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Dynamin GTPase is stimulated by crosslinking through the C-terminal proline-rich domain

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Dynamin is a 100 kDa GTPase required for endocytic-coated vesicle formation. Recombinant human neuronal dynamin (dynamin-1) was used for monoclonal antibody (mAb) production. Two mAbs, designated hudy-2 (for human dynamin) and hudy-4, were chosen for further study based on their differential ability to recognize dynamin-1 and its non-neuronal isoform, dynamin-2. Both bind to the proline-rich C-terminal domain (PRD) of dynamin and inhibit the ability of microtubules and grb2 to stimulate GTPase activity. Hudy-4 binds to an epitope within the last 20 amino acids of dynamin-1 and has no effect on its intrinsic GTPase activity. Hudy-2 binds to an epitope within amino acids 822–838 that is common to dynamin-1 and dynamin-2. Hudy-2 stimulates dynamin's intrinsic GTPase activity in a manner proportional to the valency of the immunoglobulin (Ig) G. Crosslinking IgGs with secondary antibodies caused a 2-fold increase in GTPase activity, while F(ab)s were inactive. Importantly, our findings suggest that the stimulation of dynamin GTPase activity by multivalent proteins which bind *in vitro* to the PRD may not be a valid criterion on its own for assessing the *in vivo* functional significance of these interactions.

Key words: dynamin/endocytosis/GTPase/monoclonals

Introduction

Dynamin is a 100 kDa member of the GTPase superfamily (Obar *et al.*, 1990) that regulates endocytic-coated vesicle formation (Herskovits *et al.*, 1993a; van der Bliek *et al.*, 1993; Damke *et al.*, 1994). The first insight into dynamin's function *in vivo* came from the finding that it was 70% identical to the *Drosophila shibire* gene product (Chen *et al.*, 1991; van der Bliek and Meyerowitz, 1991). Temperature-sensitive *Drosophila shibire* mutants show a pleiotropic defect in endocytosis (Grigliatti *et al.*, 1973; Kosaka and Ikeda, 1983a,b). Transient overexpression of dominant-interfering GTP binding mutants of dynamin blocked transferrin endocytosis (Herskovits *et al.*, 1993a; van der Bliek *et al.*, 1993) and firmly established a role for dynamin in receptor-mediated endocytosis in mammalian cells. The originally identified mammalian dynamin, now referred to as dynamin-1, is expressed exclusively in neurons (Nakata *et al.*, 1991; van der Bliek *et al.*, 1993; Sontag *et al.*, 1994), while a recently identified second isoform, called dynamin-2, is uniformly expressed in all tissues (Cook *et al.*, 1994; Sontag *et al.*, 1994). Dynamin-2 is >70% identical to both dynamin-1 and *shibire*, with much of the amino acid variability localized in the proline-rich C-terminal domain (PRD).

Recent studies on dynamin function in inducible transformants of HeLa cells conditionally expressing wild-type dynamin, or a mutant defective in GTP binding and hydrolysis, have revealed that it is specifically required at the late stages of coated pit invagination and endocytic-coated vesicle budding (Damke *et al.*, 1994). The data also indicate that dynamin-1 and dynamin-2 are functionally interchangeable. Membrane-bound dynamin was localized specifically to clathrin-coated pits on the plasma membrane and to clathrin-coated vesicles, suggesting that dynamin was specifically targeted to a saturable, coated pit-associated membrane binding site. While it is likely that dynamin cycles between soluble and membrane-bound pools in its role in receptor-mediated endocytosis, the mechanism by which dynamin performs its function in coated vesicle formation is unknown. As a general rule, GTP binding proteins have a regulated cycle of guanine nucleotide binding and hydrolysis, which in turn regulates a sequence of interactions between the G proteins and effectors and/or accessory factors (Nuoffer and Balch, 1994). Therefore, the regulation of its GTPase cycle should be integral to the function of dynamin.

Dynamin has a very high intrinsic rate of GTP hydrolysis which can be further stimulated *in vitro* through the interaction of effector molecules with its ~100 amino acid C-terminal, proline–arginine-rich domain. The functionally diverse group of effectors thus far identified include microtubules (MT; Maeda *et al.*, 1992; Shpetner and Vallee, 1992), acidic phospholipids (Tuma *et al.*, 1993) and a subset of SH3 domain-containing proteins (Gout *et al.*, 1993; Herskovits *et al.*, 1993b). Mechanisms that explain the activation of dynamin's N-terminal GTPase activity through the binding of specific molecules to the PRD remain speculative. Both intramolecular interactions within the folded dynamin molecule and intermolecular interactions made possible by oligomerization of dynamin have been proposed (Herskovits *et al.*, 1993b). Which, if any, of the effector molecules thus far identified *in vitro* participate in dynamin's function *in vivo* is an open question.

We have generated and characterized monoclonal antibodies (mAbs) to dynamin as specific reagents to study the function of the dynamin GTPase. Here we report that mAbs which bind to the PRD of dynamin inhibit MT- and grb2-stimulated GTPase activity. A subset of these mAbs can themselves stimulate dynamin GTPase activity. Analysis of the mAbs' epitopes and effects on dynamin's GTPase activity has suggested a model whereby crosslinking

dynamin in a specific orientation may be a mechanism by which various effectors stimulate dynamin GTPase. Finally, since a variety of disparate multivalent effectors can stimulate dynamin's GTPase *in vitro*, we question whether this activity alone is a valid criterion upon which to assess the physiological significance of dynamin−protein interactions.

Results

Expression and purification of recombinant dynamin and production of mAbs

To perform detailed biochemical studies on dynamin we developed a rapid and efficient purification scheme based on overexpression of human neuronal dynamin in Sf9 insect cells. Recombinant baculovirus encoding the cDNA for wild-type dynamin-1 (van der Bliek *et al.*, 1993) was used to drive high-level expression. Figure 1A shows the SDS−PAGE analysis of various fractions obtained during the purification of dynamin. Dynamin accounted for 5–10% of total cell-associated protein at 65 h post-infection in baculovirus-infected insect cells (compare lane 1 with lane 2). The overexpressed dynamin was purified from the cytosol of infected Sf9 cells (lane 4) by sequential chromatography on Q-Sepharose (lane 5), phosphocellulose (lane 6) and Sephacryl S300 (lane 7). Dynamin purified by this method was active as a MT-, and grb2-stimulated GTPase with a K_m for GTP of 32 μM in the presence of MT. The intrinsic rate of GTP hydrolysis by recombinant dynamin averaged 30.0 nmol/min/mg and was stimulated >30-fold by MT (to 950 nmol/min/mg) and >10-fold by grb2 (to 310 nmol/min/mg), in the range of values published for dynamin purified from bovine brain (Shpetner and Vallee, 1992; Herskovits *et al.*, 1993b; Tuma *et al.*, 1993). This procedure yielded >10 mg of dynamin purified to >98% homogeneity from a 1 l culture of infected cells.

We undertook the production of mAbs to dynamin to develop specific reagents for *in vivo* and *in vitro* studies of dynamin function (see Materials and methods). Four mAbs, designated hudy-1 to hudy-4 (for **hu**man **dy**namin), were obtained; two were chosen for further study based on their differential ability to recognize neuronal and non-neuronal dynamin. Both hudy-2 and hudy-4 recognized the major isoform of dynamin in bovine brain cytosol by Western blot analysis (Figure 1B, lanes labeled 'b'). In addition, hudy-2 also recognized a protein migrating slightly faster than the predominant dynamin band (lane 1b). To determine whether the mAbs also recognize ubiquitously expressed dynamin-2, HeLa cell lysates were analyzed by Western blot (Figure 1B, lanes labeled 'h'). Hudy-2 recognized endogenous HeLa dynamin-2, while hudy-4 did not (compare lane 1h with lane 2h). Taken together, these data indicate that hudy-2 recognizes an epitope on dynamin that is conserved between dynamin-1 and dynamin-2, while hudy-4 recognizes an epitope unique to dynamin-1.

Hudy mAbs inhibit MT- and grb2-stimulated dynamin GTPase activity

MT and grb2 are two of the effectors described so far that can stimulate the GTPase activity of dynamin (Shpetner and Vallee, 1992; Gout *et al.*, 1993; Herskovits

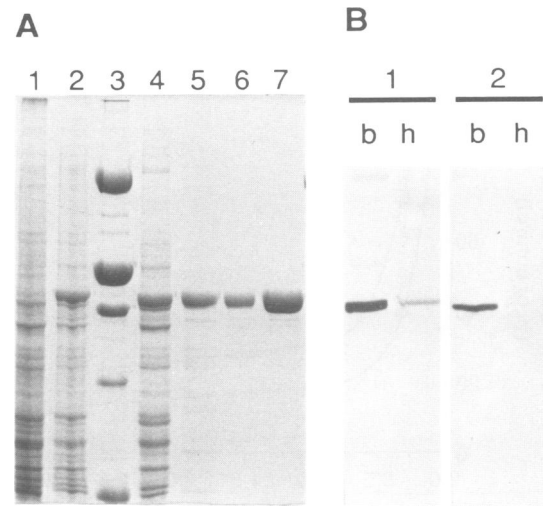

Fig. 1. Purification of recombinant dynamin-1 and specificity of anti-dynamin mAbs. (**A**) Coomassie Blue-stained SDS−PAGE of fractions from dynamin purification. Lane 1, 5×10^4 uninfected Sf9 cells; lane 2, 5×10^4 Sf9 cells harvested 65 h after infection with recombinant baculovirus encoding human dynamin-1; lane 3, molecular weight markers (from top, 200, 116, 97.4, 68 and 46 kDa); lane 4, cytosol from baculovirus-infected Sf9 cells (10 μg protein); lane 5, Q-Sepharose pool (2.5 μg protein); lane 6, phosphocellulose pool (2.5 μg); lane 7, Sephacryl-S300 pool (2.5 μg). (**B**) Western blot analysis of 25 μg bovine brain cytosol (lanes labeled 'b') or HeLa cell lysates (2×10^5 cells; lanes labeled 'h') probed with anti-dynamin mAbs hudy-2 (lanes 1) or hudy-4 (lanes 2).

et al., 1993b). To determine whether hudy-2 or hudy-4 mAbs would be useful as reagents to interfere with dynamin function, we tested their effects on both MT- and grb2-stimulated dynamin GTPase. The data in Figure 2 show that both antibodies effectively inhibited MT- (Figure 2A) and grb2- (Figure 2B) stimulated GTPase activity, while a control immunoglobulin (Ig) G1 (12CA5) specific for hemagglutinin had no effect (Figure 2, Δ). Interestingly, while hudy-4 (Figure 2, ■) reduced MT- and grb2-stimulated GTPase activities to almost intrinsic rates, a significant residual stimulated GTPase activity, corresponding to ~25% of MT-stimulated GTPase and ~50% of grb2-stimulated GTPase, remained resistant to inhibition by hudy-2 (Figure 2, □).

MT and grb2 both stimulate dynamin through interactions with its highly basic C-terminal PRD (Herskovits *et al.*, 1993b). Therefore, inhibition of stimulated GTPase activity could be due to either directly effecting the function of dynamin's GTPase domain or sterically blocking the interaction of these effectors with dynamin's PRD. To distinguish between these two possibilities we examined the effects of hudy-2 and hudy-4 on the intrinsic rate of GTP hydrolysis by purified dynamin. The data in Figure 3 show that hudy-4 had no effect on constitutive dynamin GTPase activity, suggesting that its inhibition of stimulated GTPase activity was due to steric hindrance of MT and grb2 binding. In contrast, hudy-2 stimulated dynamin GTPase activity in a concentration-dependent manner. In this experiment, dynamin's intrinsic rate of GTP hydrolysis (29.5 nmol/min/mg) was stimulated in the presence of hudy-2 to 489 nmol/min/mg, almost equivalent to the grb2-stimulated GTPase activity of 493 nmol/min/mg. These observations indicated that the inhibition of stimulated GTPase activity by hudy mAbs

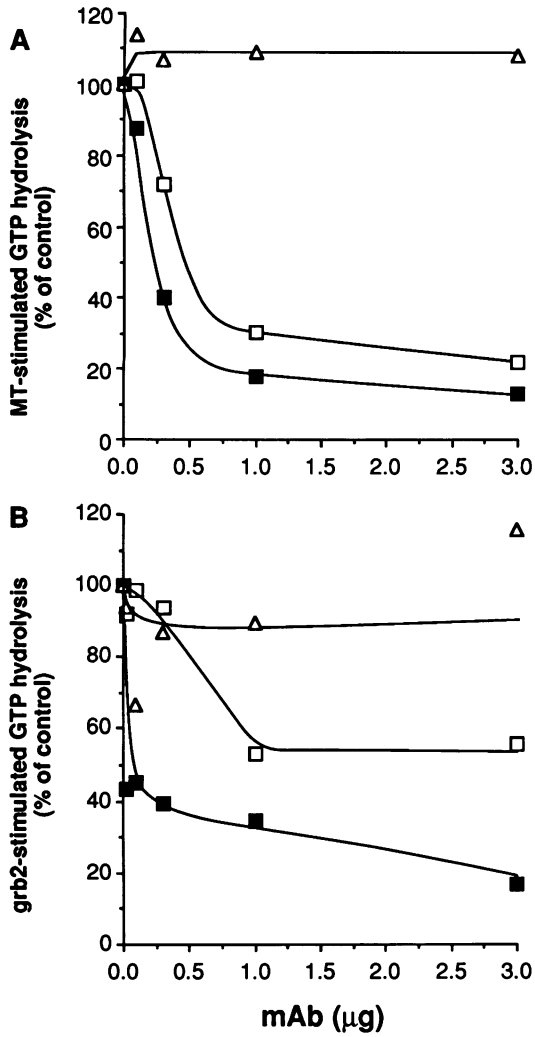

Fig. 2. Hudy mAbs inhibit MT- and grb2-stimulated dynamin GTPase. Reaction mixtures (20 μl) contained the indicated amounts of anti-dynamin (hudy) mAb and (**A**) 0.1 μg dynamin and 2 μg of taxol-stabilized MT or (**B**) 0.25 μg dynamin and 1 μg grb2. Reactions were initiated by the addition of 1 mM [α-^{32}P]GTP and incubated at 37°C under the conditions described in Materials and methods. (□) hudy-2; (■) hudy-4; (Δ) 12CA5 control IgG. The data are expressed as percentage of stimulated hydrolysis in the absence of mAb, i.e. the intrinsic rate of GTP hydrolysis by dynamin (41.6 nmol/min/mg) in the absence of MT or grb2 has been subtracted. MT-stimulated GTP hydrolysis in this experiment was 1259 nmol/min/mg; grb2-stimulated GTPase activity was 330 nmol/min/mg.

seen in Figure 2 was not a consequence of interference with the GTPase domain itself but was rather due to the interference of MT and grb2–dynamin interactions. Furthermore, the residual grb2-stimulated GTPase activity seen in the presence of hudy-2 probably reflects competition between the two effectors for binding to similar sites on dynamin.

Hudy-2 and hudy-4 bind to distinct sites in the PRD of dynamin

The ability of the hudy mAbs to interfere with MT- and grb2-stimulated GTPase activity suggested that epitopes for these antibodies lie within dynamin's C-terminal PRD. We mapped the hudy mAb binding sites to identify specific sites in dynamin's PRD that can affect the stimulation of

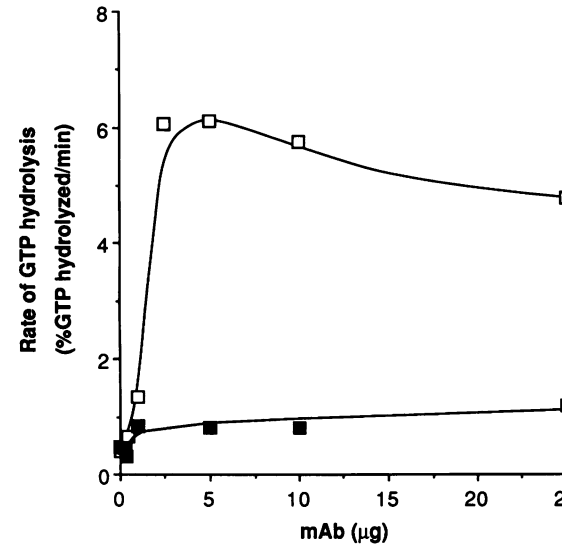

Fig. 3. Hudy-2 mAb stimulates dynamin GTPase activity. GTPase assays were performed as described in Figure 2. Reaction mixtures contained 2.5 μg dynamin, 1 mM GTP and increasing concentrations of hudy mAbs. (□) hudy-2; (■) hudy-4. The results are expressed as the rate of GTP hydrolysis.

Table I. Epitope mapping hudy mAbs

Dynamin molecule	Hudy-2	Hudy-4
Intact dynamin-1	++	++
92 kDa subtilisin product	–	–
98 kDa collagenase D product	++	–
Carboxypeptidase Y product, 30 min	++	–
Intact dynamin-2	+	–

Summarized are the results from Western blot analysis of the ability of hudy mAbs to recognize limited proteolytic digestion products of dynamin-1, or the ubiquitously expressed isoform, dynamin-2. See Materials and methods for experimental details.

GTPase activity. Since the PRD of dynamin is a ready substrate for proteolysis (Herskovits *et al.*, 1993b), it was digested with a variety of proteases and assayed by Western blot analysis to determine whether the products of these limited digests could be recognized by either of the hudy mAbs. These results are summarized in Table I. None of the mAbs recognized fragments <92 kDa, indicating that their epitopes are contained within the C-terminal PRD. Discrete high molecular weight fragments of dynamin that differed by as little as 2 kDa from the full-length molecule could be detected by Coomassie Blue staining of limited digests. Of these, an ~98 kDa collagenase D fragment was recognized by hudy-2 but not by hudy-4. To confirm that hudy-4 binds distal to hudy-2, dynamin-1 was digested with carboxypeptidase Y over a time course. Carboxypeptidase Y is well suited to such a study because its activity includes the C-terminal release of proline (Hayashi *et al.*, 1973), and five of the last 15 residues in dynamin are proline. Glycine, which is the most slowly released amino acid during carboxypeptidase Y proteolysis, is first present 17 residues from the C-terminus (Figure 4). As indicated in Table I, the recognition of dynamin by hudy-4 was almost completely lost after only 30 min of incubation with carboxypeptidase Y, while most of the hudy-2 reactivity remained, even after 6 h of

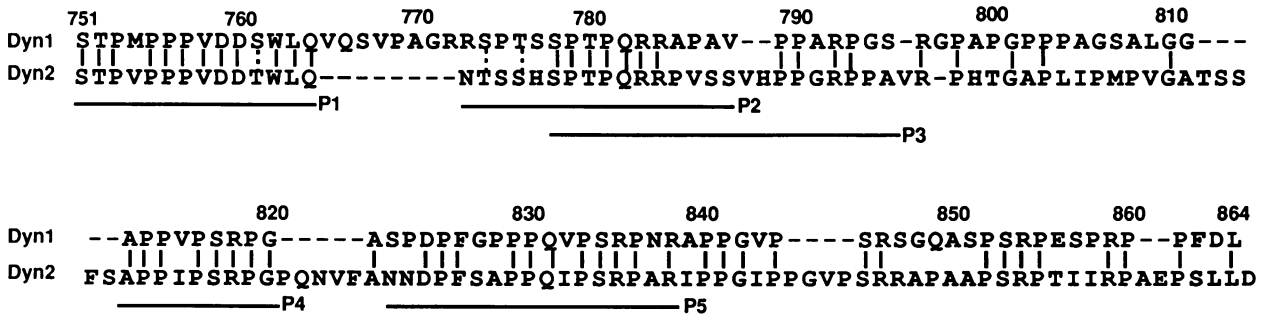

Fig. 4. Sequence comparison of the C-terminal proline-rich domain of human dynamin-1 (van der Bliek *et al.*, 1993) and rat dynamin-2 (Sontag *et al.*, 1993; Cook *et al.*, 1993). Human and rat dynamin-1 are 99% identical. Vertical dashes represent residues conserved between dynamin-1 (top) and dynamin-2 (bottom). Lines below the sequences represent peptides used to epitope-map the hudy mAb binding sites. Amino acid numbering refers to human dynamin-1 sequences. Sequence alignment was performed with Dnastar Align software (Madison, WI) using the clustal method of alignment.

digestion (results not shown). These data indicate that hudy-4 is an isoform-specific antibody that recognizes the extreme C-terminal ~20 amino acids of dynamin-1.

Hudy-2 recognizes a conserved epitope between dynamin-1 and dynamin-2. A comparison of the sequences of the PRD of dynamin-1 and dynamin-2 (Figure 4) shows that there are four regions of homology between the two PRDs. To pinpoint the epitope recognized by hudy-2, peptides corresponding to each of these conserved regions (designated P1–P5, as indicated in Figure 4) were synthesized or obtained from others (see Materials and methods). To map the epitope, the ability of these peptides to block the binding of hudy-2 to immobilized dynamin was determined using ELISAs. The data are shown in Figure 5. As expected, none of the peptides inhibited hudy-4 binding, even at mM concentrations (data not shown). Similarly, peptides designated P1–P4 failed to significantly inhibit the binding of hudy-2 to immobilized dynamin, even at mM concentrations (data not shown). Only peptide P5 inhibited hudy-2 binding, with half-maximal inhibition occurring at <10 μM peptide (Figure 5). Hudy-2 stimulation of dynamin GTPase activity through binding to amino acids 822–838 identifies this region as a site of possible effector control of dynamin function.

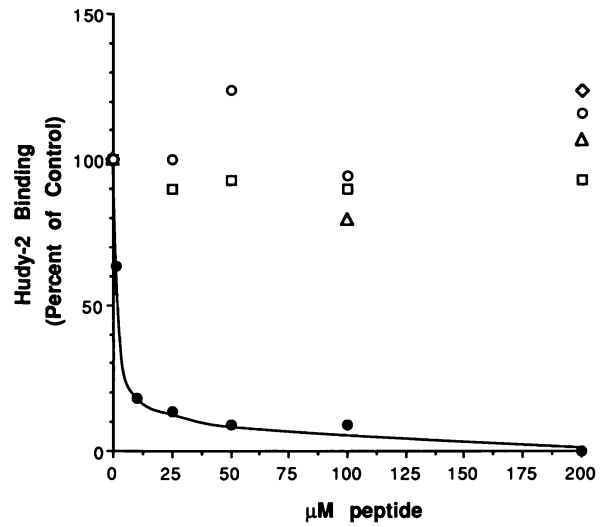

Fig. 5. Epitope mapping of hudy-2 binding using dynamin peptides. Hudy-2 was incubated in PBS containing 0.2% BSA and increasing concentrations of dynamin peptides for 1 h at 37°C in microtiter wells coated with recombinant dynamin. Wells were washed and bound antibody was determined by ELISA using HRP-conjugated rabbit anti-mouse secondary antibodies, as described in Materials and methods. Results are expressed as the percentage of total signal in the absence of peptides. Hudy-2 antibody concentrations that just saturated binding (0.13 μg/ml) were determined in preliminary titrations and used in these experiments. All assays were performed in duplicate and varied by <5%. (□) P1, amino acids 751–764; (△) P2, amino acids 773–788; (○) P3, amino acids 778–795; (◇) P4, amino acids 812–820; (●) P5, amino acids 822–838.

A crosslinking mechanism for antibody-stimulated dynamin GTPase activity

The hudy-2 epitope on dynamin between amino acids 822 and 838 corresponds to one of several possible SH3 domain binding sites (Ando *et al.*, 1994; Miki *et al.*, 1994; Scaife *et al.*, 1994; Seedorf *et al.*, 1994). Of the SH3 domain-containing proteins shown to bind to the PRD of dynamin, grb2 is the most potent effector of GTPase function. The observation that grb2 can stimulate GTPase activity as intact divalent SH3–SH2–SH3–GST constructs but not as monovalent SH3–SH2–GST molecules (Gout *et al.*, 1993), led us to the simple mechanistic hypothesis that crosslinking through the PRD stimulated dynamin's GTPase activity. To test this hypothesis, we first examined the effect of further crosslinking of hudy-2 using anti-Fc antibodies on dynamin's stimulated GTPase activity. As shown in Figure 6A, the presence of anti-Fc enhanced hudy-2-stimulated GTPase activity by ~2-fold. Anti-Fc-stimulated GTPase activity required the presence of hudy-2 and was concentration-dependent, with maximal stimulation occurring at 1:1 molar ratios of anti-Fc:hudy-2 IgG (data not shown). To strengthen this hypothesis, F(ab) fragments were next generated from hudy-2 and examined for their ability to stimulate dynamin GTPase activity. The relative affinity of hudy-2 F(ab)s for dynamin was assessed by ELISA; although they bound dynamin with lower affinity than intact IgG, the concentrations used in GTPase assays were sufficient to saturate the dynamin stimulatory site. Under these conditions, however, no stimulation of dynamin GTPase activity by monovalent hudy-2 F(ab) fragments was observed (Figure 6B). As predicted by the model, when anti-light chain antibody was included in the assay to crosslink hudy-2 F(ab) fragments, dynamin GTPase activity was stimulated to nearly those levels seen with intact IgGs. Hudy-4 was unable to stimulate GTPase activity under any of the

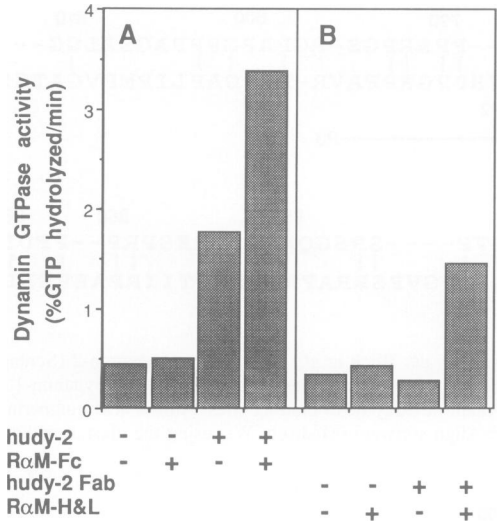

Fig. 6. The ability of hudy-2 to stimulate dynamin GTPase activity is dependent on antibody valency. GTPase assays were performed as described in Figure 3 with 2.5 μg dynamin and 1 mM GTP in the presence of (**A**) 1 μg hudy-2 IgG and/or 1 μg rabbit anti-mouse Fc antibody or (**B**) 5 μg F(ab) fragments derived from hudy-2 and/or 5 μg rabbit anti-mouse heavy and light chain specific antibody. Rates of GTP hydrolysis by dynamin are shown.

conditions tested, even though it binds to the dynamin PRD at a site separated from the stimulatory site only by ~20 amino acids (results not shown). From these data we conclude that crosslinking dynamin through the PRD in a specific orientation can stimulate dynamin GTPase activity.

Discussion

We have characterized mAbs raised against the major 864 amino acid splice variant of neuronal dynamin-1. The mAbs bind to epitopes within the proline-rich C-terminal domain of dynamin and inhibit MT-stimulated GTPase activity, probably by steric inhibition of dynamin–MT interactions. Hudy-2 binds to an epitope between amino acids 822 and 838 of neuronal dynamin-1, which is conserved in the ubiquitously expressed isoform dynamin-2. Binding of hudy-2 to this site on dynamin's PRD stimulates GTP hydrolysis rates by 5- to 8-fold. The stimulation of dynamin GTPase activity was proportional to the valency of the mAb. F(ab)s had no activity; crosslinking either F(ab)s or intact IgGs with secondary antibodies restored or increased, respectively, their ability to stimulate dynamin GTPase activity. From these data we conclude that reagents capable of crosslinking dynamin through specific sites within its rigid extended PRD can stimulate GTPase activity. Another mAb, hudy-4, which binds to an epitope located at the extreme C-terminus (between amino acids 841 and 864) of the major neuronal isoform of dynamin, does not affect its intrinsic rate of GTP hydrolysis. From this we conclude that the relative orientation of the crosslinked dynamin molecules is important. Since dynamin is an oligomer (see Materials and methods; Maeda *et al.*, 1992), antibody crosslinking could cause further oligomerization or could effect intrasubunit interactions within a dynamin oligomer to stimulate GTPase activity.

Several disparate effectors of dynamin GTPase activity have been identified through *in vitro* studies. However, there is as yet no clear mechanism that can correlate dynamin's function in endocytic clathrin-coated vesicle formation *in vivo* with the diverse functions of these effectors. It is possible that each of these effectors stimulates dynamin GTPase activity by a similar crosslinking mechanism.

Dynamin was originally isolated as a MT binding protein, and since MTs are the most potent activators of dynamin GTPase activity, they have been the leading candidate as a physiologically relevant effector of dynamin function (Maeda *et al.*, 1992; Shpetner and Vallee, 1992). However, several lines of evidence now argue that dynamin–MT interactions may not be relevant *in vivo*, specifically: (i) there is no colocalization in immunofluorescence assays between MT and dynamin (Scaife and Margolis, 1990; Damke *et al.*, 1994), (ii) in general, the disruption of MT with colchicine or nocodazole has no effect on clathrin-mediated endocytosis, and (iii) overexpression of a dominant-negative mutant of dynamin in stably transformed cells had no effect on the MT cytoskeleton (Damke *et al.*, 1994). Since dynamin binds cooperatively in patches along MTs (Maeda *et al.*, 1992; Shpetner and Vallee, 1992), it is likely, as has been suggested (Herskovits *et al.*, 1993b), that MTs can stimulate dynamin GTPase activity *in vitro* by providing a multivalent substrate for the ordered oligomeric assembly of dynamin molecules.

Grb2, a key signal transduction molecule, has also been proposed as a physiological effector of dynamin function, perhaps facilitating endocytosis of tyrosine-kinase growth factor receptors (Gout *et al.*, 1993; Herskovits *et al.*, 1993b; Ando *et al.*, 1994; Miki *et al.*, 1994; Scaife *et al.*, 1994; Seedorf *et al.*, 1994). However, recent results have established that dynamin functions at late events in coated vesicle formation, after receptor sorting has occurred (Damke *et al.*, 1994). Either of the two SH3 domains of grb2 can interact with the PRD of dynamin *in vitro* (Gout *et al.*, 1993; Miki *et al.*, 1994). Studies using peptides to inhibit grb2–dynamin interactions have implicated three of the four regions of conservation between dynamin-1 and dynamin-2 (see Figure 4) as possible sites for grb2 binding: amino acids 778–795 (Ando *et al.*, 1994), 812–820 (Seedorf *et al.*, 1994) and 822–838 (Miki *et al.*, 1994; Scaife *et al.*, 1994). This apparent lack of specificity is uncharacteristic of SH3 domain-containing proteins (see, for example, Olivier *et al.*, 1993; Ren *et al.*, 1993; Sparks *et al.*, 1994). One of these potential grb2 binding sites (amino acids 822–838) is the same site to which hudy-2 binds to stimulate dynamin GTPase activity. As grb2 is a divalent molecule, we suggest that it might also stimulate dynamin GTPase activity *in vitro* by a crosslinking mechanism through high-affinity, but physiologically irrelevant, interactions with dynamin's PRD.

Proline-rich domains are common to a variety of functionally diverse proteins, including structural proteins, actin binding proteins, transcription factors and RNA polymerase. Such regions are believed to have extended conformationally restricted structures that provide sites of rapid, reversible and often non-specific interactions with other molecules (reviewed by Williamson, 1994). It is possible, therefore, that even relatively high-affinity interactions with dynamin's PRD observed *in vitro* may not occur *in vivo* due to spatial or temporal constraints or to

the occurrence of more favorable interactions with other molecules. Thus, the identity of the physiological effector(s) of dynamin remains uncertain.

The exact function of dynamin's PRD remains to be determined. Interestingly, a number of proteins involved in the release and recycling of synaptic vesicles contain PRDs, including vesicle-associated membrane protein-1, synaptophysin, synapsin-1, p145 and dynamin. It has been demonstrated recently that grb2 binds to three of these molecules, dynamin, synapsin-1 and p145 (McPherson *et al.*, 1994a,b), suggesting some structural similarity between their PRDs. In fact, the PRDs of dynamin-1 and synapsin-1 are strikingly similar: both are ~30% proline and highly basic, both can bind to acidic phospholipids and both are phosphorylated/dephosphorylated during neurotransmission. Depolarization of the synapse causes the rapid phosphorylation of synapsin-1, releasing it from interactions through its PRD and with actin filaments allowing for synaptic vesicle release (reviewed by Greengard *et al.*, 1993). Conversely, phosphorylated dynamin-1 is rapidly dephosphorylated upon depolarization (Robinson *et al.*, 1993) and is recruited from the cytosol to the synaptic membrane (Robinson *et al.*, 1994). These data suggest that the PRDs of dynamin and synapsin-1 might regulate the function of these proteins by specifically targeting them through interactions with other, as yet unidentified, proteins to their sites of action.

How might dynamin GTPase activity be regulated in the context of endocytic-coated vesicle formation? While answers to this question remain speculative, the assembly of dynamin complexes at a coated pit in the correct orientation might trigger GTP hydrolysis to drive coated vesicle budding. Alternatively, perhaps physiological effectors will stimulate dynamin GTPase by a mechanism independent of crosslinking, e.g. by phosphorylation of dynamin's PRD (Robinson *et al.*, 1993). The identification of the physiological partners for dynamin, especially the protein(s) involved in targeting dynamin to plasma membrane-associated coated pits, will be essential in understanding the mechanism by which dynamin regulates coated vesicle formation.

Materials and methods

Materials

GTP, collagenase and elastase were obtained from Boehringer-Mannheim. Subtilisin and protease inhibitors were purchased from Sigma Chemical Co. (St Louis, MO). Carboxypeptidase Y was acquired from Calbiochem. Enzyme-conjugated secondary antibodies and rabbit anti-mouse Fc were obtained from Pierce Chemical Company. Rabbit anti-mouse heavy and light chain antibody was purchased from Cappel. The dynamin peptide P1 (amino acids 751–764) was synthesized at the TSRI Protein Chemistry Core Facility; P2 (amino acids 773–788), P3 (amino acids 778–795) and P5 (amino acids 822–838) were obtained from I.Gout and M.Waterfield (Ludwig Institute, London, UK) and P4 (amino acids 812–820) was obtained from S.Ali and A.Ullrich (Max-Planck Institute for Biochemistry, Martinsried, Germany). If not otherwise indicated below, all other chemicals were reagent grade.

Expression and purification of dynamin

Construction of recombinant baculovirus and expression of recombinant dynamin in Sf9 cells. A full-length cDNA encoding human neuronal dynamin (dynamin-1) was removed from a pBlueScript expression vector (obtained from A.van der Bliek, UCLA) using *Bam*HI and *Hin*dIII restriction enzymes. These fragments were subcloned into the polycloning site of the pBlueBacIII baculovirus expression vector (Invitrogen, San Diego, CA). Recombinant baculovirus was plaque-purified and amplified according to the instruction manual provided with the baculovirus expression vector system. For purification of dynamin, Sf9 cells (1.5×10^6 cells/ml) were infected with high titer virus stocks at ~10 p.f.u./cell and harvested 65 h after infection. At this point, dynamin represented 5–10% of total cellular protein.

Dynamin purification. Sf9 cells (1–2 l containing $1.5–3.0\times10^9$ cells) were harvested by centrifugation at 1000 *g* for 5 min and washed once with dPBS. Cell pellets were resuspended in 50 ml of HEPES column buffer (20 mM HEPES, pH 7.2, 2 mM EGTA, 1 mM $MgCl_2$, 1 mM dithiothreitol) containing 100 mM NaCl (referred to as HCB100) and a protease inhibitor cocktail consisting of 2 μM pepstatin, 2 μM chymostatin, 10 μg/ml leupeptin, 2 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were homogenized at 4°C by N_2 cavitation at 500 p.s.i. for 25 min prior to slow release. The homogenate was diluted 2-fold in HCB0 (no NaCl, to yield final 50 mM NaCl concentration) and then centrifuged at 50 000 r.p.m. for 60 min in a Beckman Ti60 rotor. The supernatant was collected and is referred to as 'cytosol'.

Cytosol was applied to a Q-Sepharose Fast Flow (Pharmacia) column (7.5×2.5 cm, ~30 ml) pre-equilibrated in HCB50 (HCB containing 50 mM NaCl). The column was washed extensively with HCB50 and then eluted with a 500 ml gradient (50–500 mM NaCl in HCB). 6 ml fractions were collected and dynamin eluted as a major protein peak early in the gradient in fractions ~17–25 at ~160 mM NaCl. The pooled fractions were diluted 5-fold in HCB0 and applied to a 5 ml phosphocellulose (P-cell) column (Whatman) pre-equilibrated in HCB50. The column was washed extensively with HCB50, and then with HCB100. A variable but relatively minor fraction of dynamin eluted in HCB100, but the major fraction of dynamin was eluted with HCB containing 300 mM NaCl. Dynamin was >95% pure at this stage and could be used for GTPase assays. For antibody production, further purification could be achieved by gel filtration chromatography on S300-Sephacryl. 2–3 ml of the P-cell pool were applied to an S300-Sephacryl column (1.5×100 cm) and eluted with HCB50. Dynamin eluted as a single broad peak between the void volume and the 667 kDa marker, thyroglobulin, suggesting that it exists as an oligomer. At this stage dynamin was >98% pure, as judged by Coomassie Blue staining following SDS−PAGE. The yield was ~20 mg of dynamin from 2×10^9 infected Sf9 cells.

mAb production

Mice were immunized by intraperitoneal injection of 50 μg of gel filtration-purified dynamin in complete Freund's adjuvant and boosted 4 weeks later by intraperitoneal injection of 50 μg dynamin in incomplete Freund's. Mice were bled and serum was tested for reactivity against dynamin in ELISAs. Briefly, microtiter wells were coated overnight with 2 μg/ml dynamin in 50 mM bicarbonate buffer, pH 9.6, and then blocked with blocking buffer (1% TX-100, 0.1% SDS, 0.2% BSA, 50 mM NaCl, 10 mM Tris, pH 7.4). A dilution series of serum was prepared in blocking buffer and incubated with the immobilized dynamin for 1 h at 37°C. Wells were washed with blocking buffer and incubated with alkaline phosphatase-conjugated rabbit anti-mouse secondary antibodies for 60 min at 37°C. A colorimetric assay for alkaline phosphatase activity was performed following the manufacturer's instructions (Sigma Chemical Co.); adsorption at 405 nm was measured using an ELISA plate reader. Mice with the highest titer antiserum were boosted by tail vein injection of 50 μg dynamin in dPBS for hybridoma production.

At 4 days after immunization, a mouse was euthanized by cervical dislocation and the spleen removed for fusion. Spleen cells were fused with SP2/Ag0 myelomas at a ratio of five spleen cells/myeloma using 1% polyethylene glycol (Boehringer Mannheim) following the manufacturer's instructions. They were plated at 10^5 myelomas/well in 96-well clusters. Culture supernatants were screened on day 8 by ELISA and the strongest were re-screened for their ability to immunoprecipitate dynamin from bovine brain cytosol. The hybridomas producing the six strongest immunoprecipitating antibodies were subcloned by limiting dilution and in soft agar. They were maintained in α-MEM containing 10% heat-inactivated fetal calf serum (HyClone), 10 mM HEPES, pH 7.2, and 100 U/ml each of penicillin and streptomycin. Four clonal hybridomas were obtained. We named these hudy-1 to hudy-4, for **hu**man **dy**namin; all are IgGs. The antibodies could be grouped in two pairs, hudy-1/hudy-2 and hudy 3/hudy-4. Within each pair the mAbs mapped to similar epitopes and displayed similar behaviors in all assays, so only data from hudy-2 and hudy-4 are reported here. The use of hudy-1 for immunolocalization studies has been reported elsewhere (Damke *et al.*, 1994).

Hudy mAbs were purified from ascites fluid by ammonium sulfate fractionation and chromatography on protein G–Sepharose (Pierce Chemical Co.). F(ab) fragments were prepared by papain digestion of purified hudy-2 IgG (5 mg/ml) at a ratio of 10 μg papain/mg IgG for 7 h at 37°C. Digestion was terminated by the addition of iodoacetamide; F(ab) fragments were purified by FPLC on Superdex-75 (Pharmacia).

Dynamin GTPase assays

Tubulin was purified from bovine brain cytosol by three rounds of polymerization/depolymerization and phosphocellulose chromatography, according to published procedures (Williams and Lee, 1982). Taxol-stabilized MT were prepared according to Shpetner and Vallee (1991). A grb2–GST expression vector was obtained from Dr Lawrence Quilliam (University of North Carolina at Chapel Hill) and the fusion protein was purified from *Escherichia coli* lysates by chromatography on glutathione-Sepharose.

GTPase assays were performed in 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM $MnCl_2$, in a final volume of 20 μl, essentially as described (Gout *et al.*, 1993; Damke *et al.*, 1994). Since grb2 was added in 1 μl of GSHM (20 mM HEPES, pH 7.4, 30 mM Na-glutamate, 5 mM $MgCl_2$, 20 mM sucrose), this buffer was added to all reactions for consistency. Reactions were initiated by the addition of 0.5–1.0 mM GTP (0.1 μCi [α-^{32}P]GTP; Amersham). 1.5 μl aliquots were removed at each time point and spotted onto cellulose polyethyleneimine thin layer chromatography (TLC) plates with fluorescent indicator (J.T.Baker, Phillipsburg, MD). Nucleotides were resolved by TLC in 1 M $LiCl_2$:2 M formic acid (1:1). Quantitation was performed on the Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Limited proteolysis of dynamin

Purified dynamin was digested with a variety of proteases which cleave the proline-rich C-terminal domain (Herskovits *et al.*, 1993b). Digestions with subtilisin, elastase and collagenase D were performed at a protease: dynamin ratio of 1:400 (w/w) in dPBS containing 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$ on ice for 0–60 min. Carboxypeptidase Y digestion was performed at a ratio of 1 μg protease:100 μg dynamin in 75 μl H_2O for up to 24 h at room temperature. Digestions were terminated by the addition of 1 mM PMSF. Following proteolysis, samples were subjected to SDS–PAGE on 6.5% acrylamide gels. Various digestion products were tested for reactivity with hudy-1 to hudy-4 mAbs using Western blot analysis.

SDS–PAGE and Western blot analysis

SDS–PAGE on either 7.5 or 6.5% acrylamide gels and Western blot analysis were performed by standard methods as described previously (Damke *et al.*, 1994). To detect dynamin, blots were incubated with hudy mAbs at 0.1 μg/ml in TBS containing 2% milk powder and 0.1% Tween-20 either overnight at 4°C or for 1–2 h at 37°C and were developed using alkaline phosphatase-conjugated secondary antibodies as described previously (Damke *et al.*, 1994).

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