Transient, Phorbol Ester-induced DOC2-Munc13 Interactions in Vivo*

(Received for publication, June 21, 1999, and in revised form, July 19, 1999)

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Munc13-1 and DOC2 have been implicated in the regulation of exocytosis. Here we demonstrate in vivo that these two proteins undergo a transient phorbol ester-mediated and protein kinase C-independent interaction, resulting in the translocation of DOC2 from a vesicular localization to the plasma membrane. The translocation of DOC2 is dependent upon the DOC2 Munc interacting domain that binds specifically to Munc13-1, whereas the association of DOC2 with intracellular membranes is dependent on its C2 domains. This is the first direct in vivo demonstration of a protein-protein interaction between two presynaptic proteins and may represent a molecular basis for phorbol ester-dependent enhancement of exocytosis.

Munc13-1 enhances neurotransmitter release in a phorbol ester (PE)1-dependent manner, most likely by increasing the size of the readily releasable pool of vesicles (1). The molecular mechanism of this action remains unclear, but it is independent of protein kinase C (PKC) (1). There are only two known PE-dependent features of Munc13-1 that may contribute to the molecular mechanism: (i) PE-dependent translocation from cytoplasm to plasma membrane (1) and (ii) PE-dependent binding of DOC2 (2).

DOC2, like Munc13-1, has been implicated in the regulation of exocytosis (1–4). DOC2 co-purifies with markers for secretory vesicles and, to a lesser extent, plasma membrane (5). Overexpression of DOC2 in PC12 cells enhances regulated vesicular localization to the plasma membrane. The subcellular distribution of DOC2 in PC12 cells is dynamic, resulting in the translocation of DOC2 from a vesicular localization to the plasma membrane (5).

In this study we use GFP reporter constructs to monitor the subcellular distribution of DOC2 in HEK293 cells. This same model system was used to great effect to demonstrate that Munc13 isoforms can translocate to the plasma membrane upon PE stimulation (1). For the present study it offers the additional advantage that neither Munc13 nor DOC2 is endogenously expressed.

Our work demonstrates directly that Munc13-1 and DOC2 interact transiently in vivo upon PE stimulation, leading to a massive, Munc13-1-dependent redistribution of DOC2. This PE-stimulated interaction may represent the molecular basis of PE-dependent, Munc13-1-mediated enhancement of neurotransmitter release and provides the first direct observation of the interaction of two presynaptic proteins in vivo.

**Experimental Procedures**

**Isolation of cDNAs**—A reverse transcription-PCR strategy was used to amplify a cDNA encoding the open reading frame of mouse brain DOC2. Briefly, total RNA was prepared from adult mouse brain following standard procedures (7), followed by mRNA enrichment using Poly(A) Quik columns (Stratagene, Cambridge, UK) according to the manufacturer’s instructions. One μg of this RNA was used as template in a first-strand and cDNA synthesis directed from an anchored oligo d(T) (5’-TTCTTAGATTCGAAGCGCCTGT(T)9N9(T)9) primer, using Superscript II reverse transcriptase (Life Technologies, Inc.). The resultant cDNA was diluted and used in a polymerase chain reaction between forward (5’-CTGCTCTATGACCTCCTCGG) and reverse (5’-CATCGCTGAGYACGCCCGTGGG) degenerate oligonucleotides using Expand polymerase mixture (Roche Molecular Biochemicals). Sequence analysis revealed that the cloned cDNA had a sequence identical to that previously published for mouse cerebellum DOC2c cDNA (9) (GenBank accession no. D85037). The cDNA constructs used in this work are represented in Fig. 1. The PCR product(s) were ligated to a T/A vector (pCR2.1, Invitrogen, Groningen, Netherlands) and completely sequenced on both strands (OSWEL DNA Services, Southampton, UK).

Rat VAMP/synaptobrevin II was isolated from rat whole brain first-strand cDNA prepared in a similar manner. Full-length cDNA was amplified using forward (5’-ATGTCGGCTACCGCTGCCACCGTCCCG-3’) and reverse (5’-AGTGCTGAAGTAAACGATGATGAG-3’) primers, using Superscript II reverse transcriptase (Life Technologies, Inc.). The resultant cDNA was diluted and used in a polymerase chain reaction between forward (5’-CTGCTCTATGACCTCCTCGG) and reverse (5’-CATCGCTGAGYACGCCCGTGGG) degenerate oligonucleotides using Expand polymerase mixture (Roche Molecular Biochemicals). Sequence analysis revealed that the cloned cDNA had a sequence identical to that previously published for mouse cerebellum DOC2c cDNA (9) (GenBank accession no. D85037). The cDNA constructs used in this work are represented in Fig. 1. The PCR product(s) were ligated to a T/A vector (pCR2.1, Invitrogen, Groningen, Netherlands) and completely sequenced on both strands (OSWEL DNA Services, Southampton, UK).

**Expression of Recombinant Proteins**—Complementary DNA encoding full-length DOC2 was subcloned from pCR2.1 into pcDNA3.1 (Invitrogen) as a Kpn1-BamHI restriction fragment. A dilution of this plasmid was used as template in a PCR as described above to generate an EcoRI-BamHI restriction fragment suitable for in-frame ligation to pEGFPN1 (CLONTECH, Cambridge, UK). A further PCR was used to generate DOC2 C2, which was ligated to pEGFPN1. MID-GFP was generated by PCR and ligation to pcDNA3.1-NT-GFP-TOPO (Invitrogen) according to the manufacturer’s instructions. All constructs were sequenced as before. Construction of Munc13-1 in pcDNA3.1 and pEGFPN1 was accomplished as previously (1). Expression in HEK293 cells was confirmed in vivo by confocal microscopy.
achieved by transfection using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions.

Cell Culture—HEK293 cells, between passages 8 and 25, were cultured on glass coverslips at 37 °C in 95% (v/v) air, 5% (v/v) CO 2 in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum, 5 mM glutamine, and 1% (v/v) penicillin/streptomycin (Life Technologies, Inc.), and transfected as described above. All experiments were performed 24–48 h after transfection.

RESULTS

DOC2β Associates with Intracellular Membranes in HEK293 Cells as Mediated by the C2 Domains—HEK293 cells transfected with cDNA encoding Munc13-1-EGFP alone showed a uniform cytoplasmic distribution of fluorescence with nuclear sparing, as described previously (Fig. 2a). Reverse transcription-PCR and immunoblot analysis failed to detect endogenous DOC2α or DOC2β mRNA or protein in HEK293 cells (not shown). Cells co-transfected with cDNAs encoding DOC2β and Munc13-1-EGFP (see below) showed a fluorescence distribution similar to that described for Munc13-1-EGFP alone. HEK293 cells transfected with DOC2-EGFP alone showed the fluorescence to be distributed throughout the cell cytoplasm in a punctate pattern with nuclear sparing, reminiscent of a vesicular distribution (Fig. 2c). Cells transfected with cDNA encoding a fluorescent fusion protein containing only the N-terminal 38 amino acids of DOC2β fused to GFP (MID-GFP) showed a diffuse cytoplasmic distribution of fluorescence with sparing of organelles and the nucleus (Fig. 2e). In contrast, a cDNA encoding a fusion between both C2 domains of DOC2β and EGFP (MID-GFP) showed a punctate vesicular distribution of fluorescence similar to the full-length protein (Fig. 2f).
orescence altered dramatically when the cells were stimulated with PMA. Previously, it had been demonstrated that Munc13-1-EGFP translocates in a C1 domain-mediated manner from a cytoplasmic localization to the plasma membrane in HEK293 cells upon PE stimulation, an observation repeated here (Fig. 2) (1). PE stimulation of cells transfected with DOC2-EGFP alone had no effect upon the intracellular localization of the fluorescence (Fig. 2, c and d). The localization of DOC2-EGFP remained unaltered in unstimulated cells in the presence or absence of Munc13-1. However, after PE stimulation the distribution of DOC2-EGFP altered dramatically from the vesicular-like pattern to a plasma membrane distribution, but only in cells co-expressing Munc13-1 (Fig. 2, e–f). In individual fields of view, most fluorescent cells (>90%; n > 50 fields of ~20 cells) showed DOC2-EGFP:Munc13-1 co-translocation after PE stimulation. Those cells that did not show the effect were presumably transfected with only DOC2β or were sub-viable at the time of stimulation.

A specific DOC2-Munc13-1 interaction was confirmed by substituting VAMP-GFP for DOC2-EGFP in the translocation assay. The VAMP-GFP fluorescence was seen spread throughout the cell in a punctate distribution both before and after PE application. These data indicate that the DOC2 translocation is directed by an interaction with Munc13-1 induced by Munc13-1-PE interaction. The intracellular localization of DOC2 protein is determined by the C2 domains of DOC2 associating with intracellular membranes, which are directed in their localization by PE-bound Munc13-1. Scale bars = 25 μm. See Fig. 1 legend for a key describing domain shading.

FIG. 3. Confocal optical sections (0.5 μm thickness) showing EGFP fluorescence in cells transiently expressing DOC2β and Munc13-1 EGFP fusion proteins before and after PMA stimulation. The constructs transfected into each cell are represented below each photograph. a, MID-GFP fusion protein shows a diffuse, cytoplasmic distribution with nuclear sparing before PE application. This distribution alters dramatically after treatment as shown in panel b. C2-EGFP domains are shown before PMA stimulation (c) and after PE treatment (d). The C2 domains of DOC2β are required for the association of the fusion protein with intracellular membranes but are not sufficient for Munc13-1 interaction and translocation. e and f, the DOC2-Munc13-1 interaction was further confirmed as specific by substituting VAMP-GFP for DOC2-EGFP in the translocation assay. The VAMP-GFP fluorescence was seen spread throughout the cell in a punctate distribution both before and after PE application. These data indicate that the DOC2 translocation is directed by an interaction with Munc13-1 induced by Munc13-1-PE interaction. The intracellular localization of DOC2 protein is determined by the C2 domains of DOC2 associating with intracellular membranes, which are directed in their localization by PE-bound Munc13-1. Scale bars = 25 μm. See Fig. 1 legend for a key describing domain shading.

FIG. 4. The specific action of PMA was confirmed by substituting the inactive PE 4α-PMA in translocation assays. No translocation was observed using the fusion protein combination represented by DOC2-EGFP and Munc13-1 (panels a and b). Incubation of the cells for 15 min before and throughout PMA application with 100 nM bisindolylmaleimide had no effect on the translocation of DOC2-EGFP directed by PE-bound Munc13-1. The localization of fluorescent DOC2 before (c) and after (d) PMA treatment were as described for Fig. 2, e and f. However, treatment as before using the C1 domain-specific PKC inhibitor chelerythrine (25 μM) abolished DOC2-EGFP:Munc13-1 co-translocation, as shown in panels e (no PMA) and f (after PMA treatment, as described for Fig. 2). Scale bars = 25 μm. See Fig. 1 legend for a key describing domain shading.
All of the above experiments were repeated using the inactive PE 4α-PMA, and no stimulation-dependent translocation was observed using any construct combination (e.g. Fig. 4, a and b). Pretreatment of the cells for 15 min before the addition of PMA and during PMA treatment with the broad spectrum PKC inhibitor bisindolylmaleimide I (which does not block the PE-induced Munc13-1 translocation (1)) had no effect upon the previously observed translocations (Fig. 4, c and d). However, a similar pretreatment of the cells with the C1 domain-specific PKC inhibitor chelerythrine (which does block PE-induced Munc13-1 translocation (1)) abolished the translocation effects for all construct combinations (Fig. 4, e and f).

**DISCUSSION**

We have taken advantage of the absence of native Munc13-1 and DOC2β in HEK293 cells to perform a systematic dissection of the interaction between heterologously expressed GFP fusion proteins of DOC2β and Munc13-1. Although previous in vitro binding studies and peptide injection assays have suggested an interaction between these two proteins, our work demonstrates clearly a transient phorbol ester-stimulated interaction in vivo. Furthermore, our work shows that DOC2β is mobile, its localization being directly regulated by Munc13-1 and phorbol ester.

The central role of Munc13-1 in mediating the DOC2β translocation is supported by the observations that (i) translocation of DOC2β occurs only when Munc13-1 is co-expressed with DOC2β; (ii) the translocation can be blocked by chelerythrine but not by bisindolylmaleimide I, as in the case of translocation of Munc13-1 (1), but not of PKC (which is blocked by both agents); and (iii) translocation depends on the presence of the MID domain in DOC2β.

Munc13-1 and DOC2β interact only after PE stimulation. We speculate that PE binding to the C1 domain of Munc13-1 triggers a conformational shift in Munc13-1, exposing the DOC-interacting domain. As the endogenous ligand in this pathway is likely to be diacylglycerol, activation of the phospholipase C pathway may be a key regulatory event. In some synapses, phospholipase C activation may lead to parallel activation of PKC, which may have additional effects on secretion (11–13). The in vivo interaction between DOC2β and Munc13-1 reported here may represent the molecular basis of PE-dependent, PKC-independent, Munc13-1-mediated enhancement of neurotransmitter release.

**Acknowledgment**—We thank Linda Sharp for expert assistance in confocal imaging.

**REFERENCES**

1. Betz, A., Ashery, U., Rickmann, M., Augustin, I., Neher, E., Sudhof, T. C., Rettig, J., and Brose N. (1998) *Neuron* 21, 123–136
2. Orita, S., Naito, A., Sakaguchi, G., Maeda, M., Igarashi, H., Sasaki, T., and Takai, Y. (1997) *J. Biol. Chem.* 272, 16081–16084
3. Orita, S., Sasaki, T., Naito, A., Komuro, R., Ohnaka, T., Maeda, M., Suzuki, H., Igarashi, H., and Takai, Y. (1995) *Biochem. Biophys. Res. Commun.* 206, 439–448
4. Orita, S., Sakaguchi, G., Naito, A., Maeda, M., Igarashi, H., Sasaki, T., Komuro, R., and Takai, Y. (1996) *Mol. Biol. Cell* 7, (suppl.) 2603
5. Verhage, M., de Vries K. J., Roshal, H., Burbach, J. P., Gispen, W. H., and Sudhof, T. C. (1997) *Neuron* 18, 453–461
6. Tanaka, C., and Nishizuka, Y. (1994) *Annu. Rev. Neurosci.* 17, 551–567
7. Mochida, S., Orita, S., Sakaguchi, G., Sasaki, T., and Takai, Y. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 11418–11422
8. Chomczynski, P., and Saachi, N. (1987) *Anal. Biochem.* 162, 156–159
9. Kojima, T., Fukuda, M., Aruga, J., and Mikoshiba, K. (1996) *J. Biochem. (Tokyo)* 120, 671–676
10. Elferlink, L. A., Trimble, W. S., and Scheller, R. H. (1989) *J. Biol. Chem.* 264, 11061–11064
11. Hilfiker, S., and Augustine, G. (1999) *J. Physiol. (Lond.)* 515, 1
12. Yawo, H. (1999) *J. Physiol.* 515, 169–180
13. Stevens, C. F., and Sullivan, J. M. (1998) *Neuron* 21, 885–893