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SH3 Domains from a Subset of BAR Proteins Define a Ubl-Binding Domain and Implicate Parkin in Synaptic Ubiquitination

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SUMMARY

Mutations in the *parkin* gene are responsible for a common inherited form of Parkinson's disease (PD). Parkin is a RING-type E3 ubiquitin ligase with an N-terminal ubiquitin-like domain (Ubl). We report here that the parkin Ubl binds SH3 domains from endocytic BAR proteins such as endophilin-A with an affinity comparable to proline-rich domains (PRDs) from well-established SH3 partners. The NMR structure of the Ubl-SH3 complex identifies the PaRK extension, a unique C-terminal motif in the parkin Ubl required for SH3 binding and for parkin-mediated ubiquitination of endophilin-A in vitro. In nerve terminals, conditions that promote phosphorylation enhance the interaction between parkin and endophilin-A and increase the levels of ubiquitinated proteins within PRD-associated synaptic protein complexes in wild-type but not *parkin* knockout brain. The findings identify a pathway for the recruitment of synaptic substrates to parkin with the potential to explain the defects in synaptic transmission observed in recessive forms of PD.

INTRODUCTION

Parkinson's disease (PD) is characterized by the degeneration of dopamine neurons in the substantia nigra and consequent neurological impairments (Lang and Lozano, 1998). Mutations in the *parkin* gene are a frequent cause of autosomal recessive juvenile Parkinson's disease (AR-JPD), an early-onset form of the disease (Kitada et al., 1998; Lücking et al., 2000). The mammalian *parkin* gene encodes a 52 kDa protein that harbors a conserved N-terminal ubiquitin-like domain (Ubl) and four zinc-binding domains that include the RING0 domain and two RING fingers that flank an In-Between-RING (IBR) domain (Hristova et al., 2009). Parkin functions as an E3 ubiquitin (Ub) ligase by recruiting E2 Ub-conjugating enzymes to its RING domains (Shimura et al., 2000; Zhang et al., 2000). A number of parkin ubiquitination substrates involved in a wide range of cellular processes have been identified (Hampe et al., 2006; Huynh et al., 2003, 2007; Ko et al., 2005). However, the precise role of individual substrates in the pathogenesis of *parkin*-linked PD and the cellular function of parkin-mediated ubiquitination remains unclear. PD-linked point mutations are found throughout the parkin protein but are most common in the RING domains (C289G, R334C, C431F, etc.) and the Ubl (R33Q, R42P, and V56E), emphasizing the critical role played by these domains (Hedrich et al., 2004).

Despite the intense interest in understanding parkin function, relatively little is known about the role of its domains at the structural level. The solution structure of the IBR suggests it assists in the recruitment of substrates or E2s to the RING domains and can bring the two RING domains in close proximity (Beasley et al., 2007). Both NMR and crystal structures of parkin Ubl show a remarkable structural similarity to Ub (Sakata et al., 2003; Tomoo et al., 2008), suggesting it has the potential to interact with Ub-binding domains (UBDs), a diverse group of modules involved in transducing the cellular effects of ubiquitination (Hicke et al., 2005). Indeed, the parkin Ubl interacts with the Ub-interacting motifs (UIMs) of Eps15, an adaptor protein involved in epidermal growth factor receptor endocytosis and signaling (Fallon et al., 2006), supporting the idea that the Ubl could serve as a key interaction module involved in targeting parkin to relevant substrates.

We report here that the parkin Ubl binds SH3 domains within a subset of proteins containing a lipid-binding BAR domain including endophilin-A, an endocytic protein with a N-terminal N-BAR domain that dimerizes and stimulates membrane curvature by virtue of its geometry and lipid-inserting elements (Gallop et al., 2006; Masuda et al., 2006). Endophilin-A contains a C-terminal SH3 domain that binds proline-rich domain (PRD)-containing proteins such as synaptojanin, dynamin, and ataxin-2 (de Heuvel et al., 1997; Nonis et al., 2008; Ringstad et al., 1997). Although SH3 domains have been extensively characterized as peptide-binding domains (Kaneko et al., 2008), recently, the SH3 domains from the endocytic proteins Sla1/Cin85 were found to also bind Ub (Stamenova et al., 2007). However, neither

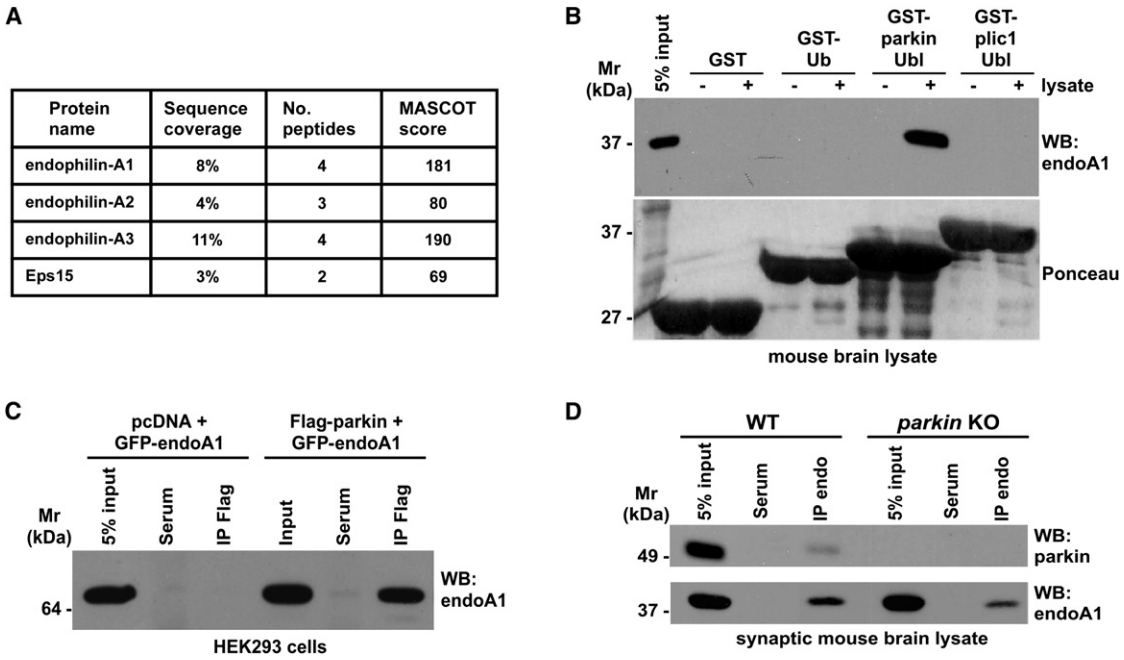


Figure 1. Parkin Interacts with Endophilin-A

(A) Mass spectrometry identification of parkin Ubl ligands. GST-parkin-Ubl (1–108) and GST bound to glutathione-Sepharose resin were incubated with mouse brain lysate, washed, and resolved on SDS-PAGE. Ubl-specific bands were excised, digested with trypsin, and identified using mass spectrometry and the program MASCOT.

(B) Brain endophilin-A1 interacts specifically with the parkin Ubl. The indicated GST fusion proteins were immobilized on glutathione-Sepharose beads, incubated with mouse brain lysate, washed, eluted with loading buffer, loaded on SDS-PAGE, and revealed by immunoblotting with an endophilin-A1 antibody (above) and by Ponceau staining (below).

(C) Parkin and endophilin-A1 interact in cells. HEK293 cells were transfected with GFP-endophilin-A1 and either pcDNA or Flag-parkin. The cell lysates were immunoprecipitated with anti-Flag or mouse serum, and the bound products were resolved on SDS-PAGE and probed with an endophilin-A1 antibody.

(D) Endogenous parkin and endophilin-A1 interact in brain. Mouse brain synaptic lysates were prepared from wild-type and *parkin* KO mice and immunoprecipitated with preimmune serum or an endophilin-A1 antibody. The bound products were resolved on SDS-PAGE and probed with parkin and endophilin-A1 antibodies.

the cellular function of the SH3 interaction with Ub nor its capacity to bind Ubl domains in addition to Ub is known. Here, we demonstrate that the endophilin-A1 SH3 domain binds the parkin Ubl domain with an affinity comparable to the PRD of synaptojanin. The structural basis for the highly selective Ubl-SH3 interaction lies in a basic C-terminal extension unique to the parkin Ubl. Finally, we show that conditions that promote phosphorylation drive the interaction between endogenous parkin and endophilin-A in synaptosomes, which in turn leads to an increase in the levels of ubiquitinated synaptic proteins in WT but not in parkin knockout (KO) mice. Together, our findings define the SH3 domain as a parkin Ubl-binding partner and implicate BAR-SH3 proteins in parkin-mediated synaptic ubiquitination.

RESULTS

Parkin Interacts with Endophilin-A

In an effort to identify parkin Ubl-binding proteins, we used a GST-Ubl fusion protein to carry out affinity chromatography in mouse brain lysates. Specific proteins that bound to GST-Ubl but not GST or GST-Ub were identified using mass spectrometry. In addition to the previously established parkin Ubl ligand Eps15 (Fallon et al., 2006), we identified endophilin-A1, -A2, and -A3 (Figures 1A and S1). The interaction with endophilin-A1 was confirmed by immunoblotting (Figure 1B). No binding was detected with Ub or the Ubl of Plic1 (another protein with an N-terminal Ubl domain), indicating that the interaction with parkin is specific. Parkin was immunoprecipitated with endophilin-A1 in transfected HEK293 cells (Figure 1C), demonstrating that the full-length proteins interact in cells. Moreover, coimmunoprecipitation from synaptic brain lysates indicates that endogenous parkin and endophilin-A1 interact in vivo (Figure 1D).

SH3 Domains from a Subset of BAR Proteins Define a Parkin Ubl-Binding Domain

The association of the parkin Ubl with endophilin-A isoforms was surprising, as the latter do not contain a classic UBD. To determine the domain that mediates the interaction with the parkin Ubl, endophilin-A1 deletions were used to pull down HA-tagged full-length or ΔUbl-parkin expressed in HEK293 cells (Figure 2A). The results showed that the endophilin SH3 domain is both necessary and sufficient for the interaction with parkin.

The extent of parkin Ubl selectivity toward different SH3 domains was investigated by testing a panel of recombinant

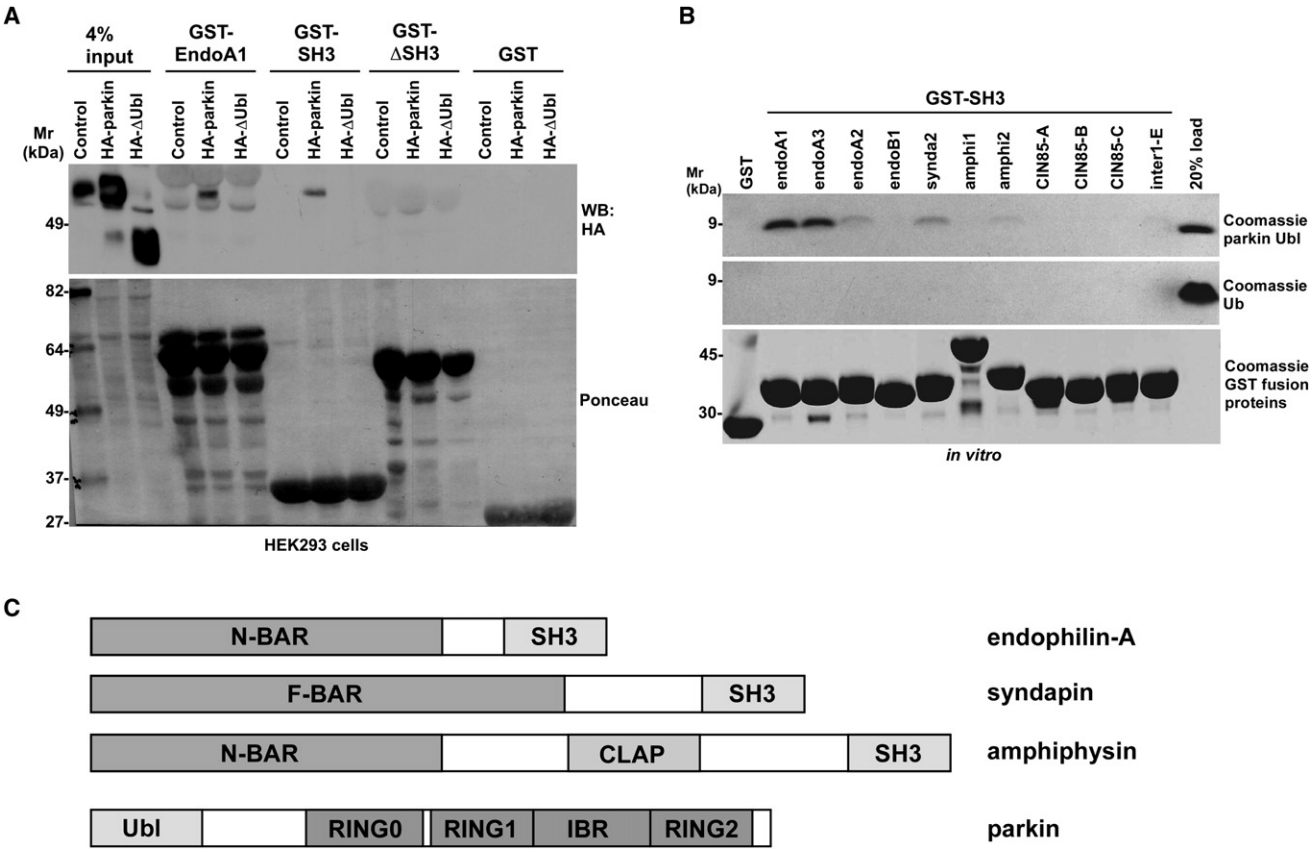


Figure 2. The Parkin Ubl Interacts with a Subset of SH3 Domains Found in BAR-Containing Proteins

(A) The SH3 and Ubl domains mediate the interaction between endophilin-A1 and parkin. HEK293 cells were transfected with HA-parkin, HA-parkin-ΔUbl, or a control vector (pcDNA). The cell lysates were incubated with the indicated GST-endophilin-A1 constructs, and the bound products were immunoblotted with an anti-HA antibody (above) or stained with Ponceau (below). The band in lane 1 is a spillover from lane 2 (see Figure 5C for similar control).

(B) The parkin Ubl interacts with a subset of SH3 domains from BAR-domain proteins. Glutathione-Sepharose-immobilized GST or GST-SH3 proteins (lower panel) were incubated with the parkin Ubl (1–76, top) or Ub (middle). The washed products were eluted with loading buffer, resolved by SDS-PAGE, and stained with Coomassie blue.

(C) Domain organization of BAR-SH3 proteins (top) that interact with the parkin Ubl domain (bottom). CLAP is a clathrin/AP-2-interacting domain.

GST-SH3 fusion proteins for their capacity to bind the parkin Ubl or Ub (Figures 2B and S2). The three isoforms of endophilin-A all bound the parkin Ubl, although the endophilin-A2 interaction was weaker. The SH3 domains from syndapin-2/PACSIN-2 and amphiphysin-2 also bound the parkin Ubl in vitro as well as in HEK293 cells (Figure S2C). A striking common link among SH3 proteins that bind the parkin Ubl is that they all contain a BAR domain (Figure 2C) and are involved in vesicle trafficking. In contrast, the parkin Ubl did not bind the SH3 domain from endophilin-B1, an endophilin-A homolog involved in mitochondrial membrane dynamics (Karbowski et al., 2004), nor did it bind the SH3 domains from Grb2, STAM, intersectin-1, and CIN85, which do not have a BAR domain. Ub has been reported to bind the SH3 domains of yeast Sla1 and its vertebrate ortholog CIN85, as well as amphiphysin-1 and -2 (Stamenova et al., 2007). However, no SH3 domains bound to Ub under the stringent conditions used to test Ubl binding. Thus, the SH3 domains from a subset of BAR-domain proteins involved in vesicle trafficking define a parkin-specific Ubl-binding domain.

The Parkin Ubl Domain Binds to the PRD-Binding Surface of the Endophilin-A1 SH3 Domain

SH3 domains are known to bind peptide ligands through the surface formed by invariant proline and tryptophan side chains (Kaneko et al., 2008). Structural biology techniques were therefore employed to assess binding of the parkin Ubl and different PRDs to the endophilin-A1 SH3 domain. The crystal structure of the free SH3 domain was determined at 1.4 Å resolution (Figure S3 and Table S1), and its NMR ^{15}N-^{1}H HSQC spectrum was assigned. Addition of parkin Ubl to ^{15}N-labeled endophilin-A1 SH3 induced large and specific NMR chemical shift perturbations on the peptide-binding surface of the SH3 domain (Figures 3A and S4). The perturbations were in the fast-intermediate exchange regime and enabled us to calculate a dissociation constant of 13 ± 4 μM (Figures 3B and S5). This affinity is significantly stronger than that of Ub toward the SH3 domains of Sla1 and CIN85 SH3-C, which have K_D (dissociation constant) values of 40 and 171 μM, respectively (Bezsonova et al., 2008; Stamenova et al., 2007). Interestingly, a similar titration experiment

carried out with Ub induced negligible chemical shift perturbations, which confirmed the absence of binding observed in pull-down assays (Figure S6).

A PRD peptide derived from the synaptojanin E2′ binding site (PPARPAPPQRPPPPS$_{1113–1127}$) induced chemical shift perturbations on the same surface of endophilin-A1 SH3 that binds the Ubl domain (Figure 3A) and bound with a similar affinity (Figures 3B and S5), suggesting they compete for binding. This was directly demonstrated by addition of synaptojanin E2′ PRD to ^{15}N-labeled parkin Ubl bound to endophilin-A1 SH3 domain (Figure S5F). A large molar excess of PRD peptide was needed to completely displace the Ubl domain, since their affinities for the SH3 domain are similar. A dynamin PRD peptide (RRAPAVPPARP$_{783–793}$) induced chemical shift perturbations similar to the E2′ PRD, but the interaction had a much weaker affinity (Figures 3B and S5). Thus, the SH3 domain binds the Ubl and PRDs via a common site, with similar affinities.

The C Terminus of the Parkin Ubl Confers SH3-Binding Selectivity

The selectivity of the parkin Ubl toward certain SH3 domains prompted us to determine the structure of the Ubl-SH3 complex. A model of the rat parkin Ubl was derived from the crystal structure of murine parkin Ubl (Tomoo et al., 2008) and docked on the SH3 crystal structure using 24 NMR intermolecular NOEs (Figure S7) and 119 ^{15}N-^{1}H residual dipolar couplings (RDCs) (Figure S8). Chemical shift perturbations (Figure 3C) and heteronuclear NOE measurements (Figures 3D and S9) suggested that the flexible C-terminal tail of the parkin Ubl (72–76) was involved in binding and became structured upon complex formation. The conformation of the C-terminal tail was therefore calculated de novo and incorporated in the structure calculation, which converged to yield an ensemble of structures with a 0.65 Å backbone root-mean-square deviation (rmsd) from the lowest energy structure (Table 1 and Figure S10A). The hydrophobic patch formed by Ile44, Gly47, His68, and Val70 of the Ubl interacts with the peptide-binding surface of the SH3 domain (Figure 3E). The alignment of the two domains is reminiscent of that observed in the Sla1- and Cin85-SH3-Ub complexes (Bezsonova et al., 2008; He et al., 2007), but the SH3:Ubl complex shows a considerable shift in the position of the two domains (Figure S10B). The side chain of Ile44 is located between the side chains of Tyr299 and Tyr343 in the SH3 domain, which moves the C terminus of the Ubl closer to the center of the peptide-binding surface of the SH3 domain (Figure 3F). The C-terminal tail of the Ubl adopts an extended β strand conformation that mediates interactions with the SH3 domain that are similar to PRD:SH3 interactions. Notably, the Ubl residue Pro73 packs between the invariant SH3 residues Pro340 and Trp327, in a manner similar to the second proline in a PXXP motif bound to the related Grb2 SH3 domain (Wittekind et al., 1997). In this specific tail conformation, the side chain of Arg75 is well positioned to interact with the acidic loop formed by Asp324 and Glu325 in the SH3 domain (Figure 3F).

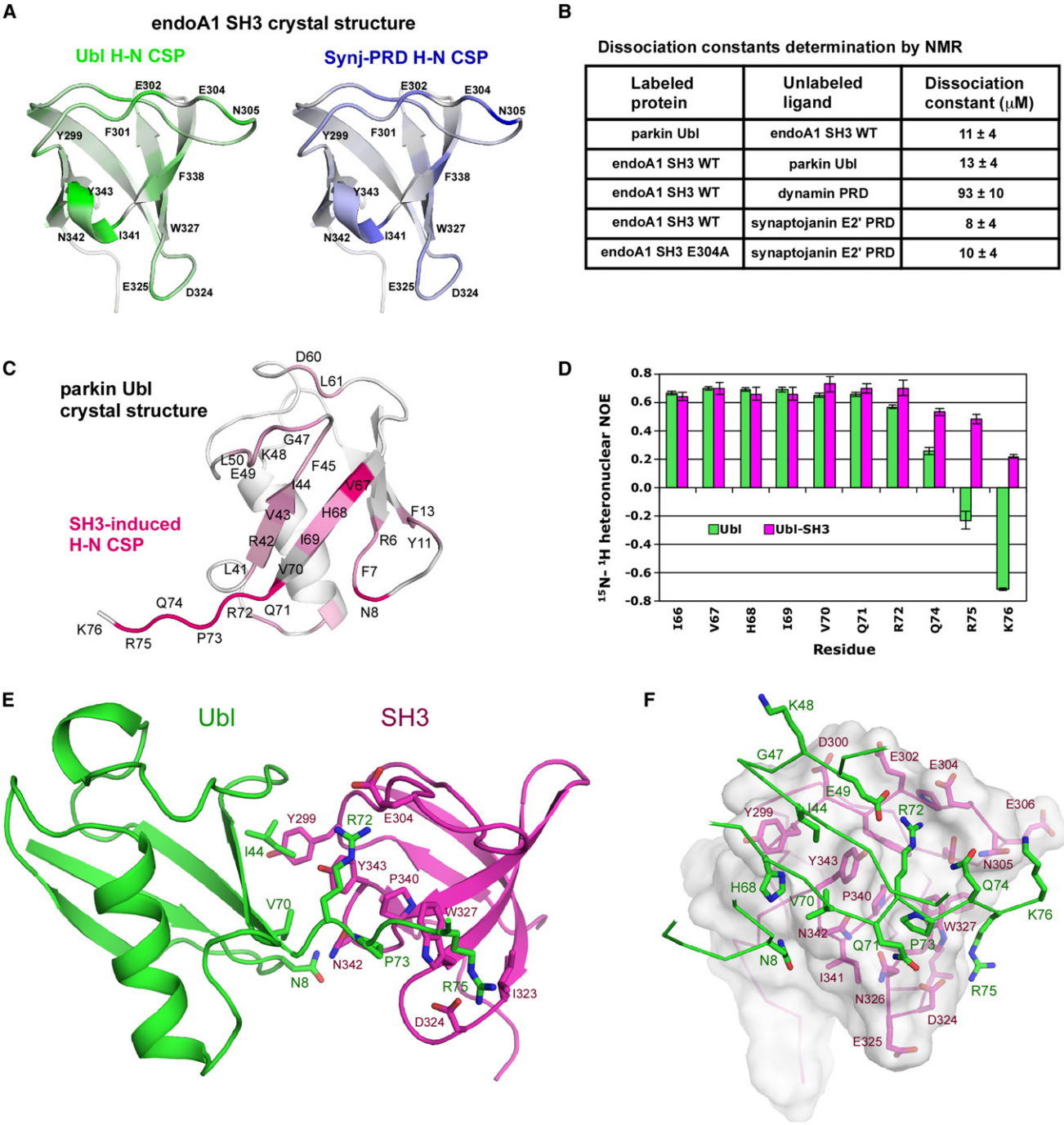
The C-terminal stretch (73–76) of the Ubl is unique to mammalian parkin and forms the consensus sequence PxRK, which we refer to as the PaRK extension (Figure 4A). The role of the PaRK extension in binding SH3 domains was tested using several constructs, including a truncation mutant lacking the two C-terminal PaRK extension residues (1–74), a chimera in which residues 73–76 (PQRK) were replaced with the corresponding residues in Ub (LRGG), and two single-site mutants of parkin 1–76 (R75G and K76G). The C-terminal truncation, the chimera, and the R75G mutation completely abolished binding to GST-endophilin-A1 SH3 (Figure 4B), whereas the K76G mutant retained some affinity for the SH3 domain. The PaRK extension is essential and specific for SH3 binding, as the R75G mutant was still able to bind the tandem UIMs from Eps15 (Fallon et al., 2006) (Figure 4C).

Additional parkin Ubl mutants were produced to test the contribution of other residues to SH3 binding. Asn8 is located in the β1-β2 loop that contacts Asn342 in the SH3 domain (Figure 3F); mutation to alanine or leucine (the residue in Ub) abolished binding to endogenous endophilin-A1 in brain lysate (Figure 4D). The PD-linked mutant R42P, which was shown to unfold the Ubl (Safadi and Shaw, 2007), was also unable to bind endophilin-A1. The I44A and K48A mutations also disrupted the interaction, in agreement with the contacts observed in the structure of the complex (Figure 3F). Ile44 and Lys48 are conserved in Ub and therefore do not constitute selectivity determinants, as opposed to Asn8 and the PaRK extension, which are unique to parkin (Figure 4A).

A Unique Glutamate Residue within Endophilin-A SH3 Domains Confers Parkin Ubl-Binding Specificity

Alignment of SH3 domain sequences (Figure 5A) and the NMR solution structure (Figures 3E and 3F) led us to test specificity determinants in the endophilin-A SH3 domain using single-site mutagenesis and in vitro pull-down assays. Three glutamic acid residues (Glu302, Glu304, and Glu325) conserved in Ubl-binding SH3 domains (Figure 5A) and located in regions that display large chemical shift perturbations (Figure 3A) were mutated to alanine. Strikingly, only the E304A mutation abrogated the interaction (Figure 5B). The three endophilin-A isoforms are the only vertebrate SH3 proteins to have an acidic residue at this position (Figure 5A). In the solution structure, the side chain of Glu304 is in proximity to that of the conserved Ubl residue Arg72 and thus could form a salt bridge with the latter. Syndapin-2 and amphiphysin-2 have polar residues and show weaker binding, whereas endophilin-B1 and amphiphysin-1 have alanine and show no binding. Despite the shared Ubl- and PRD-binding surface on the SH3 (Figure 3A), the E304A mutation, which abolishes Ubl binding (Figure 5B), did not significantly reduce the affinity for the synaptojanin E2′ PRD (Figure 3B). Glu304 is therefore a crucial selectivity determinant for Ubl binding.

Other residues were also found to contribute to the interaction. The mutation D324A, which disrupts an electrostatic interaction with the parkin-specific Arg75, reduced the interaction. The mutations N342A and Y343F reduced binding, likely through loss of a polar interaction with the Ubl. Finally, we also tested the binding of full-length and ΔUbl parkin expressed in HEK293 cells to endophilin-A1 SH3 mutants that showed reduced affinity toward the Ubl, and an identical pattern of binding was observed (Figure 5C). Together, our findings demonstrate that the endophilin-A1 residues Glu304, Asp324, Asn342, and Tyr343 constitute critical SH3 selectivity



Labeled protein	Unlabeled ligand	Dissociation constant (μM)
parkin Ubl	endoA1 SH3 WT	11 ± 4
endoA1 SH3 WT	parkin Ubl	13 ± 4
endoA1 SH3 WT	dynamin PRD	93 ± 10
endoA1 SH3 WT	synaptojanin E2' PRD	8 ± 4
endoA1 SH3 E304A	synaptojanin E2' PRD	10 ± 4

Figure 3. The Parkin Ubl Domain C-Terminal PaRK Extension Interacts with the PRD-Binding Surface of the Endophilin-A1 SH3 Domain

(A) ^{15}N-^{1}H NMR chemical shift perturbations mapping on the endophilin-A1 SH3 domain in the presence of parkin Ubl (left) and synaptojanin E2′ PRD (right). The intensity of the color (green or blue) is proportional to the magnitude of the chemical shift perturbations (figure created using ProtSkin and Pymol).

(B) K_D of the binding determined from a fit of NMR chemical shift perturbations in the ^{15}N-labeled protein upon addition of the indicated ligand. See Figure S5 for detailed curve fitting.

(C) Backbone amide ^{15}N-^{1}H NMR chemical shift perturbations of ^{15}N-labeled parkin Ubl upon addition of endophilin-A1-SH3 mapped on the rat parkin Ubl model. The intensity of the color (magenta) is proportional to the magnitude of the chemical shift perturbations.

(D) ^{15}N-^{1}H heteronuclear NOE values for the unbound (green) and bound (magenta) Ubl domain are shown for residues 66–76 (Figure S9 for the entire data set). Error bars correspond to the standard error on peak intensity measurement, estimated from background noise levels.

determinants for Ubl binding and that specific residues within endophilin-A1 (Glu304) and parkin (PaRK extension) selectively coordinate SH3:Ubl binding without affecting canonical PRD:SH3 or UBD:Ubl interactions.

Parkin Ubiquitinates Endophilin-A1 In Vitro

The tight and specific association between the Ubl and SH3 suggests that parkin could mediate endophilin-A ubiquitination. In vitro ubiquitination assays were performed with recombinant Myc-tagged Ub, E1 and E2 (UbcH7) enzymes, His_6-tagged endophilin-A1, and GST-tagged parkin in the presence of ATP. Following incubation of all components, we observed a Myc-reactive band in the pull-downs with nickel beads, migrating at 49 kDa, the predicted size of monoubiquitinated His_6-endophilin-A (Figure 6A, lane 4, top panel). The reaction was specific, as no ubiquitination was observed in the absence of parkin or UbcH7 (Figure 6A), and significantly less ubiquitination was observed with other E2s (Figure S11C). The amount of ubiquitinated E304A-, E325A-, and N342A mutant endophilin correlated closely with their ability to bind the parkin Ubl (Figure 6A, top panel). Moreover, the parkin N8A and R75G-K76G mutants, which impair the Ubl:SH3 interaction, showed reduced ubiquitination of endophilin-A (Figure 6B, top panel). The defect is not due to a decrease in parkin E3-Ub ligase activity, since these mutants still mediate parkin self-ubiquitination (Figure 6B, lower panel). The PD-linked inactive C341F mutant (Fallon et al., 2006) failed to ubiquitinate both endophilin-A1 and itself (Figure 6B, lane 10). Together, these results demonstrate that parkin-dependent ubiquitination of endophilin-A proceeds via the formation of an SH3:Ubl complex. However, comparison of the levels of unmodified and ubiquitinated endophilin-A show that only a small fraction was ubiquitinated by parkin in vitro (Figure 6C), and no ubiquitination of endogenous endophilin-A1 was detected in brain (Figure 6D). Moreover, at steady state, only a small fraction of endogenous endophilin-A1 associates with parkin in brain (Figure 1D), and no differences in endophilin-A1 levels or subcellular distribution were observed in synaptic brain fraction from parkin wild-type and KO mice (Figures 6E and S12). Together, these results demonstrate that while parkin ubiquitinates endophilin-A in vitro, additional regulatory mechanisms are likely to be at play in vivo.

Phosphorylation Regulates the Interaction between Parkin and Endophilin-A in Nerve Terminals

Calcium-dependent dephosphorylation of dephosphins, which include the PRD-containing endophilin-A ligands synaptojanin and dynamin, is an important regulatory process in neurons and affects the assembly and activity of SH3-protein-containing complexes at the synapse (Cousin et al., 2001; McPherson et al., 1994; Slepnev et al., 1998). We therefore investigated the effects of phosphorylation on the subcellular localization of endogenous parkin and endophilin-A1 in brain. At steady state, both parkin and endophilin-A were enriched in the cytosolic fraction (LS2) purified from mouse brain synaptosomes (Figure 7A). Remarkably, incubation of synaptosomes with phosphatase inhibitors and ATP, in conditions that promote the phosphorylation of endogenous proteins, induced a partial relocalization of both parkin and endophilin-A from the cytosol (LS2) to plasma membrane- (LP1) and synaptic vesicle-enriched (LP2) fractions (Figure S13A). The relocalization was specific, as phosphorylation did not affect the localization of the NR1 subunit of the N-methyl D-aspartate (NMDA) receptor, synaptophysin or actin (Figure 7A).

Given these findings and the weak steady-state interaction between parkin and endophilin-A1 (Figure 1D), we hypothesized that synaptic phosphorylation might also affect the capacity of parkin to interact with endophilin-A1. Using GST-endophilin-A1 constructs, we carried out pull-downs from synaptic brain lysates (Figure 7B). Preincubation of lysates with phosphatase inhibitors and ATP as above markedly enhanced parkin binding to endophilin-A1 (Figure 7B, top panel) as well as to its SH3 domain (Figure S13B). Consistent with a role for phosphorylation

Table 1. Structural Statistics for the Parkin Ubl-Endophilin-A1 SH3 Complex

Experimental restraints	
Residual dipolar couplings	119
TALOS ϕ/ψ angles	143
Intramolecular distance restraints (NOEs)	40
Intermolecular distance restraints (NOEs)	24
Ambiguous interaction restraints (AIRs)	3
Structural statistics for the 20 lowest-energy conformers	
Backbone rmsd from average (Å)	0.44 ± 0.22
Backbone rmsd from E_{min} (Å)	0.65 ± 0.11
Buried surface area (Å^2)	1141 ± 92
E_{vdw} (kcal/mol)	−47 ± 5
E_{elec} (kcal/mol)	−183 ± 43
RDC R-factor	0.15 ± 0.02
RDC rmsd (Hz)	1.74 ± 0.05
Distance restraints rmsd (Å)	0.02 ± 0.01
Dihedral angles rmsd (°)	0.31 ± 0.18
Rmsd from ideal values	
Bond length (Å)	0.0032 ± 0.0002
Bond angle (°)	0.44 ± 0.02
Improper angle (°)	0.45 ± 0.02
Ramachandran ϕ/ψ angles statistics (%)	
Core region	87.6
Additionally allowed region	12.3
Generously allowed region	0.1
Disallowed region	0

(E) Cartoon representation of the lowest-energy model of the NMR structure ensemble for the complex of parkin Ubl (green) with endophilin-A1 SH3 (magenta). Residues at the interface are labeled, and their side chains are displayed as sticks.
(F) Close-up view of the complex interface for endophilin-A1 SH3 (magenta sticks) bound to the parkin Ubl (green). The molecular surface of the SH3 domain is shown in transparent white.

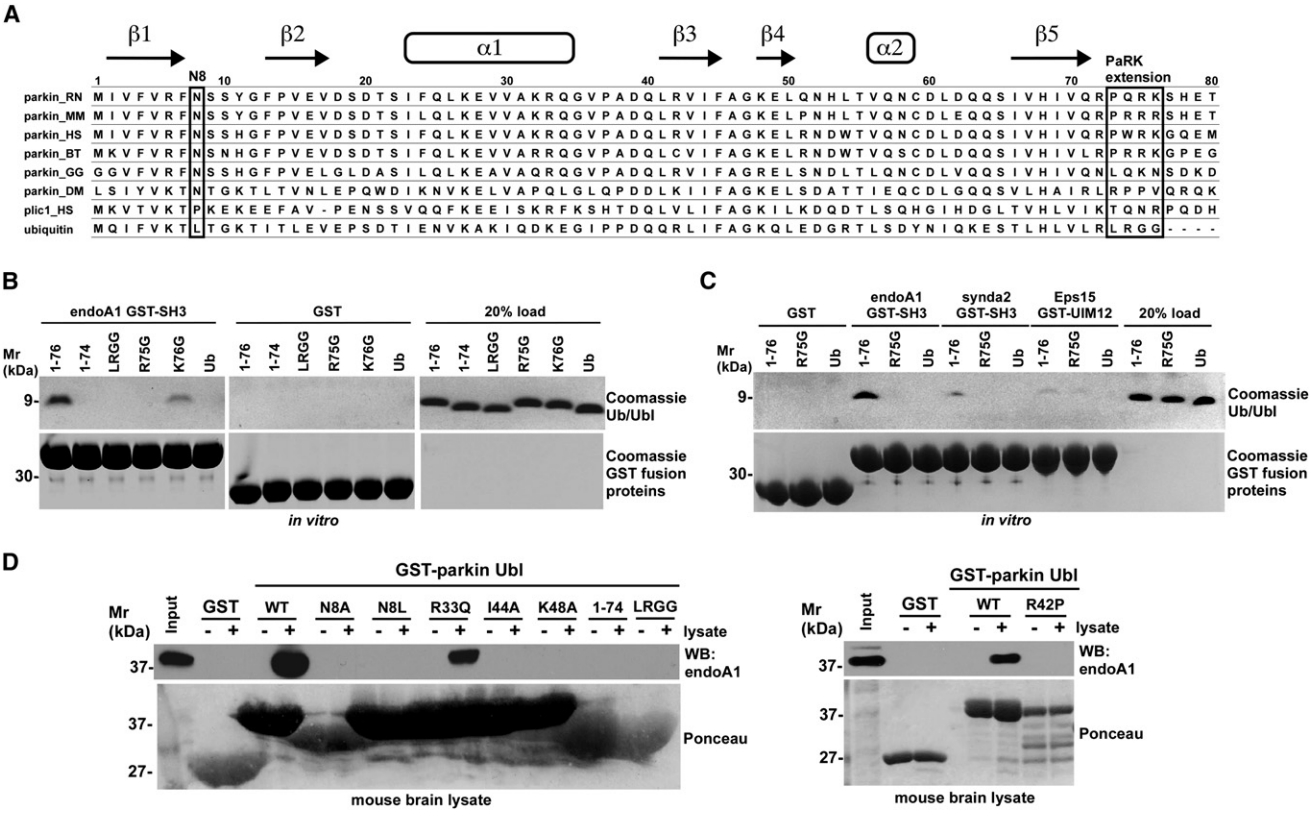


Figure 4. The Parkin Ubl C-Terminal PaRK Extension Is Required for Binding SH3 Domains

(A) Sequence alignment of the parkin Ubl domains from rat (RN, *Rattus norvegicus*), mouse (MM, *Mus musculus*), human (HS, *Homo sapiens*), chimp (PT, *Pan troglodytes*), bovine (BT, *Bos taurus*), chicken (GG, *Gallus gallus*), and fly (DM, *Drosophila melanogaster*), as well as Ub and the human plic1 Ubl. The secondary structure elements of parkin Ubl are shown above the alignment. Boxed residues are unique to mammalian parkin and required for parkin to interact with the endophilin-A1 SH3.

(B) The PaRK extension of the parkin Ubl is required for binding the endophilin-A1 SH3 domain. GST-endophilin-A1 SH3 (left panel) or GST (middle panel) was immobilized on glutathione-Sepharose beads and incubated with the parkin Ubl (1–76) wild-type or mutants ($PQRK_{73–76}$ to $LRGG_{73–76}$, R75G, and K76G), a deletion (1–74), or Ub (Ub). The procedure is the same as in Figure 3B.

(C) Arg75 of parkin is required for binding SH3 domains but not the Eps15 UIMs. The procedure is the same as in Figure 4B.

(D) Identification of parkin Ubl residues mediating interaction with endophilin-A1. GST-parkin Ubl mutants were incubated with mouse brain lysate, and retained proteins were detected by an endophilin-A1 antibody.

per se, which requires ATP hydrolysis, the enhancement was blocked by the nonhydrolyzable ATP analog AMP-PNP (Figure 7C). However, the effect did not appear to be mediated by kinases previously implicated in parkin phosphorylation and function (Rubio de la Torre et al., 2009; Yamamoto et al., 2005), as inhibitors of Cdk5, caseine kinase I, and protein kinase C all failed to block the enhancement (Figure S13C). Preincubation with phosphatase inhibitors and ATP did not affect the interaction between endophilin-A1 and its main PRD-containing ligand synaptojanin (Figure 7B, middle panel), suggesting that phosphorylation stimulates parkin-endophilin-A binding directly rather than by decreasing competition of PRD proteins for SH3 binding.

To test whether phosphorylation enhanced the interaction between endogenous endophilin-A1 and parkin in synaptosomes, we used a GST fusion protein encoding a PRD peptide from synaptojanin E2′ site (1111–1129) to purify endophilin-containing complexes from mouse brain synaptosomes. This PRD peptide has been shown previously to efficiently pull down endophilin-A from brain (Gad et al., 2000). Under basal conditions, parkin could not be detected in the PRD-purified complexes despite the efficient recruitment of endophilin-A1 (Figure 7D, lane 3). Strikingly, preincubation of synaptosomes with phosphatase inhibitors and ATP drove parkin into endophilin-A-containing complexes (Figure 7D, lane 4). Experiments with purified proteins confirmed that parkin is recruited to the PRD indirectly by endophilin-A1 and does not bind the PRD directly (Figure 7E). As the parkin Ubl and synaptojanin PRD bind the same site on endophilin-A1's single SH3 domain (Figure 3A), the mechanism is likely to involve endophilin-A1 dimerization via its N-BAR domain. Endophilin-A1 dimers can simultaneously accommodate PRD and Ubl binding via their two SH3 domains. Interestingly, the endophilin-A1 N-BAR domain binds membranes as dimers, but is predominantly monomeric in the cytosol (Gallop et al., 2006). Thus, the relocalization of endophilin-A1 from the cytosol to synaptic membrane compartments that we

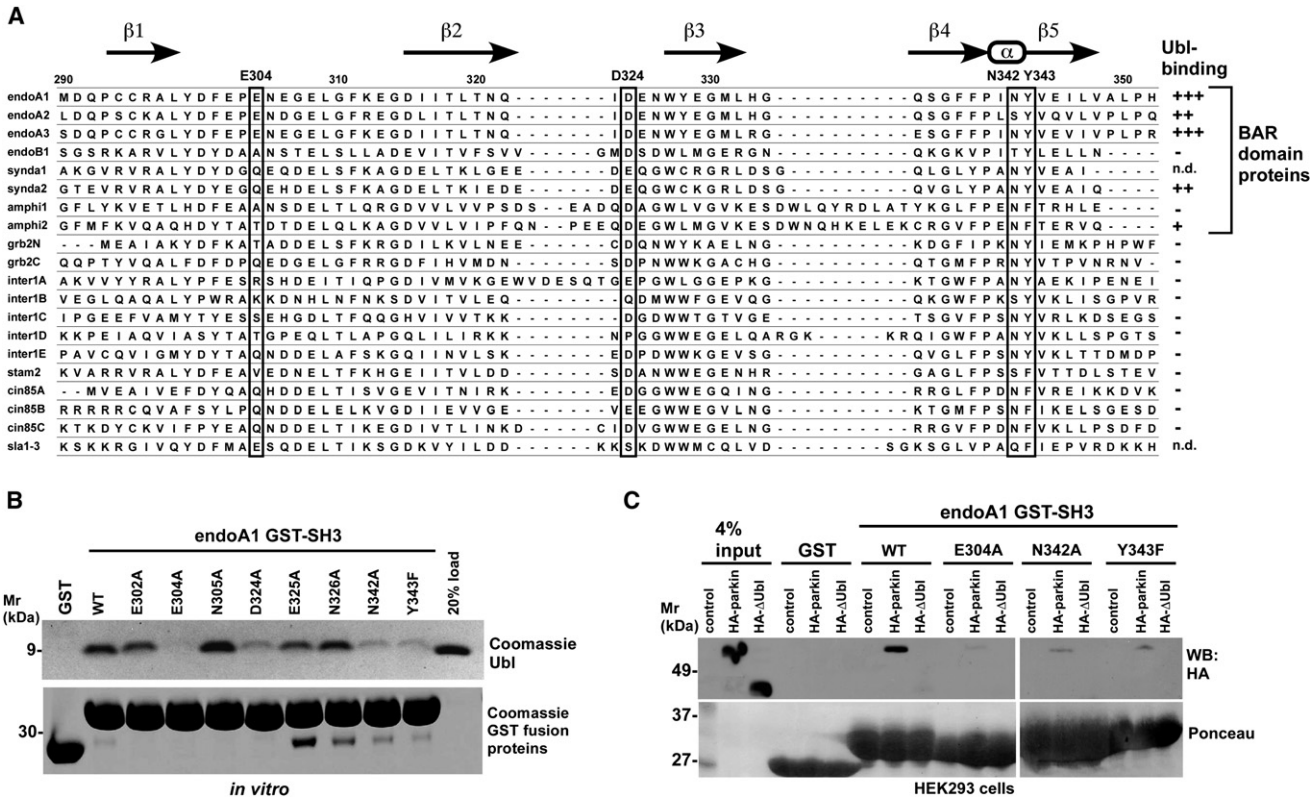


Figure 5. Selectivity for the Parkin Ubl Is Determined by a Set of Polar Residues on the PRD-Binding Surface of the SH3 Domain

(A) Sequence alignment of SH3 domains from proteins involved in endocytosis. All sequences are from *Rattus norvegicus* except the budding yeast sla1 SH3-3 domain. Residues critical for the interaction with the parkin Ubl are boxed. Secondary structure elements and characteristics loops are labeled above the alignment.

(B) Identification of specificity determinants in the endophilin-A1 SH3 domain required for parkin Ubl-binding. GST or endophilin-A1 GST-SH3 mutants were immobilized on glutathione-Sepharose beads and incubated with the parkin Ubl (1–76). The procedure is the same as in Figure 3B.

(C) HEK293 cells were transfected with HA-parkin, HA-parkin-ΔUbl, or a control vector (pcDNA). The cell lysates were incubated with GST-endophilin-A1 SH3 mutants immobilized on glutathione-Sepharose beads. The bound products were immunoblotted with anti-HA antibodies.

observe in response to phosphorylation (Figure 7A) may promote endophilin-A1 dimerization, which would allow endophilin-A-bound parkin to be indirectly recruited by GST-PRD (Figure 7D). Taken together, these experiments suggest that phosphorylation promotes the interaction of endogenous parkin with endophilin-A in synaptic membrane fractions.

Parkin Ubiquitinates PRD-Associated Proteins in Nerve Terminals

Synaptic activity has also been shown to decrease ubiquitination in nerve terminals and correlates with dephosphorylation of synaptic proteins (Chen et al., 2003). We thus sought to test whether the converse was true, specifically that phosphorylation could increase protein ubiquitination within synaptic endophilin complexes. Immunoblotting of GST-PRD-purified endophilin-A1 complexes with an antibody against Ub revealed a marked increase in the levels of Ub-protein conjugates copurified from synaptosomes incubated with phosphatase inhibitors (Figure 7D, lower panel). Importantly, this increase was attenuated in synaptosomes prepared from *parkin* KO mice (Figure 7D, lane 8), indicating that ubiquitination of proteins within synaptic endophilin-A complexes is largely parkin dependent. A long exposure of the endophilin-A1 blot showed that these ubiquitination products are probably not ubiquitinated forms of endophilin-A1 (Figure S13D). These results are consistent with a model whereby phosphorylation targets endophilin-A to membrane fractions in nerve terminals, where it dimerizes and recruits parkin to promote the ubiquitination of synaptic proteins (Figure 7F).

DISCUSSION

SH3 Domains: A Ubl-Binding Domain

Most known SH3-binding partners contain small peptide motifs such as PRDs, but there have been several recent reports describing weak interactions with Ub (Kang et al., 2008; Stamenova et al., 2007). We show here that a Ubl domain can bind specific SH3 proteins with an affinity comparable to PRDs. Most parkin Ubl residues interacting with the endophilin-A1 SH3 domain are identical to those found in Ub. Two exceptions are Asn8 and Arg75, which are conserved in all mammalian

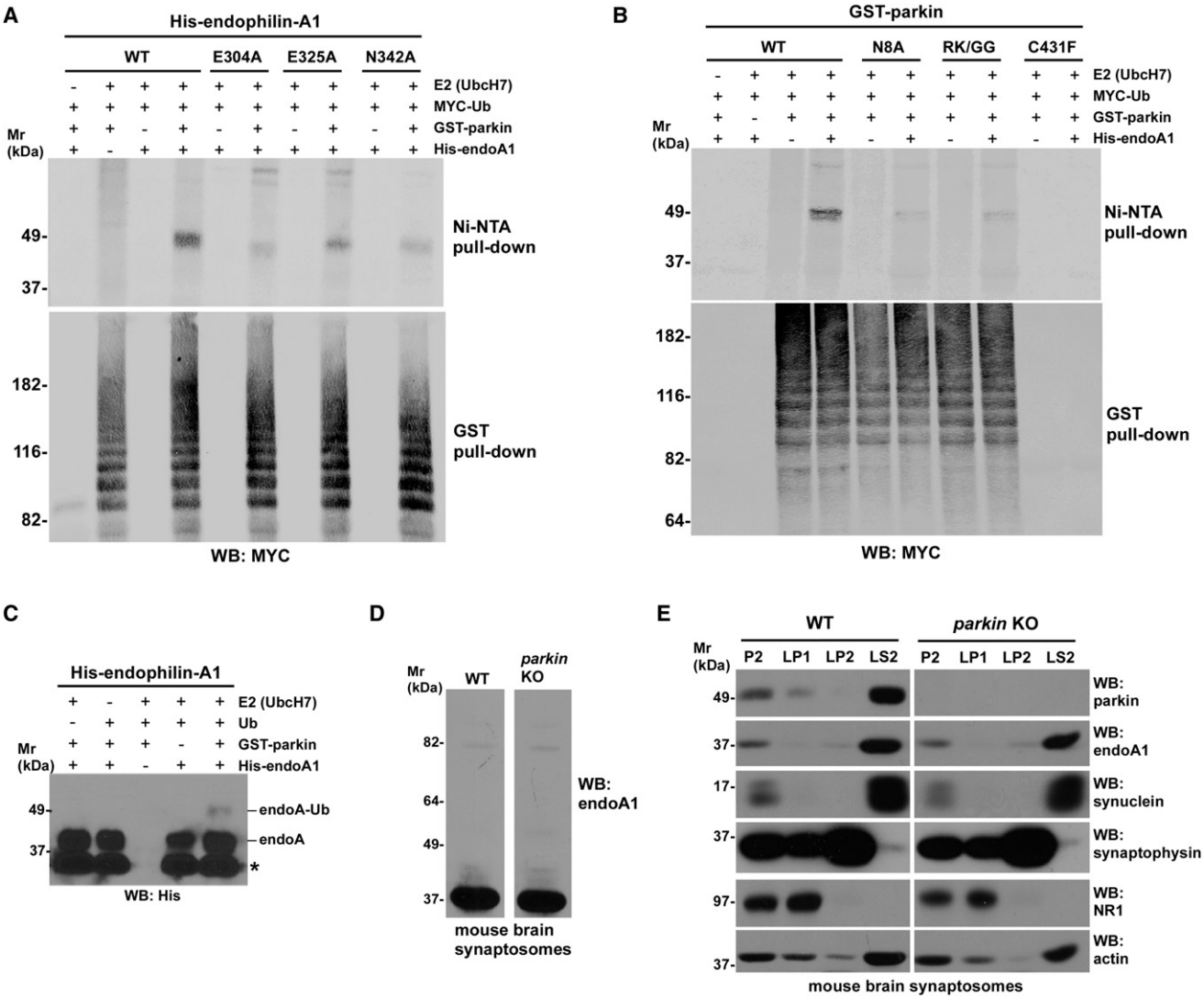


Figure 6. Parkin-Mediated Ubiquitination of Endophilin-A

(A) Endophilin-A1 ubiquitination levels correlate with the capacity of the mutant SH3 domains to bind the parkin Ubl. Ubiquitination reactions were performed in the presence of E1, ATP, $MgCl_2$, and the components labeled with (+). GST-parkin was immobilized on glutathione-Sepharose resin (bottom panel), and His_6-endophilin-A1 (supernatant of GST pull-down) was immobilized on Ni-NTA agarose (top panel). The products were resolved on SDS-PAGE and probed with an anti-Myc antibody.

(B) Endophilin-A1 ubiquitination was reduced by the N8A and RK/GG Ubl mutations that reduce SH3 binding as well as by the C341F ligase-inactive, PD-linked RING mutant (Fallon et al., 2006). Ubiquitination reactions were as above.

(C) Parkin weakly monoubiquitinates endophilin-A1 in vitro. Ubiquitination reactions were performed as described above. The products were probed with an anti-His antibody. The band indicated by an asterisk (*) represents a degradation product of endophilin-A1.

(D) Endophilin-A1 is not significantly ubiquitinated in synaptosomes. Mouse brain synaptosomes prepared from wild-type and parkin KO mice were resolved on SDS-PAGE and probed against endophilin-A1.

(E) Distribution of endophilin-A1 in subsynaptic fractions prepared from parkin WT and parkin KO mouse brain lysate. Equal amounts (50 μg) of proteins were loaded in each lane and probed against endophilin-A1, parkin, NR1 (plasma membrane marker), synaptophysin (synaptic vesicle marker), synuclein (cytosol marker), and actin (general marker).

parkin Ubls and absent in Ub and other Ubls. Both residues are essential for SH3 binding and unique specificity determinants.

Ub was shown to bind the third SH3 domain of CIN85 as well as the SH3 domains from amphiphysin-1 and amphiphysin-2 (Stamenova et al., 2007). In our binding assays, we did not detect an interaction between Ub and any of the SH3 domains (Figures 2B and S2). The high cellular levels of free Ub may compensate for the weak affinity of SH3:Ub interactions; indeed, other UBDs also bind weakly to Ub, with affinities rarely below 20 μM (Hicke et al., 2005). One of the key Ub-binding determinants in the Sla1/CIN85 SH3 domain was a phenylalanine residue located at a position equivalent to Tyr343 in endophilin-A1 (Bezsonova

et al., 2008; Stamenova et al., 2007). Mutation of this phenylalanine to tyrosine in Sla1/CIN85 SH3 abolished binding to Ub, and all Ub-interacting SH3 domains were found to have a phenylalanine at this position. Interestingly, we observed the reverse situation for parkin Ubl binding: the Y343F mutant reduces binding. The presence of Asp324, although not unique to Ubl-binding SH3 domains, is essential, as it provides an anchor point for recruiting Arg75 in the parkin Ubl C-terminal PaRK extension. Similarly, Asn342 is not unique to endophilin-A isoforms, but it does contribute to Ubl binding. Interestingly, this asparagine correlates with the ability of the two brain-enriched endophilin-A isoforms (A1 and A3) to bind the parkin Ubl better than endophilin-A2, which has a serine at this position. This observation suggests a neuronal role for the interaction between parkin and endophilin-A.

Effects of Phosphorylation on Parkin-SH3 Interactions and Synaptic Ubiquitination

In brain, relatively little parkin binds endophilin-A at steady state (Figure 1D). We find that conditions that favor protein phosphorylation in synaptosomes promote parkin and endophilin-A trafficking from the cytosol to membrane compartments and markedly enhance their association. One possible explanation is that endophilin-A is not available for parkin binding at steady state because it is constitutively bound to PRD proteins such as synaptojanin and dynamin. These PRD proteins, collectively referred to as dephosphins, are believed to trigger synaptic vesicle endocytosis upon dephosphorylation by calcineurin, a calcium-activated phosphatase (Cousin et al., 2001; Slepnev et al., 1998). Antagonistic phosphorylation of dephosphins by kinases such as Cdk5 is essential for the recycling of synaptic vesicles (Tan et al., 2003). Upon phosphorylation, dephosphins release their SH3 partners, making them available for parkin binding. Indeed, a phosphomimetic mutation in the vicinity of synaptojanin's PRD was shown to decrease its affinity for endophilin-A1 (Lee et al., 2004). Surprisingly, we found that synaptojanin binding to GST-endophilin-A1 was not affected by the addition of ATP and PPI (Figure 7B), suggesting that other mechanisms could be at play. For instance, phosphorylation could activate parkin (or inactivate a parkin inhibitor) and make the Ubl available for binding. In agreement with this model, we found that recombinant GST-Ubl can efficiently recruit endogenous brain endophilin-A1 in the absence of PPI and ATP (Figure 1B). Taken together, these observations imply that phosphorylation would release parkin or one of its interacting partners and make the Ubl available for binding. Cdk5, a candidate regulatory kinase, has been shown to phosphorylate parkin, possibly affecting its interactions, activity, and cellular localization (Avraham et al., 2007; Rubio de la Torre et al., 2009; Yamamoto et al., 2005). However, Cdk5 inhibitors did not inhibit binding to recombinant GST-endophilin-A in our assays (Figure S13C), suggesting that other kinases may be involved.

Beyond the phosphorylation dependence of the parkin-endophilin-A interaction, we observed that phosphorylation promotes the parkin-dependent ubiquitination of PRD-associated proteins in nerve terminals (Figure 7D). The wide smear of protein-Ub conjugates observed in nerve terminals appears to arise from proteins other than endophilin-A1. What are these in vivo substrates of parkin? Parkin has been shown previously to ubiquitinate synaptic vesicle-associated proteins such as CDCrel-1 (Zhang et al., 2000) and synaptotagmin (Huynh et al., 2003). Dimers of endophilin-A and other BAR-SH3 proteins may act as platforms for the ubiquitination of synaptic proteins by simultaneously recruiting parkin and PRD-containing proteins (Figure 7F). For instance, ataxin-2 has recently been shown to be a substrate for parkin (Huynh et al., 2007), and the ataxin-2 PRD binds selectively to the SH3 domains of endophilin-A1 and -A3 (Nonis et al., 2008), as is the case for parkin. The assignment of one SH3 domain to the parkin Ubl and the other to a PRD may not be stochastic and could be governed by additional factors. Moreover, a parkin substrate need not be recruited through the other SH3 domain in an endophilin-A dimer and could be recruited to endophilin-A by another, yet unknown mechanism. At present, our data cannot discriminate between these alternatives. Future work will therefore aim at identifying synaptic parkin substrates that depend on the association of parkin with endophilin-A as well as the factors involved in substrate recruitment.

Implication of Parkin-Mediated Ubiquitination in Synaptic Transmission and PD

An important clue toward understanding parkin's role comes from its ability to interact with brain-enriched SH3 proteins that harbor BAR domains. The N-BAR domains of endophilin-A and amphiphysin form homodimers that can induce curvature in lipid membrane (Gallop et al., 2006; Masuda et al., 2006; Peter et al., 2004) and are thought to regulate endocytic vesicles by recruiting enzymes at various stages of the vesicle's formation and trafficking (Dawson et al., 2006; Simpson et al., 1999). For example, in vivo studies have shown that the primary role of endophilin-A is to recruit synaptojanin, a phosphatidylinositol-4,5-biphosphate phosphatase (McPherson et al., 1996), to endocytic vesicles for clathrin uncoating (Schuske et al., 2003; Verstreken et al., 2003). The syndapin/PACSIN proteins have an F-BAR domain composed of an Fes-CIP homology and BAR domain (Halbach et al., 2007) that may serve a scaffolding function by bridging actin organization to vesicle endocytosis and trafficking (Anggono et al., 2006; Qualmann and Kelly, 2000). How could this be relevant to PD? One of the most consistent and intriguing findings associated with both dominant (Tong et al., 2009) and recessive (Nakamura and Edwards, 2007) forms of PD, including those due to *parkin* mutations (Kitada et al., 2009), has been defects in synaptic transmission, possibly related to altered synaptic vesicle endocytosis, recycling, or release. Yet the molecular mechanisms involved have remained completely unknown. Thus, by linking parkin to endophilin-A, a protein at the heart of synaptic vesicle endocytosis and recycling, our findings provide a molecular link between recessive PD genes and defects in synaptic transmission.

EXPERIMENTAL PROCEDURES

Antibodies, Reagents, Plasmids, and Recombinant Proteins

Antibodies, reagents, plasmids, and recombinant protein expression and purification are described in the Supplemental Experimental Procedures.

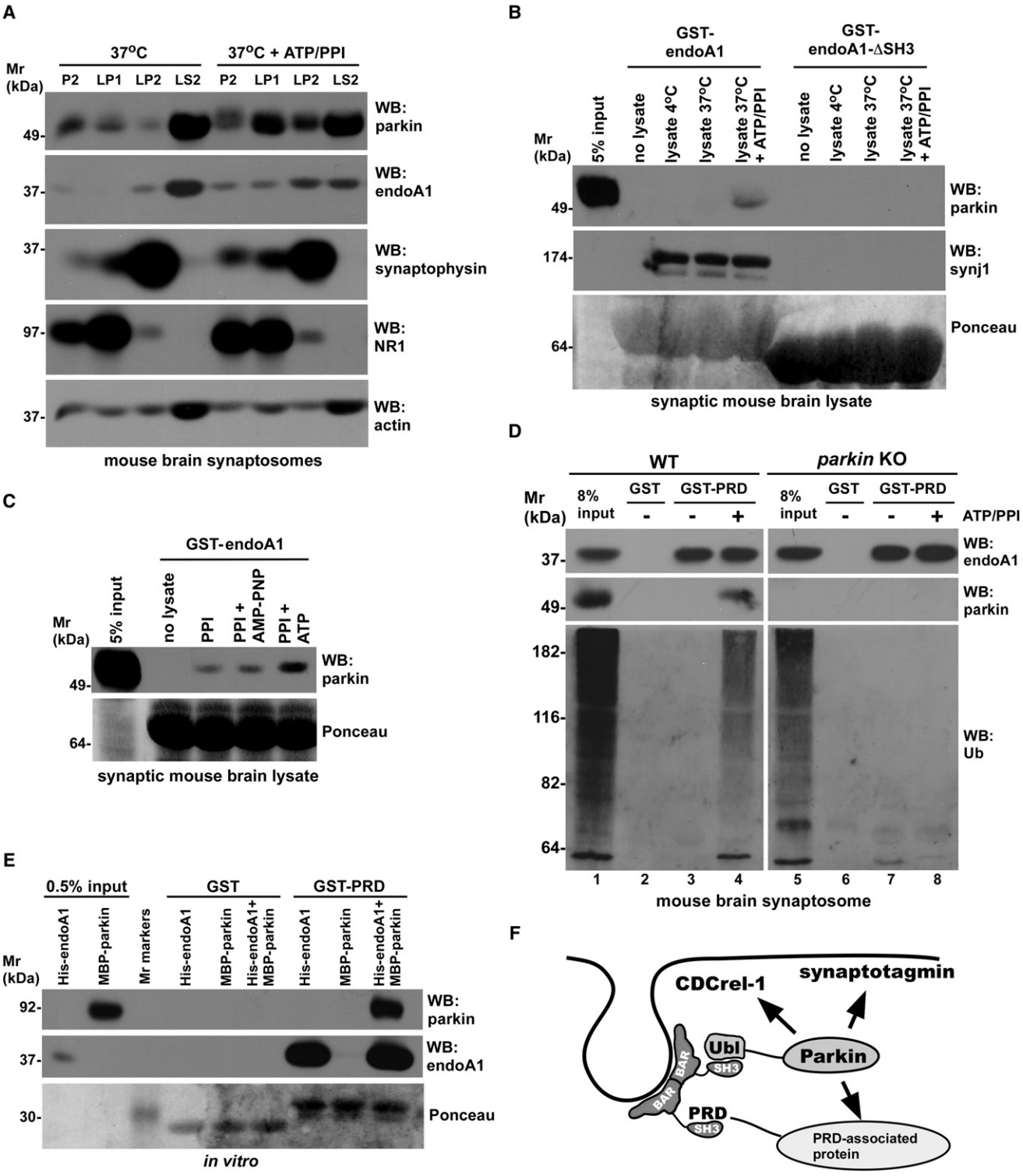


Figure 7. Phosphorylation Regulates Parkin and Endophilin-A in Nerve Terminals

(A) Phosphorylation-dependent relocalization of endogenous parkin and endophilin-A1 to the synaptic plasma membranes and vesicles. Crude mouse brain synaptosomes (P2) were incubated for 15 min at 37°C with or without phosphatase inhibitors (PPI) and 2 mM ATP. The synaptosomes were then fractionated by centrifugation to yield LP1 (plasma membrane), LP2 (vesicle), and LS2 (cytosol). Equal amounts of proteins (50 μg) were resolved on SDS-PAGE and probed with the antibodies marked on the right.

(B) Phosphorylation-dependent binding of endogenous brain parkin to the endophilin-A1 SH3 domain. GST fusion proteins were immobilized on glutathione-Sepharose beads and incubated with mouse brain LS1 that was preincubated for 15 min at 37°C with (+) or without (–) PPI in the presence of 2 mM ATP.

Mouse Brain Synaptosome Preparation

Whole mouse brain was fractionated by differential centrifugation, as previously described (Fallon et al., 2002; Huttner et al., 1983). The brain was homogenized in 0.32 M sucrose, 10 mM HEPES (pH 7.4) supplemented with protease inhibitors (0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 100 μg/ml benzamidine, 20 μg/ml PMSF). The homogenate was centrifuged for 10 min at 1,000× *g*, and the supernatant (S1) was collected and centrifuged again for 15 min at 12,000× *g* to produce a synaptic pellet (P2). P2 was resuspended in the original volume of buffer and centrifuged for 15 min at 13,000× *g* to produce the P2′ pellet. The soft, white component of P2′ was used as the crude synaptosome fraction. Purified synaptosomes were either kept on ice or incubated for 15 min at 37°C in control buffer (132 mM NaCl, 4.8 mM KCL, 2.4 mM $MgSO_4$, 10 mM glucose, 1.1 mM $CaCl_2$, 0.1 mM EGTA, 10 mM HEPES-NaOH [pH 7.4]), either with or without protein phosphatase inhibitors (1 μM okadaic acid, 0.5 μM cyclosporin A, 50 mM NaF, 2 mM Na_3VO_4) and 2 mM ATP. To further fractionate synaptosomes into subsynaptic components, P2′ was resuspended in nine volumes of water and disrupted in a glass-teflon homogenizer (three strokes). The water was adjusted to 10 mM HEPES, and the sample was centrifuged for 20 min at 33,000× *g* to yield the synaptic plasma membrane-enriched pellet (LP1). The supernatant (LS1) was centrifuged for 2 hr at 260,000× *g* to yield the synaptic vesicle-enriched pellet (LP2) and synaptic cytosol-enriched supernatant (LS2).

Immunoprecipitation and Pull-Down Assays

Mouse brain S1 fractions were incubated with 1% Triton X-100 for 30 min at 4°C, followed by centrifugation for 30 min at 260,000× *g* to yield soluble S2 fractions, which were used for GST-Ubl pull-downs (Figures 1A, 1B, and 4D). Mass spectrometry protein identification was performed at the Genome Quebec Proteomics platform (Figure 1A). LS1 fractions were used for GST-endophilin-A1 pull-down (Figures 7B and 7C). For the GST-PRD (synaptojanin-1 VAPPARPAPPQRPPPPSGA$_{1111–1129}$) pull-down (Figure 7D), purified synaptosomes were prepared from crude synaptosomes by an additional Percoll gradient purification, as described (Dunkley et al., 1986). After incubation with or without phosphatase inhibitors and ATP (see above), the synaptosomes were centrifuged at 140,000× *g* for 10 min, and the pellets were lysed in binding buffer: 50 mM Tris-HCl (pH 7.4), 20 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.5% Triton X-100, plus protease inhibitors with or without protein phosphatase inhibitors and ATP, as described above. Fractions were incubated overnight at 4°C with equivalent amounts of GST fusion protein, immobilized on glutathione-Sepharose beads. The in vitro GST-PRD pull-down (Figure 7E) was performed as described above using purified MBP-parkin and His-endophilin-A1. In all cases, the beads were rinsed four times in binding buffer, and bound proteins were eluted in SDS sample buffer at 65°C. Samples were subjected to SDS-PAGE followed by immunoblotting.

HEK293 cells were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS (heat inactivated), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. For pull-downs (Figures 2A and 5C) and immunoprecipitations (Figure 1C), HEK293 cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). Cells were lysed 48 hr posttransfection in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, plus protease inhibitors, for 30 min on ice. Lysates were cleared by centrifugation at 14,000× *g* for 10 min. For pull-downs, the supernatant was incubated at 4°C overnight with GST fusion proteins immobilized on glutathione-Sepharose beads in buffer (50 mM Tris-HCl, 20 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.5% Triton X-100 [pH 7.4]) with protease inhibitors (described above), washed three times with buffer, and eluted with SDS sample buffer. For immunoprecipitations, the supernatant was incubated for 2 hr at 4°C with Anti-Flag M2 affinity gel (Sigma, F2426), washed four times with lysis buffer, and eluted with SDS sample buffer. For coimmunoprecipitation of endogenous proteins (Figure 1D), 2 mg of LS1 fractions were incubated with goat anti-endophilin-A1 antibody overnight at 4°C. The mixture was incubated with Protein A Sepharose beads for 4 hr, followed by washing four times with lysis buffer and eluting in SDS sample buffer. Samples were subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies.

In vitro GST pull-down assays with recombinant proteins (Figures 2B, 4B, 4C, and 5B) were performed using 60 μg of GST fusion proteins and 15 μl of glutathione-Sepharose resin. The GST-bound resin was incubated for 15 min with 12 μg of Ubl or Ub (25 μl at 50 μM) in HBS-I buffer (HBS with 0.02% [v/v] Igepal 630). The resin was washed twice with 1 ml of HBS-I buffer for 30 s and eluted with 15 μl of SDS-PAGE loading buffer. The products were resolved using SDS-PAGE and stained with Coomassie blue.

NMR Spectroscopy

All data sets in H_2O were acquired at 30°C on a 600 MHz Bruker NMR spectrometer equipped with a triple-resonance (^1H, ^{13}C, ^{15}N) cryoprobe, whereas experiments in D_2O were acquired at 30°C on a 800 MHz Varian spectrometer equipped with a triple-resonance room temperature probe. Backbone assignments were performed on both free and ligand-bound ^{15}N,^{13}C-labeled proteins using standard triple-resonance NMR methods. SH3 and Ubl titrations were performed by recording HSQC spectra on 0.3 mM ^{15}N-labeled protein in 5% D_2O, to which unlabeled protein was added. PRD titrations were done with 0.4 mM ^{15}N-labeled SH3 (WT or E304A) in 5% D_2O, to which concentrated PRD peptides (10 mM) were added. Dissociation constants were estimated by least-square minimization of the mean-squared chemical shifts difference calculated from a chemical equilibrium equation. RDCs were measured using stretched polyacrylamide gels according to a published procedure (Chou et al., 2001). ^{13}C-edited HSQC-NOESY and HCCH-COSY experiments with and without ^{13}C decoupling were recorded on samples containing 1.2 mM ^{13}C-labeled SH3 or 0.8 mM ^{13}C-labeled Ubl mixed with an equimolar concentration of unlabeled ligand, lyophilized and resuspended in D_2O. Additional details can be found in the Supplemental Experimental Procedures.

Structure Calculation

The endophilin-A1 SH3 domain crystal structure determination and homology modeling of the rat parkin Ubl are described in the Supplemental Experimental Procedures. Data-driven docking of the SH3 and Ubl structures was performed with the program HADDOCK (Dominguez et al., 2003) using RDCs, NOEs, ambiguous interaction restraints (AIRs), and backbone dihedral angles (Tables 1 and S2 for details). Ubl residues 7–13 and 71–76 were defined as fully flexible. High-temperature rigid body docking, starting from randomized orientations, was run to generate 1000 structures. Structures were sorted according to an intermolecular energy scoring term equal to the sum of E_{vdw}, E_{elec}, E_{dist}, E_{dih}, and E_{sani}. The 200 lowest-energy structures were selected for semi-flexible simulated annealing and explicit water refinement. The 20 lowest energy structures were selected for the final ensemble of structures (Table 1).

The washed products were eluted with loading buffer, loaded on SDS-PAGE, and immunoblotted against parkin (top panel) or synaptojanin (middle panel) or stained with Ponceau to show loading (lower panel).

(C) ATP hydrolysis is required for parkin binding to GST-endophilin-A1. The procedure is the same as in Figure 7B but with PPI-only and PPI/AMP-PNP controls.

(D) Phosphorylation- and parkin-dependent ubiquitination of endophilin-A1-associated proteins in mouse brain synaptosomes. Synaptosomes were prepared from wild-type or *parkin* KO mouse brain and incubated for 15 min at 37°C with (+) or without (–) PPI and 2 mM ATP. Synaptosomes were lysed and incubated with GST or GST-PRD (synaptojanin 1111–1129); the bound products were resolved on SDS-PAGE and probed with antibodies against endophilin-A1 (top), parkin (middle), or Ub (bottom).

(E) The association of parkin with GST-PRD is mediated by endophilin-A1 in vitro. Recombinant MBP-parkin and/or His-tagged endophilin-A1 were incubated with GST or GST-PRD. Bound products were resolved on SDS-PAGE and probed with antibodies against parkin (top) and endophilin-A1 (bottom).

(F) Schematic model for the recruitment of the parkin E3 Ub-ligase to BAR-SH3-containing proteins such as endophilin-A. The BAR domain binds membranes as a dimer and induces curvature in lipid vesicles. It has two SH3 domains that can each interact with the Ubl and/or PRD motif, thus bringing potential synaptic substrates close to parkin.

In Vitro Ubiquitination Assays

Reactions were carried out in a total volume of 100 μl of ubiquitination buffer (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl) containing 90 nM E1 enzyme, 4 mM ATP, 0.4 mM Myc-tagged Ub, 0.04 μg/μl UbcH7, 0.04 μg/μl wild-type or mutant glutathione-Sepharose-bound GST-parkin, and 0.04 μg/μl of wild-type or mutant His_6-tagged endophilin-A1. The reactions were incubated for 2 hr at 37°C, followed by centrifugation at 3000× *g*. The pellet, containing GST-parkin, was washed once with ubiquitination buffer, boiled 10 min in SDS sample buffer, and immunoblotted for anti-Myc. The supernatant was incubated at 4°C overnight in 1 ml Ni-NTA binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole [pH 8.0]) containing 20 μl Ni-NTA beads. The Ni-NTA beads were washed once with the Ni-NTA binding buffer, boiled 10 min in SDS sample buffer, and immunoblotted with anti-His or anti-Myc. Blots were imaged with the Odyssey Infrared Imaging System according to the manufacturer's instructions.

ACCESSION NUMBERS

Atomic coordinates, structure factors and NMR data have been deposited with the Protein Data Bank under accession numbers 3IQL and 2KNB for the endophilin-A1 SH3 crystal structure and SH3:Ubl complex, respectively.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, 13 figures, and two tables and can be found online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00862-4.

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