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Specific role for the PH domain of dynamin-1 in the regulation of rapid endocytosis in adrenal chromaffin cells

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Dynamin plays a key role in the scission event common to various types of endocytosis. We demonstrate that the pleckstrin homology (PH) domain of dynamin-1 is critical in the process of rapid endocytosis (RE) in chromaffin cells. Introduction of this isolated PH domain into cells at concentrations as low as 1 μM completely suppressed RE. PH domains from other proteins, including that from the closely related dynamin-2, were ineffective as inhibitors, even at high concentrations. Mutational studies indicated that a pair of isoform-specific amino acids, located in a variable loop between the first two β-strands, accounted for the differential effect of the two dynamin PH domains. Switching these amino acids in the dynamin-2 PH domain to the equivalent residues in dynamin-1 (SL→GI) generated a molecule that blocked RE. Thus, the PH domain of dynamin-1 is essential for RE and exhibits a precise molecular selectivity. As chromaffin cells express both dynamin-1 and -2, we speculate that different isoforms of dynamin may regulate distinct endocytotic processes and that the PH domain contributes to this specificity.

Keywords: chromaffin cells/dynamin/endocytosis/PH domain

Introduction

The dynamin family of GTPases function in the pinch-off reaction underlying various types of endocytosis at the plasma membrane. For example, the *Drosophila* mutant *shibire* (Kosaka and Ikeda, 1983), later shown to encode a defective form of fly dynamin (Chen *et al.*, 1991; van der Bliek and Meyerowitz, 1991), and dominant-negative inhibition of dynamin function in fibroblasts (Herskovits *et al.*, 1993a; van der Bliek *et al.*, 1993) both lead to coated pit accumulation indicative of a block in receptor-mediated endocytosis. In addition, *shibire* mutant flies accumulate uncoated tubulovesicular membranes, suggesting that other forms of endocytosis are also dependent on a functional dynamin protein (Kosaka and Ikeda, 1983). Recently, we showed that GTP hydrolysis and dynamin are required for a very specific form of membrane retrieval in secretory cells termed rapid endocytosis (RE; Artalejo *et al.*, 1995). Introduction of GTPγS or anti-dynamin antibodies into adrenal chromaffin cells led to a complete abolition of RE. As detected by patch–clamp measurements of cell membrane capacitance, RE immediately follows stimulated exocytosis of catecholamines in these cells. RE is a kinetically complex and highly regulated event that is complete within 20 s. The speed of membrane recapture typical of RE, together with the fact that it does not involve clathrin, distinguishes the process from receptor-mediated endocytosis (Artalejo *et al.*, 1995). Like exocytosis, RE requires Ca^{2+}, but we recently showed that the divalent cation receptor for exocytosis and RE are distinguishable and that RE is regulated by calmodulin (Artalejo *et al.*, 1996). A phenomenon with kinetic properties similar to RE has been demonstrated in a number of cell types (e.g. Parsons *et al.*, 1994; Thomas *et al.*, 1994; von Gersdorff and Matthews, 1994) and is clearly of widespread occurrence (for review, see Henkel and Almers, 1996). Indeed, RE may represent the first step in synaptic vesicle recycling in the nervous system; as such, it is important to understand the molecular basis for this process in detail.

The mechanism whereby dynamin regulates any endocytotic event has not been elucidated. For example, several putative dynamin-interacting molecules have been identified *in vitro*, e.g. microtubules (Shpetner and Vallee, 1992), grb2 (Gout *et al.*, 1993), amphiphysin (David *et al.*, 1996); for a review, see Vallee and Okamoto (1995). However, the role of such interactions in dynamin function *in vivo* remains to be determined. Another complicating factor is the existence of multiple dynamin isoforms that exhibit a differential tissue distribution (Nakata *et al.*, 1993; Cook *et al.*, 1994; Sontag *et al.*, 1994). Dynamin-1 is prevalent in the CNS where it is highly concentrated in neurons (Noda *et al.*, 1993; Powell and Robinson, 1995), whereas dynamin-2 predominates in non-neuronal tissues (Sontag *et al.*, 1994) and dynamin-3 seems to be testis-specific (Nakata *et al.*, 1993). Whether some individual cell types express more than one isoform of the protein, or if different modes of endocytosis are mediated by distinct isoforms of dynamin, is not yet firmly established. Moreover, comparative analysis of the properties of different dynamin isoforms has not been made. Each family member contains a highly conserved N-terminal GTPase domain that is critical to the function of the protein. The mutations in this domain found in the *shibire* mutant fly (van der Bliek and Meyerowitz, 1991) manifest as a temperature-sensitive block in endocytosis. Introduction of similar mutations by site-directed mutagenesis of human dynamin-1 results in

a non-functional protein that acts as a dominant-negative suppressor of receptor-mediated endocytosis when over-expressed (Herskovits *et al.*, 1993a; Damke *et al.*, 1994). The C-terminal proline-rich tail of dynamin is variable between isoforms and has been implicated in the interactions of the protein with microtubules and SH3 domain-containing proteins (Gout *et al.*, 1993; Herskovits *et al.*, 1993b; Scaife *et al.*, 1994) as well as in the formation of homo-oligomers (Tuma and Collins, 1995).

All dynamins also contain a central pleckstrin homology (PH) domain. PH domains are structurally distinct modules of ~120 amino acids that have been identified in diverse (>90) signaling proteins (for reviews, see Gibson *et al.*, 1994; Saraste and Hyvönen, 1995; Lemmon *et al.*, 1996). Most of these proteins seem to have a functional requirement for membrane association and PH domains may be essential to such interactions (Lemmon *et al.*, 1996). For example, deletion of the PH domain compromises function in proteins that exhibit signal-dependent recruitment to the cell surface: e.g. Sos (Myers *et al.*, 1995), IRS-1 (McCollam *et al.*, 1995) and Dbl (Zheng *et al.*, 1996b). Recent studies with the PH domains of phospholipase (PLC)δ1 (Lemmon *et al.*, 1995) and other proteins (Harlan *et al.*, 1994; Hyvönen *et al.*, 1995; Zheng *et al.*, 1996a) suggest that some PH domains bind to membrane lipids, although protein ligands may also be involved in other cases (see Discussion).

While structural studies of the isolated PH domain from dynamin-1 have reached a refined stage (Ferguson *et al.*, 1994; Timm *et al.*, 1994; Fushman *et al.*, 1995), its role in dynamin function remains unclear. In the light of the studies summarized above, we hypothesized that dynamin recruitment to the cell surface prior to membrane retrieval might depend on its PH domain. Here, we have analyzed the contribution of the PH domain of dynamin to RE as part of our efforts to understand the molecular basis for this form of endocytosis. We find that introduction of the isolated PH domain of dynamin-1 into adrenal chromaffin cells completely blocks RE. This effect is highly specific as even the closely related PH domain from dynamin-2 is ineffective as an inhibitor. We have localized the key residues underlying the difference between these similar PH domains to a pair of amino acids in a variable loop connecting the first two β-strands in the structure.

Results

The PH domain from dynamin-1 but not from other proteins blocks RE

The involvement of the dynamin-1 PH domain in chromaffin cell RE was tested by introducing the bacterially produced recombinant protein (Ferguson *et al.*, 1994) into the cell via diffusion from the patch pipette, and measuring the effect on the membrane capacitance decrease characteristic of RE (Artalejo *et al.*, 1995, 1996). We reasoned that, if the PH domain were critical for dynamin-1 function, an excess of the isolated domain should act as a dominant-negative suppressor of RE by binding to sites normally occupied by the intact protein. As shown in Figure 1, this prediction was found to be correct. In the absence of exogenous protein, two rounds of exocytosis, each followed by RE, could be elicited reproducibly in chromaffin cells (Figure 1A; see also Artalejo *et al.*, 1995, 1996). Introduction of dynamin-1 PH domain (0.5 mg/ml; ~40 μM) into the cell totally blocked the second round of RE without significant effects on either cell Ca^{2+} currents or the rate and extent of exocytosis, including the 'excess retrieval' that accompanies many RE events (Figure 1B; Table I). These data indicate that the effect of the exogenous protein was selective and not due to a general toxic effect on the cell. Complete inhibition of RE was also found in experiments where a 40-fold lower concentration of dynamin-1 PH in the pipette (~1 μM) was used (data not shown). As discussed previously (Artalejo *et al.*, 1995, 1996), the paradigm used here permits the same cell to act as both the control and experimental sample because RE is normal immediately after patching onto the cell, prior to diffusion of significant amounts of a high molecular weight effector (such as a PH domain) into the cell interior. We next tested whether the ability of the dynamin-1 PH domain to inhibit RE was a general property of PH domains or was specific to the domain used. Introduction of PH domains from PLCδ1 (Figure 1C; Table I) or pleckstrin (N-terminal; Table I) into the cell at 0.5 mg/ml had no effect on RE, suggesting that the effect was indeed specific for the dynamin-1 PH domain.

To address the question of dynamin isoform specificity we then conducted similar experiments with isolated dynamin-2 PH domain (Figure 1D; Table I). While the PH domains of PLCδ1 and pleckstrin show only 14 and 25% amino acid sequence identity with that of dynamin-1, the PH domains of dynamin-1 and -2 are 81% identical (Sontag *et al.*, 1994). Surprisingly, introduction of the dynamin-2 PH domain into chromaffin cells had no effect on RE at the same concentration used for the dynamin-1 PH domain studies (0.5 mg/ml). Three separate batches of dynamin-2 PH, including a form with a 22-amino acid C-terminal extension, were used and all proved ineffective in this assay. These results (summarized in Table I) not only indicate that the PH domain of dynamin is essential for RE, but also strongly suggest that the role of the PH domain is highly specific and render support for the hypothesis that the mechanism of action of the isolated PH domain is dominant-negative interference with the normal binding site(s) for dynamin-1 on the membrane. Most importantly, these findings argue that dynamin-1, and not dynamin-2, mediates RE.

Chromaffin cells normally express both dynamin-1 and -2

Members of the dynamin family exhibit a differential tissue distribution (Sontag *et al.*, 1994), but whether any single cell type expresses more than one form of dynamin has not been extensively investigated. To determine which isoforms of dynamin exist in chromaffin cells we conducted RT–PCR analysis with primers specific for dynamins-1, -2 and -3 (Figure 2A). mRNAs for dynamins-1 and -2, but not dynamin-3, were found to be present by this criterion and sequencing of the PCR products showed that they represented the bovine forms of dynamins-1 and -2 (Figure 2B). Northern blot analysis with specific probes confirmed the presence of dynamin-1- and -2-specific mRNAs of ~4 kb (Figure 2C) in line with previous analyses (Sontag *et al.*, 1994). Further analysis of dynamin expression in chromaffin cells by two-

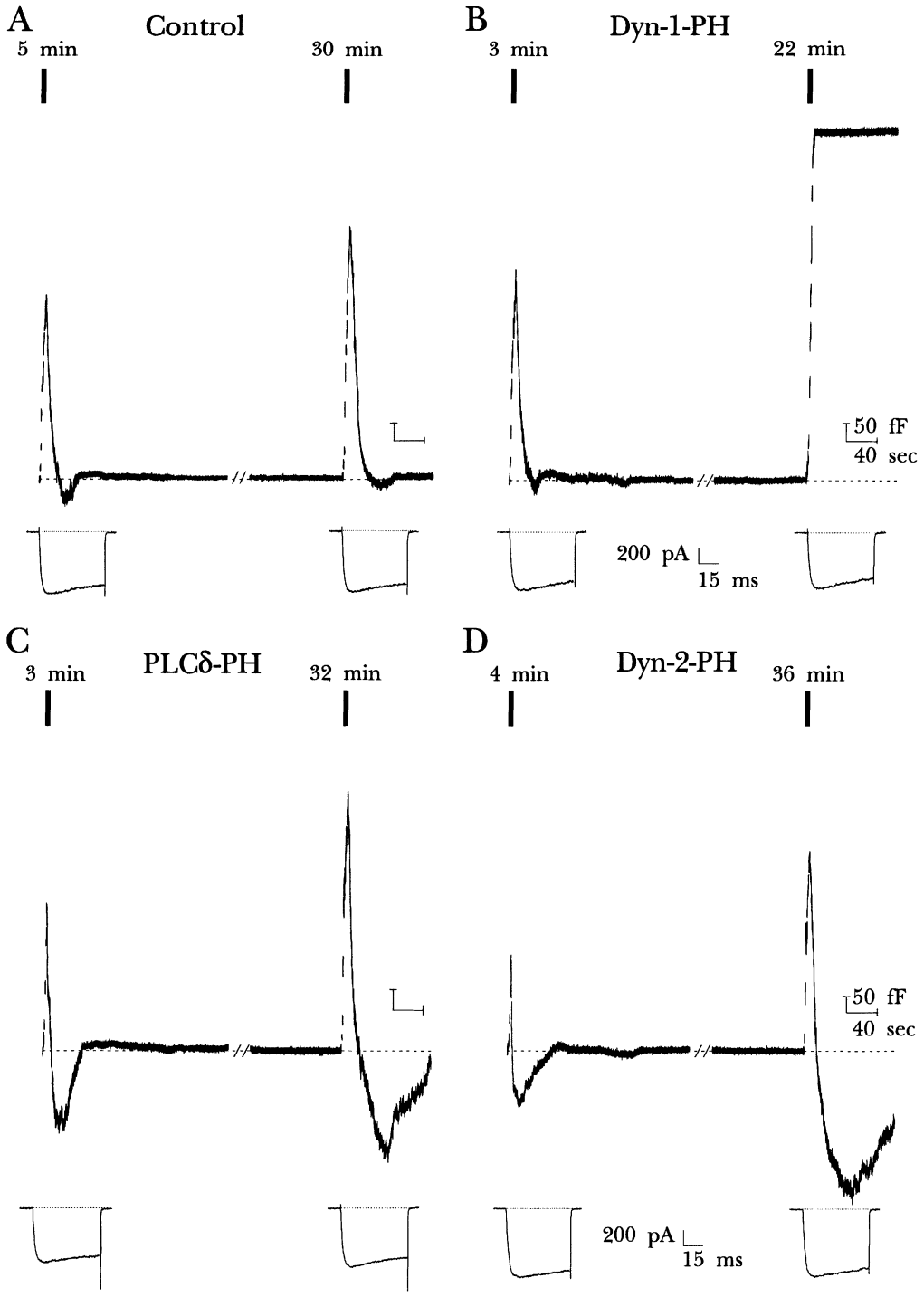

Fig. 1. Inhibition of chromaffin cell RE by dynamin-1 PH domain, but not other PH domains. Continuous membrane capacitance (C_m) recordings from individual calf adrenal chromaffin cells in which the patch pipette contained: (**A**) no protein (control); (**B**) human dynamin-1 PH domain; (**C**) rat PLCδ1-PH domain; or (**D**) rat dynamin-2 PH domain (all at 0.5 mg/ml). RE was assayed by measurement of membrane capacitance decrease (downward trace; dotted line is baseline) after triggering secretion (recorded as a capacitance increase; upward trace) with a train of ten 50-ms depolarizing pulses from a holding potential of –90 mV to +10 mV (bars above traces). Two rounds of exocytosis/RE were elicited (times above bars), one shortly after patching onto the cell and establishing the whole-cell mode, and a second 20–30 min later after allowing the various PH domains to diffuse into the cell from the patch pipette. In this paradigm, we have found that in control cells (A) the first and second rounds of exocytosis/RE are kinetically similar (see also Artalejo *et al.*, 1995, 1996). In (B), only the first round of RE is completed; after the dynamin-1 PH domain has diffused into the cell RE is blocked. The current elicited by the first test depolarization is plotted below the capacitance trace. Note that neither the C_m increase nor the Ca^{2+} current is significantly affected by time of incubation or the presence of the proteins in the pipette (means are shown in Table I), indicating that cell integrity is not affected under these conditions and that the effects of the PH domains is specific. Note also the presence of 'excess retrieval' (C_m declining below baseline value at the end of RE) followed by recovery from excess retrieval (REFER; Artalejo *et al.*, 1996) in these and subsequent records. When present, these parameters were not significantly affected by the presence of PH domains in the patch pipette (data not shown).

Table I. Statistical analysis of the involvement of PH domains in rapid endocytosis

Treatment[a]	Peak current (pA)	Peak capacitance change[b] (fF)	Max rate exocytosis[c] (fF/s)	Membrane retrieved within 20 s (%)
Control (n = 91)	−808 ± 15.6	649.4 ± 29.7	643.9 ± 15.9	105.4 ± 2.37
Dynamin-1 PH (n = 40)	−761.6 ± 17.7	1083.1 ± 51.9[d]	841.2 ± 27.8[d]	0.234 ± 0.07[d]
PLCδ1 PH (n = 30)	−849.4 ± 31.6	629.4 ± 53.9	635.2 ± 21.8	105.1 ± 3.28
Pleckstrin (n = 37)	−800.9 ± 28.6	671.5 ± 46.7	625.4 ± 16.8	103.4 ± 3.18
Dynamin-2 PH (short-form) (n = 40)	−828.4 ± 45.1	719 ± 51.2	689 ± 24.3	106.5 ± 3.35
Dynamin-2 PH (long-form) (n = 25)	−768.3 ± 68.2	696.7 ± 62	680.1 ± 30.2	106.6 ± 3.73
Dynamin-1 VL-1 Mut (n = 40)	−799.3 ± 23.9	726.5 ± 44	683.4 ± 22	107.8 ± 3.72
Dynamin-1 VL-2 Mut A (n = 26)	−717.2 ± 66.3	963.7 ± 46.4[d]	848 ± 48.7[d]	0.337 ± 0.11[d]
Dynamin-1 VL-2 Mut B (n = 19)	−784.8 ± 20.5	1052.8 ± 65.1[d]	874.6 ± 42.6[d]	0.387 ± 0.14[d]
Dynamin-1 VL-3 (n = 29)	−753.4 ± 19.2	1060.3 ± 50.2[d]	856.2 ± 41.2[d]	0.26 ± 0.10[d]
Dynamin-2→Dynamin-1 (n = 27)	−754.1 ± 23.3	1204.9 ± 59.2[d]	898.3 ± 35[d]	0.31 ± 0.10[d]

[a]Concentration of all reagents given in the text.
[b]Measured as mean peak increase in capacitance during the exocytotic burst in response to a standard 10-depolarization stimulus train.
[c]Rate of C_m change as dC_m/dt was measured as the slope of the lines connecting the increments in the capacitance trace from the beginning and end of sequential depolarization interrupts; maximum value is denoted here. Peak amplitudes and rates of C_m were each divided by the initial capacitance, and then multiplied by the average initial capacitance of the cell, to normalize data.
[d]Significantly different from the corresponding control values at $P < 0.0001$.

dimensional electrophoresis and immunoblotting with a polyclonal pan-dynamin antibody revealed two ~100 kDa species with isoelectric points corresponding to those predicted from the amino acid compositions of dynamin-1 and dynamin-2 (Figure 2D). These results show that chromaffin cells normally express both forms of dynamin and suggest that this may also be true in other cell types.

The sequence of the first variable loop determines the functional difference between dynamin PH domains in blocking RE

Structural analysis of several PH domains, including that from dynamin, reveals a strikingly similar folding pattern characterized by an anti-parallel set of seven β-strands ('β-sandwich') closed off at one corner by a C-terminal amphipathic α-helix (Ferguson *et al.* 1994; Macias *et al.*, 1994; Timm *et al.*, 1994; Yoon *et al.*, 1994; Fushman *et al.*, 1995). Further investigation of the inositol-1,4,5-trisphosphate complex with the PH domain of PLCδ1 revealed that three variable loops (VL 1–3) between β-strands are important sites of interaction between this ligand and the PH domain (Ferguson *et al.*, 1995a; Lemmon *et al.*, 1995). The equivalent three variable loops lie on the most positively charged face of each PH domain of known structure (for review, see Saraste and Hyvönen, 1994; Ferguson *et al.*, 1995b). To determine whether one or more of these loops is also critical for the inhibitory effect of dynamin-1 PH domain on RE, we produced constructs in which VLs 1–3 were individually substituted with the corresponding regions from the PLCδ1 PH domain (Figure 3) using the crystal structures of the two domains as a guide. We then assessed the effects of introducing each mutated PH domain into chromaffin cells on membrane capacitance changes. While the replacement of VL-2 (β3/β4 loop) or VL-3 (β5/β6 loop) had no effect on the efficacy of the mutant dynamin-1 PH domains to block RE, swapping VL-1 (β1/β2 loop) totally abolished the inhibitory effect (Figure 4; Table I). Again, none of these constructs had any effect on exocytosis or Ca^{2+} currents (Table I), indicating their specificity. These results show that VL-1 is important in determining the inhibitory effect of the dynamin-1 PH domain on RE.

The PH domain of dynamin-1 differs from that of dynamin-2 in VL-1 by just two amino acids (GI to SL; Figure 3). We therefore mutated these residues in dynamin-2 PH (plus a position in VL-3) to those of dynamin-1 and tested whether this altered PH domain could block RE. As seen in Figure 5A, this modification resulted in a gain-of-function in dynamin-2 PH such that it now was a fully effective inhibitor of RE. Since VL-3 of dynamin-1 PH plays no role in RE (see above) these data suggest that the GI/SL pair in VL-1 constitutes the critical difference between the PH domains of dynamins-1 and -2. Figure 5B shows a Cα trace of the dynamin-1 PH domain backbone with the variable loops highlighted and the two critical amino acids in VL-1, which are centrally positioned in the positively charged face of the domain, indicated.

Discussion

The PH domain was first identified only in 1993, but since then a large number of proteins have been found to contain this module (Gibson *et al.*, 1994; Saraste and Hyvönen, 1995). Structural analysis reveals that PH domains, while poorly conserved at the primary sequence level, exhibit a very similar tertiary organization that also extends to the recently analyzed phosphotyrosine-binding domain (Zhou *et al.*, 1995). Despite progress on this front, the function of PH domains in many proteins, including dynamin, is poorly understood. Previous studies employing deletion analysis showed that the PH domains of Sos, IRS-1 and Dbl are important for the functions of their respective host proteins (McCollam *et al.*, 1995; Myers *et al.*, 1995; Zheng *et al.*, 1996b). It is also known that point mutations in the PH domain of Bruton's tyrosine kinase lead to the disease X-linked agammaglobulinemia (Vihinen *et al.*, 1995), showing that alterations in single amino acids can modify the function of this domain. Here, we employed a novel approach to assess the function of the dynamin PH domain, i.e. suppression of RE by introduction of recombinant PH domain proteins into cells. Our earlier work showed that dynamin is essential for RE, based on the inhibition of this process by affinity-purified anti-

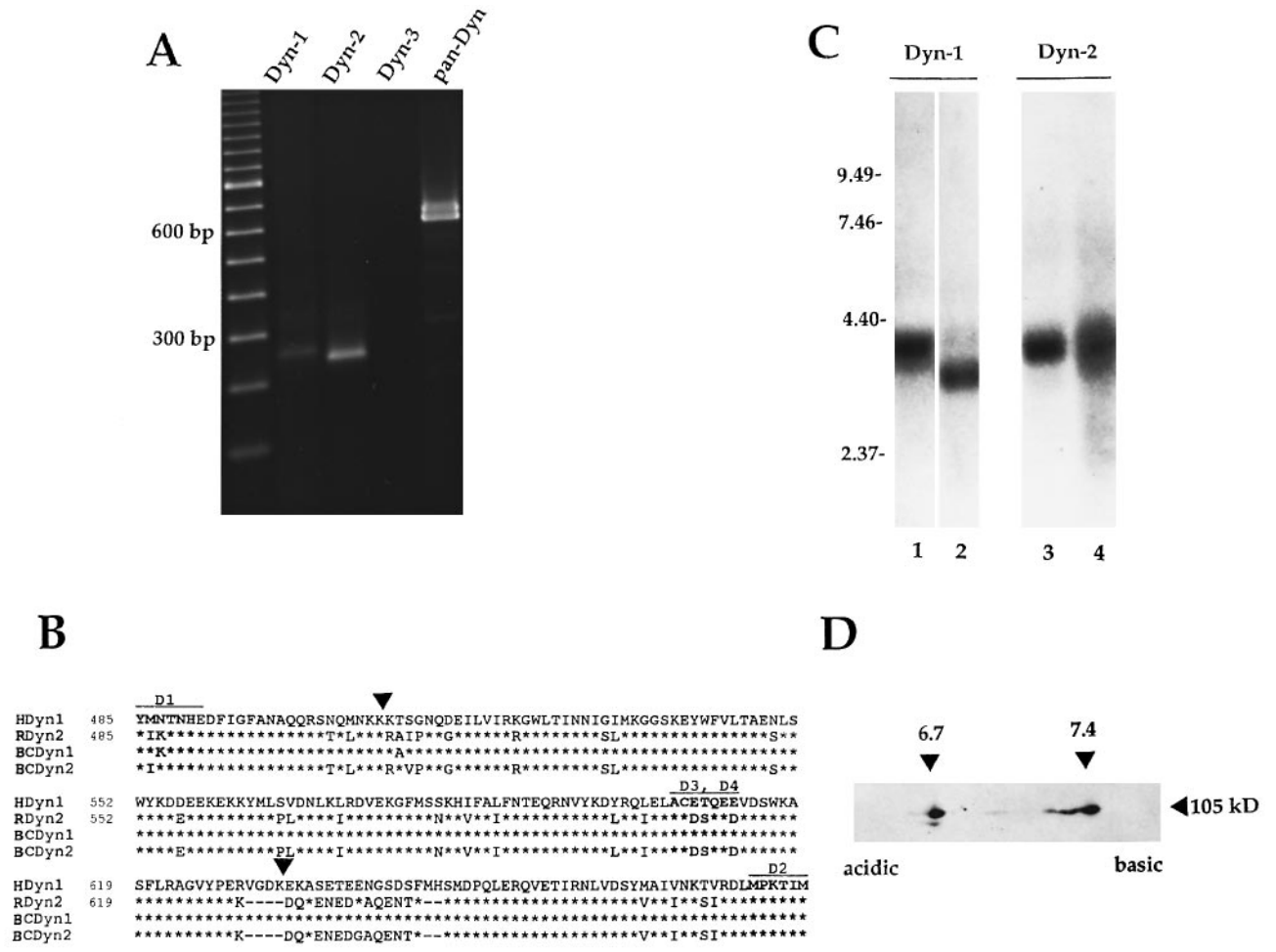

Fig. 2. Chromaffin cells express both dynamin-1 and dynamin-2. (**A**) mRNA from chromaffin cells was subjected to RT–PCR analysis to identify dynamin isoforms. The dynamin-1 (Dyn-1) and dynamin-2 (Dyn-2)-specific primers yielded products of the expected size (240 bp) while no product was evident in material amplified with the Dyn-3 primers or in control reactions without added reverse transcriptase (not shown). In addition, products of the expected size (~600 bp) were obtained with pan-Dyn primers (note a duplex suggestive of two different isoforms). (**B**) Sequencing of representative pan-Dyn cDNA clones from PCRs in (A) and alignment with human dynamin-1 (HDyn1) and rat dynamin-2 (RDyn2). 93 separate clones were sequenced of which 49 coded for dynamin-1 (BCDyn1) and 44 coded for dynamin-2 (BCDyn2). The location of the primers (see Materials and methods) used to produce the long product as well as those used to produce specific products are indicated above the sequence. The limits of the PH domain are denoted by arrowheads; * indicates residues identical with dynamin-1; – indicate gaps in relation to dynamin-1. (**C**) Northern blot analysis of mRNAs for dynamins-1 and -2 in chromaffin cells. Total RNA from chromaffin cells (30 μg; lanes 1 and 3) or bovine brain (3 μg, lane 2; 30 μg lane 4) were separated by gel electrophoresis, transferred to nylon membrane and hybridized with specific ^{32}P-riboprobes. Autoradiography was for 14 h (lanes 2–4) or 72 h (lane 1). RNA size markers (in kb) are shown on the left. (**D**) Two-dimensional IEF/SDS electrophoresis of chromaffin cell proteins and immunoblotting for dynamin isoforms. Cytosolic proteins (150 μg) from chromaffin cell extracts were separated, blotted to nitrocellulose then probed with an affinity-purified pan-dynamin antibody. Dynamin reactivity was detected at p*I*s of 6.7 and 7.4. Reaction detection was by enhanced chemiluminescence.

dynamin antibodies and their Fab fragments (Artalejo *et al.*, 1995). In the present study, we observed that the PH domain of dynamin-1 but not those of PLCδ, pleckstrin or dynamin-2, blocked RE in adrenal chromaffin cells. The most likely explanation for these results is that the exogenous PH domain is competing for sites involved in the vesicle retrieval event which are normally occupied by dynamin-1. It is less likely that the inhibitory effect is a simple non-specific interaction of the dynamin-1 PH domain with vesicular/membrane components. First, all other PH domains were ineffective, even at the high concentration of 0.5 mg/ml (~40 μM) used, while the dynamin-1 PH domain was still effective in totally blocking RE at ~1 μM. This is significant because it places an upper limit on the affinity of dynamin-1 PH for its ligand that is within the range seen for the specific binding of PLCδ to phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2; ~1.7 μM; Lemmon *et al.*, 1995). Second, the lack of activity of the closely related dynamin-2 PH domain in blocking RE, as well as the fact that substitution of just two amino acids in dynamin-2 PH converts it into a fully inhibitory molecule, is indicative of an exquisitely specific interaction between dynamin-1 PH and its target. Third, multiple experiments to detect interactions between dynamin-1 PH domain and various complex mixtures of proteins and lipids *in vivo* and *in vitro* (co-precipitation, blotting, yeast two-hybrid, etc.) have thus far proved negative, indicating that this PH domain does not have a propensity to bind non-specifically to other molecules (M.A.Lemmon and J.Schlessinger, unpublished results). The present data strongly suggest that the PH domain is critical for the function of dynamin-1 in chromaffin cell

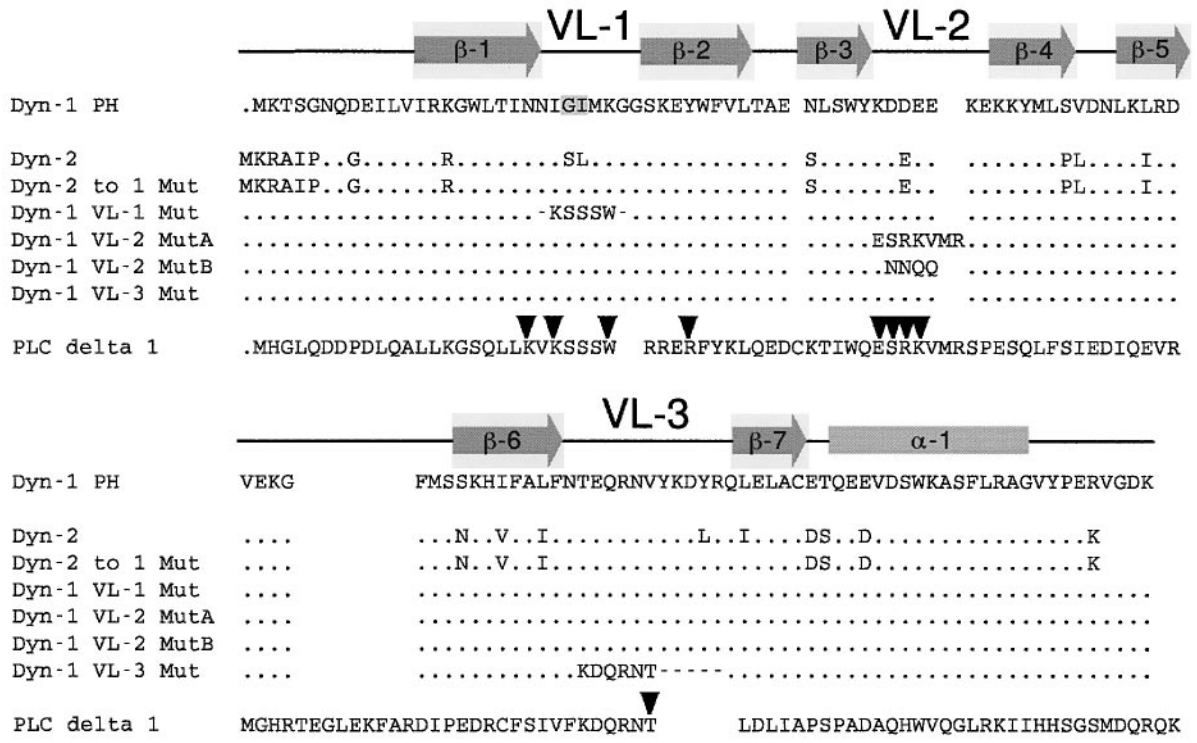

Fig. 3. Sequences of dynamin-1 and -2 PH domains and loop mutant variants. Above the sequence of human dynamin-1 PH domain (top line; residues 510–633 of human dynamin-1; Obar *et al.*, 1990) is a scheme of the secondary structure of the PH domain showing the location of the seven β-strands and C-terminal α-helix (Ferguson *et al.*, 1994). The variable loops VL-1, -2 and -3 are indicated. In the mutated forms of dynamin-1 the individual variable loops were swapped such that they resemble those in the PLCδ1 PH domain (bottom line; residues 11–140) except in Dyn-1 VL-2 Mut B, where the acidic residues in dynamin-1 VL-2 were converted to the corresponding amides. In the dynamin 2→1 mutant, four mutations were made in VL-1 and VL-3 of the dynamin-2 sequence such that it resembles dynamin-1 in these regions. Identical residues in dynamin-2 and the various mutants are indicated by dots; dashes indicate deletions. Each sequence has an additional N-terminal initiator M residue not found in the native PH domain. Residues in the PLCδ1 PH domain variable loops that are involved in the interaction with inositol-1,4,5-trisphosphate (Ferguson *et al.*, 1995b; Lemmon *et al.*, 1995) are marked with arrowheads. Key residues in the VL-1 loop important for RE are shaded.

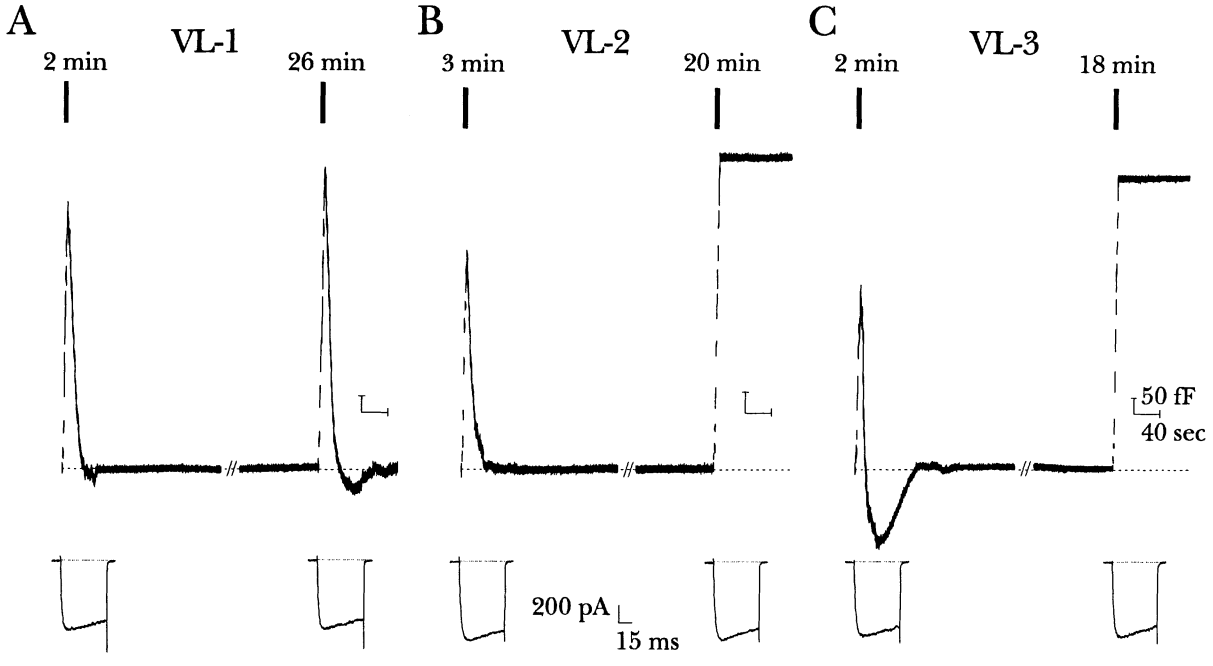

Fig. 4. The major specificity determinant in the dynamin-1 PH domain regulating RE lies in variable loop-1 between the two first β-strands. Continuous C_m records from chromaffin cells in which the pipette contained the recombinant proteins: (**A**) dynamin-1 PH VL-1; (**B**) dynamin-1 PH VL-2; or (**C**) dynamin-1 PH VL-3. Dynamin-1 PH domain mutants (see Figure 3) were introduced into cells at 0.5 mg/ml and their effects on RE were tested in a paradigm similar to that described in the legend to Figure 1. The current elicited by the first test depolarization is plotted below the capacitance trace. Statistical analyses of these results are summarized in Table I.

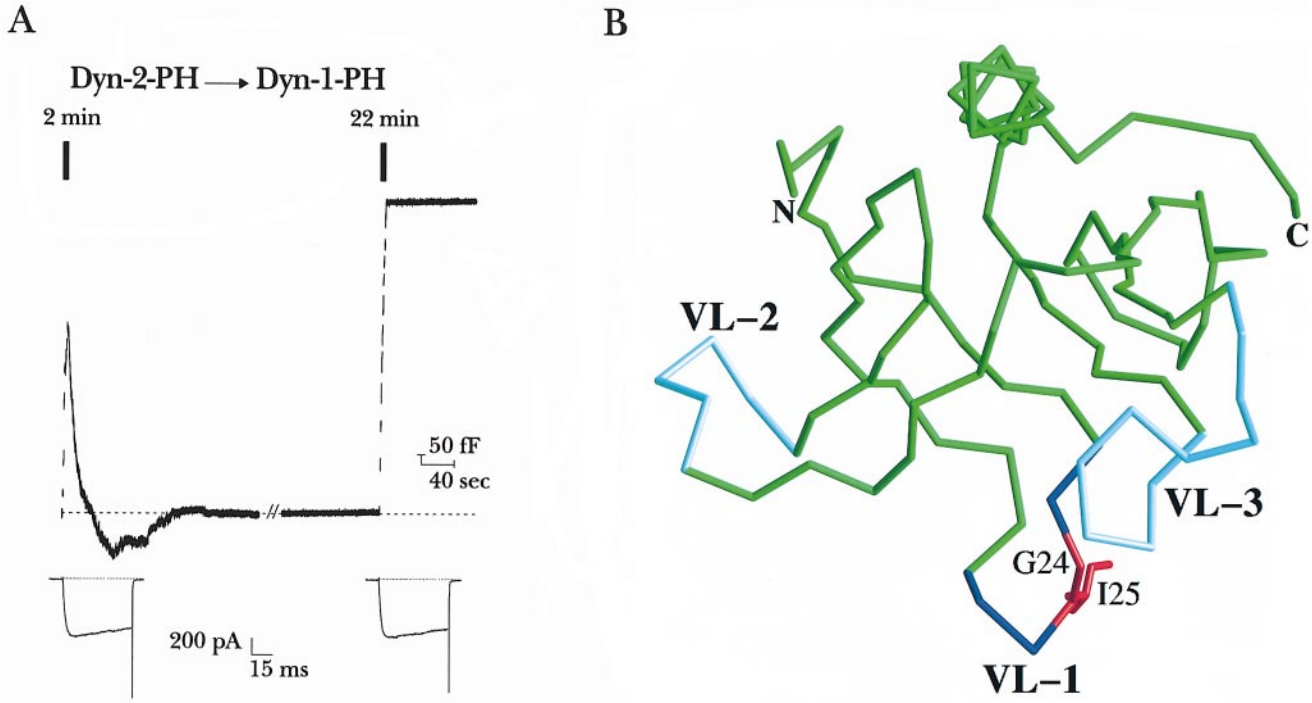

Fig. 5. The PH domain of dynamin-2 becomes an inhibitor of RE with substitution of two amino acids from dynamin-1 in the VL-1 loop. (**A**) Dynamin 2→1 switch mutant protein blocks RE. Dynamin 2→1 mutant protein (see Figure 3 for sequence; 0.5 mg/ml) was introduced into cells and its effect on RE determined as described in the legend to Figure 1. The current elicited by the first test depolarization is plotted below the capacitance trace. (**B**) A Cα trace representing the X-ray crystal structure of the PH domain from human dynamin-1 (Ferguson *et al.*, 1994). Regions corresponding to the variable loops are shown in turquoise (VL-2 and -3) or dark blue (VL-1). Residues in VL-1 that differ between the dynamin-1 and -2 PH domains (G_{24} and I_{25} of dynamin-1-PH corresponding to G_{532} and I_{533} in the dynamin-1 sequence) are colored red. These residues appear to be critical for the interaction between dynamin-1-PH and its target in the regulation of RE. The figure was generated with the program RIBBONS (Carson, 1991).

RE, the first time this region of the protein has been implicated in any form of endocytosis. For such a result to be physiologically relevant, chromaffin cells must normally express dynamin-1. We demonstrate here that this is unequivocally the case. Together with previous work showing that PC12 cells also express dynamin-1 and dynamin-2 at the mRNA level (Sontag *et al.*, 1994), our results confirm that different dynamin isoforms can be expressed in the same cell, and thus may well carry out distinct functions.

The inability of PH domains other than that from dynamin-1 to inhibit RE presages the existence of a specific ligand for this dynamin isoform. A common feature in most, if not all, proteins containing a PH domain is a requirement for membrane localization, and PH modules seem to play key roles in anchoring various proteins to their particular membrane sites (Ferguson *et al.*, 1995a; Lemmon *et al.*, 1996). By analogy, the ligand for the dynamin-1 PH domain may well be a membrane component; indeed, dynamin-1 is known to associate with membranes both *in vitro* and *in vivo* (e.g. Scaife and Margolis, 1990; Herskovits *et al.*, 1993a; Noda *et al.*, 1993). Several other PH domains have been found to bind, albeit with widely varying affinities, to inositol phospholipids, most notably PtdIns-4,5-P_2 (see Lemmon *et al.*, 1996 for discussion). Detailed studies of the PLCδ1 PH domain indicate that it binds with high affinity to the inositol 1,4,5-trisphosphate head group of PtdIns-4,5-P_2, the physiological substrate for this enzyme. PtdIns-4,5-P_2 and its hydrolysis product compete for binding to the PH domain, providing a mechanism for regulating the membrane availability (and hence activity) of PLCδ1 (Cifuentes *et al.*, 1993; Garcia *et al.*, 1995; Lemmon *et al.*, 1995). Indeed, it appears that the specificity exhibited by the PH domain of PLCδ1 is entirely recapitulated in the isolated PH domain, making it unlikely that other regions of the protein contribute to modifying the binding properties of the PH domain in this enzyme (Lemmon *et al.*, 1995). Whether this will also turn out to be true of dynamin remains to be seen. In contrast to PLCδ1, other PH domains, such as that from β-spectrin, exhibit a much lower affinity for inositol phospholipids (Harlan *et al.*, 1994; Hyvönen *et al.*, 1995) and it is uncertain if such interactions are physiologically relevant for these proteins. In any case, inositol phospholipids are unlikely to represent the membrane binding site important for RE as: (i) dynamin-1 PH does not bind to membranes containing these lipids (Lemmon *et al.*, 1995; binding to inositol phospholipids can only be detected in the presence of detergent: Zheng *et al.*, 1996a); and (ii) the PLCδ1 PH domain, which has a high affinity for PtdIns-4,5-P_2 (Garcia *et al.*, 1995; Lemmon *et al.*, 1995), fails to inhibit RE in our assay (Figure 1B; Table I).

Using a loop-swapping approach we localized the region that is apparently responsible for regulation of RE to the variable loop (VL-1) connecting the first and second β-strands in the dynamin-1 PH domain. Residues in VL-1 have been implicated in the binding of PtdIns-4,5-P_2 to PLCδ1 and other proteins (Harlan *et al.*, 1994; Ferguson *et al.*, 1995a,b; Hyvönen *et al.*, 1995; Lemmon *et al.*,

1995); thus, the dynamin PH domain appears to recognize its specific ligand through a similarly localized site. The amino acids in VL-1 that are most relevant to RE are the adjacent pair $G_{24}I_{25}$ as mutational exchange of the corresponding amino acids ($S_{24}L_{25}$) in the inactive dynamin-2 PH domain converts the latter into a molecule that can inhibit RE. As mentioned above, this remarkable gain-of-function is another strong argument that the effects of these PH domains in the present study are highly specific. One of the mutations in the PH domain of Bruton's tyrosine kinase (whose physiological ligand has also not been identified) that leads to X-linked agammaglobulinemia also maps to the analogous VL-1 loop (Vihinen *et al.*, 1995). The nature of the ligand for the dynamin-1 and -2 PH domains is presently under study and the functionally distinct mutations identified here should offer useful controls for ligand binding specificity. It is very unlikely that the VL-1 loop is involved in linking dynamin to βγ-subunits of G-proteins. Studies of the β-adrenergic receptor kinase reveal that the binding site for βγ-subunits on this enzyme is close to the C-terminus of the PH domain and includes several amino acids beyond the PH domain proper (Touhara *et al.*, 1994). While it has been speculated that other PH domains may exhibit similar properties (Touhara *et al.*, 1994; for discussion, see Saraste and Hyvönen, 1995; Lemmon *et al.*, 1996), the region of the PH domain involved in βγ-subunit interactions encompasses the α-helix and β-sheets 5, 6 and 7, and thus is structurally distant from the VL-1 loop. Furthermore, a variety of co-immunoprecipitation and other studies failed to detect association between dynamin and G-protein βγ-subunits (M.A.Lemmon and J.Schlessinger, unpublished observations).

The striking difference between dynamin-1 and dynamin-2 PH domains in the RE assay raises the possibility that these two isoforms of dynamin might carry out distinct functions. Other differences between the two species exist, most notably in the C-terminal region thought to be important in dynamin-1 interactions with microtubules, lipids and SH3 domain-containing proteins (for review, see Vallee and Okamoto, 1995). This region may also be involved in dynamin oligomerization, a process that could play a role in the membrane fission events controlled by this protein (Baba *et al.*, 1995; Tuma and Collins, 1995). It was postulated that membrane binding precedes oligomerization (Tuma and Collins, 1995); if this is correct then the PH domain might provide the membrane anchor for such oligomers. Comparative analysis of different dynamins with respect to these functions has not yet been carried out and domain analyses of dynamin-1 have hitherto been done only in cells that do not normally express this protein (e.g. Herskovits *et al.*, 1993a). One interesting difference already established is that dynamin-1 is phosphorylated by protein kinase C, a phenomenon hypothesized to play a role in synaptic vesicle recycling, while dynamin-2 is not (summarized in Robinson *et al.*, 1994).

Despite the differences in dynamin distribution and structure described above, it has been proposed that the function of the two dynamins may be redundant (Baba *et al.*, 1995). Dynamin-1 can localize to clathrin-coated pits in fibroblasts and a mutant dynamin-1 can act as a dominant-negative inhibitor of receptor-mediated endocytosis (Herskovits *et al.*, 1993a; Damke *et al.*, 1994). However, these were overexpression studies in cells that normally express only dynamin-2 and the results are not necessarily incompatible with those presented here. It may well be that dynamins exhibit two-point attachment (C-terminus and PH domain) to membranes, as intimated above. If the relevant binding site(s) for the C-terminal domain exhibits even limited cross-reactivity between different dynamin isoforms, then substantial overexpression of wild-type dynamin-1 [said to be ~100-fold in the experiments of Herskovits *et al.* (1993a)] might force dynamin-1 on to sites normally occupied by dynamin-2 and allow it to perform a similar function in receptor-mediated endocytosis. Analogously, overexpression of the GTP-site mutant dynamin-1 might compete with endogenous dynamin-2 when vastly overexpressed. In this scenario, the PH domain might then confer greater specificity on the membrane binding sites for one or both types of dynamin. Further studies with isoform-specific antibodies and parallel measurements of both types of endocytosis are needed to address these issues. If dynamin-1 is involved primarily in RE we speculate that dynamin-2, which is more generally distributed than dynamin-1, could be the isoform responsible for receptor-mediated endocytosis by clathrin-coated vesicles. The data presented here argue that subtle differences in the PH domain may direct binding of the different dynamins to distinct vesicular components involved in the two endocytotic pathways.

Materials and methods

Cell preparation, patch–clamp current and capacitance recording

Chromaffin cells were isolated from calf adrenal glands, cultured and patch–clamped as previously described (Artalejo *et al.*, 1995). Capacitance was measured by a computer program using a phase-tracking technique. A standard protocol of ten 50 ms depolarizations from a holding potential of –90 mV to +10 mV, each pulse preceded by a prepulse to +120 mV to recruit facilitation Ca^{2+} channels, was used to evoke secretion. Following the secretory phase, RE manifests as a decrease in capacitance; both the rate and extent of RE were measured, as well as the extent of exocytosis and magnitude of Ca^{2+} current (see Table I). All experiments were carried out at room temperature (21–24°C). The patch pipette solution contained: 110 mM Cs-glutamate, 0.1 mM Cs-EGTA, 40 mM HEPES, 5 mM $MgCl_2$, 2 mM ATP, 0.35 mM GTP, pH 7.2, with the addition of various PH domains as indicated in the figure legends. The external solution consisted of 2 mM $CaCl_2$, 150 mM TEA–HCl, 10 mM HEPES, 10 mM glucose and 1 μM tetrodotoxin, pH 7.2.

Preparation of PH domains

Recombinant PH domains from human dynamin-1 (residues 510–633), rat dynamin-2 (residues 509–629), rat PLCδ1 (residues 11–140) and human pleckstrin (residues 1–105) were expressed in *Escherichia coli* from pET11a and purified as described previously (Ferguson *et al.*, 1994; Lemmon *et al.*, 1995). Mutated versions of the dynamin-1 and dynamin-2 PH domains were generated by four-primer PCR. Two initial amplification reactions were performed, one used a sense mutagenic oligonucleotide and a 3′ antisense primer, while the second used an antisense oligonucleotide and a 5′ sense primer. The two PCR products were mixed, diluted and a second round of amplification was performed using only the primers defining the N- and C-terminal portions of the PH domain. The resulting product was subcloned between the *Nde*I and *Bam*HI sites of pET11a, and the DNA sequence verified by dideoxynucleotide sequencing. Mutated dynamin-1 PH domains were expressed in *E.coli* MGT7 grown at 25°C while cells expressing the dynamin-2 mutant were grown at 37°C. Mutated dynamin-1 and -2 PH domains were solubly expressed at high levels under these conditions,

and were purified exactly as described for the wild-type protein (Ferguson *et al.*, 1994). Each mutated PH domain was homogeneous by SDS–PAGE and behaved identically in gel filtration; circular dichroism spectra of the purified mutant PH domains closely resembled that of the wild-type dynamin-1 PH domain, indicating that the secondary structure was similar (data not shown). Prior to experimentation all proteins were dialyzed against nucleotide-free internal pipette solution (Artalejo *et al.*, 1995) and their concentration recalibrated by UV absorption.

Analysis of dynamin expression in chromaffin cells

mRNA was isolated from chromaffin cells and bovine brain by oligo-(dT) chromatography. For PCR analysis, degenerate oligonucleotide primers based on conserved amino acid sequences specific for all dynamins (pan-Dyn), dynamin-1 (Dyn-1), dynamin-2 (Dyn-2) or dynamin-3 (Dyn-3) were used as follows: YM(I)N(K)TNH (485–490) and MPKTIM (681–686) called D1 and D2 were used as pan-Dyn primers; two alternate forward primers for either dynamin-1 (ACETQEE; D3) or dynamin-2 (ACDSQED; D4) with D2 as the reverse primer were used for specific amplifications. For dynamin-3, D4 was used as the forward primer and IPKTIM (D5) used as the reverse primer. PCR products were cloned in pCRII and sequenced in both directions by the dideoxynucleotide technique. For RNA hybridization analysis, total RNA from bovine chromaffin cells or bovine brain was isolated by standard extraction and precipitation procedures. RNAs were electrophoresed on 1% agarose/1.2% formaldehyde gels then transferred to nylon membranes. The blot was hybridized with ^{32}P-riboprobes prepared from RT–PCR products derived from cDNA clones specific for dynamins-1 or -2. Hybridizations were carried out overnight under standard conditions with ~10^6 c.p.m./ml probe. Non-specific background was reduced by incubation of hybridized blots with 5 μg/ml RNase A in 2× SSC for 15 min prior to high-stringency rinses (0.1× SSC/0.1% SDS; 4× for 20 min at 65°C). For protein analysis, cultured chromaffin cells were extracted with a denaturing buffer and 150 μg lysate subjected to isoelectric focusing (p*I* gradient 4–9) followed by SDS–7.5% PAGE. Proteins were transferred to nitrocellulose and probed with a pan-dynamin antibody using methods described previously (Artalejo *et al.*, 1995).

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Note added

Salim,K. *et al.* (*EMBO J.*, **15**, 6241–6250, 1996) recently reported that large unilamellar liposomes containing PIP_2 stimulated the GTPase activity of dynamin-1 in a manner dependent on the PH domain and could be shown to bind to the isolated PH domain using surface plasmon resonance. No affinities were reported for these interactions. However, NMR investigation of the interaction between $InsP_3$ and the dynamin-1 PH domain yielded a K_d of 1.23 mM, suggesting a very low-affinity interaction with the PIP_2 headgroup. As stated in the discussion above, this interaction is probably irrelevant for RE, as we have shown that the dynamin-1 PH domain can inhibit this process at concentrations as low as 1 μM.