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

Takashi Nakayama[a,*], Takeshi Yaoi[b], Goro Kuwajima[b], Osamu Yoshie[a], Tsuneaki Sakata[b]

[a] *Department of Bacteriology, Kinki University school of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan*
[b] *Shionogi Institute for Medical Science, 2-5-1 Mishima, Settsu-shi, Osaka 566-0022, Japan*

Received 15 February 1999; received in revised form 6 May 1999

Abstract N-copine is a novel two C2 domain protein that shows Ca^{2+}-dependent phospholipid binding and membrane association. By using yeast two-hybrid assays, we identified OS-9 as a protein capable of interacting with N-copine. We further revealed that the second C2 domain of N-copine bound with the carboxy-terminal region of OS-9. Their interaction in vivo was also confirmed by co-immunoprecipitation from 293E cells co-expressing transfected N-copine and OS-9. In vitro binding assays showed that this interaction was Ca^{2+}-dependent. By Northern blot analysis, N-copine and OS-9 were co-expressed in the same regions of human brain. These results reveal that OS-9 is a potential target of N-copine.

© 1999 Federation of European Biochemical Societies.

Key words: N-copine; OS-9; C2 domain; Phospholipid binding; Yeast two-hybrid system

1. Introduction

The C2 domain was originally identified as a Ca^{2+}-binding domain of protein kinase C-γ (PKC-γ) [1]. Its function now includes Ca^{2+}-dependent and Ca^{2+}-independent phospholipid-binding, inositol polyphosphate binding, Ca^{2+} binding, and interaction with other proteins [2–7]. Several proteins, such as synaptotagmin, rabphilin-3A [8] and Doc2 [9,10], have two C2 domains and constitute a family designated the 'double C2 domain protein family' [11]. Members of the 'double C2 domain proteins' are known to be involved in the transport of synaptic vesicles during neurotransmitter release in neurons [12,13].

Copines constitute a novel family of Ca^{2+}-dependent phospholipid-binding proteins conserved from *Paramecium* to human [14]. In spite of the existence of two C2 domains, copines do not belong to the 'double C2 domain protein family'. Recently, we identified a novel, brain-specific member of the copine family, N-copine, and suggested that it is involved in the synaptic plasticity as a cytoplasmic Ca^{2+} sensor [15,16]. Since the localization of N-copine differs from that of 'double C2 domain proteins' such as synaptotagmin and rabphilin, N-copine must have different functions from those of 'double C2 domain proteins' [16]. However, functions of N-copine and the copine family proteins are mostly unknown yet.

As a first step to elucidate the function of N-copine, we searched for proteins interacting with N-copine by using the yeast two-hybrid system. Here, we report that OS-9, a gene frequently amplified in human sarcoma [17], is capable of interacting with the second C2 domain of N-copine through its carboxy-terminal region in a Ca^{2+}-dependent manner.

2. Materials and methods

2.1. Two-hybrid assay

The cDNA fragment corresponding to amino acids (aa) 240–428 of mouse N-copine was subcloned into pLexA (pLexA-N-copine). The yeast strain EGY48 that contains a reporter plasmid, p8op-lacZ, was transformed with pLexA-N-copine and a mouse cDNA library constructed in pB42AD (Clontech). Transformants were grown on selection plates lacking leucine. Leu^+ colonies were transferred to an X-gal indicator filter for β-galactosidase activity. Plasmids were recovered from Leu^+ and $lacZ^+$ clones through *Escherichia coli* transformation. The recovered plasmids were retransformed into EGY48 containing p8op-lacZ and pLexA-N-copine to confirm the Leu^+ and $lacZ^+$ phenotype. A clone (pB42AD-OS-9) containing the carboxy-terminal region of OS-9 was isolated. To determine the region of N-copine involved in binding with OS-9, the cDNA fragments encoding subregions of N-copine (aa 1–161, 1–270, 141–270, 141–428, 240–428, and 310–428) were amplified by PCR and subcloned into pLexA. After co-transformation with pB42AD-OS-9 into the yeast strain EGY48, β-galactosidase activity was determined by filter assays.

2.2. Co-immunoprecipitation

A DNA fragment encoding the Flag epitope (DYKDDDDK) or the HA epitope (YPYDVPDYA) with the methionine codon was inserted into the *Xba*I site of pEFBOS [18] to generate pEFBOS-Flag and pEFBOS-HA. The cDNA fragments of mouse N-copine (aa residues 1–161, 1–557, or 310–557) were amplified by PCR and subcloned into pEFBOS-Flag. The cDNA fragment encoding the carboxy-terminal region of human OS-9 (aa 536–667) was subcloned into pEFBOS- HA. 293E cells (2×10^6) were transfected with various combinations of expression plasmids (5 μg each) by using Lipofectamine Plus reagent (Gibco BRL). After 48 h, cells were lysed in 600 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% Nonidet P-40) and immunoprecipitated with anti-Flag antibody (Kodak). After washing five times with cold lysis buffer, immunoprecipitates were analyzed by immunoblotting using anti-hemagglutinin (anti-HA) (Boehringer Mannheim).

2.3. In vitro binding assay

The cDNA fragments corresponding to two regions of mouse N-copine (C2A region, aa residues 4–161; C2B region, aa residues 141–270) were amplified by PCR and subcloned into pGEX3T-3 to express as glutathione *S*-transferase (GST) fusion proteins (Pharmacia). The GST fusion proteins (GST-C2A, GST-C2B) were produced in *E. coli* (DH5α) upon IPTG induction and affinity-purified with glutathione-Sepharose 4B beads following the instruction manual coming with pGEX3T-3. The cDNA fragment encoding the carboxy-terminal region of human OS-9 (aa 536–667) was subcloned into pRSET-A (In-

*Corresponding author. Fax: (81) (723) -67-3606.
E-mail: nakayama@med.kindai.ac.jp

Abbreviations: PKC-γ, protein kinase C-γ; Leu, leucine; HA, hemagglutinin; GST, glutathione *S*-transferase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

0014-5793/99/$20.00 © 1999 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(99)00700-0

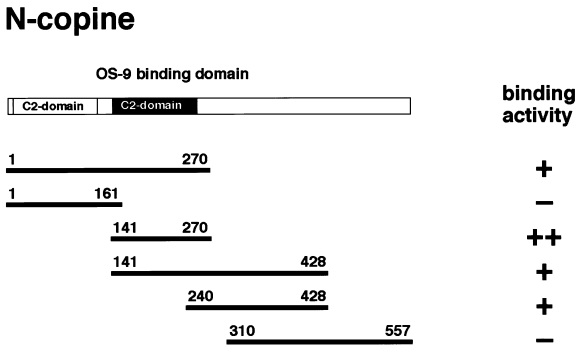

Fig. 1. Binding of N-copine and OS-9 in the yeast two-hybrid system. The domain structure of N-copine is depicted with the relative locations of the C2 domains and the OS-9 binding domain. Various truncated N-copines were used to determine the OS-9-binding domain. Binding activities of truncated N-copines and OS-9 were examined by β-galactosidase assay. The number of plus signs corresponds to blue color intensity on the X-gal indicator filter.

vitrogen). The carboxy-terminal region of OS-9 labeled with [^{35}S]methionine was prepared using TNT T7-coupled reticulocyte lysate system (Promega). GST-C2A or GST-C2B (4 μg) was immobilized onto 20 μl of glutathione-Sepharose 4B beads. The beads were suspended in 500 μl of buffer A (150 mM NaCl and 50 mM HEPES, pH 7.4) containing the [^{35}S]methionine-labeled carboxy-terminal region of OS-9 and gently mixed at 4°C for 4 h in the presence of 1 mM $CaCl_2$ or 1 mM EGTA. The beads were washed four times with buffer A and bound proteins were eluted with 40 μl of buffer A containing 20 mM glutathione. The eluates were subjected to SDS-PAGE and autoradiography.

2.4. Northern blot analysis

Multi Tissue Northern Blot membrane (Clontech) was hybridized with ^{32}P-labeled DNA probe corresponding to nucleotides 509–2019 of human N-copine cDNA. The same membrane was reprobed with the cDNA corresponding to nucleotides 1690–2785 of human OS-9 cDNA.

3. Results

Using the yeast two-hybrid system, we searched for proteins from a mouse brain cDNA library that were capable of binding to the region of aa 240–428 of N-copine. Among 1×10^7 total yeast transformants, 28 clones showed Leu$^+$ and lacZ$^+$. The cDNA derived from one of them encoded an amino acid sequence with 90% identity to the carboxy-terminal region (aa 536–667) of human OS-9, which was originally identified as a gene frequently amplified in human osteosarcoma [17]. Therefore, we conclude that the isolated cDNA encodes a part of the mouse counterpart of human OS-9 (GenBank accession number U41635). However, the binding activity appeared to be rather weak as indicated by the β-galactosidase assay. Therefore, we further examined other regions of N-copine for binding with the carboxy-terminal region of mouse OS-9 by the yeast two-hybrid system. As shown in Fig. 1, the second C2 domain of N-copine had a strong binding activity. Furthermore, all the fragments containing the second C2 domain were positive. Neither the first C2 domain nor the carboxy-terminal region of N-copine had such binding activities. From these results, we conclude that the second C2 domain of N-copine binds to the carboxy-terminal region of OS-9.

To confirm the binding between N-copine and OS-9 in vivo, 293E cells were transiently co-transfected with Flag-tagged N-copine or its subregions and the HA-tagged carboxy-terminal region of OS-9. The N-copine proteins were immunoprecipitated from the cell lysates with anti-Flag antibody, and immunoprecipitates were analyzed by immunoblot using anti-HA antibody. As shown in Fig. 2, HA-tagged OS-9 was co-immunoprecipitated with Flag-tagged N-copine. In contrast, the first C2 domain or the carboxy-terminal region of N-copine did not co-precipitate OS-9. These results confirm that N-copine, most probably through the second C2 domain, physically interacts with OS-9 in mammalian cells.

Next, we wished to test whether the binding between the second C2 domain of N-copine and the carboxy-terminal region of OS-9 was Ca^{2+}-dependent. We prepared fusion proteins by coupling the first or the second C2 domain of N-copine with GST and immobilized them by glutathione-Sepharose beads. The beads were incubated with the carboxy-terminal region of OS-9 labeled with [^{35}S]methionine in the presence or absence of Ca^{2+}. Bound proteins were eluted with glutathione and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3, the carboxy-terminal region of OS-9

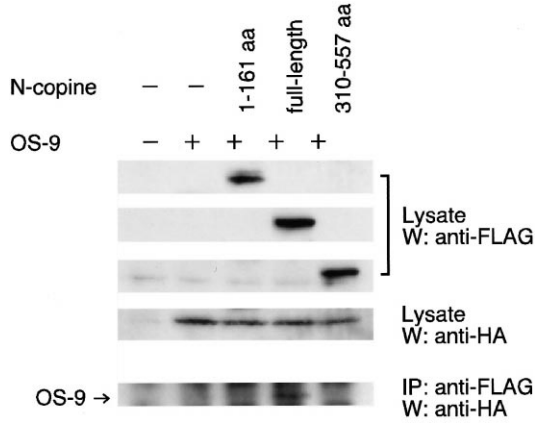

Fig. 2. Interaction of N-copine and OS-9 in vivo. 293E cells were transiently transfected with expression vectors for the indicated tagged proteins. The cell lysates were subjected to immunoprecipitation with anti-Flag antibody. Immunoprecipitates and the whole cell lysates were immunoblotted with anti-Flag or anti-HA antibody. IP, immunoprecipitation; W, Western blotting.

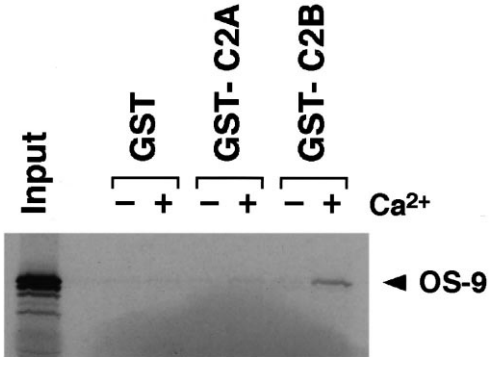

Fig. 3. Ca^{2+}-dependent binding of N-copine and OS-9. GST or GST fused with the first or the second C2 domain of N-copine was immobilized on glutathione-Sepharose beads and incubated with the [^{35}S]methionine-labeled carboxy-terminal region of OS-9 (aa 536–667) in the presence or absence of Ca^{2+}. Bound proteins were eluted with glutathione and subjected to SDS-PAGE followed by autoradiography. Input shows the in vitro translated products of the carboxy-terminal region of OS-9.

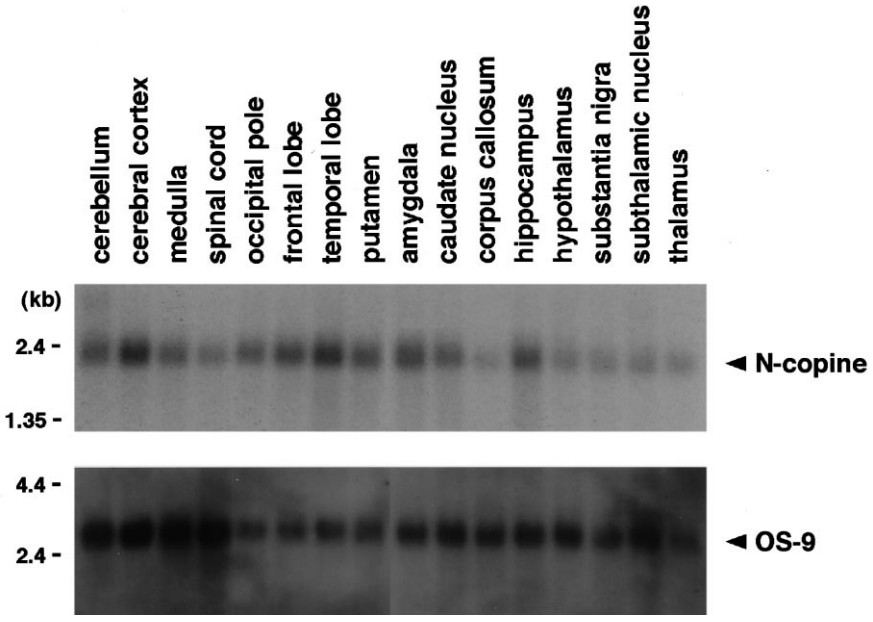

Fig. 4. Expression of N-copine and OS-9 mRNA in human brain. Multi-tissue Northern blot membrane of various regions of human brain was hybridized with ^{32}P-labeled N-copine (upper panel) or OS-9 (lower panel). Positions of N-copine (2.2 kb) and OS-9 (2.8 kb) mRNAs are indicated. Positions of RNA markers are shown on the left.

bound to the second C2 domain of N-copine only in the presence of Ca^{2+}. No binding was seen without Ca^{2+}. Furthermore, the carboxy-terminal region of OS-9 did not bind to the first C2 domain of N-copine even in the presence of Ca^{2+}. Thus, the second C2 domain of N-copine shows a Ca^{2+}-dependent binding to the carboxy-terminal region of OS-9.

Previously, we have shown that the expression of N-copine mRNA is brain-specific both in mouse and human [15]. Therefore, we examined the expression of OS-9 and N-copine mRNA in various regions of human brain by Northern blot analysis. The N-copine-specific 2.2-kb transcript was detected in all regions of human brain (Fig. 4). The OS-9-specific 2.8-kb transcript was also expressed ubiquitously in human brain. These results show that N-copine and OS-9 mRNAs are co-expressed in human brain.

4. Discussion

N-copine is a novel brain-specific two C2 domain protein and a member of a copine family which shows Ca^{2+}-dependent phospholipid binding [15,16]. The C2 domain was originally identified as a Ca^{2+}-binding domain of PKC-γ [1]. Its function now includes Ca^{2+}-dependent and Ca^{2+}-independent phospholipid binding, inositol polyphosphate binding, Ca^{2+} binding, and interaction with other proteins [2–7]. Here, we show that the second C2 domain of N-copine interacts with the carboxy-terminal region of OS-9 in the presence of Ca^{2+}. Furthermore, N-copine and OS-9 are co-expressed in the same regions of human brain. N-copine shows Ca^{2+}-dependent phospholipid binding and is localized in the membrane in the presence of Ca^{2+} [16]. Therefore, N-copine may recruit OS-9 at the plasma membrane in the presence of Ca^{2+}. The N-copine gene was first identified as a gene whose expression was up-regulated by neuronal activity such as kainate stimulation and tetanus stimulation that evoke hippocampal CA1 long-term potentiation [15]. This suggests that N-copine is involved in the synaptic plasticity in CNS. Therefore, N-copine and OS-9 may play a role in a Ca^{2+}-dependent step of synaptic plasticity.

Even though N-copine is specifically expressed in the brain, other members of the copine family are expressed ubiquitously. Furthermore, the two C2 domains are well conserved in all copine members. On the other hand, OS-9 mRNA has been shown to be expressed in various human tissues [19]. Therefore, OS-9 may interact not only with N-copine in the brain but also with other copine family proteins in other tissues. OS-9 was identified as a gene frequently amplified in human sarcoma [17]. However, little is known about its function. Also the role of the copine family proteins are mostly unclear. The Ca^{2+}-dependent binding of N-copine and OS-9 may give us a clue to clarify their biological functions.

Acknowledgements: We are grateful to Dr. Masakazu Hatanaka for encouragement of this work. We thank Harukazu Suzuki, Keiko Matsuda, and Satoshi Kojima, and Toshio Imai for helpful discussion.

References

[1] Nishizuka, Y. (1988) Nature 334, 661–665.
[2] Fukuda, M., Aruga, J., Niinobe, M., Aimoto, S. and Mikoshiba, K. (1994) J. Biol. Chem. 269, 29206–29211.
[3] Sheng, Z.H., Rettig, J., Takahashi, M. and Catterall, W.A. (1994) Neuron 13, 1303–1313.
[4] Ullrich, B., Li, C., Zhang, J.Z., McMahon, H., Anderson, R.G., Geppert, M. and Südhof, T.C. (1994) Neuron 13, 1281–1291.
[5] Chapman, E.R., Hanson, P.I., An, S. and Jahn, R. (1995) J. Biol. Chem. 270, 23667–23671.
[6] Sugita, S., Hata, Y. and Südhof, T.C. (1996) J. Biol. Chem. 271, 1262–1265.
[7] Schiavo, G., Stenbeck, G., Rothman, J.E. and Sollner, T.H. (1997) Proc. Natl. Acad. Sci. USA 94, 997–1001.
[8] Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M. and Takai, Y. (1993) Mol. Cell. Biol. 13, 2061–2068.
[9] Orita, S., Sasaki, T., Naito, A., Komuro, R., Ohtsuka, T., Maeda, M., Suzuki, H., Igarashi, H. and Takai, Y. (1995) Biochem. Biophys. Res. Commun. 206, 439–448.

[10] Sakaguchi, G., Orita, S., Maeda, M., Igarashi, H. and Takai, Y. (1995) Biochem. Biophys. Res. Commun. 217, 1053–1061.
[11] Südhof, T.C. and Rizo, J. (1996) Neuron 17, 379–388.
[12] Geppert, M., Goda, Y., Hammer, R.E., Li, C., Rosahl, T.W., Stevens, C.F. and Südhof, T.C. (1994) Cell 79, 717–727.
[13] Orita, S., Sasaki, T., Komuro, R., Sakaguchi, G., Maeda, M., Igarashi, H. and Takai, Y. (1996) J. Biol. Chem. 271, 7257–7260.
[14] Creutz, C.E., Tomsig, J.L., Snyder, S.L., Gautier, M.C., Skouri, F., Beisson, J. and Cohen, J. (1998) J. Biol. Chem. 273, 1393–1402.
[15] Nakayama, T., Yaoi, T., Yasui, M. and Kuwajima, G. (1998) FEBS Lett. 428, 80–84.
[16] Nakayama, T., Yaoi, T. and Kuwajima, G. (1999) J. Neurochem. 72, 373–379.
[17] Elkahloun, A.G., Krizman, D.B., Wang, Z., Hofmann, T.A., Roe, B. and Meltzer, P.S. (1997) Genomics 42, 295–301.
[18] Mizushima, S. and Nagata, S. (1990) Nucleic Acids Res. 18, 5322.
[19] Su, Y.A., Hutter, C.M., Trent, J.M. and Meltzer, P.S. (1996) Mol. Carcinogen. 15, 270–275.