Cyclic-AMP-DEPENDENT PROTEIN KINASE PHOSPHORYLATION OF Drp1 REGULATES ITS GTPase ACTIVITY AND MITOCHONDRIAL MORPHOLOGY
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Running title: PKA phosphorylation of Drp1
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Mitochondria in cells comprise a tubulovesicular reticulum shaped by dynamic fission and fusion events. The multimeric dynamin-like GTPase Drp1 is a critical protein mediating mitochondrial division. It harbors multiple motifs including GTP-binding, middle, and GTPase effector (GED) domains that are important for both intra- and intermolecular interactions. As for other members of the dynamin superfamily, such interactions are critical for assembly of higher-order structures and cooperative increases in GTPase activity. Though the functions of Drp1 in cells have been extensively studied, mechanisms underlying its regulation remain less clear. Here, we have identified cAMP-dependent protein kinase-dependent phosphorylation of Drp1 within the GED domain at Ser\textsuperscript{637} that inhibits Drp1 GTPase activity. Mechanistically, this change in GTPase activity likely derives from decreased interaction of GTP-binding/middle domains with the GED domain, since the phospho-mimetic S637D mutation impairs this intramolecular interaction, but not Drp1-Drp1 intermolecular interactions. Using the phospho-mimetic S637D substitution, we also demonstrate that mitochondrial fission is also prominently inhibited in cells. Thus, protein phosphorylation at Ser\textsuperscript{637} results in clear alterations in Drp1 function and mitochondrial morphology that are likely involved in dynamic regulation of mitochondrial division in cells.

INTRODUCTION
Mitochondria are critical for a number of cellular functions because of their roles in producing energy, buffering calcium, and regulating apoptosis. In contrast to their common depiction as sausage-shaped organelles in schematic diagrams, they form a dynamic reticulum in the cell, continuously dividing and fusing with one another (1-4). The steady-state equilibrium of these opposing processes is altered during critical cellular events such as apoptosis and cell division, and perturbation of this balance has been directly implicated in a number of inherited neurological disorders including Charcot-Marie-Tooth neuropathy and optic atrophy type 1 (3, 5) as well as a neonatal lethal syndrome of microcephaly, abnormal brain development, optic atrophy, and lactic acidemia (6).

The dynamin-related GTPase Drp1 is an evolutionarily conserved protein that mediates mitochondrial division, and its functional impairment results in aggregates of large, interconnected mitochondria within cells. Drp1 is also involved in the mitochondrial scissioning that occurs during apoptosis and cell division (5, 7). However, mechanisms by which Drp1 function is regulated during such critical cellular events are less well understood. Frequently, cellular responses to changing conditions are mediated by reversible covalent modifications of existing molecules, and Drp1 is modified post-translationally by protein phosphorylation, ubiquitylation and sumoylation (7-10). However, in most cases neither the direct
impact of these modifications on Drp1 functions nor the sites of modification have been thoroughly investigated.

Like many dynamin-related proteins, Drp1 has an N-terminal GTP-binding domain, a middle assembly domain, a short insert (insert B), and a C-terminal interaction domain dubbed the GTPase effector domain (GED)\(^1\); the GED domain folds back to interact in cis with the GTP-binding and middle domains and also interacts intermolecularly in trans with GED domains in other Drp1 proteins (11-15). Here, we demonstrate phosphorylation of Drp1 by cAMP-dependent protein kinase (protein kinase A; PKA) at a specific serine residue within the C-terminal GED domain of Drp1. We further show that this phosphorylation alters the intramolecular domain associations of the Drp1 GTPase, resulting in decreased GTPase activity and impairment of mitochondrial fission.

### EXPERIMENTAL PROCEDURES

**Metabolic Labeling** -- HeLa cells were transfected with pGW1-Myc-Drp1 (splice variant 1; GenBank Accession Number NM_012062) as described previously (15). After 24 h, cells were incubated in serum-free DMEM for 3 h and then labeled with 1 mCi/ml 32P-orthophosphate (8500-9120 Ci/mmol; PerkinElmer, Waltham, MA) in phosphate-free media for 3.5 h. Dimethyl sulphoxide (DMSO) or 20 \(\mu\)M forskolin (Sigma-Aldrich, St. Louis, MO) and 10 \(\mu\)M 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich) prepared in DMSO were added 20 min before cells were lysed in modified radioimmunoprecipitation (RIPA) buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich; P8340) and phosphatase inhibitor cocktail (Sigma-Aldrich; P2850). Myc-Drp1 protein was immunoprecipitated using anti-Myc antibodies immobilized on agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA), with extensive washing in 20 mM phosphate buffer (pH 7.4) supplemented with 500 mM NaCl. Immunoprecipitates were then resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and 32P-labeled Drp1 was visualized by autoradiography using Kodak BioMax MR film with a BioMax MS intensifying screen.

**Phosphopeptide Mapping** -- 32P-labeled Drp1 was excised and digested with trypsin (20 ng/ml in 50 mM NH\(_4\)HCO\(_3\)) overnight at 37 °C. Samples were then dried in a Savant SpeedVac Concentrator, washed with H\(_2\)O, and dried again. Resulting tryptic fragments were resuspended in 10 \(\mu\)l H\(_2\)O and spotted onto thin-layer cellulose sheets (EMD Chemicals, Gibbstown, NJ). Separation of the phosphopeptides in the first dimension was by electrophoresis using an HTLE 7002 system at 1000 V in formic acid : acetic acid : H\(_2\)O (25:78:897; pH 1.97) for 30 min. Next, sheets were air dried, and ascending chromatography was performed in the second dimension in isobutyric acid : pyridine : butanol : acetic acid : H\(_2\)O (1250:96:38:58:558) for 16 cm. Sheets were again air dried, and 32P-labeled phosphopeptides were visualized by autoradiography.

**In Vitro Phosphorylation** -- Glutathione S-transferase (GST)-Drp1 fusion proteins were purified using glutathione Sepharose 4B resin (GE Healthcare, Piscataway, NJ) and then incubated with 50 ng purified PKA catalytic subunit (Promega, Madison, WI) in 10 mM HEPES (pH 7.0) with 20 mM MgCl\(_2\), 50 mM ATP, and 1 pmol of [\(\gamma\)-32P]ATP EasyTides (3000 Ci/mmol; PerkinElmer) at 30 °C for 30 min. Reactions were terminated by adding Laemmli sample buffer, and samples were resolved by SDS-PAGE and visualized by autoradiography.

**Immunoblotting** -- HeLa cells were transfected as described previously (15), washed twice with phosphate-buffered saline (PBS; pH 7.4), then solubilized with RIPA buffer. Where indicated, alkaline phosphatase (1 U/\(\mu\)l; New England Biolabs, Beverly, MA) or \(\lambda\) protein phosphatase (1 U/\(\mu\)l; New England Biolabs) were added to cell lysates and incubated at 37 °C for 1 h. Proteins were resolved by SDS-PAGE and then immunoblotted (15). Affinity-purified rabbit polyclonal anti-peptide antibodies against Drp1 (number 2457) were described previously (15). Rabbit polyclonal antibodies that specifically recognize Drp1 phosphorylated at Ser\(^{637}\) were generated.
commercially (Quality Controlled Biochemicals, Hopkinton, MA) with a synthetic phosphopeptide Ac-CPVARKL[pS]AREQD-amide corresponding to Drp1 residues 631–643 (splice variant 1; GenBank Accession Number NP_036192). Mouse monoclonal anti-actin ascites was obtained from Sigma-Aldrich (1:2000; clone AC-40; IgG2a).

Yeast Two-Hybrid Analyses -- Full-length Drp1 and Drp1 truncation fragments were cloned into pGAD10 and pBHA vectors as described previously (15). Yeast two-hybrid tests were performed in the L40 strain, which contains LexA operators upstream of HIS3 and lacZ reporter genes. Interactions were semi-quantified based on growth on His/Leu/Trp drop out plates supplemented with 10 mM 3-amino-1,2,4-triazole (Sigma-Aldrich) and time for colonies to turn blue in X-gal filter lift assays from Leu/Trp drop out plates (15), with similar results obtained in at least three experiments.

GTPase Activity Assays -- Calmodulin-binding peptide (CBP)-Drp1 fusion proteins were purified from E. coli BL21 (DE3) expressing CBP-tagged wild-type Drp1, Drp1S637A, or Drp1K38A as described previously (15). Affinity-purified CBP-Drp1 fusion proteins were dialyzed against assay buffer (20 mM HEPES [pH 7.2], 2 mM MgCl2, and 1 mM dithiothreitol). The reaction mixture for the GTPase assay at 25 °C included 1.45 μg dialyzed CBP-Drp1 protein with 0.05% bovine serum albumin and 165 nM [α-32P]GTP EasyTides (3000 Ci/mmol; PerkinElmer) in assay buffer. For assay of in vitro phosphorylated CBP-Drp1 fusion proteins, 3.0 μg CBP-Drp1 was first incubated with 50 ng purified PKA catalytic subunit in 10 mM HEPES (pH 7.0) with 20 mM MgCl2 and 500 mM ATP for 30 min at 30 °C before diluting into GTPase assay buffer. Samples of the reaction mixtures were spotted onto polyethyleneimine cellulose on polyester thin layer chromatography (TLC) plates (Sigma-Aldrich) at various times. Guanine nucleotides were separated by ascending chromatography in 1.0 M formic acid : 1.2 M LiCl. [32P]GDP and [32P]GTP spots were identified, and intensities were quantified using a Storm 860 Phosphorimager with ImageQuant software (GE Healthcare). GTPase activity was expressed as the ratio of GDP to total guanine nucleotides (GDP + GTP) at each time point. All data points represent the averages of at least three independent experiments.

Immunostaining -- HeLa cells were grown on glass coverslips and transfected with Myc-Drp1 as described previously (15). Drp1 mutants were produced using the QuikChange method (Stratagene, La Jolla, CA). After 36-48 h of transfection, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 min. After three washes with PBS, cells were incubated in blocking buffer (5% horse serum, 0.1% bovine serum albumin, and 0.1% Triton X-100 in PBS) for 1 h. Goat anti-Myc epitope (1:500; Bethyl Laboratories, Montgomery, TX) and mouse monoclonal anti-cytochrome c (1:500; BD Biosciences Pharmingen, San Diego, CA) antibodies were incubated in blocking buffer overnight at 4 °C. After three washes with PBS, Alexa Fluor 488 and/or Alexa Fluor 568 secondary antibodies (1:500; Invitrogen, Carlsbad, CA) were added for 1 h. After three washes with PBS, coverslips were mounted using Gel/Mount (Biomeda, Burlingame, CA). Images were acquired using a Zeiss LSM-510 confocal microscope. For determination of mitochondrial morphology, three trials were performed, with 100 transfected HeLa cells in each trial classified into normal, elongated and fragmented mitochondrial groups.

Where indicated, HeLa cells were incubated with 20 μM forskolin and 10 μM IBMX for 10, 20, or 30 min before fixation and subsequent immunostaining for cytochrome c.

RESULTS AND DISCUSSION

Drp1 is Phosphorylated by PKA at Ser637

We examined whether Drp1 is a phosphoprotein under basal conditions by labeling HeLa cells overexpressing Myc-tagged Drp1 with 32P-orthophosphate. After immunoprecipitation with anti-Myc antibodies, we clearly detected the Drp1 phosphoprotein at ~82 kDa. We tested multiple different kinase activators, and found...
that forskolin and IBMX, activators of PKA, significantly increased phosphorylation levels (Fig. 1A). To determine whether this stimulated phosphorylation occurred at the same or different sites from the basal phosphorylation, we performed two dimensional tryptic phosphopeptide mapping. This revealed that there was a single novel site of phosphorylation in response to PKA activation that was distinct from sites of basal phosphorylation (Fig. 1B).

To confirm that the phosphorylation was directly mediated by PKA, we performed in vitro phosphorylation assays with [γ-32P]ATP using purified PKA in combination with GST-Drp1 fusion proteins, and we observed a prominent increase in Drp1 phosphorylation. A number of fusion proteins comprising Drp1 truncation constructs were tested, narrowing down the region sufficient for PKA phosphorylation to residues 490-646; phosphoamino acid analysis identified the phosphorylated residue(s) as serine (data not shown). One of the sites within this region, Ser637, comprised a consensus sequence for PKA phosphorylation (-R-R/K-X-S/T-). Site-directed mutagenesis of this site, S637A, resulted in loss of PKA-mediated phosphorylation in vitro, both with full-length GST-Drp1S637A and GST-Drp1(490-646)S637A (Fig 1C and data not shown). After two dimensional tryptic phosphopeptide mapping, the PKA-stimulated phosphopeptide from wild-type GST-Drp1WT migrated essentially the same as that identified in intact cells. It was selectively abolished in the GST-Drp1S637A mutant, confirming the PKA phosphorylation site as Ser637 (Fig 1D). Finally, we developed anti-phosphopeptide antibodies against phospho-Ser637 in Drp1; the phosphorylation site was detected in Drp1WT only after in vitro phosphorylation with PKA, and not at all with Drp1S637A, confirming the specificity of the antibodies for phospho-Ser637. Also, the immunoreactive signal was markedly diminished after treatment with broad spectrum protein phosphatases (Fig 1E). Similarly, in extracts from HeLa cells treated with activators of PKA there was appearance of a phospho-Ser637 band at ~82 kDa, representing endogenous phosphorylated Drp1, that was similarly sensitive to protein phosphatase treatment (Fig. 1F).

Importantly, the consensus site for PKA phosphorylation at Ser637 of human Drp1 is highly conserved in a variety of species (Suppl. Fig. S1). This evolutionary conservation suggests that phosphorylation at this site may be a common means of regulation Drp1 function across species.

**Phosphorylation at Ser637 Impairs Drp1 Intramolecular Associations and GTPase Activity**

Since the Ser637 phosphorylation site is within the GED domain that has been shown to mediate both intramolecular and intermolecular interactions (15), we examined whether this modification changed Drp1 interactions using a phospho-mimetic substitution, S637D, in yeast two-hybrid tests. We found that this mutation significantly inhibited the intramolecular interactions between GED and GTP-binding/middle domains (Fig. 2A) but not intermolecular interactions between full-length Drp1 proteins (Fig. 2B).

Since this intramolecular interaction has previously been shown to be important for GTPase activity (15), we examined the effects of Ser637 phosphorylation on Drp1 GTPase activity. Using in vitro GTPase assays with purified CBP-Drp1 fusion proteins, we found that PKA phosphorylation caused a significant reduction of GTPase activity (Fig. 3A). Similarly, the phosphomimetic GST-Drp1S637D exhibited decreased GTPase activity as well (Fig. 3B), though not so robust an effect as that seen with the more highly negatively-charged phospho-Ser637 modification. This loss of activity likely occurs through effects of the phospho-Ser637 modification and S637D mutation on the intramolecular GED domain interaction with GTP-binding/middle domains, since there are no effects on oligomerization as assessed by yeast two hybrid assays (Fig. 2B) and chemical cross-linking studies (data not shown). Indeed, recent studies examining dynamin have suggested that the middle domain is critical for both tetramerization and higher-order self-assembly (16).
Importantly, our data identify a key regulatory mechanism for the GED domain interaction with middle and GTP-binding domains. Though this interaction has been intensively studied for other dynamin superfamily members, phosphorylation at Ser⁶³⁷ in Drp1 represents the first demonstration of dynamic regulation of this intermolecular interaction with functional changes in GTPase activity.

**Phosphorylation of Drp1 at Ser⁶³⁷ Inhibits Mitochondrial Division**

We examined the effects of IBMX and forskolin, which increase cAMP levels and activated PKA, on mitochondrial morphology in HeLa cells 10-30 minutes after application, but saw no consistent morphology changes. Since cAMP has pleiotropic effects in cells that may mask any specific effects of Drp1 phosphorylation on mitochondrial morphology, we next evaluated the effects of the phospho-mimetic S637D mutation on mitochondrial morphology. Overexpression of this mutant form resulted in mitochondria that were more elongated (Fig. 4). Interestingly, another GED mutation in Drp1 that decreases GTPase activity, K679A, inhibited mitochondrial fission as well (15), reminiscent of our results with Drp1S⁶³⁷D. Importantly, these effects on mitochondrial morphology are less prominent than those seen upon overexpression of a well-characterized Drp1 mutation, K38A, which lacks detectable GTPase activity (15). Thus, effects on mitochondrial fission seem to mirror the extent of changes in GTPase activity for these mutations. Overexpression of Drp1⁵⁶³⁷A that cannot be phosphorylated by PKA showed no clear differences in mitochondrial morphology, consistent with a number of reports that found no increase in fission upon overexpression of wild-type Drp1.

A very recent study identified a cdk1/cyclin B phosphorylation site at a nearby residue, Ser⁶¹⁶ in human Drp1 splice variant 1 (Ser⁵⁸⁵ in the variant reported in Ref. 7). However, a phospho-mimetic substitution, S616D, did not change the intramolecular GTP-binding/middle domain interactions with the GED domain (data not shown), nor did it affect GTPase activity or mitochondrial morphology in cells (7). Thus, Ser⁶¹⁶ phosphorylation likely regulates Drp1 through other mechanisms.

**Functional Implications**

In this study we have shown that PKA phosphorylates Drp1 on Ser⁶³⁷ and inhibits its GTPase activity, likely through changes in the intramolecular association among the GED and GTP-binding/middle domains of Drp1, though higher-order oligomerization may also be affected. This represents the first reported Drp1 modification to have a direct effect on Drp1 GTPase activity.

Furthermore, in keeping with the impaired Drp1 GTPase activity, we have found that a phospho-mimetic substitution, S637D, inhibits mitochondrial division. However, the functional effects of GED domain mutations in general have been controversial in the dynamin superfamily, since some studies have seen facilitation of fission in response to changes in conserved amino acid residues for both dynamin and Drp1 (17, 18), while others have not (15, 19). On the other hand, since very recent studies support a two-step model for dynamin function (20), with an early regulatory GTPase-like function preceding a late, assembly-dependent step during which GTPase activity is required for fission, different GED mutations may give different results. In addition, the phospho-mimetic substitution is a permanent one that lacks the dynamic aspects of phosphorylation in vivo, and thus we suspect that the timing of phosphorylation would be critical. Further identification of the temporal aspects of the phosphorylation/dephosphorylation cycle will help unravel the cellular role of this modification.

**ACKNOWLEDGMENTS**

We thank C. Smith (NINDS Light Imaging Facility) and J. Stadler for technical assistance.
REFERENCES


FOOTNOTES

*This work was supported by the Intramural Research Program of the National Institute of Neurological Disorders and Stroke, NIH.

1The abbreviations used are: GED, GTPase-effector domain; PKA, cAMP-dependent protein kinase; DMSO, dimethyl sulphoxide; IBMX, 3-isobutyl-1-methylxanthine; GST, glutathione S-transferase; CBP, calmodulin-binding peptide; TLC, thin layer chromatography; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation
FIGURE LEGENDS

FIGURE 1. Drp1 is phosphorylated at Ser\(^{637}\) by protein kinase A. A, Myc-Drp1 was immunoprecipitated from \(^{32}\)P-orthophosphate-labeled HeLa cells treated with DMSO (control) or else forskolin and IBMX. Immunoprecipitates were resolved by SDS-PAGE, and \(^{32}\)P-labeled Myc-Drp1 was visualized by autoradiography. Migrations of molecular mass standards (in kDa) are indicated to the left. B, Tryptic phosphopeptides of \(^{32}\)P-labeled Myc-Drp1 were resolved by two dimensional TLC. Origins are identified with filled circles (●). The major phosphopeptide induced by forskolin and IBMX treatment is indicated with an arrow. C, GST-Drp1\(^{WT}\) and Drp1\(^{S637A}\) (0.5 μg protein/lane) were phosphorylated \textit{in vitro} using PKA in the presence of [\(^\gamma\)-\(^{32}\)P]ATP and resolved by SDS-PAGE. \(^{32}\)P-labeled GST-Drp1 is indicated by an arrow, and an asterisk (*) identifies autophosphorylated PKA. No GST protein was included in the control shown. D, Samples from \textit{in vitro} Drp1 phosphorylation assays were subjected to phosphopeptide mapping. A prominent phosphopeptide difference between Drp1\(^{WT}\) and Drp1\(^{S637A}\) maps is indicated by arrows. E, \textit{In vitro} phosphorylated GST-Drp1\(^{WT}\) and Drp1\(^{S637A}\) (0.5 μg/lane) were immunoblotted with phosphospecific antibodies against phospho-Ser\(^{637}\). Protein phosphatases (PPases) were added as indicated. F, HeLa cells (25 μg/lane) were treated with DMSO (control) or else forskolin and IBMX, and total cell lysates were immunoblotted with anti-Drp1 phospho-Ser\(^{637}\) (pS637) antibodies. Protein phosphatases (PPases) were added as indicated. Equal protein loading was monitored by immunoblotting for total endogenous Drp1.

FIGURE 2. Drp1\(^{S637D}\) phospho-mimetic mutation impairs intramolecular Drp1 interactions, but not intermolecular interactions. A, Schematic of Drp1 structure; boundary amino acids of constructs used are indicated. Intramolecular interactions between Drp1 fragments were assayed using the \textit{HIS3} reporter (10-fold yeast dilutions shown) and \(\beta\)-galactosidase activity (assessed by determining the time for colonies to turn blue in X-gal filter lift assays: ++++; -30 min; ++, 30-60 min; +, 60–120 min; -, no significant activity). B, Strengths of intermolecular interactions in the yeast two-hybrid system between full-length Drp1\(^{WT}\) and Drp1\(^{S637A}\) were assayed as in A.

FIGURE 3. Drp1 GTPase activity is attenuated by phosphorylation at Ser\(^{637}\). Autoradiograms of TLC plates showing the time course of GTP hydrolysis by CBP-Drp1\(^{WT}\), with or without addition of PKA (A), as well as CBP-Drp1\(^{WT}\) and phospho-mimetic CBP-Drp1\(^{S637D}\) (B). In (B), equimolar bovine serum albumin (Control) and CBP-Drp1\(^{K38A}\) were used as negative controls. Migrations of \(^{[32]}\)P\(\text{GDP}\) and \(^{[32]}\)P\(\text{GTP}\) are indicated. \(^{[32]}\)P\(\text{GDP}\) and \(^{[32]}\)P\(\text{GTP}\) intensities were quantified at each time point, and percentages of GTP hydrolysis were calculated as described previously (15). Means of three trials for each condition (± S.E.M.) are shown graphically.

FIGURE 4. Overexpression of phospho-mimetic Drp1\(^{S637D}\) results in elongated mitochondria. A, HeLa cells were transfected with Myc-tagged Drp1\(^{WT}\) or Drp1\(^{S637D}\) and immunostained for Myc-epitope (for Drp1) and cytochrome \(c\) (for mitochondria). Mitochondria are elongated in cells expressing Myc-Drp1\(^{S637D}\) (arrow). Scale bar, 10 μm. B, HeLa cells expressing Myc-tagged Drp1\(^{WT}\) or Drp1\(^{S637D}\) were categorized into one of three groups based on mitochondrial morphology. Values represent means ± S.D. for three trials; \(n=100\) cells per trial.
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J. Biol. Chem. published online June 6, 2007

Access the most updated version of this article at doi: 10.1074/jbc.C700083200

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