Phosphorylation of dynamin II at serine-764 is associated with cytokinesis

Megan Chircop a,⁎, Boris Sarcevic b, Martin R. Larsen c, Chandra S. Malladi a, Ngoc Chau a, Michael Zavortink a, Charlotte M. Smith a, Annie Quan a, Victor Anggono a, Peter G. Hains a, Mark E. Graham a, Phillip J. Robinson a

a Children’s Medical Research Institute, The University of Sydney, 214 Hawkesbury Road, Westmead, NSW 2145, Australia
b St. Vincent’s Institute of Medical Research and Department of Medicine, St. Vincent’s Hospital, The University of Melbourne, Fitzroy, Victoria, Australia
c Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense, Denmark

⁎ Corresponding author. Tel.: +61 2 9687 2800; fax: +61 2 9687 2120.
E-mail address: mchircop@cmri.org.au (M. Chircop).

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Abstract
Calcineurin is a phosphatase that is activated at the last known stage of mitosis, abscission. Among its many substrates, it dephosphorylates dynamin II during cytokinesis at the midbody of dividing cells. However, dynamin II has several cellular roles including clathrin-mediated endocytosis, centrosome cohesion and cytokinesis. It is not known whether dynamin II phosphorylation plays a role in any of these functions nor have the phosphosites involved in cytokinesis been directly identified. We now report that dynamin II from rat lung is phosphorylated to a low stoichiometry on a single major site, Ser-764, in the proline-rich domain. Phosphorylation on Ser-764 also occurred in asynchronously growing HeLa cells and was greatly increased upon mitotic entry. Tryptic phospho-peptides isolated by TiO2 chromatography revealed only a single phosphosite in mitotic cells. Mitotic phosphorylation was abolished by roscovitine, suggesting the mitotic kinase is cyclin-dependent kinase 1. Cyclin-dependent kinase 1 phosphorylated full length dynamin II and Glutathione-S-Transferase-tagged—dynamin II—proline-rich domain in vitro, and mutation of Ser-764 to alanine reduced proline-rich domain phosphorylation by 80%, supporting that there is only a single major phosphosite. Ser-764 phosphorylation did not affect clathrin-mediated endocytosis or bulk endocytosis using penetratin-based phospho-deficient or phospho-mimetic peptides or following siRNA depletion/rescue experiments. Phospho-dynamin II was enriched at the mitotic centrosome, but this targeting was unaffected by the phospho-deficient or phospho-mimetic peptides. In contrast, the phospho-mimetic peptide displaced endogenous dynamin II, but not calcineurin, from the midbody and induced cytokinesis failure. Therefore, phosphorylation of dynamin II primarily occurs on a single site that regulates cytokinesis downstream of calcineurin, rather than regulating endocytosis or centrosome function.

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1. Introduction
Dynamin II (dynII) is a large GTPase enzyme that is essential for clathrin-mediated endocytosis (CME). It also plays roles in centrosome cohesion and cytokinesis [1–4]. It is one of three “classical” dynamins (I, II and III) and with a variety of dynamin-related proteins (DRPs) forms the dynamin super family, which is conserved throughout eukaryotes [5]. All members appear to be involved in membrane fission or fusion events. Dyn is neuron-specific, dynII is ubiquitously expressed and dynIII has restricted expression primarily in brain and testis [6]. DynII appears to be the ancestral form most closely related to the DRPs found in lower organisms. All dynamin super family proteins contain an N-terminal GTPase domain and a middle domain, while most also have a GTPase effector domain (GED). Interaction between these domains allows for dynamin oligomerisation [7]. The classical dynamins contain two additional domains: a pleckstrin homology (PH) domain for phospholipid binding and a proline-rich domain (PRD) that is the main site of phosphorylation and interacts with SH3 domains from other proteins [8].

Dyn and dynII are phosphorylated in nerve terminals on Ser-774 and Ser-778 (equivalent to Ser-759 and Ser-763 in dynIII) in the phospho-box region of their PRD [9]. DynII is phosphorylated on these sites in vivo by the cyclin-dependent kinase (cdk), cdk5 [8] and on Ser-857 by minibrain kinase/Dyrk1A [10]. In addition, we used mass spectrometry to confirm Ser-857 as well as identify Ser-512, Ser-851, Ser-822 and Ser-347 as in vivo phosphosites [9]. Whereas dynI and dynIII are phosphorylated on only one additional phosphosite, Ser-853, which is equivalent to Ser-851 in dynI [11] and immediately stimulates activity-dependent bulk endocytosis (ABDE) for later formation of new synaptic vesicles by budding from the endosomes [6,12]. These phosphosites regulate protein–protein interactions: Ser-774/Ser-778 dephosphorylation facilitates or phospho-mimetic peptides. In contrast, the phospho-mimetic peptide displaced endogenous dynamin II, but not calcineurin, from the midbody and induced cytokinesis failure. Therefore, phosphorylation of dynamin II primarily occurs on a single site that regulates cytokinesis downstream of calcineurin, rather than regulating endocytosis or centrosome function.

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phosphorylation appears to reduce dynl binding to amphiphysin I (amplh) and Grb2, although it is not located within their known binding site [10]. The roles of the other dynl sites or the three phosphosites in dynll are unknown.

In non-neuronal cells, overexpressed dynl and endogenous dynl are phosphorylated by the tyrosine kinase c-Src at Tyr-231 and Tyr-597 following growth factor stimulation [15,16] and this appears to regulate Golgi integrity and vesicle secretion from the Golgi [17]. Previous systematic phosphosite analysis experiments in nerve terminals revealed no in vivo phosphorylation sites identified in rat brain dynl, despite that the protein was readily isolated and detected, indicating that it is not appreciably phosphorylated in nerve terminals despite good protein abundance [9]. Of the seven dynl phosphorylation sites in rat brain, only two are conserved in the dynll amino acid sequence, Ser-347 and Ser-778. This corresponds to rat/human dynll amino acids Ser-347 and Ser-764. Ser-347 phosphorylation accounts for <2% of total dynll phosphorylation and is not dephosphorylated following depolarisation, suggesting it is not a major player in regulating dynll function for ADP-HE [9]. In contrast, Ser-778 is one of the two major phosphorylation sites that are dephosphorylated following depolarisation. The analogous site, Ser-764, in the dynll phospho-box region is located within a predicted amino acid consensus motif for potential phosphorylation by cdk.

We recently reported that CaN activity is required for cytokinesis and that dynl is one of its substrates at the midbody [18]. Dynll is required for the abscission phase of cytokinesis which is delayed or blocked in the presence of small molecular inhibitors and siRNA targeting dynl [4]. A recent manuscript indicated that dynll is phosphorylated by Cdk1/cyclin B1 on Ser-764 and/or Thr-768 during mitosis and that this reduces its ability to bind microtubules [19]. The role of dynll phosphorylation in cytokinesis is unknown. At least three other endocytic proteins are mitotically phosphorylated by cdkl, including epsin, eps15 and amphiphysin I [20,21] of which all are CaN targets in nerve terminals [6]. Two large-scale phosphoproteomic screens identified additional endocytic proteins (e.g. synaptotagmin, clathrin light chain) that may be mitotically phosphorylated in HeLa cells [22,23]. This raises the question of whether phosphorylation of endocytic proteins during mitosis affects their endocytic and/or mitotic functions. However, shotgun phosphoproteomic screening data require validation of each protein and phosphosite, as the false discovery rate of assigning the correct phosphosite to a peptide is often unacceptably high. Therefore, our first aim was to use several approaches to directly identify the in vivo dynll serine/threonine phosphorylation sites and to confirm which are genuine in vivo mitotic phosphosites. We found that it is mitotically phosphorylated by cdk1 in HeLa cells at a single detectable site in the phospho-box, Ser–764. We then aimed to reveal the role of dynll–Ser–764 phosphorylation during mitosis since the analogous site in dynl controls endocytosis. Surprisingly, phosphorylation was not involved in CME or centrosome localisation but specifically targets dynll to the midbody and is involved in cytokinesis.

2. Materials and methods

2.1. siRNA and plasmid construction

Stealth siRNAs targeting human dynamin (dyn, 5′-UCUCUCUCCCG-GACUCCUCAAAGGCU-3′) and luciferase (luc, 5′-CGUCCCGGAAUAU-CUCCGATT-3′) were purchased from invitrogen. GFP-α-tubulin was obtained from Jenny Stow (Institute for Molecular Biosciences, Australia). A rat dynl II cDNA encoding the “aa” splice variant was isolated from a brain cDNA library (invitrogen) and cloned into pEGFP-C1 as an EcoRI and BamHI fragment to generate GFP–dynllwt. The proline-rich domain (PRD) of rat dynl was amplified using pGFP-dynllwt as a template, then cloned into pGEX–1X7 as a BamHI fragment to generate wild-type GST–dynll–PRD (amino acids 740–870). Phospho-deficient (Ser–764A and Ser–764A/T766A) and phospho-mimetic (Ser–764E and Ser–764E/T766E) mutant forms of GFP–dynll (full length and PRD) and a phospho-deficient (Ser–764A) form of GST–dynll–PRD were generated using the QuickChange site-directed mutagenesis kit (Stratagene). The identity of plasmid constructs were confirmed by sequencing and details of primers are available upon request. GST–human amphiphysin II (amplh)–SH3 domain (residues 499–593), in a pGEX-2T plasmid as described [24], GST–rat dynll–PRDwt (amino acids 740–870) and GST–dynll–PRD–Ser–764A fusion proteins were expressed in Escherichia coli and purified using glutathione–sepharose beads (Amersham Biosciences) according to the manufacturer’s instructions.

2.2. Cell culture and transfection

HeLa cells were maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) and grown at 37 °C in a humidified 5% CO2 atmosphere. Cells were seeded at 50%–60% confluence and transfected with the 1.5 μg of the indicated GFP-tagged plasmid/well of a 6-well plate using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For siRNA analysis, HeLa cells were transfected with 1000 pmol of siRNA (per 10 cm2 dish) using Lipofectamine 2000 (Invitrogen). Cells were collected five days post-transfection for immunoblotting. For siRNA rescue assays, cells (2.5×105) were co-transfected with 200 pmol of siRNA (against human dynll) and 1.2 μg DNA (from rat dynll) using 4 μl of Lipofectamine 2000 (Invitrogen) following the manufacturer’s general protocol. This rat dynll sequence varies in 6 positions from the human siRNA target region sequence, making it immune from the siRNA treatment. Cells were trypsinized and re-seeded at 48 h after transfection then assayed for transferrin (Tfn) uptake the following day.

2.3. Cell synchronisation and treatments

Cells grown on coverslips were synchronised at the G1/S border by double thymidine block. Cells were then released into the cell cycle following thymidine wash-out as previously described [25]. At 8 h post-release cells were treated with 100 μM of the dynll peptide for 10 h then fixed for immunofluorescence microscopy analysis. For mitotic synchronisation, cells were treated with 0.5 μg/ml nocodazole for 16 h. Mitotic arrested cells were collected by "mitotic shake-off" for immunoblot analysis. For cdkl inhibition, cells were treated with 10 μM roscovitine either alone or in combination with nocodazole. Under these conditions only mitotically rounded cells were collected and analysed. Alternatively, cells were synchronised at the G2/M border by treatment with the selective cdkl small-molecule inhibitor, RO–3306 (9 μM) for at least 18 h [26]. Cells were allowed to progress through mitosis following RO–3306 wash-out. Where indicated, cells were treated with 100 μM of the dynll peptide immediately following RO–3306 wash-out and incubated at 37 °C/5% CO2 for 1.5 h (meta-phase localization analysis), 3 h (cytokinesis localization analysis) or 6 h (multiplication scoring).

2.4. Antibodies

The following antibodies were used: anti-dynll (C-18, Santa Cruz Biotechnology), anti-dynl, anti-dynl phospho-Ser–774 and anti-dynl phospho-Ser–778 [13], anti-cyclin B1 (V152; Cell Signaling), anti-α-tubulin (clone DM1A; Sigma) and anti-γ-tubulin (GTU88; Sigma).

2.5. Immunoblotting

Cellular extracts were prepared by incubating HeLa cells in ice-cold lysis buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% Triton X-100, and EDTA-free Complete
protease inhibitor cocktail (Roche) and the supernatant was collected following centrifugation at 13,000 rpm for 30 min at 4 °C. Dynl protein was enriched by incubating the cellular extracts with purified GST–amphII–SH3 domain bound to glutathione-sepharose beads for 1 h at 4 °C. Beads were washed extensively with ice-cold lysis buffer. Bound proteins were fractionated by SDS–PAGE for immunoblot analysis. Primary antibody was applied then detected by horseradish peroxidase-conjugated secondary antibody (Sigma). Blotted proteins were visualised using the ECL detection system (Pierce).

2.6. Antarctic phosphatase treatment

For dephosphorylation, mitotic HeLa cell lysates were prepared and the sample was enriched for dynl protein by performing a pull-down with 100 μl of a 50% slurry of GST–amphII–SH3 as described above. Beads were washed twice with ice-cold wash buffer (20 mM ammonium bicarbonate, pH 8.0), then resuspended in ice-cold reaction buffer (20 mM ammonium bicarbonate, 1 mM MgCl₂, pH 8.0). Antarctic phosphatase (AP, 5 μl, New England Biolabs) was added and the reaction was incubated for 2 h at 15 °C before the beads were washed to remove the phosphatase.

2.7. In vitro protein phosphorylation

Dynl was purified by a GST–amphII–SH3 pull-down from total sheep brain extract, as described previously for rat brain [13]. Dynl (100 μg) was purified from baculovirus-infected SF9 insect cells (provided by SL Schmid, the Scripps Research Institute, CA) but had a high level of endogenous phosphorylation that required prior removal. Dynl and dynll were diluted in Tris buffer (20 mM Tris–HCl pH 7.4, 1 mM DTT, 150 mM NaCl and EDTA-free Complete protease inhibitor cocktail), bound to GST–amphII–SH3 beads, washed twice and incubated overnight with AP at 4 °C and dephosphorylated dynl and dynll were eluted twice with Tris–HCl buffer containing 1.2 M NaCl. The eluted fractions were pooled and micro-dialysed (Pierce) against Tris buffer containing 20% glycerol and 100 mM NaCl. Dephosphorylated native dynl and dephosphorylated baculovirus expressed dynl were then used as in vitro substrates for purified recombinant cdk1/cyclin B1, cdk1/cyclin A, cdk2/cyclin A, cdk2/cyclin E, cdk4/cyclin D1 and MAPK kinase as described previously [27]. Where indicated, purified recombinant rB and dynll purified from rat liver were used as substrates. Alternatively, purified bacterially expressed wild-type and phospho-deficient (Ser-764A) forms of GST–dynll–PRD were used as substrates for cdk1/ cyclin A.

2.8. Nano-liquid chromatography mass spectrometry (nanoLC–MS/MS)

Lungs were excised from two rats or 10⁷ mitotic synchronised HeLa cells were used to make lysates from which dynl protein was enriched by performing a GST–amphII–SH3 pull-down as described above. Proteins were resolved by SDS–PAGE and visualised on the gel by staining with colloidal Coomassie blue. Dynl SDS–PAGE gel bands, estimated at 1–5 pmol protein from human mitotic cells or 10–20 pmol from rat lung, were digested with trypsin and phospho-peptides were enriched using titanium dioxide as described previously [28]. The rat lung dynl tryptic digest was further digested with Asp–N because the tryptic phospho-box peptide was considered too long for correct assignment of the phosphorylation site. An aliquot of the phospho-peptide-enriched human or rat dynll digest was injected into a nano–HPLC system (LC Packings Ultimate HPLC system, Dionex, Netherlands) and the eluate was analysed by a QSTAR XL quadrupole–TOF MS (Applied Biosystems, USA) or QTOF Premier (Waters, UK) as described previously [29]. Spectra were recorded using information-dependent data acquisition. Phosphopeptide tandem mass spectra were exported to Mascot 2.2 (Matrix Science, UK) for automated database searching and identification. They were also manually examined to determine the phosphorylation sites.

2.9. Immunofluorescence microscopy

Cells were fixed in ice-cold 100% methanol for 10 min at −20 °C and then blocked in 3% bovine serum albumin/PBS for 45 min before the required primary antibody was applied. Cells that were transfected with GFP–dynll–PRD plasmids were fixed in 4% paraformaldehyde/PBS. Fluorescein- or Texas Red dye-conjugated AffiniPure secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) were then applied. Cell nuclei were counterstained with DAPI (4′,6′-diamidino-2-phenylindole; Sigma). Cells were washed three times with PBS between each step except after blocking. Cells were viewed and scored with a fluorescence microscope (Zeiss). Fluorescence images were captured under an Olympus IX80 inverted microscope using 100× oil immersion lenses and deconvolved using AutoDeblur v9.3 (AutoQuant Imaging, Watervliet, NY).

3. Results

3.1. Dynl phosphorylation at Ser-764 by cdk1/cyclin B1

Of the two main dynl phosphosites (Ser-774 and Ser-778), only Ser-778 is conserved in the dynl phospho-box at position Ser-764 (Fig. 1A), but this site is not detectably phosphorylated in dynll from nerve terminals [9]. Ser-764 precedes a proline residue. Therefore, we used in vitro protein kinase phosphorylation assays to ask whether a cdk could phosphorylate dynll–Ser–764. Dynll was phosphorylated in vitro by cdk1/cyclin A, cdk1/cyclin B1, cdk2/cyclin A and cdk2/cyclin E complexes with similar efficiency using [γ-32P]ATP labelling (Fig. 1B and D). This phosphorylation was specific, as cdk4/cyclin D1 did not phosphorylate dynll yet was fully active on a reference substrate—RB protein (Fig. 1D). Another proline-directed kinase, mitogen-activated protein kinase (MAPK), also phosphorylated both dynamin proteins to a lesser extent (Fig. 1B and C). To determine if dynll–Ser–764 was a site of phosphorylation we used a phospho-specific antibody. The phospho-peptide amino acid sequence used to generate the dynl phospho-Ser–778 antibody was conserved in dynll (underlined in Fig. 1A) except for the single N-terminal residue. We have previously shown that this antibody recognises dynll phosphorylated at Ser–764 [18]. This antibody recognised cdk1-mediated phosphorylated dynll in vitro, indicating that cdk targets Ser–764 (Fig. 1B). As a control, dynll was phosphorylated on Ser–778 in vitro by both cdk1/cyclin A and cdk1/cyclin B1 as indicated by both [γ-32P]ATP labelling and Western blot analysis with the phosphosite-specific antibody (Fig. 1C), consistent with previous reports [8]. Phosphorylation detected by both [γ-32P]ATP labelling and the phosphosite-specific antibody matched for each protein kinase, suggesting dynll is phosphorylated on the analogous site, Ser–764 in the PRD. This was confirmed by MS analysis (see below).

We next aimed to determine whether other phosphosites were targeted by cdk1 in the dynll PRD. As found for full length dynl (Fig. 1B), GST–dynll–PRDwt protein was efficiently phosphorylated
DynII is phosphorylated by the mitotically active kinase cdk1/cyclin B1 on Ser-764. (A) Dynamin consists of four major domains: the GTP hydrolysis domain (GTPase), a pleckstrin homology (PH) domain, a GTPase effector domain (GED) and a proline-rich domain (PRD). DynII is phosphorylated by cdk5 at Ser-774 and Ser-778 (bold amino acids) in vivo in the ‘phospho-box’ region. Of these two phosphorylation sites, only Ser-778 is conserved in dynII as an SP motif, corresponding to residue Ser-764 (bold). The amino acid sequence underlined in dynI was the peptide used to generate the phospho-Ser-778 specific antibody [13]. (B and C) In vitro protein kinase assay. Purified cdk1/cyclin A, cdk1/cyclin B1 and MAPK were examined for their ability to phosphorylate purified dynII and dynI at Ser-764 and Ser-778, respectively, in an in vitro protein kinase assay. DynII (B) and dynI (C) phosphorylation were determined by γ-32P-ATP labelling (upper panel) and Western blotting (middle panel) with the phospho-specific antibody anti-dynI–Ser-778. (D) In vitro phosphorylation of dynII by Cdk/cyclin complexes. Purified cdk1/cyclin A, cdk1/cyclin B1, cdk2/cyclin A, cdk2/cyclin E and cdk4/cyclin D1 kinases were examined for their ability to phosphorylate dynII, which had been purified from rat liver (0.1 μg, lanes 1–6) and from baculovirus (0.5 μg, Sf9 cells; lanes 7–12) in an in vitro protein kinase assay. Rb (2 μg) was phosphorylated with the same kinases (lanes 13–18) and served as a positive control. Phosphorylation was determined by γ-32P-ATP labelling. DynII was phosphorylated by all cdk/cyclin protein kinase complexes except for cdk4/cyclin D1. Blots shown in (B–D) are representative of at least two independent experiments.

by cdk1 in vitro using [γ-32P]-ATP labelling and phosphorylation increased in a time-dependent manner (Fig. 2). Phosphorylation was greatly reduced when Ser-764 was mutated to alanine (Fig. 2). Less than 20% of the total phosphorylation remained in this mutant, suggesting that Ser-764 is the major in vitro phosphorylation site by cdk1 within the dynII–PRD.
Mutation of Ser-764 reduces cdk1-mediated phosphorylation of dynII in vitro. GST–dynII–PRD wt was phosphorylated by cdk1/cyclin A as determined by $\gamma^{32}$P-ATP labelling and this increased with time. Cdk1/cyclin A-mediated phosphorylation was significantly reduced by mutation of Ser-764 to alanine. NK, no kinase. The upper panels are autoradiographs ($^{32}$P) of phosphorylated GST–dynII–PRD wt (left) or GST–dynII–PRD–S764A (right), with the corresponding Coomassie stained gels in the panels below (Coomassie). Blots are representative of two independent experiments.

Fig. 3. DynII is phosphorylated at Ser-764 during mitosis. (A) Asynchronous and mitotically synchronised HeLa cells were treated with the cdk inhibitor, roscovitine (10 $\mu$M) for 16 h. Whole cell lysates (100 $\mu$g) were blotted for the phosphorylation status of dynII at Ser-764 and for dynII levels. (B) Lysates from HeLa cells synchronised in prometaphase were treated with or without Antarctic phosphatase (AP). DynII purified from baculovirus-infected Sf9 cells served as a control. Samples were blotted for the phosphorylation status of dynII and for dynII levels. Blots shown in panels A and B are representative of three independent experiments. (C) Native dynII was purified from rat lung and doubly digested with trypsin followed by Asp-N. Analysis of the titanium dioxide enriched fraction of this digest revealed a single phospho-peptide that was sequenced by tandem mass spectrometry. Fragmentation of the triply charged precursor at m/z 626.6 produced a spectrum matching the sequence of rat dynII754–769 where Ser-764 is phosphorylated (shown as pS). The phosphorylation site was readily revealed as the molecular mass of a dehydroalanine residue (69 Da) between y5 and y6-98 ions resulting from the neutral loss of phosphoric acid (−98 Da) from the phosphoserine residue. (D) Phosphorylated dynII was isolated from mitotically synchronised HeLa cells. A single phosphorylated tryptic peptide was found and its phosphorylation site was sequenced by tandem mass spectrometry. Fragmentation of the quadrupally charged precursor at m/z 1046.3 produced a spectrum matching the sequence of human dynII731–769 where Ser-764 is phosphorylated. Although this peptide was longer than the rat dynII peptide, the fragmentation information from the C-terminal end was the same. The phosphorylation site was unequivocally revealed between the y5 and y6-98 ions.
3.2. DynII is mitotically phosphorylated at Ser-764 by cdk1

Several components of the endocytic machinery are mitotically phosphorylated—eps15, amphiphysin [21] and Drp1 [32]. Since dynII is phosphorylated by the mitotic kinase, cdk1/cyclin B1, and dephosphorylated by CaN during cytokinesis [18], we asked whether phosphorylation is increased during mitosis in HeLa cells. DynII phosphorylation at Ser-764 was barely detectable in asynchronously growing cells but was markedly increased in mitotically synchronised cells (Fig. 3A). This was prevented by treatment with the cdk inhibitor, roscovitine (Fig. 3A), suggesting that cdk1 is the responsible in vivo protein kinase. The dynI phospho-Ser-774 antibody did not recognise any HeLa cell proteins in the 90–110 kDa region, consistent with undetectable expression of dynI in these cells (data not shown) and lack of conservation of Ser-774 in the dynII sequence (Fig. 1A). Antarctc phosphatase treatment of lysates from mitotically synchronised HeLa cells abolished phospho-Ser-778 antibody labelling (Fig. 3B), confirming that the band detected during mitosis represented bona fide dynII phosphorylation. Thus, Ser-764 on dynII is mitotically phosphorylated.

3.3. Identification of Ser-764 phosphorylation by mass spectrometry

We used comprehensive mass spectrometry analysis of dynII to determine the number and site(s) of phosphorylation on dynII. Tandem mass spectrometry on a QTOF Premier (Waters, UK) revealed that native dynII purified from two rat lungs was phosphorylated on a single site in vivo at Ser-764 (Fig. 3C). This identification required a large amount of tissue and extracted dynII to be detectable, thus, is likely to be very low stoichiometry. To directly identify the mitotic phosphorylation site in human dynII, the protein was enriched from mitotically synchronised HeLa cells by binding to GST–amphII–SH3 attached to GSH sepharose, which quantitatively binds all three classical dynamins [9]. Tryptic digests analysed by tandem mass spectrometry exclusively detected dynII phosphorylation at Ser-764 in these cells. In at least six independent experiments, a single phospho-peptide was found and the site was assigned to Ser-764 in dynII (Fig. 3D). At no point were other phospho-peptides or phosphosites identified.

3.4. Phosphorylation at Ser-764 is not required for clathrin-mediated and bulk endocytosis

To gain insight into the biological significance of dynII–Ser-764 phosphorylation, we sought to determine its effect on endocytosis by using a penetratin-based membrane permeable peptide strategy [12,13,33]. Phospho-mimetic peptides were designed to mimic the amino acid sequence of the dynII phospho-box and were tagged with a short penetratin heptapeptide to facilitate their delivery into cells (Fig. 4A). This strategy prevents indirect or long-term effects caused by overexpressing dynII phosphosite mutants and avoids variability in transfection efficiency. It has been validated in several previous studies, such as in determining the role of dynI dephosphorylation in...
stimulating recruitment of the dynl partner syndapin[12,13]. In those studies, the homologous phospho-box AA peptide designed against the sequence of dynl (Fig. 4A) potently inhibits ADBE by preventing the dynl/syndapin I interaction, while it is without effect on transferrin endocytosis in U2OS cells which lack dynl. Thus, it was possible that the analogous dynll peptide would inhibit CME. However, neither the dynll759–775EE phospho-mimetic peptide nor the dynll759–775AA phospho-deficient peptide had significant effects on endocytosis of transferrin (Fig. 4B) or EGF (Fig. 4C) in U2OS cells. Only at extremely high concentrations was endocytosis of transferrin slightly inhibited; however, this was not significant. Endocytosis of dextran, the fluid phase-marker of bulk endocytosis, was also not affected by either dynll phospho-peptide (Fig. 4D). Similar findings were observed in HeLa cells (Supplementary Fig. 1). This suggests that Ser-764 phosphorylation does not regulate the endocytic function of dynll.

To independently ask whether there is a role for Ser-764 in endocytosis, we performed a rescue experiment following depletion of dynll by siRNA. An siRNA sequence common to human dynl, II and III resulted in >90% depletion of dynll and a block in endocytosis of transferrin (data not shown and Fig. 4E). We aimed to rescue this phenotype by overexpressing rat GFP-dynll since the rat sequence varies in 6 positions from the human siRNA target region sequence and is resistant to the siRNA treatment. Ectopic expression of wild-type rat GFP–dynll completely restored endocytosis (Fig. 4E). In contrast, endocytosis was not restored in cells expressing GFP-α-tubulin. Consistent with the results obtained with the phospho-peptide strategy, endocytosis was completely restored in cells ectopically expressing either the phospho-deficient (Ser-764A/Thr-766A) or phospho-mimetic (Ser-764E/Thr-766E) forms of GFP-dynll (Fig. 4E). This indicates that Ser-764 phosphorylation is not required for the endocytic function of dynll. Thus, dynll–Ser-764 phosphorylation may be specifically involved in a mitosis-specific role that is independent of endocytosis.

3.5. Dynll localisation to the mitotic centrosome is not dependent on Ser-764 phosphorylation

Dynll has been implicated in centrosome cohesion [3] and cytokinesis [1,2,4,34,35]. The centrosome is a critical component for mitosis as it forms the poles of the mitotic spindle. Dynll localises to the interphase centrosome and participates in centrosome cohesion [3]. We now show by immunostaining with the dynll–Ser-764 phospho-specific antibody that dynll localises to the mitotic centrosome and is primarily in the phosphorylated form (Fig. 5A). As cells progress through mitosis (metaphase to anaphase/telophase) phospho-dynll remains associated with the centrosome until a late stage, cytokinesis (see below). Centrosomal localisation of phospho-dynll was not disrupted by the dynll759–775EE or dynll759–775AA peptides (Fig. 5B). Therefore Ser-764 phosphorylation appears not to target or tether dynll to the mitotic centrosome. Unfortunately, attempts to address this question by transfection with GFP-tagged dynll were unsuccessful since, in contrast to native dynll, none of our ectopically expressed proteins (wt. S764A or S764E; C-terminal GFP-tagged dynll, N- or C-terminal FLAG-tagged dynll) were associated with the centrosome possibly due to different expression levels, processing or subcellular distribution. Despite this, a GFP-tagged middle domain of dynll did localise at the centrosome as previously observed [3], supporting our findings that the PRD is not required for centrosome tethering. Thus, full length GFP-dynll does not always mimic the behaviour of endogenous dynamin in all roles.

3.6. Ser-764 phosphorylation is associated with dynll midbody localisation and cytokinesis

CaN and phospho–dynll localise to the midbody during cytokinesis [18] and both are required for the abscission stage of cytokinesis [1,2,4]. The phosphatase activity of CaN is specifically associated with completion of cytokinesis and dynll is one of its substrates [18]. Thus, we next asked whether this involves dynll–Ser–764 phosphorylation since phospho-dynll is enriched at the midbody 2.5 h after the onset of mitosis [18]. We now show that midbody localisation was unaffected by treatment with the phospho-deficient peptide (Fig. 6A). In contrast, phospho-dynll was undetectable at the midbody

Fig. 5. Ser-764 phosphorylation is not required for targeting dynll to the mitotic centrosome. (A) Phospho-dynll localises to the mitotic centrosome. Asynchronously growing HeLa cells were stained for endogenous phospho-dynll–Ser-764 (green) and total dynll protein (red). Representative cells from different stages of mitosis are shown, illustrating phosphorylated dynll initially at the mitotic centrosome and later at the midbody during cytokinesis. The position of phospho-dynll is shown with arrows (all panels). (B) Representative microscopy images of HeLa cells in metaphase treated in the absence and presence of the Dynll-AA and Dynll-EE peptide. Phospho-dynll–Ser-764 mitotic centrosome localisation is not disrupted by treatment with either peptide. γ-tubulin (red) served as an independent centrosome marker.
of cells treated with the phospho-mimetic peptide dynII\textsubscript{759–775}EE (Fig. 6A, upper panels). CaN and γ-tubulin midbody localisation was unaffected (Fig. 6B, lower panels). This suggests that phospho-dynII localization to the midbody is dependent in its phosphorylation status, while CaN localization to the midbody is not dependent on dynII. Again, full length GFP–dynII did not target the midbody when ectopically expressed in HeLa cells and therefore could not be used to address this question. The results suggest a role for Ser-764 phosphorylation in regulating dynII localisation during cytokinesis.

To test whether dynII phosphorylation is involved in cytokinesis, we examined multinucleation, which is a characteristic of failed cytokinesis. The phospho-mimetic dynII\textsubscript{759–775}EE peptide significantly increased multinucleation, indicating cytokinesis failure due to displacement of dynII rather than CaN (Fig. 6C). The phospho-deficient peptides from either dynII or dynI were without effect, suggesting that the observation is specific.

We initially chose the EE mutant to increase the net negative charge in this region since phosphorylation carries two negative charges while E mutants carry only one. Similar results were obtained with an identical dynII peptide wherein only the Ser-764 was mutated (Fig. 6D, peptides are named AT or ET to indicate that the Thr-766 is wild-type). This is consistent with the major role being ascribed only to the Ser-764 phosphosite.

![Figure 6](image_url)
We next aimed to confirm the link between dynII phosphorylation at Ser-764 and cytokinesis by an independent approach. Again, full length GFP-dynII did not target the midbody when ectopically expressed in HeLa cells. Therefore, it could not be used to address the question. However, analogous to endogenous dynII, the dynII–PRD region (GFP–dynII–PRD) when ectopically expressed, localised to the midbody. Overexpression of this form of dynII containing phospho-mimetic mutations (S764E or S764E/T766E) caused a specific increase in multinucleation compared to the equivalent phospho-deficient (S764A or S764A/T766A) and wild-type forms (Fig. 6E). This confirms that Ser-764, not Thr-766, is the phosphosite that is associated with cytokinesis in a cellular context. The results suggest that dynII phosphorylation is important for cytokinesis.

4. Discussion

CaN triggers events late in mitosis that appear to trigger cellular abscission at the end of cytokinesis [18]. This involves dephosphorylation of dynII and potentially many other CaN substrates at the midbody. DynII has recently been shown to be mitotically phosphorylated on Ser-764 and/or Thr-766 [19]. We have now significantly extended these observations by showing that dynII is phosphorylated exclusively at Ser-764 specifically during mitosis and that this phosphosite appears to be involved in cytokinesis rather than endocytosis or centrosome cohesion. Thus, phosphorylation switches the function of dynII from a traditional to a novel role. Our findings strongly suggest that not just CaN but CaN-mediated dynII dephosphorylation is an essential molecular component of cytokinesis, as CaN alone at the midbody is insufficient to complete cytokinesis in the absence of dynII. To our surprise, dynII phosphorylation did not affect other known biological roles for dynII such as CME or bulk endocytosis, suggesting that phosphorylation is specific to its cytokinesis role. This is surprisingly analogous to dynl, the phosphorylation of which switches the function of dynl from clathrin-dependent synaptic vesicle endocytosis to activity-dependent bulk endocytosis in neurons [6,11,12]. Our findings suggest that phosphorylation of dynamin directs it to a new role in cytokinesis that is independent of its well-characterised role in classical endocytosis. This is the first report of directly validated dynII phosphorylation on serine or threonine residues. Mass spectrometry analysis suggests that there is only a single phosphosite in dynII at the onset of mitosis, although non-detectable minor sites may exist, strongly contrasting to multi-site phosphorylation of dynI and III in neurons [9]. Phosphorylation at Ser-764 was independently confirmed by three approaches: MS analysis on dynII in rat lung, HeLa cells and using phosphosite-specific antibodies. Neither dynl protein nor phosphorylation of its abundant site at Ser-774 was detected with any approach. No dynII phosphorylation on tyrosine was detected in our study, but this cannot be ruled out due to its lability. In contrast, other studies suggest a role for tyrosine phosphorylation at the Golgi during vesicle trafficking [17], which is normally stopped during mitosis but resumes during cytokinesis [36–38]. Given that dynl is phosphorylated at 7 sites and dynII on 3 sites in synaptosomes [9], it is perhaps surprising that we found only 1 phosphosite in dynII in rat lung or HeLa cells within methodological detection limits, despite our extensive efforts to search for more. Mono-phosphorylation of a protein in cells is far less common than multi-site phosphorylation. However, among the phosphosites identified in the other classical dynamins, only two (Ser-347/778) are conserved in the sequence of dynl (as dynII-347 and Ser-764). Ser-347 does not have the amino acid consensus motif required for mitotic phosphorylation, resides in an acidic amino acid environment (IEGSDQVD) and is the lowest abundance site in dynl. Therefore, it is not surprising that its phosphorylation in rat lungs or HeLa cells was undetectable.

Two large-scale phosphoproteomic studies reported that dynII from mitotic cells is phosphorylated on a single tryptic phosphopeptide (dynII31–769), in agreement with our work [22,23]. However, these studies listed all four phosphorylatable sites (S/T/Y) between Ser-761 and Thr-766 as phosphorylated in HeLa cells (the tryptic peptide is EALNIIDSTSVSTPPDDTDWLSASSHPSPQPSR–31–769 sites are underlined; Ser-764 is in bold). All four phosphosite results were subsequently transferred to a public phosphosite database (www.phosphosite.org). We believe that these sites in dynII were prematurely given the status of genuine phosphorylation sites. Ser-764 is the only genuine phosphorys and evidence supports this conclusion (see Supplementary information for a detailed discussion).

Our phosphosite assignment has been validated by detection of phospho-dynII–Ser-764 using phospho-specific antibodies to this site both after in vitro phosphorylation and in cells. Additionally, the recombinant form of dynII where this site was mutated to Ala lost over 80% of its in vitro phosphorylation by cdk, indicating that Ser-764 is the main phosphosite in vitro as well as in cells. Finally, endogenous dynII did not localise to the midbody in cells treated with the dynII phospho-mimetic peptide whereby only the Ser-764 was mutated to Glu and the Thr-766 was wild-type (data not shown), indicating that the functional role is only for Ser-764 in cells. Note that in vitro phosphosites are only indicative of phosphosites that might be found in cells but cannot be used as evidence for the presence or not of that site in cells.

The mitotic phosphorylation site in dynII clearly did not play an essential role in CME or bulk endocytosis as determined by two independent strategies of peptide competition and siRNA depletion/rescue. For example, we found that both phospho-deficient and phospho-mimetic GFP–dynII constructs could completely rescue CME. This is consistent with the known ability of such wild-type constructs to rescue endocytosis [1] and suggests that Ser-764 is not required. This observation is consistent with recent studies on mitotic endocytosis. At least three other endocytic proteins are phosphorylated upon mitotic entry: epsin, eps15, and amphiphysin I. While their phosphorylation in nerve terminals appears to be related to inhibiting their role in endocytosis until they are dephosphorylated by calcineurin, they were originally proposed to be mitotically phosphorylated in order to inhibit endocytosis during mitosis. However, a recent study suggests that endocytosis is not specifically inhibited in mitotic cells [39]. This suggests that mitotic phosphorylation of key endocytic proteins may be a common mitotic event with a role in functions other than endocytosis. In support of this idea, epsin induces membrane curvature for proper mitotic spindle organisation and this role is independent of its endocytic function and mitotic phosphorylation [40]. In an analogous manner to dynl–Ser-764, it is also possible that these other mitotically phosphorylated endocytic proteins might prove to be CaN targets during cytokinesis.

Phosphorylation of dynII at Ser-764 in the phosopho-box region by cdk1 coincided with its localisation to the centrosome where it resided until late in mitosis. It is unclear whether there is any role for phosphorylation here since we observed no specific centrosome defects in dynII-depleted cells (data not shown). Like dynII, many other proteins are phosphorylated upon mitotic entry and locate to the mitotic centrosome [41]. This localisation is thought to be driven by phosphorylation. However, our data suggest that Ser-764 phosphorylation is not required for targeting dynII to the mitotic centrosome. This is consistent with previous findings demonstrating that the dynamin middle domain binds the centrosome component, γ-tubulin [3]. We confirmed that the middle domain of dynII, when fused to GFP, targets the centrosome, while the full length protein did not. This suggests that full length dynII constructs (GFP or Flag-tagged) do not fully mimic all of the biological roles of endogenous dynII. Many cytokinesis proteins, such as Cep55, reside at the centrosome during mitosis without any known centrosome role [41,42]. The role of phospho-dynII on the mitotic centrosome is unclear; however, one possibility is that it is an intermediate “holding” location for certain proteins that are involved in later mitotic events. In contrast to phospho-dynII, CaN does not locate to
the mitotic centrosome [18]. Therefore, the centrosome location of phospho-dynII may provide a mechanism of physically separating it from CaN at this point in mitosis. For example, Ca2+ activates CaN and acts at a second point in mitosis, prior to cytokinesis during anaphase progression [43,44]. The lack of CaN/dynII colocalisation at this stage may conceivably protect dynII from being prematurely dephosphorylated and hence triggering mitotic exit prematurely.

Collectively, our observations highlight a molecular signalling pathway that is associated with completion of the abscission stage of cytokinesis that involves Ca2+, CaN and phospho-dynII–Ser–764. This is strikingly analogous to dyn where an analogous signalling pathway and phosphosites regulate the switching of the standard synaptic vesicle endocytosis mode to a second unique bulk endocytosis role [6,11,12]. Our two recent discoveries demonstrate that CaN activity [18] and dynII [1,2,4,34,35] are required for the completion of the abscission stage of cytokinesis. Here, we now show that it is the phosphorylated form of dynII that is a key part of the cytokinosis mechanisms downstream of CaN rather than playing a role in endocytosis. We found phospho-dynII is concentrated at the midbody at the time of cytokinesis [18]. In contrast to the centrosome, this localisation was disrupted by the phospho-mimetic peptides but not by phospho-deficient peptides. It is possible that Ser–764 phosphorylation is a signal to target or tether dynII to the midbody during cytokinesis with its dephosphorylation releasing it into partnership with a specific, as yet unidentified, phospho-dependent binding partner. The phospho-mimetic peptides specifically induced cytokinesis failure, as indicated by a significant increase in multinucleation, a primary characteristic of failed cytokinesis. The dynII phospho-mimetic peptides had no effect on CaN midbody localisation; thus, CaN alone at the midbody is not sufficient to complete cytokinesis. Instead, these findings collectively suggest that CaN-mediated dephosphorylation of dynII–Ser–764 is associated with successful completion of cytokinesis. We also found that a GFP–dynII–PRD construct targeted the midbody and that cells expressing GFP–dynII–PRD phospho-mimetic mutants, not phospho-deficient mutants, increased cytokinesis failure. Overall, our data show that dynII is mitotically phosphorylated primarily on a single site, Ser–764 by cdk, and that this phosphorylation site is not involved in endocytosis or centrosome cohesion but rather in the regulation of cytokinesis.

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All authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbrc.2010.12.018.

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