Communication

Physical and Functional Interactions of Doc2 and Munc13 in Ca\(^{2+}\)-dependent Exocytotic Machinery*

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Doc2 has two C2 domains that interact with Ca\(^{2+}\) and phospholipid. Munc13 has two C2 domains and one C1 domain that interacts with phorbol ester or diacylglycerol (DAG) and phospholipid. Both Doc2 and Munc13 are implicated in Ca\(^{2+}\)-dependent neurotransmitter release, but their modes of action still remain unclear. We show here that Doc2 interacts with Munc13 both in a cell-free system and in intact PC12 cells during the high K\(^+\)-induced Ca\(^{2+}\)-dependent exocytosis. The Doc2-Munc13 interactions are stimulated by phorbol ester through the C1 domain of Munc13. Overexpression of the Doc2-interacting domain of Munc13 reduces the Ca\(^{2+}\)-dependent exocytosis from PC12 cells, and co-expression with Doc2 suppresses this reduction. These results, together with the earlier findings that secretagogues produce DAG and elevate cytoplasmic Ca\(^{2+}\), suggest that the DAG-induced Doc2-Munc13 interactions play an important role in Ca\(^{2+}\)-dependent exocytotic machinery.

We have isolated Doc2 as a novel protein having two C2 domains that interact with Ca\(^{2+}\) and PL. Doc2 consists of two isoforms, Doc2α and Doc2β (1, 2). Doc2α is specifically expressed in neuronal cells, whereas Doc2β is ubiquitously expressed (1–3). Both isoforms have at least the N-terminal Doc2-specific region and C-terminal two C2 domains. We have moreover shown that overexpression of the N-terminal fragment of Doc2α or its C-terminal fragment including the C2 domains in PC12 cells inhibits Ca\(^{2+}\)-dependent exocytosis (4).

The Abbreviations used are: PL, phospholipid; PE, phospholester; DAG, diacylglycerol; aa, amino acids; GST, glutathione S-transferase; HA, hemagglutinin; TPA, 12-O-tetradecanoylphorbol-13-acetate; PDII, 46-phorbol dibutyrate; PAGE, polyacrylamide gel electrophoresis; PSS, physiological salt solution; GH, growth hormone; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SNARE, SNAP receptor.

These results suggest that Doc2α is involved in Ca\(^{2+}\)-dependent exocytosis and interacts with another component of Ca\(^{2+}\)-dependent exocytotic machinery. To clarify the mode of action of Doc2α in Ca\(^{2+}\)-dependent exocytosis, it is important to isolate its interacting protein(s). We have attempted here to isolate a Doc2α-interacting protein from a rat brain cDNA library by use of the yeast two-hybrid system and isolated Munc13 as a Doc2α-interacting protein.

Munc13 has been isolated as a mammalian homologue of Caenorhabditis elegans unc-13, which is implicated in Ca\(^{2+}\)-dependent neurotransmitter release (5, 6). Munc13 has three isoforms, Munc13-1, -2, and -3. All the isoforms have two C2 domains and Munc13-1 has another atypical C2 domain. They have moreover one C1 domain that interacts with PE or DAG and PL (5–7). Munc13 is specifically expressed in neuronal cells, and Munc13-1 is localized at the presynaptic plasma membrane (6).

We describe here that Doc2α directly interacts with Munc13-1 in a DAG-dependent manner and that the Doc2α-Munc13-1 interactions play an important role in Ca\(^{2+}\)-dependent exocytotic machinery.

EXPERIMENTAL PROCEDURE

Two-hybrid Assay—The N-terminal fragment (1–90 aa) of human Doc2α cDNA (1) was inserted into the pBTH16 (pLexA-Doc2αN). The yeast reporter strain L40 was transformed with pLexA-Doc2αN and a rat brain cDNA library constructed in pGAD10 (CLONTECH). Library plasmids from positive clones were analyzed by transformation tests with yeast strain L40,β-galactosidase activity was assayed by liquid and filter assays (8, 9).

Preparation of Recombinant Proteins—The cDNA fragments encoding the N-terminal fragment (1–90 aa) of human Doc2α (1) and Munc13-1 (851–1461 aa) were inserted into pGEX vectors, expressed in Escherichia coli as GST fusion proteins, and purified on glutathione-Sepharose 4B columns (Pharmacia Biotech Inc.).

Construction of Expression Vectors—Mammalian expression plasmids pEFBOS-HA and pEFBOS-myc were generated to express fusion proteins with the N-terminal HA and myc epitopes, respectively (4, 10). In vitro and in vivo expression plasmids pGEM-HA and pBluescript-myc were generated to express fusion proteins with the N-terminal HA and myc epitopes, respectively. The cDNA fragments encoding human Doc2α (1) and its deletion mutants were inserted into pEFBOS-HA and pGEM-HA. The cDNA fragments encoding Munc13-1 and its deletion mutants were inserted into pEFBOS-myc and pBluescript-myc.

Assay for Doc2α-Munc13-1 Interactions in a Cell-free System—The cDNA fragments, which were inserted into pGEM-HA or pBluescript-myc, were translated in vitro using TNT T7-coupled reticulocyte lysate system (Promega). 2 μg of GST-Doc2α (1–90 aa) or GST-Munc13-1 (851–1461 aa) were immobilized onto 20 μl of glutathione-Sepharose 4B beads. The immobilized beads were added to 500 μl of Buffer A (150 mM NaCl, 50 mM HEPES, pH 7.4, and 1 mM EGTA) containing 20 mM glutathione. The eluates were subjected to SDS-PAGE followed by autoradiography.

Assay for Doc2α-Munc13-1 Interactions in an Intact Cell System—PC12 cells were plated at a density of 5 × 10^5 cells/60-mm dish and were incubated for 18 h. PC12 cells were infected for 30 min with T7 RNA polymerase recombinant vaccinia virus (LO-T7) and then cotransfected with 2 μg of pGEM-HA encoding Doc2α or its deletion...
and incubated for 10 min with a high K
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clones containing the C-terminal portion of Munc13-1 lacked
to 840–1743 aa residues of Munc13-1, except that the isolated
all of which encoded the parts of the sequences corresponding
clones had cDNA inserts ranging from 2.0 to 3.1 kilobase pairs,
(1–90 aa) of Doc2
m
m

pXGH5 encoding human GH (11) and 2
m
m

The structures of Doc2a and Munc13-1 are depicted with the
relative locations of the C1 domain (C1), C2 domain (C2), atypical C2
domain (C2a*), Munc13-1-Did (Did), and Doc2a-Mid (Mid). The number
of plus signs corresponds to blue color intensity on the X-gal indicator
filter. β-Galactosidase activities are represented as the means ± S.E.
obtained by three independent transformants. a, mapping of the site of
Munc13-1 interacting with the N-terminal region of Doc2a. b, mapping
of the site of Doc2a interacting with Munc13-1. c, interactions of the
Doc2 and Munc13 isoforms.

RESULTS

We first attempted to isolate a Doc2a-interacting protein by
use of the yeast two-hybrid system with the N-terminal region
(1–90 aa) of Doc2a as a bait from a rat brain cDNA library.
Screening of I × 10
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transfected with 2 μg of pBlueScript-myc encoding Munc13-1 or its deletion
mutants, by use of LipofectAMINE reagent (Life Technologies, Inc.).
Immunoprecipitation was performed 5 h after the transfection. In some
cases, before performing the immunoprecipitation, PC12 cells were
washed with PSS (140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM
MgSO4, 1.2 mM KH2PO4, 20 mM HEPES, pH 7.4, and 11 mM glucose)
and incubated for 10 min with a high K
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mutations and 2 μg of pBlueScript-myc encoding Munc13-1 or its deletion
mutants, by use of LipofectAMINE reagent (Life Technologies, Inc.).
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MgSO4, 1.2 mM KH2PO4, 20 mM HEPES, pH 7.4, and 11 mM glucose)
and incubated for 10 min with a high K
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Assay for GH Release—PC12 cells were co-transfected with 2
μg of pXGH5 encoding human GH (11) and 2 μg of pEF-BOS bearing the
indicated cDNA by use of LipofectAMINE reagent (4). After 48 h, PC12
cells were then stimulated by each agonist for 10 min. The amounts of
GH released into the medium and retained in the cells were meas-
ured by radioimmunoassay kit (Nichols Institute).

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GH released into the medium and retained in the cells were meas-
ured by radioimmunoassay kit (Nichols Institute).

The Doc2a-Munc13 interactions were furthermore confirmed by
co-immunoprecipitation from cultured PC12 cells of
myc-tagged full-length Munc13-1 and HA-tagged full-length Doc2a and of
myc-tagged Munc13-1-Did and HA-tagged full-length Doc2a (Fig. 2, c and d).
The Doc2a-Mid and Munc13-1-Did deletion mutants did not interact with the respective partner proteins.

The Doc2a-Munc13-1 interactions were confirmed by the binding of in vitro translated, [35S]methionine-labeled Doc2a and Munc13-1 to GST-tagged recombinant Munc13-1-Did and the GST-tagged Doc2a Mid-containing region, respectively (Fig. 2, a and b). The Doc2a-Mid and Munc13-1-Did deletion mutants did not interact with the respective partner proteins.

Doc2β. Doc2a-Mid showed striking sequence homology to Doc2β-Mid (92% identity). Munc13-1-Did also showed striking sequence homology to Munc13-2-Did (51% identity). These inter-
actions were estimated by the yeast two-hybrid system.

The Doc2a-Munc13-1 interactions were confirmed by the
binding of in vitro translated, [35S]methionine-labeled Doc2a and Munc13-1 to GST-tagged recombinant Munc13-1-Did and the
GST-tagged Doc2a Mid-containing region, respectively (Fig. 2, a and b). The Doc2a-Mid and Munc13-1-Did deletion
mutants did not interact with the respective partner proteins.

The Doc2a-Munc13-1 interactions were confirmed by
co-immunoprecipitation from cultured PC12 cells of
myc-tagged full-length Munc13-1 and HA-tagged full-length
Doc2a and of myc-tagged Munc13-1-Did and HA-tagged full-
length Doc2a (Fig. 2, c and d). The Doc2a-Mid and Munc13-1-Did
deletion mutants were not co-immunoprecipitated with the respective partner proteins. The co-immunoprecipitation of full-length Munc13-1 with Doc2a from PC12 cells was mark-
edly enhanced when the cells were stimulated by TPA or high
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in the absence of extracellular Ca
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Munc13-1 and its deletion mutants are shown in the positions of Munc13-1 and Munc13-1(D). The binding of PE to the C1 domain of Munc13-1 causes the enhancement of the in vitro interactions of the N-terminal fragment of Doc2α (1–90 aa) and Munc13-1 by TPA or PDBu. Affinity-purified GST-Doc2α (1–90) immobilized on glutathione-Sepharose beads was incubated in vitro translated, [35S]methionine-labeled Munc13-1 in the presence of TPA or PDBu. The specifically bound proteins were detected by SDS-PAGE followed by autoradiography.

Munc13-1. With the C1 domain deletion mutant, the co-immunoprecipitation of full-length Munc13-1 with Doc2α was observed even without the stimulation of PC12 cells by high K+ or TPA. Consistent with these cell level experiments, the interactions of in vitro translated, [35S]methionine-labeled Munc13-1 and the recombinant GST-tagged Doc2α-Mid-containing region were also stimulated by TPA or PDBu in a cell-free binding assay system (Fig. 3b). The Doc2α-Munc13-1 interactions were not observed with the Doc2α-Mid and Munc13-1-Did deletion mutants of the respective proteins irrespective of the presence or absence of TPA (data not shown).

It was finally examined whether the Doc2α-Munc13-1 interactions are functionally relevant for Ca2+-dependent exocytosis. For this experiment, we took advantage of the GH coexpression assay system of PC12 cells (12). In this system, human GH and a sample to be tested were coexpressed. Expressed GH is known to be stored in dense core vesicles and to be released in response to high K+ and TPA in the presence of extracellular Ca2+ (13, 14). The Northern blot analysis indicated that both Doc2α and Munc13-1 were expressed in PC12 cells (data not shown). Overexpression of Doc2α enhanced not only the high K+-induced GH release (4) but also the TPA-induced GH release (Fig. 4). Overexpression of Munc13-1-Did reduced both the high K+- and TPA-induced GH release. Co-expression with Doc2α suppressed this reduction.

**DISCUSSION**

We have shown here that Doc2α interacts with Munc13-1 in a cell-free system and that these interactions are stimulated by PE. These results, together with the earlier findings that PE directly interacts with the C1 domain of unc-13 (5, 7), indicate that the binding of PE to the C1 domain of Munc13-1 causes the Doc2α-Munc13-1 interactions. We have moreover shown here that the Doc2α-Munc13-1 interactions are observed in intact PC12 cells and enhanced during the high K+ or TPA-induced Ca2+-dependent exocytosis, and that these interactions are observed even without the stimulation of PC12 cells by high K+ or TPA when the C1 domain deletion mutant of Munc13-1 is used. These results, together with the earlier findings that high K+ induces DAG formation (15), suggest that the Doc2α-Munc13-1 interactions are induced by DAG produced during Ca2+-dependent exocytosis through the C1 domain of Munc13-1. Finally, we have demonstrated by use of the GH co-expression assay system of PC12 cells that Doc2α and Munc13-1 functionally interact with each other during Ca2+-dependent exocytosis.

Many systems and components are implicated in Ca2+-dependent exocytosis, such as neurotransmitter release. These include N-ethylmaleimide-sensitive factor/SNAP/SNARE, Rab, protein kinase C, and Ca2+-binding protein systems (for reviews see Refs. 16 and 17). Of these systems, the SNARE system is implicated in docking of synaptic vesicles with the presynaptic plasma membrane through the v-SNARE (vesicle-associated membrane protein)-t-SNARE (syntaxin and SNAP-25) interactions (for a review see Ref. 18). In C. elegans, unc-13 belongs to a group of genes defined by mutations with a paralytic phenotype and accumulation of acetylcholine (19), suggesting that Munc13-1 is also involved in neurotransmitter release in mammals. Doc2α is involved in Ca2+-dependent exocytosis from PC12 cells (4). Munc13-1 is located on the presynaptic plasma membrane (6), and Doc2α is concentrated on synaptic vesicles (1). Our present results together with these earlier findings suggest that the Doc2α-Munc13-1 system is another docking machinery controlled by DAG. It has recently been shown that Munc13-1 interacts directly with syntaxin (20) and that Munc18, a mammalian homologue of C. elegans unc-18 (21), directly interacts with Doc2 (22). Munc18 directly interacts with syntaxin, and Munc18 is dissociated from syntaxin when syntaxin forms a complex with vesicle-associated membrane protein and SNAP-25 (23). Therefore, the Doc2-Munc13 and Doc2-Munc18 systems may function in cooperation with syntaxin in docking processes. It is likely that the mutual interactions among syntaxin, Munc18, Munc13, and Doc2, play a crucial role in docking process. It is important to examine the effects of the DAG-induced Doc2-Munc13 interactions on the Doc2-Munc18, Munc13-syntaxin, and Munc18-syntaxin interactions.

Another recent analysis indicates that PE increases the size of the readily releasable pool of secretory granules in bovine adrenal chromaffin cells (24). It has been suggested that this action of PE is mediated through protein kinase C, but the properties of Munc13-1 suggest that it is a better candidate for this action of PE. Moreover, because Doc2α and Munc13-1 interact with Ca2+, they may serve as Ca2+ sensors for Ca2+-dependent exocytosis.
dependent exocytosis in cooperation with other Ca$^{2+}$-binding proteins. Many proteins having two C2 domains have been identified. These include Doc2 (1), Munc13 (6), synaptotagmin (25), and rabphilin-3A (26), all of which are implicated in Ca$^{2+}$-dependent exocytosis. The C2 domain of PKC has been shown to interact with membrane PL, particularly phosphatidylserine, in the presence of Ca$^{2+}$ (for a review see Ref. 27). The precise role of Ca$^{2+}$ in Ca$^{2+}$-dependent exocytosis still remains unclear, but it could be speculated that the proteins having two C2 domains constitute a big complex to form a scaffold-like structure and play a critical role in the fusion process in cooperation with Ca$^{2+}$ and membrane PL in addition to the docking process.

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