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IDENTIFICATION AS β-ADDUCIN OF A PROTEIN INTERACTING WITH RABPHILIN-3A IN THE PRESENCE OF Ca^{2+} AND PHOSPHATIDYLSERINE[1]

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Summary: Rabphilin-3A is a putative target protein for *Rab*3A small GTP-binding protein implicated in neurotransmitter release. We have previously identified a Rabphilin-3A-interacting protein with a Mr of about 115 kDa in bovine brain. We have attempted here to purify this protein and to determine its primary structure. Amino acid sequence analysis has revealed that this protein is a bovine counterpart of human β-adducin which is known to be a good substrate for protein kinase C. The Rabphilin-3A-interacting protein also binds to protein kinase C in the presence of Ca^{2+} and phosphatidylserine. These results indicate that Rabphilin-3A binds to β-adducin in the presence of Ca^{2+} and phosphatidylserine.

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The abbreviations used are: G protein, GTP-binding protein; GDI, GDP dissociation inhibitor; GRF, guanine nucleotide-releasing factor; GAP, GTPase-activating protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TNMD, 20 mM Tris/HCl at pH 7.9, 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol.

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*Rab*3A small G protein, a member of the *Rab* family, is implicated in regulated secretion, particularly in neurotransmitter release (for reviews, see Refs. 1-3). Although the definitive role of *Rab*3A has not yet been clarified, available evidence suggests that *Rab*3A increases the efficiency of translocation and docking of synaptic vesicles to the presynaptic plasma membrane (4-7).

Rabphilin-3A is a putative target protein for this *Rab*3A (8,9). Rabphilin-3A is a single polypeptide with a calculated Mr of 77,967 having two functionally different domains: the N-terminal GTP-*Rab*3A-binding domain and the C-terminal two repeated C_2-like domains interacting with Ca^{2+} and phospholipid, particularly phosphatidylserine (10). Rabphilin-3A is a peripheral membrane protein of synaptic vesicles (11). Our current working model for the modes of action of *Rab*3A and Rabphilin-3A in neurotransmitter release is as follows: GDP-*Rab*3A complexed with *Rab* GDI stays in the cytosol of the presynapse (12-14). GDP-*Rab*3A dissociates from this complex and is converted to GTP-*Rab*3A by the action of a GDP/GTP exchange protein, such as *Rab*3A GRF and MSS4 (15-17). GTP-*Rab*3A binds to Rabphilin-3A on synaptic vesicles, resulting in translocation of the vesicles to the presynaptic plasma membrane. The *Rab*3A-Rabphilin-3A complex binds to a putative acceptor protein on the plasma membrane and the vesicles dock to the plasma membrane. After the Ca^{2+}-influx causes fusion of the vesicles to the plasma membrane, GTP-*Rab*3A is converted to GDP-*Rab*3A by the action of *Rab*3A GAP (18). Then, GDP-*Rab*3A is complexed with *Rab* GDI and returns to the cytosol.

On the other hand, it has been shown that translocation of synaptic vesicles to the presynaptic plasma membrane is regulated by interactions of proteins associated with the vesicles and the cytoskeletal network as described for synapsin I (2). We have recently identified a Rabphilin-3A-interacting protein in bovine brain (19). The Rabphilin-3A-interacting protein binds to Rabphilin-3A in the presence of Ca^{2+} and phosphatidylserine through its C_2-like domains. The minimum Mr of this protein is estimated to be about 115 kDa on SDS-PAGE. We have attempted here to purify this protein and to determine its primary structure.

EXPERIMENTAL PROCEDURES

Materials and Chemicals——The Rabphilin-3A-interacting protein was purified from bovine brain as described (19). Protein kinase C was purified from rat brain as described (20). PVDF membrane sheets (Problott™), 0.45-μm pore size, were obtained from

Applied Biosystems (Tokyo, Japan). *Achromobacter* protease I (API) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phosphatidylserine was obtained from Funakoshi (Tokyo, Japan). [γ-^{32}p]ATP (222 TBq/mmol) was purchased from Amersham Corp.

Peptide Map and Amino Acid Sequence Analyses of the Rabphilin-3A-Interacting Protein——The Rabphilin-3A-interacting protein purified from bovine brain (about 100 μg of protein) was accumulated by four times of the purification. About 5 μg of the Rabphilin-3A-interacting protein was resolved by SDS-PAGE and then electrophoretically transferred to a PVDF membrane sheet (9). After staining of the Rabphilin-3A-interacting protein with Ponceau S, the corresponding band was cut out with a razor for peptide map analysis. The rest of the sample was treated in the same way. Each sample of the Rabphilin-3A-interacting protein on the PVDF membrane sheet was digested with API at a molar ratio of 1:50 (API:the Rabphilin-3A-interacting protein) for 24 h at 37°C in 300 μl of 0.2 M Tris/HCl at pH 9.0 containing 8% acetonitrile. When the peptides were released from the sheet, the samples were combined and subjected to a C18 column chromatography as described previously (9). More than eleven peptides were separated. Four of them were subjected to amino acid sequencing as described (9).

Assay for the Binding of the Rabphilin-3A-Interacting Protein to ^{32}P-Labeled Protein Kinase C——The Rabphilin-3A-interacting protein was subjected to SDS-PAGE in 10% polyacrylamide gels, and then transferred to a nitrocellulose sheet. The sheet was incubated with ^{32}P-labeled protein kinase C in the presence or absence of 20 μg/ml of phosphatidylserine, 0.1 mM $CaCl_2$, and 5 mM EGTA as described (19). After washing with 0.05% Tween 20 in TNMD Buffer, the sheet was dried and exposed to a film.

Other Procedures——Protein kinase C was phosphorylated by its autophosphorylation as described (21). SDS-PAGE was performed as described (22). Protein concentrations were determined with bovine serum albumin as a reference protein as described (23).

RESULTS AND DISCUSSION

The Rabphilin-3A-interacting protein was highly purified from bovine brain in an amount sufficient for amino acid sequencing. The accumulated protein was then subjected to SDS-PAGE and transferred to a PVDF membrane sheet. The band with a Mr of about 115 kDa corresponding to the Rabphilin-3A-interacting protein was digested by lysyl-endopeptidase, API. The resulting peptides were fractionated by a C18 column chromatography. More than eleven peptides were separated (**Fig. 1**). Four of them were subjected to amino acid sequencing. The amino acid sequences were almost identical to those of human β-adducin which is known to be a good substrate for protein kinase C (**Fig. 2**) (24-26). Moreover, the Rabphilin-3A-interacting protein bound to protein kinase C in the presence of Ca^{2+} and phosphatidylserine (**Fig. 3**), consistent with the recent report that the adducin homologue shows the protein kinase C- and phosphatidylserine-interacting activity (27).

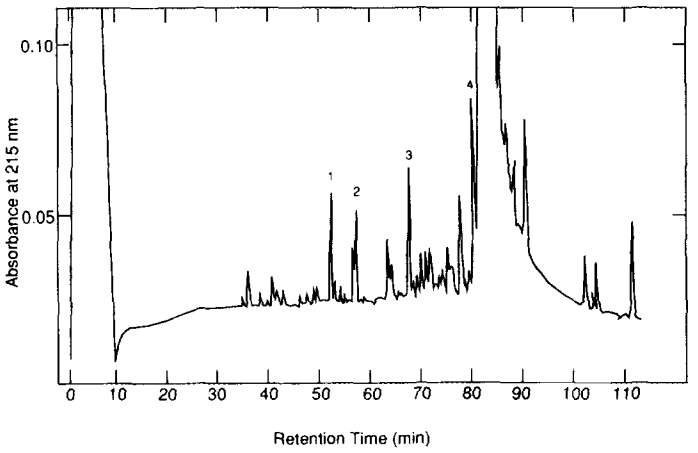

Fig. 1. Peptide map analysis of the Rabphilin-3A-interacting protein. Peaks 1 to 4 were subjected to amino acid sequence analysis.

The minimum Mr of human β-adducin is estimated to be about 100 kDa on SDS-PAGE (28,29). It binds to protein kinase C in the presence of Ca^{2+} and phosphatidylserine (21,28). It has been shown that a protein with a Mr of about 115 kDa in rat brain, which interacts with protein kinase C in the presence of both Ca^{2+} and phosphatidylserine and interacts with phosphatidylserine even in the absence of Ca^{2+}, is rat β-adducin (21,28). Judging from these observations together with the Mr of the Rabphilin-3A-interacting protein, it is most likely that this protein is bovine β-adducin. Although Rabphilin-3A and protein kinase C interact with β-adducin in a cell-free system, it remains to be clarified whether these interactions occur in intact cells.

β-Adducin is known to interact with calmodulin and to be phosphorylated by protein kinase C (29-31). β-Adducin stimulates the binding of spectrin to F-actin and tightly bundles F-actin (29,32,33). This action of β-adducin is regulated by calmodulin and Ca^{2+} (32). β-Adducin is associated with spectrin-actin-band 4.1 complexes extracted from erythrocyte membrane (33). Although an *in vivo* role of β-adducin remains uncertain, these observations suggest that β-adducin plays a role in cytoskeletal assembly and stabilization in response to Ca^{2+} signal, and serves as an anchoring molecule of the cytoskeleton for certain proteins.

In the presynapse, most synaptic vesicles are assumed to be associated with the F-actin-based cytoskeletal network presumably through synapsin I (2). Since Rabphilin-3A is associated with

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MSEETVPEAASPPPPQGQPYFDRFSEDDPEYMRLRNRAADLRQDFNLMEQ  50

KKRVTMILQSPSFREELEGLIQEQMKKGNNSSNIWALRQIADFMASTSHA 100

VFPTSSMNVSMMTPINDLHTADSLNLAKGERLMRCKISSVYRLLDLYGWA 150

QLSDTYVTLRVSKEQDHFLISPKGVSCSEVTASSLIKVNILGEVVEKGSS 200

CFPVDTTGFCLHSAIYAARPDVRCIIHLHTPATAAVSAMKWGLLTVSHNA 250

LLVGDMAYYDFNGEMEQEADRINLQKCLGPTCKILVLRNHGVVALGDTVE 300

EAFYKIFHLQAACEIQVSALSSAGGVENLILLEQEKHRPHEVGSVQWAGS 350
                                        3 PHEVGXVQ

TFGPMQKSRLGEHEFEALMRMLDNLGYRTGYTYRHPFVQEKTKHKSEVEI 400

PATVTAFVFEEDGAPVPALRQHAQKQQKEKTRWLNTPNTYLRVNVADEVQ 450

RSMGSPRPKTTWMKADEVEKSSSGMPIRIENPNQFVPLYTDPQEVLEMRN 500
      2 TLWMK          4 GMPIRIENPNQFVTPYT

KIREQNRQDVKSAGPQSQLLASVIAEKSRSPSTESQLMSKGDEDTKDDSE 550

ETVPNPFSQLTDQELEEYKKEVERKKLELDGEKETAPEEPGSPAKSAPAS 600

PVQSPAKEAETKSPLVSPSKSLEEGTKKTETSKAATTEPETTQPEGVVVN 650

GREEEQTAEEILSKGLSQMTTSADTDVDTSKDKTESVTSGPMSPEGSPSK 700
                                  1 TESVTSGPMSPEGSP

SPSKKKKKFRTPSFLKKSKKKEKVES
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Fig. 2. Comparison of the partial amino acid sequences of the Rabphilin-3A-interacting protein and the amino acid sequence deduced from the cDNA of human β-adducin. The amino acid sequences determined from the purified Rabphilin-3A-interacting protein are underlined. Numbers below these sequences correspond to the peak numbers in Fig. 1. Dots between amino acids designate identities.

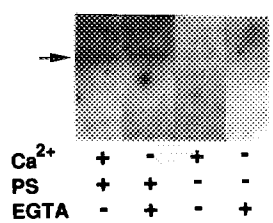

Fig. 3. Binding of protein kinase C to the Rabphilin-3A-interacting protein. The nitrocellulose-immobilized Rabphilin-3A-interacting protein was blotted with 10 nM ^{32}P-labeled protein kinase C in the presence or absence of 20 μg/ml of phosphatidylserine, 0.1 mM $CaCl_2$, and 5 mM EGTA as indicated. An arrow indicates the position of the Rabphilin-3A-interacting protein. Results are representative of three independent experiments.

synaptic vesicles (11), it is tempting to speculate that Rabphilin-3A on the vesicles binds to β-adducin and consequently promotes the docking of the vesicles to the cytoskeletal network in the presynapse as described for synapsin I. Much work is necessary to fully address this problem.

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