Doc2 is not associated with known regulated exocytotic or endosomal compartments in adrenal chromaffin cells

Nathalie CHARVIN, Geoff WILLIAMS and Robert D. BURGOYNE¹

The Physiological Laboratory, University of Liverpool, Crown Street, Liverpool L69 3BX, U.K.

Doc2 is a C2-domain-containing protein that is highly expressed in the nervous system and has a constitutively expressed isoform. It has been implicated as a potential Ca^{2+} sensor in regulated exocytosis, and has been suggested to be associated with synaptic vesicles. To examine whether Doc2 is associated with synaptic-like microvesicles (SLMVs) or dense-core granules in neuroendocrine cells, we examined the distribution of Doc2 in subcellular fractionation of chromaffin cells of the adrenal medulla and in PC12 cells. Doc2 did not co-distribute with SLMVs from either cell type, but did appear to co-distribute with dense-core granules from PC12 cells. In contrast, it was not associated with

the dense-core granules during subcellular fractionation of the adrenal medulla, and nor did it appear to be associated with endosomes, *cis*-Golgi or the *trans*-Golgi network. In contrast, Doc2 co-distributed under all conditions with a mitochondrial marker. We conclude that Doc2 is not a general component of regulated secretory vesicles, but may instead be associated with mitochondria.

Key words: calcium, exocytosis, mitochondria, PC12 cells, secretory granule.

INTRODUCTION

Regulated exocytosis, in synapses, endocrine and other cell types, triggered by an elevation of the cytosolic Ca^{2+} concentration, is a multistage process involving secretory vesicle recruitment and priming, vesicle attachment to the plasma membrane, vesicle docking, and finally membrane fusion [1]. Functional evidence suggests that several of these stages are regulated by Ca^{2+} , and are likely to involve distinct Ca^{2+} -binding proteins with differing Ca^{2+} affinities [2]. While a number of candidate Ca^{2+} -binding proteins have been implicated in Ca^{2+} -regulated exocytosis, definitive evidence for their assignment to one or other stage is still not available [2].

Several C2-domain-containing proteins have been suggested to function as Ca^{2+} sensors in one or other stage of the regulated exocytotic pathway. These include the synaptotagmins [3], rabphilin [4], Rim [5] and Doc2 [6], all of which are expressed at high levels in neuronal tissues. Doc2 contains two C2 domains, which are likely to allow its Ca^{2+} -dependent binding to phospholipids [7], and it has been implicated in regulated exocytosis as a result of its ability to bind munc-18 (nsec-1) [8] and munc-13 [9]. These two proteins are known to have a key function in neurotransmitter release, based on molecular genetic evidence [10,11]. In addition, overexpression of Doc2 increased the release of co-transfected growth hormone (as a marker of secretory granule contents) from neuroendocrine PC12 cells, and co-transfection with N- or C-terminal fragments of Doc2 was inhibitory [12]. Doc2 is present, but not highly enriched, in synaptic vesicle fractions [6,8], and so it is unclear whether or not it is a *bona fide* secretory vesicle protein.

PC12 and chromaffin cells possess two well-characterized regulated secretory vesicle populations, dense-core secretory granules and synaptic-like microvesicles (SLMVs). In addition, the existence of a novel Ca^{2+} -dependent secretory vesicle population, that may be present in all cell types, has been revealed by patch-clamp capacitance recording in fibroblasts [13,14]. We

decided, therefore, to examine the subcellular distribution of Doc2 in PC12 and adrenal chromaffin cells, two widely studied model neuroendocrine cell types, in order to determine with which secretory vesicles Doc2 is associated. Since SLMVs are generated from endosomal intermediates [15], we also examined the possibility that Doc2 may be associated with endosomes. The data from the present study show that Doc2 is not associated with SLMVs in either cell type. In PC12 cells Doc2 appears to co-distribute with dense-core granules and mitochondria, but in chromaffin cells it is not present on dense-core granules or endosomes, and instead may be associated with mitochondria. These data suggest that Doc2 is unlikely to be directly involved in dense-core-granule exocytosis.

MATERIALS AND METHODS

Subcellular fractionation of adrenal medulla

For the preparation of subcellular fractions, including a large-granule fraction [16], bovine adrenal medullas were dissected and homogenized in buffer A (0.3 M sucrose, 5 mM EDTA, 5 mM Hepes, pH 7.3), and a post-nuclear supernatant (PNS) was prepared and fractionated by differential centrifugation as previously described [17] to prepare cytosol, microsome and granule membrane fractions. For the fractionation of the PNS on sucrose gradients, the PNS was centrifuged at 100 000 *g* for 60 min, and the pelleted organelles were resuspended in buffer A, loaded on a linear 0.3–2.0 M sucrose gradient and centrifuged at 100 000 *g* for 90 min. A total of 14 fractions of 1 ml each were collected for analysis. For the fractionation of the lysed granule fraction [17], a large-granule fraction was lysed by resuspension in 5 mM EDTA/20 mM Hepes, pH 7.3, followed by freezing and thawing. After washing, the membranes were loaded on a linear 0.3–1.2 M sucrose gradient, centrifuged at 115 000 *g* for 90 min, and 14 fractions of 1 ml were collected.

Abbreviations used: D β H, dopamine- β -hydroxylase; GDH, glutamate dehydrogenase; PNS, post-nuclear supernatant; SLMVs, synaptic-like microvesicles; TGN-38, *trans*-Golgi network protein of 38 kDa.

¹ To whom correspondence should be addressed (e-mail burgoyne@liverpool.ac.uk).

Fractionation of PC12 cells

A PNS was prepared from PC12 cells [18], loaded on a linear 0.6–1.8 M sucrose gradient [19] and centrifuged at 100 000 *g* for 6 h at 4 °C, and 13 fractions of 1 ml were collected. For the fractionation of lysed organelles, fractions from this initial gradient were lysed by resuspension in 5 mM EDTA/20 mM Hepes, pH 7.3, followed by freezing and thawing. The membranes were pelleted by centrifugation at 100 000 *g* for 60 min, washed, resuspended and loaded on a linear 0.3–1.8 M sucrose gradient, which was centrifuged at 115 000 *g* for 90 min.

SDS/PAGE and immunoblotting

Subcellular and gradient fractions were separated by SDS/PAGE on 12.5% (w/v) gels. Immunoblotting was carried out as previously described [20] using the following antibodies at the dilutions indicated. Monoclonal antibodies were against dopamine β -hydroxylase (D β H) (1:100; a gift from Dr. D. Apps, Department of Biochemistry, University of Edinburgh, U.K.), synaptophysin (1:1000; Sigma Chemical Co., Poole, Dorset, U.K.), Doc2 (1:250; Transduction Laboratories, Lexington, KY, U.S.A.), munc-18 (1:250; Transduction Laboratories) and cathepsin B (1:100; Calbiochem-Novabiochem, Nottingham, U.K.). Rabbit polyclonal antibodies were against glutamate dehydrogenase (GDH) (1:400; a gift from Dr. R. Lighthowler, University of Newcastle, U.K.), protein disulphide isomerase (1:500; Stressgen Biotechnologies Corp., Victoria, British Columbia, Canada), α -mannosidase II (1:400; a gift from Dr. K. Moremen, University of Georgia, Athens, GA, U.S.A.), Rab5 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), Rab7 (1:500; a gift from Dr. M. Zerial, EMBL, Heidelberg, Germany), secretogranin II (1:1000; a gift from Dr. D. Cutler, University College London, U.K.) and TGN-38 (*trans*-Golgi network protein of 38 kDa) (1:400; a gift from Dr. G. Banting, University of Bristol, U.K.). In some cases, blots were probed several times with different primary antibodies with or without prior stripping of antibodies by incubation for 30 min in 20 mM glycine, 137 mM NaCl, pH 2.5. Bound antibodies were detected by incubation with anti-(mouse IgG) or anti-(rabbit IgG) conjugated to horseradish peroxidase, followed by enhanced chemiluminescence (Amersham). All antibodies detected single polypeptides of the expected molecular mass, and no polypeptides were detected under the same conditions with non-immune antisera.

RESULTS

A monoclonal antibody raised against the Doc2-specific N-terminal domain recognized a major polypeptide in a rat brain homogenate of the expected molecular mass of 47 kDa and a smaller minor polypeptide that may be a proteolytic fragment (Figure 1A). A single polypeptide was detected in chromaffin cells that co-migrated with the major polypeptide in brain (Figure 1A) and with recombinant Doc2 α . Previous work has shown that it is the Doc2 β isoform that is expressed in adrenal medulla [8]. In a conventional subcellular fractionation of the adrenal medulla, Doc2 was not detected in the cytosol fraction, but was substantially enriched in membranes prepared from a large-granule fraction (enriched in chromaffin granules) compared with microsomes (Figure 1B). The microsomal fraction is believed to contain plasma membranes, endoplasmic reticulum and Golgi membranes that may, therefore, contain relatively little Doc2.

In order to examine further whether the Doc2 in the large-granule fraction was associated with chromaffin granules or with another organelle, its distribution was examined following sep-

Figure 1 Detection of Doc2 in brain and chromaffin cells, and its presence in subcellular fractions from adrenal medulla

(A) A rat brain homogenate and a sample of cultured adrenal chromaffin cells at the same level of protein loading were separated and probed with anti-Doc2. The position of molecular mass markers is shown on the left. (B) Cytosol, microsome and granule membranes were prepared from bovine adrenal medulla and probed with anti-Doc2. The arrows indicate the position of Doc2.

aration of post-nuclear organelles in a PNS of the adrenal medulla on a 0.3–2.0 M sucrose gradient, and compared with that of chromaffin-granule and other organelle markers on this sucrose gradient. Doc2 showed a peak of distribution in fractions 7–9 (Figure 2). In contrast, D β H, a soluble and membrane-bound component of chromaffin granules, was distributed across the gradient, but was present at highest levels in fractions 10–14, where the very dense intact chromaffin granules sediment (Figure 2), indicating that the distribution of Doc2 did not follow that of chromaffin granules on this gradient. In order to examine whether Doc2 is associated with SLMVs, its distribution can be compared

Figure 2 Distribution of Doc2 and other markers in the fractionation of post-nuclear organelles from adrenal medulla

A PNS from adrenal medulla was fractionated on a 0.3–2.0 M sucrose gradient, and the distributions of Doc2 and various marker proteins were determined using immunoblotting with specific antisera. The positions of SLMVs and intact dense-core granules, based on the distributions of D β H and synaptophysin, are indicated. Man II, α -mannosidase II.

Figure 3 Distribution of Doc2 in sucrose-gradient fractionation of lysed dense-core granules

(A) A large-granule fraction from the adrenal medulla enriched in chromaffin granules was lysed and fractionated on a 0.3–1.2 M sucrose gradient, and fractions were probed with specific antisera as indicated. Following lysis, predominantly the 43 kDa form of cathepsin B remained membrane-associated. (B) Quantification of DβH, GDH and Doc2 immunoreactivity across the gradient, showing the clear separation of DβH and the co-distribution of Doc2 and GDH.

with that of synaptophysin. Synaptophysin is known to be present in both SLMVs and chromaffin-granule membranes [21]. A peak of synaptophysin in fractions 3–6 represents the sedimentation of SLMVs (Figure 2). These fractions contained little or no Doc2, indicating its absence from SLMVs.

The possibility that Doc2 was associated with other known vesicular organelles sedimenting in the dense region of the sucrose gradient was examined. Markers for the *cis*-Golgi (mannosidase II) and the *trans*-Golgi network (TGN-38) were enriched near the top of the gradient, in fractions 2–6 and 2–3 respectively. In addition, the distribution of Doc2 differed from that of the lysosomal marker cathepsin B, which is recognized by the antiserum as 31 and 43 kDa polypeptides, and showed a peak of distribution in fraction 9. Rab5 (a marker for early endosomes) and Rab7 (a marker for late endosomes) both showed peaks of distribution across the gradient in the same fractions as Doc2, but Rab5 was distributed over a wider range of fractions than Doc2 (Figure 2). In contrast, a marker for mitochondria, GDH, showed a distribution essentially identical with that of Doc2, suggesting that Doc2 might be associated with mitochondria.

Low levels of Doc2 were present in some dense fractions containing DβH, and some Doc2 was also present along with

cathepsin B in fraction 13. Therefore, in order to further examine the possible co-fractionation of Doc2 in lysosomes and endosomes, and whether Doc2 co-distributes with GDH under other conditions, the distribution of markers was examined after fractionation of lysed organelles from a large-granule fraction. Chromaffin granules and certain other organelles run in dense fractions on sucrose gradients due to their content. We have previously shown that, in order to shift the distribution of chromaffin-granule membranes, the organelles in a large-granule fraction can be lysed; the granule membranes are then distributed in markedly less dense fractions when run on a 0.3–1.2 M sucrose gradient [17]. Following this procedure, granule membranes, indicated by the presence of DβH and synaptophysin (Figures 3A and 3B), were found in less dense fractions than expected for intact granules, and were clearly separated from membranes containing Doc2, which remained in the denser fractions (9–14) on this gradient (Figures 3A and 3B). There was little evidence for any intact granules remaining on the gradient, as little or no synaptophysin (an integral membrane component of SLMVs and chromaffin granules) was detected in fractions 12–14. These data indicate that little, if any, Doc2 was associated with chromaffin-granule membranes. Around 30 % of cellular Rab5 and Rab7 was found in the large-granule fraction. It is clear from Figure 3 that, following lysis, membranes from early and late endosomes, indicated by associated bound Rab5 or Rab7, were now also clearly resolved from Doc2-containing vesicles, indicating that these endocytotic organelles also did not contain any substantial amounts of Doc2. The lysosomal marker cathepsin B peaked in fractions 8 and 9, and thus showed a different distribution compared with that seen for Doc2. Significantly, the mitochondrial marker GDH was detected in the same fractions as Doc2 (Figure 3A), and indeed these two proteins showed exactly overlapping distributions (Figure 3B).

These data indicate that Doc2 is associated with organelles distinct from chromaffin granules, SLMVs, early and late endosomes and lysosomes in adrenal medullary chromaffin cells; these organelles are most probably mitochondria. The density of the Doc2-containing organelles on sucrose gradients also excludes the presence of Doc2 on Golgi compartments (*cis*-Golgi and *trans*-Golgi network), which run in much lighter fractions near the top of the gradients used for the analysis of intact post-nuclear organelles (see also [22,23]).

The only functional data on Doc2 in regulated exocytosis comes from a study on PC12 cells [12], and so we also examined the distribution of Doc2, compared with SLMVs, dense-core granules, mitochondria and endosomes, in this cell type. This was initially examined by fractionation of a PC12 PNS on a 0.6–1.8 M sucrose gradient. As shown in Figure 4(A), the peak of Doc2 distribution in fractions 7 and 8 was clearly distinct from the position of SLMVs, shown by synaptophysin in fractions 3–5, again indicating that Doc2 is not associated with this class of secretory vesicles. The Doc2 distribution was, however, similar to that of PC12 dense-core granules, which are indicated by the presence of secretogranin II, a protein present in dense-core granule contents. The distribution of Doc2 differed from that of the endosomal markers Rab5 (distributed over fractions 1–8) and Rab7 (fractions 5 and 6), but was again very similar to that of the mitochondrial protein GDH (Figure 4A). In addition, the distribution of Doc2 was not identical with that of its proposed binding partner munc-18, which showed a peak in fractions 6 and 7 and had a more widespread presence in lighter fractions compared with Doc2. To examine the significance of the co-distribution of Doc2, secretogranin and GDH, peak fractions 6–9 were pooled and lysed to prepare granule membranes which would be shifted in density compared with the intact granules.

Figure 4 Distribution of Doc2 on fractionation of a PNS from PC12 cells and lysed dense-core-granule fractions

(A) A PNS from PC12 cells was fractionated on a 0.6–1.8 M sucrose gradient, and the fractions were probed with specific antisera as indicated. The distribution of SLMVs and dense-core granules, based on the distribution of the secretogranin II and synaptophysin markers, is indicated. (B) Fractions containing dense-core granules (6–9) from the post-nuclear gradient were lysed and re-run on a 0.3–1.8 M sucrose gradient, and fractions were probed for synaptophysin, GDH and Doc2.

The lysed material was run on two different gradients, a 0.3–1.2 M sucrose gradient and subsequently a 0.3–1.8 M sucrose gradient. On both gradients, Doc2 and GDH co-distributed along with the granule membrane marker synaptophysin. As illustrated for the 0.3–1.8 M sucrose gradient, we were unable to resolve dense-core granules from mitochondria and the co-distributing Doc2 (Figure 4B).

DISCUSSION

The data presented in this paper show that, in adrenal chromaffin cells, Doc2 does not co-distribute with SLMVs, with dense-core granules or with known endocytotic organelles or Golgi compartments, but instead shows a marked co-distribution with mitochondria. Doc2 has been described as a synaptic vesicle protein [6,8]. Previous work has detected Doc2 in a synaptic vesicle fraction from rat brain, but without the marked enrichment during purification expected for a *bona fide* synaptic vesicle protein [6,8]. The data presented here show that Doc2 is clearly not associated with SLMVs in either chromaffin or PC12 cells, despite these organelles containing many synaptic vesicle proteins, or with chromaffin granules. Our data cannot rule out the presence of Doc2 on dense-core granules in PC12 cells, but we could not resolve dense-core granules from mitochondria in these cells, and therefore Doc2 may also be associated with

mitochondria in this cell type. We were unable to further examine the localization of Doc2 using immunocytochemistry. The anti-Doc2 antibody used in this study gave poor signals in immunocytochemistry, precluding its use in this technique, and no other anti-Doc2 antibodies have so far been found to be useful for immunocytochemical localization [8,9]. Transfection of haemagglutinin-tagged Doc2 has allowed examination of the immunofluorescence distribution of the overexpressed protein, using anti-haemagglutinin, which showed a diffuse localization in PC12 [12] and BHK [24] cells that was not characteristic of any particular organelle with a restricted distribution. The data presented here constitute the only detailed analysis so far of the distribution of Doc2 and, significantly, clearly show the absence of this protein from known secretory vesicles and granules in adrenal chromaffin cells. This suggests that Doc2 is not a general exocytotic vesicle protein, and is unlikely to function in the well-characterized dense-core-granule exocytosis in chromaffin cells, but may instead play some role in mitochondrial function.

The specific interaction of the C2-domain-containing protein Doc2 with munc-18 [7] and munc-13 [8] provided strong but indirect evidence that this Ca^{2+} -binding protein functions in the regulated exocytosis machinery. While munc-18 is detectable in fractions containing Doc2, on fractionation of both PC12 cells (the present paper) and adrenal chromaffin cells (L. P. Haynes, A. Morgan and R. D. Burgoyne, unpublished work) its overall distribution differed from that of Doc2, and in the fractionation of lysed chromaffin granules munc-18 was not detected in dense Doc2-containing fractions (L. P. Haynes, A. Morgan and R. D. Burgoyne, unpublished work). Transfection studies with PC12 cells suggested that overexpression of Doc2 or Doc2 fragments stimulated or inhibited respectively the regulated release of growth hormone synthesized from a co-transfected plasmid and incorporated into dense-core granules [12]. That study, however, examined the effects on release due to stimulation of intact cells with high KCl concentrations and, therefore, could not distinguish between a direct effect on exocytosis and some indirect effect via channels, calcium signalling or a general effect on cell function. Another possible explanation is that Doc2 overexpression has some effect on packaging of the reporter growth hormone in secretory granules. The finding that Doc2 is likely to be present on mitochondria may provide a clue as to the mechanism of the effects of overexpression of Doc2 or Doc2 fragments on growth hormone release, and suggest that this may be indirect and due to changes in mitochondrial function.

Recent work has shown that Doc2 can interact with a light chain of cytoplasmic dynein and that the overexpression of Doc2 fragments disrupts transport from the *trans*-Golgi network to late endosomes in BHK cells [24], consistent with a non-exocytotic function for Doc2. The α -isoform of Doc2 appears to be expressed predominantly in the nervous system, but the β -isoform is ubiquitously expressed [7,8,25] and is present at similar levels even in tissues (e.g. skeletal muscle) not known to have cells with conventional regulated secretory pathways. This raised the possibility that Doc2 β may have functions in other aspects of vesicular traffic. Doc2 β appears to be the main or only isoform expressed in the adrenal medulla [8]. We did not find Doc2 co-distributed with early or late endosomal markers in chromaffin cells, arguing against a role in this pathway. Another possibility is that it is associated instead with a novel Ca^{2+} -regulated exocytotic vesicle. Evidence for such an organelle comes from the observation of a third uncharacterized vesicle type in PC12 cells that expresses GLUT4 after transfection [26], and the fact that fibroblasts can demonstrate Ca^{2+} -regulated exocytosis via some unknown vesicle population [13,14]. The data presented here suggest, in contrast, that Doc2 is associated

with mitochondria, and its exact cellular function remains to be established.

We are grateful to Dr. D. Apps, Dr. G. Banting, Dr. D. Cutler, Dr. K. Moremen and Dr. M. Zerial for gifts of antibodies. This work was supported by the Wellcome Trust.

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Received 2 February 1999/6 April 1999; accepted 28 April 1999