Dynamin function is mediated in part through association of its proline-rich domain (PRD) with the Src homology 3 (SH3) domains of several putative binding proteins. To assess the specificity and kinetics of this process, we undertook surface plasmon resonance studies of the interaction between isolated PRDs of dynamin-1 and -2 and several purified SH3 domains. Glutathione S-transferase-linked SH3 domains bound with high affinity (KD ~ 10 nM to 1 μM) to both dynamin-1 and -2. The simplest interaction appeared to take place with the amphipathic-SH3 domain; this bound to a single high affinity site (KD ~ 10 nM) in the C terminus of dynamin-1 PRD, as predicted by previous studies. Binding to the dynamin-2 PRD was also monophasic but with a slightly lower affinity (KD ~ 25 nM). Endophilin-SH3 binding to both dynamin-1 and -2 PRDs was biphasic, with one high affinity site (KD ~ 14 nM) in the N terminus of the PRD and another lower affinity site (KD ~ 60 nM) in the C terminus of dynamin-1. The N-terminal site in dynamin-2 PRD had a 10-fold lower affinity for endophilin-SH3. Preloading of dynamin-1 PRD with the amphipathic-SH3 domain partially occluded binding of the endophilin-SH3 domain, indicating overlap between the binding sites in the C terminus, but endophilin was still able to interact with the high affinity N-terminal site. This shows that more than one SH3 domain can simultaneously bind to the PRD and suggests that competition probably occurs in vivo between different SH3-containing proteins for the limited number of PXXP motifs. Endophilin-SH3 binding to the high affinity site was disrupted when dynamin-1 PRD was phosphorylated with Cdk5, indicating that this site overlaps the phosphorylation sites, but amphipathic-SH3 binding was unaffected. Other SH3 domains showed similarly complex binding characteristics, and substantial differences were noted between the PRDs from dynamin-1 and -2. For example, SH3 domains from c-Src, Grb2, and intersectin bound only to the C-terminal half of dynamin-2 PRD but to both the N- and C-terminal portions of dynamin-1 PRD. Thus, differential binding of SH3 domain-containing proteins to dynamin-1 and -2 may contribute to the distinct functions performed by these isoforms.

The dynamin family of GTPases participates in a variety of cell behaviors (for reviews, see Refs. 1–3). While they are best known for their role as "pinchases" in endocytic events at the plasma membrane, it is clear that interactions with other subcellular structures widen the influence of this group of proteins. For example, the discovery of dynamin participation in phenomena like endosomal "rocketing," membrane ruffling, and podosomes has highlighted the potential role of dynamin in regulating actin-based structures (4). Dynamin-1, -2, and -3 are multidomain proteins with an N-terminal GTPase domain, central association and pleckstrin homology (PH) domains, and a C-terminal proline-rich domain (PRD). The latter plays a vital role in targeting dynamin to a diverse set of binding partners that may cooperatively enable it to fulfill its function. Whereas dynamin isoforms were initially thought to have redundant functions, particularly in endocytosis, it is now apparent that isoform-specific functions exist. For example, we have shown that dynamin-1 regulates clathrin-independent rapid endocytosis, whereas dynamin-2 mediates clathrin-dependent slow endocytosis in adrenal chromaffin cells (5, 6). Indeed, the PRD of dynamin is the least conserved part of the protein, and dynamin-2 is only ~50% identical to dynamin-1 in this domain, whereas in other domains identity runs to >75%.

The list of putative dynamin partners is long and includes amphiphysin, endophilin, intersectin, c-Src, Grb2, phosphatidylinositol 3-kinase, phospholipase Cδ, cofilin, and several others. All of these proteins contain SH3 domains and interact with dynamin by binding to the PRD. The PRD contains several motifs characteristic of a type II polyproline helix, a specialized structural element that repeats every 3 amino acids, forming a triangular structure in cross-section (7–9). Such motifs fall into two classes, dependent on whether critical basic residues are N-terminal (as in RXXPXXP; class I) or C-terminal (as in PXXPRX; class II) to the core PXXP motif. SH3 domains bind in opposite orientations to these two motifs, because the basic residue forms a key salt bridge with appropriate acidic residues in the SH3 domain (10). Hydrophobic interactions make up the predominant binding forces in the remainder of the binding interface. Clearly, the specificity and affinity of SH3 domain binding to proline-rich sequences plays a key role in determining the functional association of dynamin with its partner proteins.

Gout et al. (11) were the first to demonstrate specificity in the binding of SH3 domains to dynamin using a GST pull-down approach. They found that SH3 domains from phosphatidyl-

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‡ The abbreviations used are: PH, pleckstrin homology; PRD, proline-rich domain; SH3, Src homology 3; SPR, surface plasmon resonance; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; RU, arbitrary response units; GST, glutathione S-transferase; amph-SH3, GST-amphiphysin-SH3 domain.

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"Kinetics of Src Homology 3 Domain Association with the Proline-rich Domain of Dynamins: SPECIFICITY, OCCLUSION, AND THE EFFECTS OF PHOSPHORYLATION*"

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23147
inositol 3-kinase (p85 subunit), phospholipase Cε, c-Src, and Grb2 were able to precipitate full-length dynamin but that SH3 domains from spectrin, N-Src, Crk, NADPH oxidase, or Csk could not. Attempts were made using peptides derived from the dynamin PRD to map the sites of interaction of various SH3 domains, but these only covered one potential region of interaction (residues 778–795). Subsequently, Okamoto et al. (12) mapped the binding sites of several SH3 domains in the dynamin PRD using deletion constructs. They established three binding regions: region 1 (residues 785–794 in dynamin-1) contains a type II polyproline helix class I consensus sequence and binds c-Src SH3, whereas regions 2 (residues 809–826) and 3 (residues 827–839) have a class II conforming sequence. Region 2 bound p85, whereas region 3 bound to both amphiphysin and Grb2 SH3 domains. Few differences between dynamin-1 and -2 were apparent (12). Further work confirmed that amphiphysin-SH3 bound exclusively to region 3 within the sequence PARXRPR (13) and that endophilin-SH3 probably bound at two sites, one overlapping that of amphiphysin and another site possibly within region 1 (14). These studies have been largely qualitative, although it was shown that the amphiphysin-SH3-dynamin interaction was of relatively high affinity ($K_D$ ~190 nM) based on pull-down titration (13). Alongside these observations, it has been suggested that phosphorylation regulates the interaction between members of endocytotic complexes, particularly those involving dynamin-1, amphiphysin, endophilin, and synaptojanin (14–16). Since dynamin-1 is principally phosphorylated in its PRD, this modification could affect the binding of SH3 domains, but few quantitative studies have analyzed this possibility.

In this study, we have determined the kinetics and specificity of SH3 binding to the dynamin PRD using surface plasmon resonance (SPR). This method enables accurate measurement of association and dissociation kinetics from which determinations of $K_D$ and stoichiometry can be readily derived (17, 18). We show that SPR can detect occlusion and the effects of phosphorylation on binding to the dynamin PRD. In addition, we demonstrate significant differences between the binding properties of dynamin-1 and -2. These results have a bearing on protein-protein interactions in endocytosis and the role of phosphorylation in the process.

**MATERIALS AND METHODS**

**Expression and Purification of Recombinant Proteins**

**Dynamin-1 PRD.—**The dynamin-1 PRD (b splice variant; residues 751–851) was excised by PCR from a pSVL-dynamin 1 plasmid obtained from Drs. P. Okamoto and R. B. Vallee (University of Massachusetts) using the primers D1-PRD forward (5′-GACATCAACACGACA-GATCTCAACCC-3′) and D1-PRD reverse (5′-CTGATCGTGATGTA-GCTAGGCTACT-3′) complementary to nucleotides 2230–2259 and 2545–2571, respectively, of the rat coding sequence. BglII and SalI restriction sites were introduced at the N and C termini of the PRD, respectively, and the PCR product was inserted into pCR2.1-TOPO-TA shuttle vector (Invitrogen) and amplified. The dyn-1 PRD was then eluted with 0.5 mM imidazole, 0.5 mM NaCl, pH 8.0. The eluate was dialyzed against BIAcore buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and stored in the presence of 10% glycerol at −80 °C. The purity of protein samples was analyzed by SDS-PAGE and assessed to be >95% by Coomassie Blue staining.

**Phosphorylation of Dynamin-1 PRD Domains by Cdk5 Kinase**

Dynamin-1 PRD domain was phosphorylated with recombinant Cdk5. Both Cdk5 and its regulatory subunit p25 (plasmids obtained from Dr. H. Pant, National Institutes of Health) were expressed and purified separately and then recombined prior to phosphorylation assays, as previously described (20). Cdk5 kinase was activated for 3 h by the addition of p25 at a 1:2 ratio in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM MgCl₂, 2 mM β-glycerophosphate, 100 nM calyculin A. The standard assay mixture for dynamin phosphorylation (final volume of 100 μl) contained 25 μM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM CaCl₂. Thrombin (Amer sham Biosciences) was added at a concentration of 0.2 units/μg protein, and the reaction was incubated at room temperature for 15 min. 4-(2-Aminoethyl)-benzenesulfonyl fluoride was added to a final concentration of 2 mM, and the mixture was incubated for an additional 5 min. The supernatant was separated from the beads and subjected to phosphoamino acid analysis by thin-layer chromatography. The mixture was incubated for 30 min at room temperature, and the supernatant containing free SH3 domain was separated from the beads by centrifugation and stored at ~80 °C or dialyzed versus HBS running buffer and used immediately. The purity of cleaved protein was confirmed by analysis on 4–20% gradient SDS-PAGE.

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The eluate was dialyzed against BIAcore buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and stored in the presence of 10% glycerol at −80 °C. The purity of protein samples was analyzed by SDS-PAGE and assessed to be >95% by Coomassie Blue staining.

To obtain the Grb2-SH3(N) domain free of GST, the fusion protein was cleaved on the glutathione column matrix. An aliquot of GST-SH3-beads was extensively washed with 100 bed volumes of phosphate-buffered saline and resuspended in 1 ml of thrombin cleavage buffer (25 mM Tris-HCl, pH 7.5, 0.25 mM NaCl, 2 mM CaCl₂, 0.1% Triton X-100, 0.1 mM EDTA, 0.1 mM EGTA, 1 μg/ml pepstatin, 50 μM leupeptin, and 1 mM benzamidine). The beads were washed three times with 20 ml Tris-HCl, pH 7.5, 0.25 mM NaCl, and bound protein was eluted with 20 ml glutathione, 20 ml Tris-HCl, 0.25 mM NaCl, pH 8.0. The eluate was dialyzed against BIAcore buffer and stored in the presence of 10% glycerol at −80 °C. The purity of protein samples was analyzed by SDS-PAGE and assessed to be >95% by Coomassie Blue staining.

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out at 30 °C for up to 60 min. Reactions were terminated by boiling in SDS sample buffer, and phosphate incorporation was determined by autoradiography of the SDS-polyacrylamide gels followed by scintillation counting of labeled bands. Up to 0.41 mol of P/mol of dynamin-1 PRD was incorporated. For the BIAcore analysis of phosphorylated PRD, reactions were scaled up 10-fold and conducted without labeled ATP. Samples were then processed by IMAC phosphoprotein affinity chromatography (using columns and reagents from Qiagen) under native conditions. The reaction mixture was dialyzed versus phosphoprotein lysis buffer in the presence of 0.25% Chaps prior to the addition to the column. The column was washed three times with 3 volumes of lysis buffer, and the phosphorylated dynamin-1 PRD was recovered with phosphoprotein elution buffer in the presence of 0.25% Chaps. The separation of phosphorylated from unphosphorylated proteins was checked using 32P-labeled PRD and autoradiography and was found to be >95% complete (i.e. <5% of applied labeled protein passed through the column).

**Surface Plasmon Resonance**

The affinity and the rates of interaction between dynamin and different SH3 domains were measured with a BIAcore 3000 biosensor through SPR. Various dynamin constructs were bound to the dextran matrix of a sensor chip containing nickel-nitrilotriacetic acid to immobilize the His6-tagged dynamin PRD domain as target (21). All steps in the immobilization procedure were carried out at a flow rate of 5 µl/min. A surface injection of 25-50 RU was preceded similarly except that the running buffer was injected instead of NiCl2 solution. All kinetic experiments were performed at 4 °C in HBS (10 mM HEPES, pH 7.4, 0.15 M NaCl, 50 µM EDTA, 0.05% Tween 20) using flow rates of 5–20 µl/min. These conditions prevented significant leakage of bound target protein from the chip. Different SH3 domains (analyte) at various concentrations (see "Results") were added in the flow during the binding phase. The amount of protein bound to the sensor chip was monitored by the change in refractive index (given in arbitrary response units (RU)). For the measurements of kinetic parameters, the theoretical maximum binding (Rmax) of SH3 domains to the full-length, N, or C termini of both PRD domains and the actual response observed. The theoretical maximum binding (Rmax) in RU is given by the following.

\[
R_{\text{max}} = \frac{\text{MW}_{\text{GST-SH3}} \cdot \text{MW}_{\text{PRD}}}{\text{MW}_{\text{PRD}}} \quad (\text{Eq. 2})
\]

where MW_{GST-SH3} is the molecular weight of GST-SH3 domain, MW_{PRD} is the molecular weight of dynamin PRD or N- and C-terminal fragment, n is the stoichiometry of binding, and R_{PRD} is the level of immobilized His6-PRD domain expressed in RU.

**RESULTS**

**Monophasic Binding of GST-Amphiphysin-SH3 to Dynamin PRDs**—Sequence alignment of the primary structure of dynamin-1 and -2 PRDs reveals the presence of several canonical PXPF sequences that can be distinguished as class I or II type II polyproline helix motifs (Fig. 1A; see also Ref. 12). Note that dynamin-2 lacks a typical PXPF sequence in its N-terminal half and has additional motifs in its C-terminal tail that are not present in dynamin-1. Of the motifs identified in both dynamins, one with high conservation is the class II sequence PSKPRN in dynamin-1 and PSRPRV in dynamin-2. The former has been identified as the major amphiphysin interaction site in dynamin-1 (13). To confirm this result and test the ability of dynamin-2 to bind amphiphysin, we assessed the kinetics of association and dissociation of GST-amphiphysin-SH3 to both PRDs using the BIAcore 3000 SPR system. Typical sensorgrams are shown in Fig. 1, B–E. Quantitative analysis of binding as in Fig. 1, B and C, revealed monophasic association and dissociation kinetics for both PRDs with calculated Kd values of 9 and 25 nM, respectively (Table I). The apparent affinity is ~20-fold higher than that previously estimated using pull-down methodology (13). The higher Kd for dynamin-2 was predicated on much slower uptake and release kinetics. Qualitative analysis, such as that shown in Fig. 1D, revealed that GST alone or a mutant amphiphysin-SH3 domain that lacks affinity for dynamin was unable to bind to the PRD. In addition, amphiphysin bound well to the isolated C-terminal fragments of each PRD but exhibited no interaction with the N-terminal fragments (Fig. 1E). These results confirm that amphiphysin-SH3 binds to a single site in the C terminus of the PRD of dynamin-1 and show that this is also true of dynamin-2, albeit with a lower affinity.

**Binding of Other Endocytosis-related SH3 Domains to Dynamin PRDs**—Two other proteins that are thought to participate in dynamin-mediated endocytosis are endophilin and intersectin. Both proteins associate with dynamin in cells as determined by immunoprecipitation experiments (23, 24), and elimination of either protein or expression of dominant-negative constructs affects endocytosis (25, 26). Endophilin-SH3 binding to the dynamin PRD was more complex than that of amphiphysin-SH3. In both cases, the interaction was well described by a two-site model with high affinity and low affinity sites (Fig. 2A, Table I). Analysis of residuals, as shown in Fig. 2B, resulted in a much better fit with the two-site model than the single-site model. In order to better resolve the two sites, we analyzed binding to the separated N- and C-terminal fragments of dynamin PRDs (Fig. 2C). Endophilin-SH3 was found to bind monophasically to both fragments of dynamin-1 with different affinities (~60 nM to the C-terminal fragment and ~14 nM to the N-terminal fragment). Binding to dyn-2-PRD(C) was of about the same affinity as that to dyn-1 PRD(C), but the affinity for dyn-2 PRD(N) was 10-fold lower than that for dyn-1 PRD(N) (Table I). One binding site for endophilin has previously been speculated to overlap that of amphiphysin (27, 28), whereas another was located in the N-terminal half of the domain.
fragments of D1PRD and D2PRD. Binding was conducted as in critical for PRD binding showed no interaction with either PRD, as did GST alone. Sensorgrams were fitted globally, and the rate constants and affinities were compared. —, no binding observed.

For derived kinetic parameters, see Table I. C. residuals from a single-site binding model (see “Materials and Methods”) indicate excellent fit. D, comparison of amph-SH3 binding to dynamin-1 or -2 (D1 or D2) PRDs. Binding was conducted as in B except that 500 nM analyte was used. An amph-SH3 variant (amph mut) mutated in residues critical for PRD binding showed no interaction with either PRD, as did GST alone. E, amph-SH3 binding to the isolated C- and N-terminal fragments of D1PRD and D2PRD. Binding was conducted as in A, using 500 nM analyte. Note that no interaction was obtained with the N-terminal fragment of either PRD.

| SH3 domain | N-terminal site | C-terminal site |
|------------|----------------|----------------|
|            | $k_a$ | $k_d$ | $K_D$ |
|            | $1/s$ | $1/s$ | ns | $1/s$ | $1/s$ | ns |
| Dynamin-1 PRD | | | | | | |
| Amph | $1.5 \pm 0.3 \times 10^4$ | $2.2 \pm 0.4 \times 10^{-4}$ | $13.9 \pm 1.9$ | $1.9 \pm 0.7 \times 10^3$ | $1.7 \pm 0.6 \times 10^{-3}$ | $9.9 \pm 2.1$ |
| Endo | $2.6 \pm 0.4 \times 10^3$ | $4.3 \pm 0.1 \times 10^{-2}$ | $164.5 \pm 29$ | $5.5 \pm 0.4 \times 10^4$ | $3.2 \pm 0.3 \times 10^{-3}$ | $59.2 \pm 5.7$ |
| Inters | | | | | | $73.5 \pm 7.4$ |
| Dynamin-2 PRD | | | | | | |
| Amph | $7.9 \pm 0.4 \times 10^3$ | $9.3 \pm 0.8 \times 10^{-4}$ | $117.7 \pm 11.7$ | $1.7 \pm 0.7 \times 10^4$ | $5.4 \pm 1.8 \times 10^{-3}$ | $25.7 \pm 0.5$ |
| Endo | | | | | | $4.0 \pm 0.2 \times 10^4$ | $2.9 \pm 0.1 \times 10^{-3}$ | $72.5 \pm 5.1$ |
| Inters | $1.5 \pm 0.1 \times 10^3$ | $5.9 \pm 0.1 \times 10^{-3}$ | $39.2 \pm 0.3$ |

Dynamin-1 PRD (14). In order to verify this supposition, we first prebound either amphiphysin-SH3 or endophilin-SH3 to the dynamin PRD and then assessed what influence this would have on the subsequent binding of the other protein. As shown in Fig. 2D, preincubation of dynamin-1 PRD with amphiphysin-SH3 partially ablated subsequent binding of endophilin-SH3, whereas preincubation with endophilin-SH3 completely abolished subsequent binding of amphiphysin-SH3. In addition, prebinding of amphiphysin to dynamin-1 PRD(C) eliminated endophilin binding to this fragment (data not shown).
These results suggest that the lower affinity endophilin binding site in the C terminus of dynamin-1 PRD overlaps that of amphiphysin, causing steric occlusion. The location of the higher affinity binding site in the N-terminal fragment was tentatively identified as indicated below.

Intersectin is a far more complex protein than endophilin and has five tandem SH3 domains as well as Dbl homology, PH, and guanine nucleotide exchange factor domains. Here we made a preliminary assessment of the binding characteristics of the SH3-C domain, reported previously to interact with dynamin (29) and to inhibit receptor-mediated endocytosis (26). Intersectin-SH3 binding to dynamin-1 PRD was best fit with a two-site model as assessed by residuals from curve-fitting models and by stoichiometry measurements, but binding to dynamin-2 PRD was monophasic, with the C-terminal fragment carrying the binding site (Table I). In contrast to a previous report (15), but in agreement with others (16), we found little effect of Cdk5 phosphorylation on the kinetics of amphiphysin-SH3 binding (Table II).

Binding of Signaling Protein SH3 Domains and Evaluation of Avidity Effects—We tested the SPR kinetics of two other SH3 domains, those from c-Src and Grb2, proteins that have both been reported to interact with, and possibly modify the function of, dynamin (1). As shown in Fig. 2, D and E, endophilin-SH3 binding was approximately halved in the phosphorylated dynamin-1 PRD (Pdyn-1 PRD) and ablated completely when amphiphysin was prebound to the Pdyn-1 PRD. In contrast to a previous report (15), but in agreement with others (16), we found little effect of Cdk5 phosphorylation on the kinetics of amphiphysin-SH3 binding (Table II).
of, dynamin (31–34). With whole dynamin-1 PRD, c-Src exhibited kinetics consistent with binding to more than one site (Fig. 3A; Table III). We conducted stoichiometry measurements to estimate the number of sites (Ns) in the whole PRD. In the case of dynamin-1, Ns = 3, but with dynamin-2, Ns = 2. To clarify the situation further, we analyzed binding to isolated N- and C-terminal fragments (Fig. 3B). With dyn-1 PRD(N), binding was consistent with a single site of KD = 126 nM, whereas binding to dyn-1 PRD(C) was consistent with two sites of KD = 150 nM and 1.3 μM (Fig. 3, B and C; Table III). With dyn-2 PRD(N), no binding was observed (Fig. 3C), in agreement with the lack of a consensus PXXP motif in this region (Fig. 1A). Similar to dyn-1 PRD(C), two sites with nanomolar and micromolar affinity were found on dyn-2 PRD(C) (Fig. 3B; Table III).

Binding to all sites could be blocked by preincubation of the SH3 domain with optimized c-Src-selective or -specific peptides (Fig. 3A; see Ref. 35), indicating specificity of binding. The same peptides had no effect on the binding of endophilin-SH3 (Fig. 3A).

Grb2 also exhibited kinetics consistent with at least two binding sites on the PRD of both dynamin-1 and -2 (Fig. 4A; Table III), but the situation was slightly different than with c-Src. The stoichiometry of association with both PRDs yielded Ns = 1. A single very low affinity site was located in dynamin-1 PRD(N), and a single high affinity site was located in dynamin-1 PRD(C). The latter site probably overlaps the amphipathic-SH3 site in that prebinding of Grb2-SH3 blocked amphipathic-SH3 binding (data not shown). By contrast, dynamin-2 PRD(N) exhibited no Grb2-SH3 binding, as with c-Src, consistent with the lack of a PXXP consensus, but binding to dynamin-2 PRD(C) was well fit by a two-site model with high (KD = 24 nM) and low (KD = 2.7 μM) affinity sites. Binding of c-Src and Grb2 SH3 domains was significantly lower in phosphodynamin-1 PRD, suggesting that, as with endophilin, phosphorylation interferes with binding to the N-terminal region 1 site in this isoform (data not shown).

One phenomenon that could confound KD measurements with solid phase assays such as SPR is the possibility of avidity effects due to the dimeric nature of GST fusion proteins. A well known example of avidity caused by cross-linking of polyvalent ligands to individual binding sites is seen in antibody-antigen interactions (e.g. Ref. 36). Ladbury et al. (37) showed that binding of GST-Src homology 2 domains to tyrosine phospho-epitopes immobilized on Biacore chips sometimes yielded KD values that were 2 orders of magnitude different from those derived from a solution technique like isothermal titration calorimetry. To evaluate the contribution of avidity effects to the KD values seen with GST-linked proteins (due to the tendency of GST domains themselves to dimerize), we cleaved the Grb2-SH3 domain from its GST fusion partner using thrombin and compared its binding characteristics with those of the fusion protein. Virtually identical kinetics were obtained, suggesting that under the conditions used in the present study, avidity effects play little if any role in the binding of GST-SH3 domains to either of the dynamin PRDs (Fig. 4B).

**DISCUSSION**

SH3 domains are a prominent feature of many signaling proteins and much work has been devoted to elucidating their specificity for proline-rich and other sequences. Peptide library studies have revealed that, for many SH3 domains, recognition of PXXP sequences is of low affinity (middle to high micromolar KD values) and specificity (e.g. see Ref. 27). Some have argued that low affinity may be advantageous to signaling pathways where rapid switching is required (38), but in at least one case it has been demonstrated that lowered affinity considerably weakens signaling in vivo (39), and several studies have shown a high degree of discrimination between various PXXP motifs by different SH3 domains (e.g. see Ref. 40). In analyzing the binding of specific SH3 domains, it may be important to consider interactions outside the minimal class I or II motifs generally used in peptide library studies. The recognition site for several SH3 domains may be more complex than these motifs alone and involve loops in the SH3 domain interacting with other elements of the specific PRD in question (e.g. c-Src SH3 binding to the human immunodeficiency virus protein Nef PRD (10); Fyn-SH3 binding to the p85 subunit of phosphatidylinositol 3-kinase (42); the C-terminal Src kinase-SH3 interaction with proline-enriched phosphatase-PRD (43); and the p67phox-SH3 interaction with p47phox-PRD (44)). A specific example relevant to the present work is the case of Grb2 binding to dynamin. Vidal et al. (34) found a high affinity interaction of Grb2-SH3(N) with intact dynamin-1 (KD = 25 nM) but very low affinity binding to peptides corresponding to the presumed

**TABLE II**

| Dynamin-1 PRD domain | kₐ | kᵣ | KD |
|----------------------|----|----|----|
| Phospho-PRD          | 6.57 × 10⁴ | 6.15 × 10⁻⁴ | 9.36 |
| PRD                  | 1.89 × 10⁵ | 1.70 × 10⁻³ | 9.90 |

**FIG. 3. Binding of c-Src-SH3 to dynamin-1 and -2 PRD.** A, sensorgrams of c-Src-SH3 interaction with dynamin-1 PRD compared with endophilin-SH3. Control binding reactions for c-Src and endophilin are shown as solid lines, whereas reactions carried out in the presence of c-Src binding peptides are shown as broken lines. c-Src-SH3 (1 μM) was preincubated with a 100 μM concentration of either the Src family-selective peptide KGGRSLRLPPLPPLPP (curve 2) or the Src family-specific peptide LSSRPFLTLPSF (curve 3). The mixture was then injected over immobilized dynamin-1 PRD. This resulted in 71.5 and 93.7% inhibition of binding, respectively. For comparison, the lack of effect of the Src-specific peptide on endophilin-SH3 domain binding is shown (curve 5). B, binding of c-Src-SH3 to dynamin-1 and -2 PRD N- and C-terminal fragments (left and right sensorgrams, respectively). Note the failure of the SH3 domain to bind to the N-terminal fragment of dynamin-2.
Kinetics of SH3 Domain Association with the PRD of Dynamins

Analysis was conducted as described in the legend to Table I. Where indicated, A and B refer to two sites found in the isolated C-terminal PRD. —, no binding observed.

| SH3 domain | N-terminal site | C-terminal site |
|------------|----------------|-----------------|
|            | $k_a$ | $k_d$ | $K_D$ | $k_a$ | $k_d$ | $K_D$ |
| Dynamin-1 PRD |       |       |       |       |       |       |
| c-Src      | 7.8 ± 0.1 × 10^4 | 9.9 ± 0.2 × 10^{-4} | 126.7 ± 3.0 nM | (A) 1.5 ± 0.2 × 10^4 | 2.2 ± 0.3 × 10^{-3} | 150.0 ± 36.6 nM |
| Grb2       | 2.4 ± 0.1 × 10^4 | 3.9 ± 0.2 × 10^{-2} | 1.7 ± 0.1 μM | (B) 1.3 ± 0.2 × 10^{-3} | 1.6 ± 0.3 × 10^{-3} | 1.3 ± 0.2 μM |
|           |       |       |       | (A) 6.4 ± 0.1 × 10^{-1} | 1.9 ± 0.2 × 10^{-3} | 29.0 ± 3.0 nM |

FIG. 4. Binding of Grb2-SH3(N): Lack of avidity effect. A, binding of GST-Grb2-SH3(N) to individual N- and C-terminal fragments of dynamin-1 PRD. B, overlaid sensograms showing the binding of GST-Grb2-SH3 and Grb2-SH3 to the dynamin-1 PRD. GST-Grb2-SH3 at 200 nM was injected over 840 RU of PRD, whereas Grb2-SH3 (cleaved from GST tag and prepared as described under “Materials and Methods”) at 10 μM was injected over 840 RU of dynamin-1 PRD. The association and dissociation phases were monitored for 600 and 300 s, respectively. The signals were then normalized.

Grb2 binding site ($K_D$ as low as 300 μM for the dynamin-1-derived peptide $\text{GPPQYPSPRNP}^{287}$). These data prompted the speculation that Grb2-SH3 binding to dynamin involved interactions beyond the canonical SH3 hydrophobic groove (47). Substantial specificity is also reflected by in vivo experiments, where introduction of particular SH3 domains, but not others, have dominant-negative effects on a cellular process. In the case of endocytosis, it has been shown that introduction of the SH3 domain of amphiphysin, but not those of spectrin or phospholipase Cβ, can disrupt receptor internalization (48). These considerations make it critical that binding studies be carried out with entire PRDs to complement findings with short peptides.

Here we assessed the binding of several SH3 domains to the full-length or bisected dynamin PRD in order to evaluate both affinity and specificity (data are summarized in Fig. 5). SPR is an effective technique to evaluate such interactions, because precise measurements of on and off rates can be achieved, and a variety of kinetic parameters can be varied (17, 18). Amphiphysin-SH3 displayed the simplest pattern, binding to only a single site with a high affinity, confirming previous results obtained by other methods (13, 27, 49). The core amphiphysin binding motif represents a variant class II binding site in that two basic residues (PXRPXR) are involved instead of just one (PXXPXR). There are corresponding crucial acidic residues in the amphiphysin-SH3 that when mutated abolish PRD binding and the in vivo dominant negative effects of the isolated domain on endocytosis (49). We found that binding to dynamin-2 PRD was slightly weaker than to dynamin-1 PRD and exhibited 10-fold slower association and dissociation kinetics. These subtle effects may reflect the difference between valine and asparagine in the PSRPXR motif or be associated with variations in flanking sequences. Amphiphysin also binds to the inositol 5'-phosphatase synaptojanin at a site (PIRPSR) that resembles that in dynamin (27). The exceptional specificity of these binding sites is in keeping with the notion that this protein could provide a link between the dynamin oligomer at the neck of the retracting endocytic vesicle and the clathrin-based machinery that forms the vesicle coat. Thus, in addition to dynamin and synaptojanin interactions via the SH3 domain, amphiphysin-I and IIa can bind to both AP-2 and clathrin itself via a separate domain (50). Consistent with this hypothesis are in vitro studies showing that mixtures of amphiphysin and dynamin exhibit a more efficient vesiculation of lipid preparations than dynamin alone (51). However, the importance of amphiphysin has been called into question by recent gene ablation data that show no effect of amphiphysin knockout on synaptic vesicle recycling in Drosophila and only a modest effect in mice (52–54). It remains to be determined whether this lack of effect is due to substitution by other proteins with similar function. Whatever the outcome, it seems clear that members of the amphiphysin family are involved in diverse processes unrelated to endocytosis, suggesting that further binding partners should be sought for these other functions.

Other SH3 domains showed more complex interactions with the dynamin PRD, indicating binding to more than one site. For example, endophilin-SH3 bound to two distinct high affinity (nM $K_D$) sites on full-length dynamin-1 and -2 PRDs. Individual sites were located on the N- and C-terminal halves of both PRDs as determined by independent analysis of the fragments. These data support the conclusion that the C-terminal higher affinity site overlaps the class II site recognized by amphiphysin, as suggested by previous analyses of pull-down assays (24) and peptide binding experiments (27). The nature of the higher affinity N-terminal site is less certain. In a peptide scanning study (27), binding of endophilin-SH3 to the peptide $\text{SPTQPQRAPAV}^{388}$ from region I in dynamin-1 was observed by blotting (no affinity determined), and in a second study, the 18-mer peptide $\text{Q}^{729}$RAPPVPAPSGSRGPA$^{989}$ (underlined PXRPXR motif corresponds to the recognition sequence in synaptojanin) was shown to bind, but only with a low affinity ($K_D$ ~80 μM; see Ref. 28). Thus, the binding site for endophilin-SH3 in the N-terminal half of dynamin-1 is not definitively identified, and we have only tentatively assigned it in Fig. 5. The vastly higher affinity we observe for the N-
terminal site (14 nM in dynamin-1) compared with the peptides alluded to above is probably due to interactions outside the 18-mer sequence. This conjecture is supported by the inhibitory effect on endophilin-SH3 binding manifest by Cdk5 phosphorylation of sites just N-terminal to the 782–799 peptide. Further experiments with peptide competition will be needed to more precisely define the endophilin binding site in the dynamin-1 N terminus. Given that dynamin-2 lacks a class I or II consensus in the N-terminal fragment, we speculate that endophilin-SH3 may also bind to a core PXXP motif (778PPGRP782) in this isoform, as suggested for dynamin-1 (28).

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The ~10-fold lower affinity of endophilin-SH3 for this region of dynamin-2 compared with dynamin-1 again may reflect differences in flanking sequences. Like amphiphysin, endophilin I forms dimers, has a BAR domain, and can thereby sense membrane curvature (55). It is thus interesting that these two proteins compete for binding to the C-terminal region of the dynamin PRD, whereas endophilin can bind alone to a site in the N-terminal region. Simultaneous binding of both proteins could then occur (see Ref. 24 for a similar analysis using full-length proteins), but in a situation where dynamin-1 has amphiphysin-SH3 associated and is phosphorylated by Cdk5, endophilin would not be able to bind to dynamin at all.

SH3 domains of proteins possibly related to a signaling function of dynamin, like those from c-Src and Grb2, also showed a complex pattern of binding. Moreover, the interaction patterns of the two dynamin isoforms were qualitatively distinct. c-Src has been reported to not only bind to dynamin-1 (32) but to phosphorylate both dynamin-1 and -2 in the PH domain (31). This modification was claimed to alter dynamin self-association and to be involved in epidermal growth factor receptor internalization (31). Thus, interaction of the c-Src SH3 domain with the PRD might activate c-Src enzyme activity and possibly orient the kinase domain toward appropriate residues for phosphorylation. Peptide library studies show that c-Src-SH3 has the ability to associate with both class I and II PXXP motifs with preferred sequences RXXXPXP and PXVPXR, where Ψ is a hydrophobic residue (38, 56) (e.g. RPLPSP (p130Cas) and RALPSIP (focal adhesion kinase)) (57). Nevertheless, c-Src-SH3 can associate with sequences that may lack a PXXP motif entirely (e.g. the internal binding site on c-Src itself or the tail domain of β3 integrin) (35), although these sequences may adopt a type II polyproline like helix. In dynamin-1, region 1 has the sequence 782RKRAPAVP788, which is one putative c-Src-SH3 binding motif (58). By contrast, dynamin-2 lacks a comparable motif (Fig. 1A). This was confirmed by SPR analysis of both the stoichiometry and affinity of c-Src-SH3 binding to the independent N and C termini of both PRDs. c-Src-SH3 could interact with either fragment from dynamin-1 but only with dynamin-2 PRD(C). The two binding sites on the C termini of dynamin-1 and -2 had similar low and high affinities and may correspond to the region 2 and 3 class II consensus sites, respectively. Further studies will be needed to assess this possibility, but the fact that dynamin-2 lacks one c-Src binding site may preclude different functional relationships between the kinase and dynamin isoforms in vivo.

Grb2 has also been claimed to associate with dynamin during receptor-mediated endocytosis of receptor tyrosine kinases like the epidermal growth factor receptor (33) (but see also Ref. 59). The Grb2-SH3(N) consensus 9DXPLPXL (where Ψ represents an aromatic residue) derived from phage display peptide libraries suggested a preference for a class I motif (38), but it turns out that Grb2 binding sites in proteins conform largely to class II consensus motifs (e.g. Sos sites, PPXVPR). In agreement with this preference, we observed only weak binding of Grb2-SH3 to the class I PXXP motif in dynamin-1 PRD(N) and much stronger binding to the class II motif(s) in the PRD(C) fragment. The higher affinity C-terminal sites probably involve the core sequences 829PQVPSR834 (dynamin-1) and 829PQIPSR834 (dynamin-2), which overlap the amphiphysin binding sites, as confirmed here by occlusion studies. The KD value of this site agrees well with previous determinations of Grb2-SH3(N) binding to whole dynamin-1 using SPR (KD ~ 25 nM) (34), although these authors did not comment on other sites. The N-terminal fragment of dynamin-2 that lacks a class I or II consensus failed to bind Grb2-SH3, yet two binding sites were apparent on the C-terminal fragment according to stoichiometric analysis. The nature of the low affinity site in the
dynamin-2 PRD C terminus remains uncertain. It could be $^{807}$PPFR$^{912}$ in region 2, but since the homologous $^{813}$PPFPFR$^{918}$ in dynamin-1 apparently does not bind Grb2, it is tempting to speculate that Grb2 may interact with one of the class II sites at the unique C terminus of dynamin-2 (Fig. 5). These sites have not been shown to interact with any species so far, and future studies should probe whether this region confers further specificity on the dynamin-2 PRD in terms of protein-protein interactions.

In the present study, we made significant effort to counter possible avidity effects in binding due to the tendency of GST to dimerize: (i) the concentration of PRD domains on the chip was kept low to avoid bridging of adjacent bound moieties by the GST-SH3 domains; (ii) the $K_d$ values of several SH3 domains were tested on the separated N- and C-terminal fragments of the dynamin PRD and were found to be quite comparable with those found in the whole PRD (particularly important for SH3 domains with more than one binding site); and (iii) free Grb2 SH3 exhibited similar kinetics to GST-Grb2-SH3 with dynamin-1 PRD. However, avidity effects may actually play a significant role in the in vivo function of these protein complexes. For example, dynamin is a native tetramer and can assemble into higher order oligomers (1–3). Since this probably brings the PRD domains into proximity, it could increase protein binding particularly of those partners that are themselves multimeric like amphiphysin and endophilin. An analogous situation may hold with the PH domain of dynamin. Individual dynamin-PH domains have a low affinity for PIP$_2$, but linked PH domains bind with much higher apparent affinity, possibly mimicking the situation with dynamin tetramers and oligomers in vivo (60). In view of the present data, it is likely that joint targeting of dynamin via PH domain-phosphoinositide and PRD domain-SH3 domain interactions could be highly synergistic and specific.

The existence of multiple SH3 domain binding sites on dynamin may promote the clustering of components involved in the endocytotic mechanism, possibly to mediate their own interactions. Alternatively, different SH3 proteins may bind in a time- or compartment-specific manner to dynamin and mediate different functions of the protein (1). A parallel example of this behavior can be seen in the guanine nucleotide exchange factor Sos. Interaction of Grb2-SH3 with the PRD of Sos promotes activation of Ras and the downstream MAP kinase pathway, whereas interaction with the SH3 domain of E3b1 promotes the activation of another small G-protein Rac and increases membrane ruffling (for a review, see Ref. 61). In this study, we have shown that simultaneous binding to region 1 and region 3 sequences can occur, but region 2 remains to be studied in this context. This region contains a class 2 PXXP consensus that has been previously shown to preferentially bind the SH3 domains of the signaling proteins phospholipase C$\delta$ and the p85 subunit of phosphatidylinositol 3-kinase (12). These molecules may participate in entirely distinct functions of dynamin at internal membranes or in signaling phenomena associated with endocytosis. Our demonstration of significant differences in the binding properties of dynamin-1 and -2 adds to the distinctions previously noted for these isoforms. Biochemically, the two dynamins diverge in their self-assembly and regulation of GTPase activity (1–3), and they are differentially distributed in various cell types (1) as well as having distinct properties in regulating endocytosis (5, 6, 41). It is likely that discrete interaction of the PRD with downstream partners is responsible for many of these functional differences.
Kinetics of SH3 Domain Association with the PRD of Dynamins

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