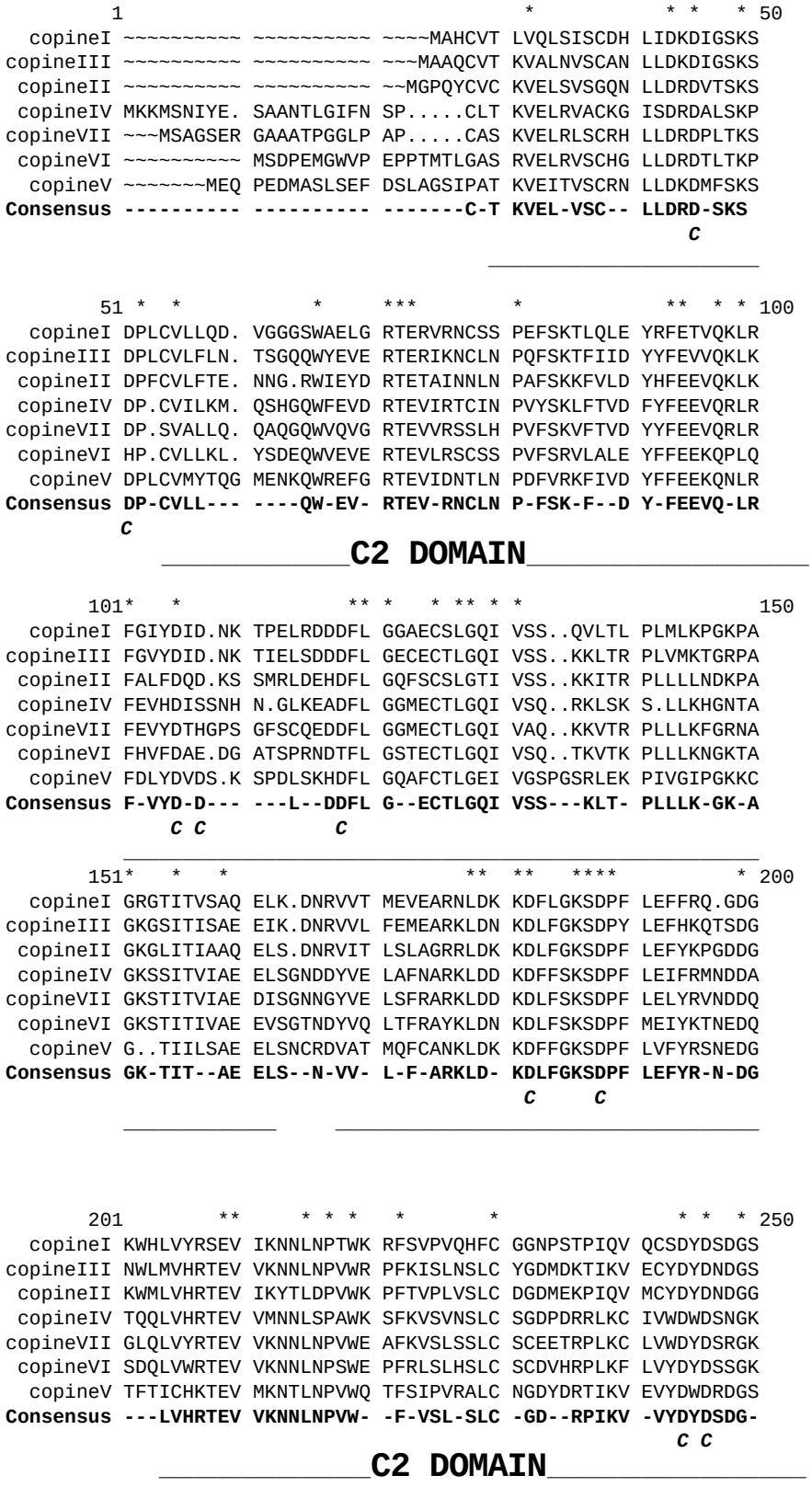

Figure 1 A (continued)

```
      401 *    *     *   *      *****  *     **  *           *450
  copineI YCAGIQGIVD AYRQALPQVR LYGPTNFAPI INHVARFAAQ AAHQGTASQY
copineIII YCNGIQGIVE AYRSCLPQIK LYGPTNFSPI INHVARFAAA ATQQQTASQY
 copineII FCSGVDGIAQ AYSACLPHIR FYGPTNFSPI VNHVARFAAQ ATQQRTATQY
 copineIV ECAGIQGVVE AYQSCLPKLQ LYGPTNIAPI IQKVAKSASE ETNTKEASQY
copineVII ECEGIQGVVE AYQNCLPRVQ LYGPTNVAPI ISKVARVAAA EESTGKASQY
 copineVI ECEEISGVIA SYRRCLPQIQ LYGPTNVAPI INRVAEPAQR EQSTGQATKY
  copineV SCCGIDGILE AYHRSLRTVQ LYGPTNFAPV VTHVARNAAA VQD...GSQY
Consensus -C-GIQGIVE AY--CLP--Q LYGPTNFAPI INHVAR-AA- ------ASQY
                               M
```

A DOMAIN

```
       451  **  ***   *   *  *  * *  ****  ****** *  *  *  ** *500
  copineI FMLLLLTDGA VTDVEATREA VVRASNLPMS VIIVGVGGAD FEAMEQLDAD
copineIII FVLLIITDGV ITDLDETRQA IVNASRLPMS IIIVGVGGAD FSAMEFLDGD
 copineII FILLIITDGV ISDMEETRHA VVQASKLPMS IIIVGVGNAD FAAMEFLDGD
 copineIV FILLILTDGV ITDMADTREA IVHASHLPMS VIIVGVGNAD FSDMQMLDGD
copineVII YILLILTDGV VTDMADTREA IVRASRLPMS IIIVGVGNAD FTDMQVLDGD
 copineVI SVLLVLTDGV VSDMAETRTA IVRASRLPMS IIIVGVGNAD FSDMRLLDGD
  copineV SVLLIITDGV ISDMAQTKEA IVNAAKLPMS IIIVGVGQAE FDAMVELDGD
Consensus F-LLILTDGV ITDMA-TREA IV-AS-LPMS IIIVGVGNAD F-AM--LDGD
          M
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```
      501        *     ******* * *               **   **** * *550
  copineI GGPLHTRSGQ AAARDIVQFV PYRRFQN...  ...APREALA QTVLAEVPTQ
copineIII GGSLRSPLGE VAIRDIVQFV PFRQFQN...  ...APKEALA QCVLAEIPQQ
 copineII SRMLRSHTGE EAARDIVQFV PFREFRN...  ...AAKETLA KAVLAELPQQ
 copineIV DGILRSPKGE PVLRDIVQFV PFRNFKH...  ...ASPAALA KSVLAEVPNQ
copineVII DGVLRSPRGE PALRDIVQFV PFRELKN...  ...ASPAALA KCVLAEVPKQ
 copineVI DGPLRCPRGV PAARDIVQFV PFRDFKD...  ...AAPSALA KCVLAEVPRQ
  copineV DVRI.SSRGK LAERDIVQFV PFRDYVDRTG NHVLSMARLA RDVLAEIPDQ
Consensus DG-LRSP-GE -A-RDIVQFV PFR-F-N---  ---A---ALA K-VLAEVP-Q
```

```
      551 * *           *                            590
  copineI LVSYFRAQGW APLKPLP..P SAKDPAQAPQ A~~~~~~~~~
copineIII VVGYFNT..Y KLLPPKN..P ATKQQKQ~~~ ~~~~~~~~~~
 copineII VVQYFK...H KNLPPTNSEP A~~~~~~~~~ ~~~~~~~~~~
 copineIV VVDYYNGKG. ..IKPK.CSS EMYESSRTLA P~~~~~~~~~
copineVII VVEYYSHRG. ..LPPRSLGV PAGEASPGCT P~~~~~~~~~
 copineVI VVEYYASQG. ..ISPGAPRP CTLATTPSPS P~~~~~~~~~
  copineV LVSYMKAQGI RPRPPPAAPT HSPSQSPART PPASPLHTHI
Consensus VV-Y-----G- ---LPP----P ---------- P---------
```

Figure 1 A (continued)

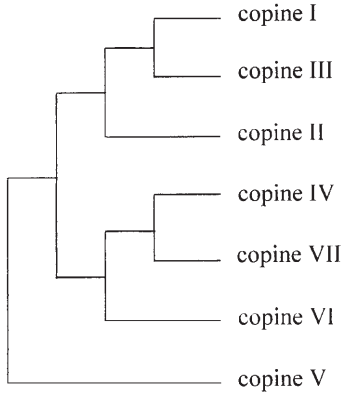

Figure 1 B. Multiple sequence alignment dendrogram for the seven human copines, as generated by the PILEUP program of the Genetics Computer Group Wisconsin Package.

Biochemistry of mammalian copine

Sequence analysis of the core domain of human copine showed that a distant similarity exists between this portion of the copine molecule and the A domain of certain integrins. Therefore, the core domain was named the 'copine A domain' and the copine prototype molecule was described as having two Ca^{2+}-dependent, phospholipid-binding domains and one A domain of unknown function. Integrins are plasma membrane proteins whose extracellular domain functions as a protein binding domain. They bind extracellular proteins or other integrins usually in Ca^{2+}-, Mg^{2+}- and/or Mn^{2+}-dependent fashion [8]. This similarity prompted the hypothesis that the copine A domain may also be a protein-binding domain. Moreover, the critical residues for Mg^{2+} chelation present in the integrin A domain are conserved in the copine A domain, further supporting this idea [1].

The uniqueness of the copine A domain makes it an ideal immunogen to produce anticopine antibodies. Confirming this prediction, polyclonal antibodies raised using a recombinant form of the human A domain of copine I as an antigen exhibited excellent specificity and allowed the identification of copine in mammalian adult organs [9]. Copine was detected as a 58-kDa band in all the rat adult organs tested: brain, heart, intestine, kidney, liver, lung, striated muscle, spleen and testis, and in bovine adrenal medulla, liver and spleen. In fact, adrenal medullary copine was observed in 1983 [10] as a member of a group of proteins that bind chromaffin granules in a Ca^{2+}-dependent manner, known as chromobindins. At that time, it was described as a 58-kDa protein with an isoelectric point of 5.8 and catalogued as 'chromobindin 17.' We recently demonstrated the identity of chromobindin 17 and copine [1]. Analysis of preparations of Ca^{2+}-dependent, phospholipid-binding proteins showed that the spleen and, to a lesser extent, the liver are the richest sources of copine. As shown in figure 2, a protocol was designed to purify to homogeneity copine from bovine spleen by binding to phospholipid vesicles, ammonium sulfate precipitation and anion exchange chromatography [9]. Purified copine from bovine spleen and liver were demonstrated to be copine I by mass spectrometry/liquid chromatography. An approximate estimation of the copine content of the bovine spleen indicates a lower limit of 1–2 µg/g of tissue.

Studies on purified copine from bovine spleen show that it retains its Ca^{2+}-dependent, phospholipid-binding activity after purification and, similar to *Paramecium* copine, binds phosphatidylserine but not phosphatidylcholine vesicles. Additional studies on lipid specificity showed that mammalian copine binds mainly negatively charged phospholipids resembling the specificity of synaptotagmin and PKC and contrasting with other C2 domain proteins such as $cPLA_2$ that prefer zwitterionic phospholipids. Interestingly, mammalian copine was observed to bind phosphatidic acid in a Ca^{2+}-independent manner which may provide a link to signal transduction [11] second-messenger mechanisms [12] mediated by phosphatidic acid. Quantitative studies showed that mammalian copine exhibits an affinity for Ca^{2+} in the micromolar range (3.16 µM $Ca^{2+} < K_{50} < 10$ µM Ca^{2+}) in the presence of phosphatidyl serine/phosphatidyl choline vesicles. Divalent cation specificity studies using similar vesicles showed that promotion of lipid binding was as follows: $Ca^{2+} > Sr^{2+} \gg Ba^{2+} > Mg^{2+} = 0$. Studies on the aggregation state of native purified mammalian copine showed that in the absence of Ca^{2+}, copine I is a monomer but in the presence of millimolar Ca^{2+} forms multimolecular aggregates. This, however, does not affect its behavior on SDS-PAGE gels. Regardless of the presence of Ca^{2+}, mammalian copine always migrates as a 58-kDa band which is very close to its calculated molecular mass of 58,928 Da.

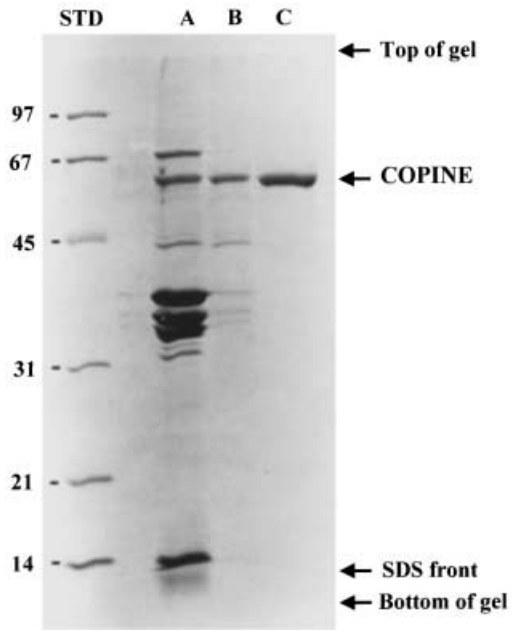

Figure 2. Identification and purification of mammalian copine. Lanes A, B and C show the analysis by SDS-PAGE of copine-containing fractions obtained from bovine spleen. Lane A corresponds to Ca^{2+}-dependent, phospholipid-binding proteins extracted from the soluble fraction of the spleen by binding to phospholipid vesicles in the presence of Ca^{2+}. Copine appears as a 58-kDa band that is a minor component (~5%) of the protein pool. Analysis by liquid chromatography/mass spectrometry indicates that this band is copine I. Most of the other proteins are annexins. Lane B shows the fraction obtained from material shown in lane A by ammonium sulfate precipitation between 35 and 52% saturation. A fairly pure preparation can be obtained by this simple method. Finally, lane C shows a more purified preparation obtained from material shown in lane B by FPLC/ion exchange chromatography. This purified protein retains its Ca^{2+}-dependent, phospholipid-binding properties after these purification steps. The STD lane corresponds to standards whose molecular masses (in kDa) are indicated on the left. The gel was stained with Coomassie blue [data from ref. 9 reproduced with permission].

The possibility that copine may exhibit properties similar to those of integrins given the similarity of its core domain with the integrin A domain was also tested using purified copine from bovine spleen [9]. In particular, binding of Mn^{2+} was explored. Mn^{2+} is a potent cofactor for ligand binding to integrins. It is usually regarded as a non-physiological substitute for Mg^{2+} although a possible physiological role may not be completely excluded. Experiments consisting of overlay of copine spots with $^{54}Mn^{2+}$ showed that copine exhibits Mn^{2+}-binding activity that can be displaced with Mg^{2+} or Ca^{2+} supporting the hypothesis that the copine A domain is a divalent cation-dependent ligand-binding site. A caveat, however, is that these experiments were carried out with the entire copine molecule and, thus the possibility that the C2 domain portion of the molecule may be responsible for the observed Mn^{2+}-binding activity cannot be entirely ruled out. Although appropriate controls using the rat synaptotagmin C2A domain showed no Mn^{2+} binding [9], it is still possible that the copine C2 domains may bind Mn^{2+} as well as Ca^{2+}. An overlay assay using recombinant copine A domain would be the crucial experiment to resolve the matter. Unfortunately, at the time that these observations were made, recombinant soluble copine was not available. Insolubility of copine constructs is a problem that we have observed in several prokaryotic expression systems. However, we have been able to express human copine I in *Saccharomyces cerevisiae* with excellent results: a recombinant protein is obtained that shows the same Ca^{2+}-dependent, phospholipid-binding properties and affinity for Ca^{2+} as native copines and protein phosphorylation [13].

Copines and protein phosphorylation

Studies on copine III have demonstrated the possibility that this member of the copine family may be a kinase [14]. Evidence is based on phosphorylation experiments with native and recombinant copine III. Studies on native copine III demonstrate that immunoprecipitates from extracts of K562 and Daudi cells obtained using anticopine III antibodies show kinase activity. In vitro assays showed phosphorylation of myelin basic protein, whereas in-gel kinase assays using the same substrate show a phosphorylated band of molecular mass similar to copine III. To further support the idea that copine III is in fact the kinase responsible for the observed phosphorylation, immunoprecipitates were purified by ion-exchange chromatography and affinity chromatography using anticopine III antibodies. Fractions from the last purification step (affinity chromatography) were silver stained, immunostained for copine III and tested for kinase activity. Similar patterns of elution were observed for copine III and kinase activity, this time measured by an in vitro assay using histone H1 as a substrate. In a related study, the activity of copine III expressed in *S. cerevisiae* as a His- and V5-tagged protein was explored. Extracts from transformed yeasts were immunoprecipitated with anti-V5 antibodies and subjected to an in vitro phosphorylation assay. Gel radioautographs show that in addition to the phosphorylation of myelin basic protein, a band of similar size to copine III also undergoes phosphorylation, suggesting that copine III may autophosphorylate. In this context, studies of the native phosphorylation state of copine III in Daudi cells show that this protein is phosphorylated on serine and threonine residues but not tyrosine residues. Finally, distribution studies by Northern blot hybridization showed that copine III was present in all organs tested although tissue expression was variable (heart > kidney > pancreas > placenta = lung > brain = liver > skeletal muscle).

Copine VI or N-copine

A sixth member of the copine family of proteins has been identified as a protein whose mRNA expression in the mouse hippocampus is upregulated by neural stimulation [5]. This phenomenon was observed in the hippocampus of animals injected with kainate and in hippocampal slices subjected for 6 h to high-frequency electric stimulation. As a result, neurons in hippocampal slices undergo long-term potentiation phenomena. Kainate-dependent upregulation was mediated by the NMDA receptor, since injection of the antagonist MK801 before kainate injection prevented copine VI upregulation. Cloning and sequencing studies show that this is a 61.7-kDa protein that, similar to other copines, possesses two C2 domains in its amino terminal. Overall identity with human copine I is 49%. Tissue distribution studies by Northern blot analysis indicate that this protein is expressed exclusively in the brain (heart, placenta, lung, liver, skeletal muscle, kidney and pancreas were the other organs tested). Accordingly, it has been named N-copine (short for neural-copine). A protein having similar distribution and molecular mass was also found in humans. Further immunohistochemical studies in the mouse brain [15] showed that copine VI is located in neurons. Specifically, copine VI is located in postsynaptic elements, i.e. dendrites and cell bodies, of the hippocampus and olfactory bulb.

A mouse protein highly similar to human OS-9 – a protein often amplified in human sarcoma cells – has been recently identified as a binding partner for copine VI [16]. This protein was detected in a yeast two-hybrid assay using a copine VI fragment (amino acids 240–428) as a bait to screen a mouse brain cDNA library, and is assumed to represent the mouse counterpart of human OS-9. In vitro studies show that the interaction between these proteins is Ca^{2+} dependent and occurs by binding of the carboxy-terminal region of OS-9 to the C2B domain of

copine VI [16]. The interaction was also confirmed to occur in vivo in cultured cells cotransfected with Flag-tagged copine VI and HA-tagged OS-9. Immnunoprecipitation of cell lysates with anti-Flag antibodies results in the coprecipitation of OS-9 as revealed by immunostaining with anti-HA antibodies. The expression of these two proteins in different anatomical regions of the human brain was analyzed to detect a possible colocalization that would further support the idea that the interaction between copine VI and OS-9 has biological meaning [16]. Results are inconclusive, since OS-9 is present in all regions tested whereas copine VI is present in most regions but almost absent in others.

Copines in plants

Studies of the effects of environmental factors on plants have led to the identification of two *Arabidopsis* mutants, *bon1* [17] and *cpn1-1* [18], with characteristic phenotypes that include impaired growth and defective repression of cell death. These defects occur only under certain environmental conditions and, most importantly, both can be traced to anomalous expression of copine genes [17, 18]. Two genes, *BON1* and *CPN1*, were identified as responsible for impaired growth at 22 °C and anomalous cell death and other defects under low-humidity conditions, respectively. We have compared these two genes and found that they are, in fact, the same gene. Because these findings were published almost simultaneously by independent groups, the identity of these two genes was not acknowledged in the original reports. Although the same gene is affected in both cases, the observed phenotypes are not identical because they were observed under different environmental conditions. The two mutants are quite likely to have similar phenotypes when tested under similar conditions. As explained below, the phenotype observed at low temperature is simpler and more dramatic and therefore seems to be more amenable to experimental exploration aimed to understand the nature of the defects and, ultimately, the biological role of copines. We summarize here the observed phenotypes and the information provided by these studies on the altered copine gene. We prefer the name *CPN1* for this gene because the name *BON1* is derived from the word 'bonzai' which stresses only the miniature phenotype observed at low temperature and therefore does not take into account the fact that other important defects, such as anomalous regulation of cell death, occur in copine mutants.

Studies on genes responsible for the ability of plants to maintain constant size and shape over a wide range of temperatures led to the identification of the *Arabidopsis* mutant *bonzai1* (*bon1*) [17]. These mutant plants show the striking feature of growing to a miniature size at the normal growth temperature of 22 °C without showing any other major deviation from the wild-type phenotype (fig. 3). At 28 °C, however, they grow normally, and their phenotype is indistinguishable from that of the wild type grown at this temperature. Microscopic examination of miniature *bon1* specimens by scanning electron microscopy shows a reduction of the size and number of epidermal cells. The primary effect is likely to be a diminished cell size, since the number of epidermal cells is related to the diameter of underlying cells. Most importantly, *bon1* mutants are unrelated to other previously identified miniature mutants and cannot be rescued by hormones such as gibberellin and brassinosteroid or mutations in hormonal signalling, indicating that the mechanism by which growth at 22 °C is affected is a novel one.

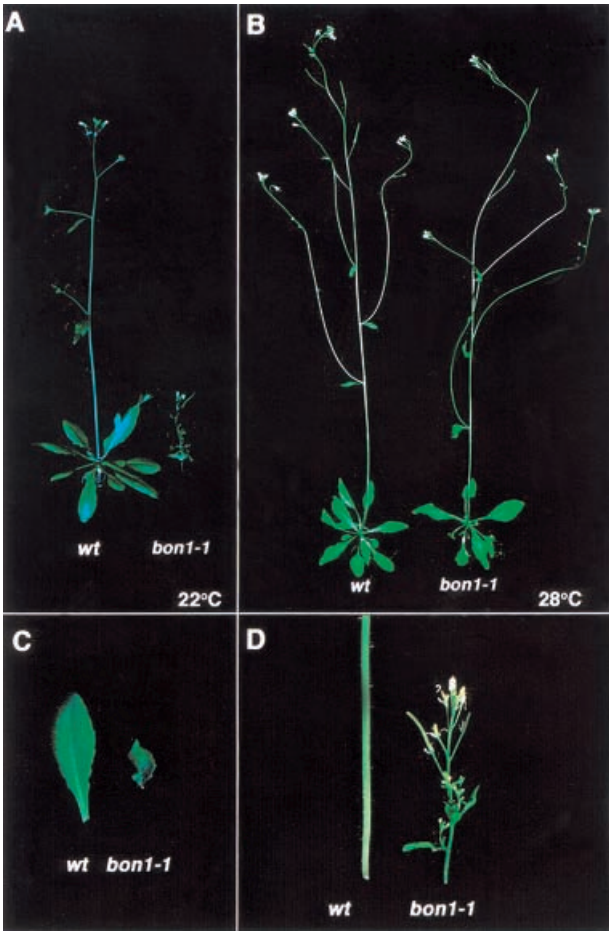

Figure 3. *Arabidopsis* mutant *bon1-1*. The phenotype of this mutant is characterized by a dramatic reduction in size when grown at 22 °C in comparison to the wild type (wt) (*A*). At 28 °C, both the *bon1* mutant and the wild type grow to similar size (*B*). At 22 °C, the leaves of the *bon1* mutant are also greatly reduced in size and present a curved morphology (*C*) and the stem is shorter and thinner (*D*). These mutants produce relatively normal flowers, are fertile and exhibit only minor changes in their development process. The growth defect of these mutants is not corrected by growth hormones or related to that of previously known mutants. Genetic studies show that the underlying cause of this phenotype is a mutation of a copine gene [data from ref. 17 reproduced with permission].

The genetic defect underlying the *bon1* phenotype was identified by characterizing *bon1* mutants produced by the T-DNA insertional mutagenesis method. A gene, *BON1*, was identified for which there are no wild-type RNA transcripts in the mutants indicating that *bon1* is a loss-of-function mutant. Studies in wild-type *Arabidopsis* show that expression of *BON1* mRNA is more intense in young, growing tissues and, as expected, is temperature dependent: expression is greatly diminished when specimens grow at 28 °C. These observations together with the dramatic impact on growth associated with the loss of *BON1* gene expression in *bon1* mutants are compelling evidence for a role for BON1 in cell division and growth. The simplest explanation to account for these phenomena is that high temperature makes BON1 no longer required for normal growth and, as a consequence, the *BON1* gene is downregulated. Adaptive mechanisms of this sort in response to temperature changes are known to occur in plants. For example, some plants develop tolerance to low temperatures by adjusting the composition of their cell membranes, in particular their lipid complement, so they remain fluid even at low temperatures. In the case of the *BON1* gene, the BON1 protein may be required at low temperatures to accelerate a process or processes that occur at normal rate at higher temperature. Membrane fusion may be a candidate process given its temperature dependence. BON1 might act either as a catalyst of membrane fusion or as a preserver of membrane function at low temperature. The diminished growth observed in *bon1* mutants at low temperature has been speculated to be due to alterations of membrane biogenesis and cell wall remodelling by exocytosis or membrane trafficking in general [17].

In a related development, a search for BON1-binding proteins by a yeast two-hybrid assay using a BON1 fragment (amino acids 319–578) as a bait to screen an *Arabidopsis* cDNA library produced a 192-amino acid protein that was named BAP1 (BON1-association protein) [17]. Although the amino-terminal region of this protein shows sequence and predicted structural homology to a C2 domain, the full-length protein is not a copine homolog. Interestingly, expression of BAP1 shows temperature dependence similar to that of BON1 and, most importantly, overexpression of BAP1 in *bon1* mutants suppresses to varying degrees the *bon1* phenotype. Studies on BAP1 may prove useful to understand the biological role of BON1 and copines in general.

A second *Arabidopsis* mutant whose genetic defect can be traced back to a copine gene has been recently reported [18]. In this case, however, the mutant phenotype consists of multiple traits that are mostly humidity sensitive, i.e. are expressed when mutants grow in low relative humidity (35–45%). Basically, this mutant, named *cpn1-1*, grows to a smaller size than the wild type and shows some malformations that are more apparent when the daily period of darkness is reduced from 16 to 8 h. Mutants also show necrotic lesions in their leaves that are presumably spontaneous since they are observed even in the absence of pathogens. This suggests that *cpn1-1* plants may have defective regulation of cell death mechanisms. To test this hypothesis, *cpn1-1* and wild-type plants were inoculated with bacteria that produce a characteristic plant defense response known as the hypersensitivity response. Mutants exhibit earlier and more intense hypersensitivity responses in comparison to wild-type plants although they are somewhat humidity independent. In addition, *cpn1-1* mutants show increased resistance to infection by *Pseudomonas syringae* and *Peronospora parasitica* and express defense-related genes constitutively, i.e. under both low and high humidity. Molecular studies based on the localization of T-DNA in *cpn1-1* mutants pointed to a gene dubbed *CPN1*. Reverse transcriptase-PCR analysis showed that *CPN1* is expressed in wild-type plants under low-humidity conditions and to a lesser extent under high-humidity conditions, whereas *cpn1-1* mutants show no expression either under low or high humidity. Similar to *bon1* mutants, the altered phenotype seems to arise from the inability of the mutant to produce the copine gene product in specific environments: low temperature and low humidity, respectively. In the case of *cpn1-1* mutants, the plethora of traits observed makes it more difficult to give a unifying explanation. The necrotic lesions seem to indicate that the CPN1 protein is a negative regulator of cell death. This hypothesis may also explain the increased hypersensitivity response of the mutants and perhaps their small size under conditions of low humidity. In this case, water and electrolyte imbalances may lead to cell damage and increased cell death due to the absence of CPN1 that results in small-size plants. The increased resistance to infection and expression of defense-related genes may be secondary to the development of systemic acquired resistance by *cpn1-1* mutants. This is a condition triggered by localized necrosis that produces enhanced resistance to a broad spectrum of pathogens. Therefore, these traits of *cpn1-1* mutants may not be directly related to CPN1 although the expression of defense-related genes under conditions of high humidity, e.g. in the absence of necrotic lesions, is difficult to explain unless a repressive effect of *CPN1* on the expression of defense-related genes is postulated [18].

In both cases, analysis of the cloned genes responsible for the phenotypes showed that they encode the same 578-amino acid, 63.1-kDa protein that shows extensive homology to the copine family of proteins including the presence of two C2 domains in the amino-terminal region and a region showing similarity to the A domain of integrins. Overall comparison of the sequences of BON1 and human copine I shows 50% identity and 67% similarity [17]. Biochemical studies [17] show that the BON1 protein binds and aggregates phosphatidylserine vesicles in

a Ca^{2+}-dependent manner as expected for a protein that belongs to the copine family.

Copine genes

In addition to the information on the biological role of copines, *Arabidopsis* studies have provided some information about the structure of the copine genes. Both *BON1* and *CPN1* have 16 exons and 15 introns. The transcription start site of *CPN1* is located 82 bp upstream of the ATG start codon and the cDNA contains a 192-bp 3′ untranslated region downstream of the TAG stop codon. The *CPN1* gene produces a single-transcript species which, as mentioned above, is expressed in wild-type *Arabidopsis* but not in *cpn1-1* mutants.

A search for tumor suppressor genes in chromosomal regions characterized by loss of heterozygosity in human tumor tissues led to the discovery of a new member of the copine family: copine VII [6]. Loss of heterozygosity is considered to be suggestive of the presence of tumor suppressor genes and in the case of sporadic breast tumors, this defect is commonly observed on the long arm of chromosome 16. A novel gene that shows homology to the copine family gene was found in region 16q24.3 of chromosome 16 and therefore named copine VII. Mutation analysis ultimately ruled out copine VII as a tumor suppressor gene, at least in the population of human tumors studied that were selected for loss of heterozygosity in the 16q24.3 region. This study has nevertheless provided information about the copine VII gene. The full-length copine VII cDNA (CPNE7) is 2654 nucleotides long and encodes a 633-amino acid protein with the prototype primary structure of copines: two C2 domains and an A domain in the carboxy-terminal end. The CPNE7 gene contains 17 exons and a polyadenylation signal (AATAAA) 17 nucleotides upstream of the poly(A). Using a probe corresponding to the 3′ untranslated region of copine VII in human tissue Northern blots, a 2.6-kb band was observed in the brain lane but not in the other tissues tested (heart, placenta, lung, liver, skeletal muscle, kidney and pancreas), and thus the distribution of copine VII appears similar to that of copine VI.

Sequencing of the human genome has revealed that the seven copine genes are not clustered: CPNE genes 1, 2, 3, 4, 5, 6 and 7 are on chromosomes 20, 16, 8, 3, 6, 14 and 16, respectively. All the human copine genes appear to have a complex intron-exon structure, but current EST data are insufficient to allow a complete, detailed analysis.

The biological role of copines

The biological role of copines is the most immediate and interesting question that can be asked about this family of proteins at this time. The widespread distribution of copines in both the animal and vegetal kingdoms and their high degree of conservation suggest that they play an important role in eukaryotic biology. The presence of C2 domains points to membrane trafficking and/or signal transduction phenomena related to Ca^{2+}. Biochemical studies show that the mechanism of membrane binding is similar to that of PKC and synaptotagmin in the sense that the copines also bind negatively charged phospholipids. Therefore, copines may be more functionally related to these proteins than to C2 domain proteins such as $cPLA_2$ that prefer neutral phospholipids. The micromolar affinity of copines for Ca^{2+} suggests that copines may interact with membranes in stimulated rather than resting cells. A key to our understanding of copines seems to be elucidating the functional role of the copine core domain. The homology between the A domain of the integrins and the copine core domain however distant is one of the few elements available for analysis. As mentioned, the binding of Mn^{2+} to native copine is not a definitive observation but shows that the A domain may be a divalent cation-dependent ligand-binding site similar to the A domain of integrins. In fact, BAP1 binds the A domain of BON1 although whether the interaction is divalent cation dependent is not known. The fact that the binding of OS-9 to the C2B domain of copine VI is Ca^{2+} dependent is an interesting observation, although anticipating the biological meaning, if any, of this interaction, is difficult.

Tissue distribution studies have not provided major clues about the biological role of copines. The localization of copine VI in neurons may indicate that it is involved in functions unique to neurons, such as neurotransmission, although this does not necessarily have to be the case. Further support for a neural role for copines is provided by the fact that the promoter of a *C. elegans* gene (ORF B0495.10, GenBank entry AAA62529) homologous to the human copine I gene is active only in eight neurons [Sanger Center Wormbase (http://www.wormbase.org/), expression pattern report Expr130]. The observation that copine is the major Ca^{2+}-dependent, phospholipid-binding protein in *Paramecium* and the relative abundance of copine I in the mammalian spleen and to a lesser extent the liver may somehow be related. The most immediate connection among these preparations seems to be that *Paramecium* is a highly phagocytic cell whereas the spleen – an organ involved in hemocatheresis or the phagocytosis of aged red blood cells – contains large numbers of phagocytic cells, and so does the liver, in the form of endothelial phagocytes or Kupffer cells. We tested for a possible relationship between phagocytic cells and copines by analyzing human neutrophils, a highly phagocytic cell type, for their copine I content [unpublished observations]. Our results show that neutrophils do in fact express copine I as revealed by the staining of Western blots of purified neutrophils with an-

ticopine I antibodies. The presence of copine in neutrophils, in this case copines I and III, was also observed in cytosolic fractions of human neutrophils purified by hydrophobic chromatography [7]. In both cases, however, it was not clear whether or not neutrophils are particularly rich in copine. Further tests on the copine content of phagocytic cells are necessary to explore a possible relationship between copines and phagocytosis.

Confirmation of the proposed kinase properties of copine III would be a major advance not only in our understanding of the biological role of copines but also in our understanding of protein phosphorylation, since copine III possesses no classic kinase domains, and copines would, therefore, be the first members of a novel kinase family. As Caudell et al. [14] have pointed out, their data indicate that '...copine III has an associated, and possibly intrinsic, kinase activity', i.e. that copine III may in fact not be a kinase. The fact that cell extracts immunoprecipitated with anticopine III antibodies show kinase activity is an indication that copine III may be a kinase or, at least, that it coprecipitates with a kinase. Both possibilities are interesting since association of copine III with a kinase may also be an indication for a role of copines in protein phosphorylation. In-gel kinase assays show that the kinase has a molecular mass similar to copine III, thus supporting the idea that copine III is itself the kinase unless both the kinase and copine III happen to have the same molecular mass or, more unlikely, they are not dissociated by treatment with SDS. Further purification of immunoprecipitates by successive ion exchange and affinity chromatography using anticopine III antibodies showed that copine III and kinase activity both elute simultaneously from the affinity column. However, as Caudell et al. [14] pointed out, the impurity of the fractions makes less certain the conclusion that copine III is the kinase in the fraction and, therefore, more studies are required to demonstrate the kinase activity of copine III. A corroboratory experiment showing that a fraction obtained by immunoprecipitation of recombinant copine III from yeast also exhibits kinase activity is also suggestive but is subject to the same caveats. We have explored a potential kinase activity of purified native copine I by binding to azido-[α-^{32}ATP] [1] and ATP-agarose columns and by an in vitro phosphorylation assay similar to that reported by Caudell et al. [14] using both histone H1 and myelin basic protein as substrates [unpublished observations]. In all cases we obtained negative results. Our observations of course do not rule out the possibility that copine may be a kinase. They may simply mean that assays were not carried out under the optimal conditions. In this connection, a promising line of investigation would be further analysis of the immunoprecipates obtained by Caudell et al. [14] which, unlike our copine I preparations, do show kinase activity. Ideally, two or more methods of purification based on different physicochemical principles should be used to obtain more purified samples and show that copine III and the kinase activity cannot be separated. In conclusion, evidence presented by Caudell et al. [14] strongly suggests the possibility that copine III may be a kinase but does not provide enough information to decide whether copine III itself or an associated kinase or a contaminant is the actual kinase.

The upregulation of copine VI in the hippocampus after electric stimulation or treatment with kainate is certainly an interesting phenomenon that deserves further exploration. Electric stimulation of hippocampal slices produced not only copine VI upregulation but, as expected, long-term potentiation phenomena as demonstrated by extracellular recording of the pyramidal cell layer of the CA1 region. As a result, it has been speculated that copine VI may be involved in long-term potentiation [5]. This may certainly be the case although another possibility is that upregulation of copine VI may be just an epiphenomenon of long-term potentiation, i.e. both are the result of electric stimulation but they are not causally related. If a causal relation does in fact exist, whether upregulation of copine VI is a causal factor or a consequence of long-term potentiation is not yet clear. There has also been speculation that copine VI may be involved in synaptic plasticity and memory formation because of its relation to long-term potentiation [5, 15]. Because the relationship between higher brain functions and cellular phenomena such as long-term potentiation is not as straightforward as once thought [19], a possible involvement of copine VI in higher brain functions is at this time most uncertain.

Without a doubt, the most exciting observations concerning the biological role of copines are the *Arabidopsis* mutants described by the groups of Fink and McNellis [17, 18]. As mentioned above, by sequence comparison, we found that the same gene is responsible for both mutants, a fact of which the authors were unaware at the time of the almost simultaneous publication of the reports. The fact that the same copine gene is responsible for the occurrence of both mutants further supports the idea that copines play an important role in eukaryotic biology. Further studies exploring the nature of the defects exhibited by these mutants as well as a search for new traits under more diverse environmental conditions are certainly warranted. So far, the phenotype observed at low temperature appears as very promising because it is so dramatic and at the same time so simple that further studies of the mechanisms of impaired growth at low temperature will probably reveal important information about the biological role of copines. The humidity-sensitive phenotype is no less important, although changes under this condition are somewhat more complex and thus less amenable to exploration. As the authors have pointed out, the primary phenomenon appears to be a lack of negative regulation of cell death mechanisms. As mentioned above, a third avenue would be to look for additional phenotypes under

a broader range of environmental conditions. New phenotypes may help in understanding the nature of the already observed temperature- and humidity-dependent changes or may point to new defects with perhaps an even more obvious relationship to altered cellular processes. A less promising aspect of the study of these mutants is the possibility that findings, however relevant to plant physiology, cannot be easily extrapolated to other species, in particular mammals. Or perhaps the affected cellular processes are unique to plants. In any event, these are very important lines of research and should be pursued further.

1 Creutz C. E., Tomsig J. L., Snyder S. L., Gautier M. C., Skouri F., Beisson J. et al. (1998) The copines, a novel class of C2 domain-containing, calcium-dependent, phospholipid-binding proteins conserved from *Paramecium* to humans. J. Biol. Chem. **273:** 1393–1402

2 Gerke V. and Moss S. E. (2002) Annexins: from structure to function. Physiol. Rev. **82:** 331–371

3 Nalefski E. A. and Falke J. J. (1996) The C2 domain calcium-binding motif: structural and functional diversity. Prot. Sci. **5:** 2375–2390

4 Rizo J. and Südhof T. C. (1998) C2-domains, structure and function of a universal Ca^{2+}-binding domain. J. Biol. Chem. **273:** 15879–15882

5 Nakayama T., Yaoi T., Yasui M. and Kuwajima G. (1998) N-copine: a novel C2-domain-containing protein with neuronal activity-regulated expression. FEBS Lett. **428:** 80–84

6 Savino M., d'Apolito M., Centra M., Beerendonk H. M. van, Cleton-Jansen A. M., Whitmore S. A. et al. (1999) Characterization of copine VII, a new member of the copine family, and its exclusion as a candidate in sporadic breast cancers with loss of heterozygosity at 16q24.3. Genomics **61:** 219–226

7 Carter D. J., Cowland J. B., Johnsen A. H., Demant E. J. F., Borregaard N. and Lollike K. (2000) Expression profiles of copines, a novel family of C2-domain proteins, and identification of two members in the cytosol of human neutrophils. Sixth European Symposium on Calcium Binding Proteins in Normal and Transformed Cells, Paris, June 14–17

8 Michishita M., Videm V. and Arnout M. A. (1993) A novel divalent cation-binding site in the A domain of the beta 2 integrin CR3 (CD11b/CD18) is essential for ligand binding. Cell **72:** 857–867

9 Tomsig J. L. and Creutz C. E. (2000) Biochemical characterization of copine: a ubiquitous Ca^{2+}-dependent, phospholipid binding protein. Biochemistry **39:** 16163–16175

10 Creutz C. E., Dowling L. G., Sando J. J., Villar-Palasi C., Whipple J. H. and Zaks W. J. (1983) Characterization of the chromobindins: soluble proteins that bind to the chromaffin granule membrane in the presence of Ca^{2+}. J. Biol. Chem. **258:** 14664–14670

11 Waggoner D. W., Xu J., Singh I., Jasinska R., Zhang Q. and Brindley D. N. (1999) Structural organization of mammalian lipid phosphate phosphatases: implications for signal transduction. Biochim. Biophys. Acta **1439:** 299–316

12 English D., Cui Y. and Siddiqui R. A. (1996) Messenger functions of phosphatidic acid. Chem. Phys. Lipids **80:** 117–132

13 Tomsig J. L. and Creutz C. E. (2000) Purification and biochemical characterization of native and recombinant copine. Sixth European Symposium on Calcium Binding Proteins in Normal and Transformed Cells, Paris, June 14–17

14 Caudell E. G., Caudell J. J., Tang C. H., Yu T. K., Frederick M. J. and Grimm E. A. (2000) Characterization of copine III as a phosphoprotein with associated kinase activity. Biochemistry **39:** 13034–13043

15 Nakayama T., Yaoi T. and Kuwajima G. (1999) Localization and subcellular distribution of N-copine in mouse brain. J. Neurochem. **72:** 373–379

16 Nakayama T., Yaoi T., Kuwajima G., Yoshie O. and Sakata T. (1999) Ca^{2+}-dependent interaction of N-copine, a member of the two C2 domain protein family, with OS-9, the product of a gene frequently amplified in osteosarcoma. FEBS Lett. **453:** 77–80

17 Hua J., Grisafi P., Cheng S. H. and Fink G. R. (2001) Plant growth homeostasis is controlled by the *Arabidopsis* BON1 and BAP1 genes. Genes Dev. **15:** 2263–2272

18 Jambunathan N., Siani J. M. and McNellis T. W. (2001) A humidity-sensitive *Arabidopsis* copine mutant exhibits precocious cell death and increased disease resistance. Plant Cell **13:** 2225–2240

19 Shors T. J. and Matzel L. D. (1997) Long-term potentiation: what's learning got to do with it? Behav. Brain Sci. **20:** 597–614