Doc2 Enhances Ca\textsuperscript{2+}-dependent Exocytosis from PC12 Cells*

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We previously isolated a new protein having two C2-like domains which interacted with Ca\textsuperscript{2+}- and phospholipid and named Doc2 (Double C2). Because Doc2 was abundantly expressed in brain where it was highly concentrated on the synaptic vesicle fraction, we have examined here whether Doc2 is involved in Ca\textsuperscript{2+}-dependent exocytosis from cultured PC12 cells. For this purpose, we took advantage of the growth hormone (GH) co-expression assay system of PC12 cells in which GH is stored in dense core vesicles and released in response to high K\textsuperscript{+} in an extracellular Ca\textsuperscript{2+}-dependent manner. Northern and Western blot analyses indicated that Doc2 is present in PC12 cells. Overexpression of hemagglutinin-tagged Doc2 stimulated the Ca\textsuperscript{2+}-dependent, high K\textsuperscript{+}-induced release of co-expressed GH without affecting the basal release. In the PC12 cells transfected with a plasmid with the coding sequence of Doc2 in the antisense orientation, the high K\textsuperscript{+}-induced release of co-expressed GH was inversely inhibited. The Doc2 mutant expressing an N-terminal fragment or a C-terminal fragment containing two C2-like domains inhibited the high K\textsuperscript{+}-induced release of co-expressed GH. These results indicate that Doc2 enhances Ca\textsuperscript{2+}-dependent exocytosis of dense core vesicles from PC12 cells.

The C2 domain was first found in protein kinase C activated by Ca\textsuperscript{2+} and phospholipid (1, 2) (for a review, see Ref. 3). The C2-like domain was subsequently found in many other important intracellular signaling elements, including phospholipase C\textsubscript{2} and phospholipase A\textsubscript{2} (6). Synaptotagmin was originally found to be specifically located on synaptic vesicles (8, 12) and shown by genetic and biochemical analyses to regulate neurotransmitter release as a Ca\textsuperscript{2+} sensor (10, 13) (for reviews, see Refs. 14 and 15). Rabphilin-3A was isolated as a downstream target molecule of Rab3A, which specifically interacts with GTP-Rab3A (9, 16). Rabphilin-3A is specifically expressed in neuron, where it is highly concentrated on synaptic vesicles (17, 18). Synaptotagmin has a transmembrane segment and is anchored on synaptic vesicles through this segment (8, 19), but rabphilin-3A has no transmembrane segment and anchors on synaptic vesicles through an anchoring protein (20). These properties of rabphilin-3A, together with the observation that Rab3A is involved in Ca\textsuperscript{2+}-dependent exocytosis for a review, see Ref. 21, strongly suggest that rabphilin-3A may serve as both a downstream target molecule of Rab3A and a Ca\textsuperscript{2+} sensor for neurotransmitter release. Consistently, Chung et al. (22) took advantage of the GH co-expression assay system, which was originally developed by Wick et al. (23), and showed evidence that rabphilin-3A is indeed involved in Ca\textsuperscript{2+}-dependent exocytosis from bovine adrenal chromaffin cells. In this assay system, expressed GH is stored in dense core vesicles of chromaffin cells and released in response to various agonists in an extracellular Ca\textsuperscript{2+}-dependent manner (23, 24).

We recently isolated a third protein having two C2-like domains, C2A (127 amino acids, 90–216 amino acids) and C2B (132 amino acids, 240–371 amino acids), and named Doc2 (Double C2) (25). Doc2 is a protein with a calculated molecular mass of 44,071 and 400 amino acids. Doc2 has neither a transmembrane nor an anchoring protein (26). These properties of rabphilin-3A, together with the observation that Rab3A is involved in Ca\textsuperscript{2+}-dependent exocytosis for a review, see Ref. 21, strongly suggest that rabphilin-3A may serve as both a downstream target molecule of Rab3A and a Ca\textsuperscript{2+} sensor for neurotransmitter release. Consistently, Chung et al. (22) took advantage of the GH co-expression assay system, which was originally developed by Wick et al. (23), and showed evidence that rabphilin-3A is indeed involved in Ca\textsuperscript{2+}-dependent exocytosis from bovine adrenal chromaffin cells. In this assay system, expressed GH is stored in dense core vesicles of chromaffin cells and released in response to various agonists in an extracellular Ca\textsuperscript{2+}-dependent manner (23, 24).

EXPERIMENTAL PROCEDURES

Materials and Chemicals—PC12 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 5% horse serum at 37°C in 10% CO\textsubscript{2} as described (26). A rabbit antiserum was generated against a fusion protein of glutathione S-transferase and the N-terminal fragment (1–85 amino acids) of mouse Doc2 and affinity-purified by using the same fusion protein and glutathione-Sepharose as described (27). Human GH was expressed using pXGH5 in which GH expression is driven by the mouse metallothionein-I promoter (28).

Northern and Western Blot Analyses—Northern blot analysis was performed using 4 μg of poly(A)\textsuperscript{+} RNA isolated from rat cerebrum, rat liver, and PC12 cells. The blot was hybridized with a 32P-labeled 1.2-kilobase DNA fragment of the mouse Doc2 cDNA. Several tissues of adult female rat and PC12 cells were homogenized as described (29). Twenty μg of the membrane fractions of rat cerebrum, rat liver, and PC12 cells were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analysis by use of the anti-Doc2 polyclonal antibody.

Construction of Expression Vectors—A DNA fragment encoding the HA (YPYDVPDYA) epitope with the metallothionein-I promoter was inserted into the XbaI site of pEF-BOS (30) to express the HA-tagged fusion proteins (pEF-HA). To generate sense (1–400 amino acids, pEF-HA) and antisense (1–400 amino acids, pEF-HA-αS), and deletion mu-
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**RESULTS**

Expression of Doc2 in PC12 Cells—In Northern blot analysis, a 2.2-kilobase transcript was detected in PC12 cells and rat cerebrum but not in rat liver (Fig. 1A). In Western blot analysis, one immunoreactive band with the property of Doc2 that it has two C2-like domains and phospholipid, 1,2-dimyristoyl phosphatidylcholine, was detected in PC12 cells and rat cerebrum but not in rat liver (Fig. 1B). These results indicate that Doc2 is indeed expressed in PC12 cells as well as in rat cerebrum.

Co-expression of Doc2 with Human GH in PC12 Cells—The plasmid encoding human Doc2 (pEF-HA-Doc2) was co-transfected with pXGH5 (encoding human GH) in PC12 cells. An epitope tag (HA) was attached to the N terminus of the Doc2 protein to detect it in the cells. When transfected Doc2 and GH were analyzed by immunocytochemistry using the respective antibodies, both proteins were detected in the same cell (Fig. 2). About 90% of the GH-expressing cells expressed detectable amounts of the HA-tagged Doc2 protein as determined by fluorescence microscopy.

**FIG. 1. Expression of Doc2 in PC12 cells.** A, Northern blot analysis of Doc2 mRNA in PC12 cells. Lane 1, rat cerebrum; lane 2, PC12 cells; lane 3, rat liver. The arrowhead indicates the position of Doc2. B, immunoblot analysis of Doc2 in PC12 cells by use of the anti-Doc2 polyclonal antibody. Lane 1, recombinant human Doc2 (9–400 amino acids) expressed as a fusion protein with the N-terminal HA epitope using the insect/baculovirus system (10 ng of protein); lane 2, the membrane fraction of rat cerebrum (25 \(\mu\)g of protein); lane 3, the membrane fraction of PC12 cells (25 \(\mu\)g of protein); lane 4, the membrane fraction of rat liver (25 \(\mu\)g of protein).

In our preceding paper, we have shown that Doc2 is abundantly expressed in brain where it is enriched in the synaptic vesicle fraction. Consistently, we have shown here that Doc2 is also expressed in PC12 cells. These results, together with the property of Doc2 that it has two C2-like domains responsible for interaction with Ca\(^{2+}\) and phospholipid, strongly suggest that Doc2 as well as synaptotagmin and rabphilin-3A plays a role in Ca\(^{2+}\)-dependent exocytosis.

**DISCUSSION**

In our preceding paper, we have shown that Doc2 is abundantly expressed in brain where it is enriched in the synaptic vesicle fraction. Consistently, we have shown here that Doc2 is also expressed in PC12 cells. These results, together with the property of Doc2 that it has two C2-like domains responsible for interaction with Ca\(^{2+}\) and phospholipid, strongly suggest that Doc2 as well as synaptotagmin and rabphilin-3A plays a role in Ca\(^{2+}\)-dependent exocytosis.
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Fig. 3. Ca\(^{2+}\)-dependent, high K\(^+\)-induced release of expressed GH from PC12 cells. A, time course for release of expressed GH in the presence of extracellular Ca\(^{2+}\). The cells were incubated for the indicated periods of time. ●, with the high K\(^+\) solution; ○, with the low K\(^+\) solution. B, effect of extracellular Ca\(^{2+}\) on the high K\(^+\)-induced release of GH. Bar 1, with the high K\(^+\) solution in the presence of 2.5 mM CaCl\(_2\); bar 2, with the high K\(^+\) solution in the presence of 1 mM EGTA instead of 2.5 mM CaCl\(_2\); bar 3, with the low K\(^+\) solution in the presence of 2.5 mM CaCl\(_2\); bar 4, with the low K\(^+\) solution in the presence of 1 mM EGTA instead of 2.5 mM CaCl\(_2\). Data are expressed as the average percentage released of the total GH stores. The values are representative of three independent experiments.

Fig. 4. Effect of overexpression of Doc2 on release of expressed GH from PC12 cells. A, effect of pEF-HA-Doc2 and pEF-HA-AS on the high K\(^+\) solution. B, dose-response effect of pEF-HA-Doc2 and pEF-HA-AS. ●, with pEF-HA-Doc2; ○, with pEF-HA-AS. C, effect of pEF-HA-Doc2 and pEF-HA-AS on the low K\(^+\) solution. Bar 1, with pEF-HA; bar 2, with pEF-HA-Doc2; bar 3, with pEF-HA-AS. Data are expressed as the average percentage released of the total GH stores. The values are representative of three independent experiments.

Fig. 5. Effect of Doc2 mutants on release of expressed GH from PC12 cells. A, effect of pEF-HA-Doc2N and pEF-HA-Doc2C on the high K\(^+\)-induced release of expressed GH. B, effect of pEF-HA-Doc2N and pEF-HA-Doc2C on the low K\(^+\)-induced release of expressed GH. Bar 1, with pEF-HA; bar 2, with pEF-HA-Doc2N; bar 3, with pEF-HA-Doc2C. Data are expressed as the average percentage released of the total GH stores. The values are representative of three independent experiments.

followed by its exocytosis. To clarify this issue, we measured internalization of surface-bound \(^{125}\)I-labeled GH in PC12 cells. The cells were incubated for the indicated periods of time. ●, with the high K\(^+\) solution; ○, with the low K\(^+\) solution. Bar 1, with the high K\(^+\) solution in the presence of 2.5 mM CaCl\(_2\); bar 2, with the high K\(^+\) solution in the presence of 1 mM EGTA instead of 2.5 mM CaCl\(_2\); bar 3, with the low K\(^+\) solution in the presence of 2.5 mM CaCl\(_2\); bar 4, with the low K\(^+\) solution in the presence of 1 mM EGTA instead of 2.5 mM CaCl\(_2\). Data are expressed as the average percentage released of the total GH stores. The values are representative of three independent experiments.

These domains into PC12 cells inhibit the Ca\(^{2+}\)-dependent exocytosis (36). The N-terminal fragment of rabphilin-3A interacts with Rab3A (11), and overexpression of this fragment in chromaffin cells inhibits the Ca\(^{2+}\)-dependent exocytosis (22). These earlier results, together with the present results that overexpression of the N-terminal fragment or the fragment containing the C2-like domains of Doc2 in PC12 cells also inhibits the Ca\(^{2+}\)-dependent exocytosis, suggest that these three proteins having two C2-like domains are involved in and play different roles in Ca\(^{2+}\)-dependent exocytosis. Doc2 may interact with specific components involved in Ca\(^{2+}\)-dependent exocytosis, which are different from those which synaptotagmin and rabphilin-3A interact with, and introduction of the N- or C-terminal fragment of Doc2 into PC12 cells may disrupt these interactions. It is important to isolate these Doc2-interacting molecules in the future.

Electrophysiological studies demonstrated that Ca\(^{2+}\)-dependent exocytosis requires several Ca\(^{2+}\) sensors (37) (for a review, see Ref. 38). One of the most probable Ca\(^{2+}\) sensors is synaptotagmin (10, 13–15). However, there are several lines of evidence that Ca\(^{2+}\) sensors other than synaptotagmin are also involved in Ca\(^{2+}\)-dependent exocytosis (13, 39). Doc2 and rabphilin-3A, which have two C2-like domains, may be possible candidates for these Ca\(^{2+}\) sensors. Further studies are necessary to clarify the role of Doc2 as a Ca\(^{2+}\) sensor.

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