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Association of three small GTP-binding proteins with cholinergic synaptic vesicles

Walter Volknandt[a], Jonathan Pevsner[b], Lisa A. Elferink[b] and Richard H. Scheller[b]

[a]*AK Neurochemie, Zoologisches Institut der Universität, Siesmayerstr. 70, D-6000 Frankfurt/M. 11, Germany* and [b]*Howard Hughes Medical Institute, Department of Molecular and Cellular Physiology, Stanford University, Stanford, CA 94305, USA*

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Several small (low molecular weight) GTP-binding proteins are associated with cholinergic synaptic vesicles derived from the electric organ of electric ray. Using GTP overlay techniques and direct micro sequencing we analyzed the association of small GTP-binding proteins with synaptic vesicles. Both experimental procedures revealed the specific occurrence of multiple small GTP-binding proteins with this organelle. Moreover, direct amino acid sequence analysis assigned at least three different small GTP-binding proteins, ora3, o-ral and o-rab3, to the vesicular compartment. Furthermore, the data reflect the relative abundance of these three proteins on the vesicle membrane, thereby demonstrating the predominant occurrence of o-rab3, the only exclusively synaptic vesicle specific small GTP-binding protein.

GTP-binding protein; Cholinergic synaptic vesicle; Electric ray electric organ

1. INTRODUCTION

Synaptic vesicles play a key role in neurotransmission. To fulfill their tasks they contain a unique set of proteins. Biochemical and molecular genetic approaches using the electric organs of electric rays as a model system lead to the identification of numerous proteins of cholinergic synaptic vesicles. These include VAMP (synaptobrevin) [1], svp25 [2], synaptophysin [3], p65 (synaptotagmin) [3], synapsin [4], SV2 [5], proteoglycan [6] and the proton pumping vacuolar ATPase [7]. Many of these proteins are shared with synaptic vesicles derived from mammalian brain. Recently, small GTP-binding proteins have been assigned to this organelle [9–11].

So far more than 40 members of the ras superfamily of G-proteins have been described, and they regulate a diverse spectrum of elementary cellular processes. Via signal transduction pathways members of the ras and rho subfamilies serve in proliferation, differentation [12,13] and in the assembly of cytoskeletal elements [14,15]. In contrast, members of the rab and arf families are involved in regulating the transport of vesicles between intracellular compartments [16]. Binding and hydrolysis of GTP has been implicated as a regulatory switch, responsible for the vectorial flow of vesicles between compartments [17]. Genetic and biochemical evidence has implied the involvement of small GTP-binding proteins in secretion [18,19]. Regulated secretion by neurons involves the vectorial transport of the synaptic vesicle membrane from the cell soma to the presynaptic membrane. LMW GTP-binding proteins of the rab3 type subfamily have been characterized in bovine and rat brain and invertebrate neuronal tissues [20–22]. Recently, we have described the primary structure and subcellular localization of an electric ray rab3 homolog, o-rab3 [11,23]. Moreover, multiple GTP-binding proteins have been reported to be associated with cholinergic synaptic vesicles derived from electric ray electric organ [9,10]. In this study, by direct microsequencing of vesicular proteins we describe the identification and relative abundance of three small GTP-binding proteins at the vesicular membrane.

Correspondence address: W. Volknandt, AK Neurochemie, Zoologisches Institut der Universität, Siesmayerstr. 70, D-6000 Frankfurt/M. 11, Germany. Fax: (49) (69) 798 4820.

2. MATERIALS AND METHODS

2.1. *Isolation of small GTP-binding proteins derived from cholinergic synaptic vesicles*

Slightly different protocols were used for the isolation of cholinergic synaptic vesicles from the electric organ of individual electric ray species. Purification of synaptic vesicles from *Discopyge ommata* was performed according to the procedure of Carlson et al. [24] with slight modifications as described recently [9]. As a final purification step synaptic vesicles obtained after column chromatography on controlled pore glass (CPG) were pelleted at 180 000 × *g* for 1 h at 4°C. Purified cholinergic synaptic vesicles from frozen *Torpedo marmorata* electric organ were extracted as described previously [25]. Purified synaptic vesicles were subjected to SDS-PAGE and proteins migrating in the apparant molecular mass range of LMW GTP-binding proteins (around M_r 25 kDa) were electroeluted from gel slices. LMW GTP-binding proteins from synaptic vesicles derived from *Torpedo californica* electric organ were kindly provided by Dr. Stanley M. Parsons, Santa Barbara.

2.2. *Electrophoretic techniques and GTP-binding assay*

SDS-PAGE was carried out on minigels [26], and two-dimensional

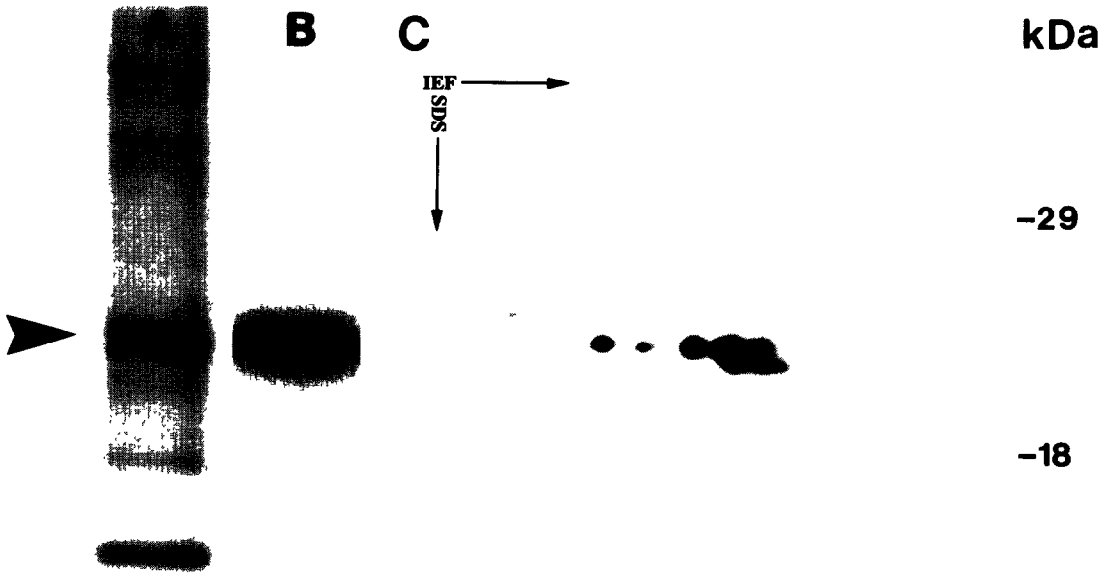

Fig. 1. Small GTP-binding proteins are associated with cholinergic synaptic vesicles. (A) Silver stained polypeptide pattern of synaptic vesicles derived from the electric organ of electric ray *Torpedo marmorata* purified on a Sephacryl-1000 column. (B) Autoradiograph of the same protein fraction after transfer to nitrocellulose and incubation with radiolabelled GTP indicating the position of small GTP-binding proteins. (C) Autoradiograph using a GTP overlay technique and synaptic vesicle proteins separated by two-dimensional gel electrophoresis. Note the presence of multiple spots labelled by bound [α-^{32}P]GTP. On the right the position of standard proteins (molecular mass in kDa) is shown. Arrows indicate the starting point in the first and second dimension.The arrowhead points to the position of small GTP-binding proteins.

gel electrophoresis was performed essentially as described by O'Farrell [27]. Proteins were identified by Coomassie brilliant blue or silver staining [28]. After gel electrophoresis and transfer to nitrocellulose [29] a GTP overlay technique according to the following GTP-binding protocol was performed. In brief, immediately after transfer blots were rinsed twice for 10 min in binding buffer (50 mM sodium phosphate buffer, pH 7.5 containing 10 μM $MgCl_2$, 2 mM DTT, 0.3% Tween 20 and 4 μM ATP). Blots were then incubated for 2 h in binding buffer containing 2 μCi/ml [α-^{32}P]GTP (3000 Ci/mmol, Amersham). Blots were washed 6 times for 3 min with binding buffer, and subsequently dried and exposed to X-ray film with intensifying screen. Finally, the blots were stained with Ponceau S to detect proteins and standards.

2.3. *Amino acid sequence analysis*

Direct amino acid sequence analysis by Edman degradation was performed as described recently [11]. Briefly, purified proteins as described above were subjected to cyanogen bromide cleavage. Protein fragments were separated by SDS-PAGE and blotted onto PVDF membranes. Furthermore, isolated proteins were also cleaved with lysyl endopeptidase (Wako). The resulting peptides were separated by reverse phase chromatography with a linear gradient from 5 to 60% acetonitrile in 0.1% trifluoroacetic acid.

3. RESULTS

Analysis of the polypeptide pattern of cholinergic synaptic vesicles revealed synaptic vesicle specific bands at M_r 25 kDa with one prominent band and a fainter second band below (Fig. 1A) indicating the presence of more than one protein in these bands. Moreover, there is a faint broad halo around the bands which is more pronounced after Coomassie brilliant blue than after silver staining (not shown). When synaptic vesicle proteins were separated by SDS-PAGE followed by transfer onto nitrocellulose and incubated with [α-^{32}P]GTP only the bands around 25 kDa efficiently bind GTP (Fig. 1B). Due to the limited resolution of the autoradiograph these proteins are represented by a single broad band. For a higher resolution and in order to visualize masked proteins synaptic vesicle proteins were subjected to two-dimensional gel electrophoresis and transferred proteins were incubated according to the GTP overlay protocol. Even after treatment with high molar urea in the first dimension and SDS in the second dimension several proteins indicated by spots are still able to bind GTP (Fig. 1C). Corresponding to the bands in Fig. 1A,B GTP-binding proteins migrate closely together revealing slightly different molecular masses but different isoelectric points. Whereas GTP-binding proteins with higher and lower apparent molecular masses are represented by one or two spots, the GTP-binding proteins migrating in between them are resolved in 5 different isoelectric spots.

To identify the synaptic vesicle proteins at M_r 25,000 Da electroeluted proteins were subjected to cyanogen bromide cleavage or enzymatic degradation with lysyl endopeptidase C. Protein fragments were separated either by SDS-PAGE or FPLC and used for direct micro sequencing by Edman degradation. To avoid the detection of proteins which might be minor contaminants of the highly purified cholinergic synaptic vesicle fraction and for the purpose of approximating the relative abundance of protein derived fragments, the strongest bands and highest peaks were analyzed, respectively. Isolated

Table I

Amino acid sequences of vesicle associated LMW GTP-binding proteins derived from the electric organ of electric rays

Peptides	Amino acid sequence	Protein/species	Residues in the predicted sequence	[Ref.]
CNBr-cleavage peptides:				
1.	(M)YDEFVEDYEPTGAD	o-ral/*T. californica*	35– 49	[10]
2.	(M)xxITNQ-NFNAVID	o-rab3/*D. ommata*	101–115	[11]
3.	(M)xxITNQDAFGQVED	o-rab3/*D. ommata*	101–115	[11]
Lysyl-endopeptidase-cleavage peptides:				
1.	(K)DAVDQNFDYMfK	o-rab3/*T. marmorata*	12– 24	[11]
2.	(K)DAVVLVFDYMFK	o-rab3/*T. marmorata*	12– 24	[11]
3.	(K)LLIIGNssVGK	o-rab3/*T. marmorata*	24– 35	[11]
4.	(K)TYSLDNAQVILVGNK	o-rab3/*T. marmorata*	121–136	[11]
5.	(K)xALTLQFMYDEFVEDYEPTK	o-ral/*T. marmorata*	27– 47	[10]
6.	(K)IPLDQmGNK	o-ral/*T. marmorata*	120–129	[10]
7.	(K)AEExGVQYVETsAK	o-ral/*T. marmorata*	146–159	[10]
8.	(K)SNLLSRFTRNEFNLESK	ora3/*T. marmorota*	23– 41	[10]

Synaptic vesicle proteins of about M_r 25,000 were purified from electric organs of various species of electric rays. Peptides obtained by treatment with CNBr or lysyl endopeptidase were sequenced as described. The peptides and their positions in the cDNA-derived sequences of LMW GTP-binding proteins are shown. Positions without amino acid assignment are donated by x, ambiguous amino acid residues are represented by small letters. Differences in the amino acid sequence obtained by direct microsequencing versus predicted sequence are underlined. Preceding amino acid residue depending on cleavage procedures is in parentheses.

and cleaved proteins from three different species of electric ray were evaluated. All amino acid sequences obtained were compared and aligned by computer search to protein sequence data bases. Table I lists 11 amino acid sequences which match the predicted sequences of cDNA derived nucleic acids coding for small GTP-binding proteins. In six instances we obtained partial amino acid sequences derived from o-rab3. Amino acid sequences derived from o-ral were obtained in 4 cases and a stretch of 14 amino acid residues with a hundred percent identity to ora3 in one case. According to the cleavage methods used the preceding amino acid residue can be added (in parentheses) and is found in the predicted sequences. The position of the polypeptide fragments in the respective protein sequences is indicated. Moreover, we obtained 7 sequences of 11 or more amino acid residues which did not match any of the amino acid sequences contained in the protein data bases (not shown).

4. DISCUSSION

Similar to the results obtained in this study, Ngsee et al. [9] showed three bands around 25 kDa when using a GTP overlay technique and synaptic vesicles derived from the electric ray *Discopyge ommata*. The limited resolution of GTP-binding proteins in one dimensional electrophoresis can be overcome by two-dimensional separation and subsequent GTP-binding overlay techniques. The number of GTP-binding proteins presumably is underestimated because after denaturing chemical treatment not all GTP-binding proteins may still be able to bind GTP. But not all GTP-binding proteins might be represented by one single spot only. In this respect it is unclear whether the spots with identical apparent molecular mass reflect different proteins or proteinaceous microheterogeneities of one or more proteins.

In order to identify small GTP-binding proteins which are associated with cholinergic synaptic vesicles we performed amino acid sequence analysis using Edman degradation. Therefore, vesicular proteins comprising the GTP-binding proteins as identified by GTP overlay techniques were isolated and subjected to direct protein sequencing. The data obtained not only demonstrate the specific association of at least three small GTP-binding proteins with the synaptic organelle but also reflect the relative abundance of these proteins on the vesicular membrane. Most of the amino acid sequences were derived from o-rab3 irrespective of the electric ray species. The exclusive association with the synaptic vesicle compartment of o-rab3, a rab3 homolog of the electric rays, has recently been described [11,23]. Four amino acid sequences have been obtained which match the predicted amino acid sequence for o-ral [10] suggesting the specific association of this small GTP-binding protein with cholinergic synaptic vesicles, and one amino acid sequence has been obtained for ora3. Similarly, previous reports describe the associaton of more than one GTP-binding protein with cholinergic synaptic vesicles derived from the electric organ of *Discopyge ommata* [9,10]. Although, the direct approach by protein sequencing resulted in N-terminal amino acid sequence of only one small GTP-binding protein present in the synaptic vesicle fraction (o-Krev) [10]. It can-

not completely be ruled out however, that the cholinergic synaptic vesicle fraction used for identification of small GTP-binding proteins might contain other populations of vesicles with a different set of GTP-binding proteins. Therefore we only analyzed fragments giving a high signal either in polypeptide staining or on HPLC separation. Furthermore, it was previously shown by Western blotting and antibodies against six different small GTP-binding that ora3 and o-ral gave the highest immunodetection signal in the synaptic vesicle fraction compared to the void volume of the column (see Fig. 4 in [10]). Whereas o-rab3 is associated with cholinergic synaptic vesicles in the peripheral nervous system it is restricted to subpopulations of neurons in the CNS [23] reflecting the highest specificity in cellular allocation so far reported for a member of the rab3 subfamily.

Several amino acid sequences obtained in this study did not belong to any of the known GTP-binding proteins or any protein sequence contained in the data bases. In this context it is noteworthy, that we have recently described the transmembrane glycoprotein svp25 which is abundant on cholinergic synaptic vesicles [2]. The amino acid sequences or at least part of it might be derived from this protein, although this has to be confirmed by cloning and sequencing svp25.

The function of o-rab3, o-ral and ora3 is still unresolved. A role for rab3A in the elementary process of exocytosis has been discussed [30]. The insertion of small GTP-binding protein is mediated by postranslational isoprenylation at conserved carboxy-terminal residues [22] whereas the hypervariable C-terminal domain acts as a targeting signal [31]. The mode of interaction of o-rab3, o-ral and ora3 with the vesicular membrane might be different. Whereas the insertion of o-rab3 and o-ral into the synaptic vesicle membrane presumably is mediated by one or two geranylgeranyls attached to the C-terminal cysteines, the type of isoprenylation serving as an anchor for ora3 is not defined yet [32].

Our results provide direct evidence for the association of at least three small GTP-binding proteins with the membrane of cholinergic synaptic vesicles. These three GTP-binding proteins might work in concert, where o-rab3 might be involved in exocytosis the other two might regulate other steps in vesicle membrane transport in the nerve terminal by mediating membrane-membrane interaction.

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