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A Role for Tumor Protein TPD52 Phosphorylation in Endomembrane Trafficking During Cytokinesis

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Abstract

Tumor Protein D52 is expressed at high levels in exocrine cells containing large secretory granules where it regulates Ca²⁺-dependent protein secretion; however, D52 expression is also highly induced in multiple cancers. The present study investigated a role for the Ca²⁺-dependent phosphorylation of D52 at the single major phospho-acceptor site serine 136 on cell division. Ectopic expression of wild type D52 (D52wt) and the phosphomutants serine 136/alanine (S136A) or serine 136/glutamate (S136/E) resulted in significant multinucleation of cells. D52wt and S136/E each resulted in a greater than 2 fold increase in multinucleated cells compared to plasmid-transfected controls whereas the S136/A phospho-null mutant caused a 9 fold increase in multinucleation at 48 h post-transfection. Electron microscopy revealed D52 expression induced a marked accumulation of vesicles along the mid-line between nuclei where the final stages of cell abscission normally occurs. Supporting this, D52wt strongly colocalized on vesicular structures containing the endosomal regulatory protein vesicle associated membrane protein 81 (VAMP 8) and this colocalization significantly increased with elevations in cellular Ca²⁺. As VAMP 8 is known to be necessary for the endo-membrane fusion reactions that mediate the final stages of cytokinesis, these data indicate that D52 expression and phosphorylation at serine 136 play an important role in supporting the Ca²⁺-dependent membrane trafficking events necessary for cytokinesis in rapidly proliferating cancer cells.

Keywords

Tumor Protein D52; cytokinesis; Ca²⁺-dependent phosphorylation; VAMP 8

¹**Abbreviations:** TPD52, Tumor Protein D52; VAMP 8, vesicle associated membrane protein 8; SNARE, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor; LAMP1, lysosome associated membrane protein; HA, hemagglutinin; pfu, plaque forming units;

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Introduction

Tumor protein D52¹ (TPD52) (also known as CRHSP-28) is a highly charged, acidic, cytosolic and peripheral membrane protein [1] found to be overexpressed in multiple cancers including lung [2-3], prostate [4-7], colon [8], ovary [9], breast [10-13], B cell malignancies [14] and tumor-derived cell lines [15]. D52 is the founding member of a small protein family which includes D52, D53 and D54. These proteins all contain a coiled-coil motif that mediates homomeric and heteromeric interactions among family members [15]. TPD52 family members have been shown to interact with a number of proteins; MAL2, an integral membrane protein localized to lipid rafts in epithelial cells [16] that is also known to be overexpressed in certain cancers [13], annexin VI, a Ca²⁺-regulated phospholipid binding protein [17], synaptobrevin 2 and syntaxin 1, members of the soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor¹ (SNARE) family [18] and 14-3-3, a multifunctional cytoplasmic, negative regulator of the G2-M transition phase in mitosis [19].

Aside from the high expression reported in cancer cells, D52 is normally expressed at high levels in exocrine cells that contain large secretory granules [20,21] where it was shown to directly regulate Ca²⁺-dependent digestive enzyme secretion from pancreatic acini [1]. Recently we reported that when ectopically expressed in CHO-K1 cells, D52 localized to an endo-lysosomal secretory pathway marked by a distinct colocalization with adaptor protein 3, endocytosed dextran, Rab 27A, vesicle associated membrane protein 7 and lysosomal membrane protein 1¹ (LAMP1) [22]. D52 was also shown to undergo Ca²⁺-dependent phosphorylation at serine 136 [21,22]. Further, site specific mutations of serine 136 indicated that D52 directly regulates LAMP1 exocytosis in a phosphorylation-dependent manner. Notably, mutation of serine 136 to alanine abrogated the Ca²⁺-stimulated accumulation of LAMP1 at the plasma membrane whereas phosphomimetic mutants constitutively induced LAMP1 plasma membrane accumulation independent of elevated intra-cellular Ca²⁺ [22].

Aside from the effects of D52 on membrane trafficking and exocytosis, the highly induced expression of D52 in multiple cancers has prompted investigation of its potential role in cell growth. Boutrous and Byrne [15] showed that D53 is a cell-cycle regulated protein that is expressed maximally at the G2-M transition phase. Further, transient expression of either D52 or D53 in MDA-MB-231 breast cancer cells caused abnormalities in mitosis resulting in the aberrant accumulation of multinucleated cells and increased cell death. Moreover, immunofluorescence analysis revealed endogenous D52 protein expression increases during mitosis implicating D52 as a cell cycle regulated protein. Other studies indicate that overexpression of D52 in 3T3 fibroblasts induces cell transformation as evidenced by growth in soft agar and tumor propagation when implanted in mice [23].

Given the effects of D52 phosphorylation on endosomal and lysosomal membrane trafficking, the present study examined a potential role for D52 phosphorylation in regulating cell division. Results revealed that a phospho-null mutation at serine 136 strongly inhibits cytokinesis. Because cytokinesis is known to be regulated by acute changes in cellular Ca²⁺ [24], these findings suggest that D52 phosphorylation plays an important role in the vesicle trafficking events that direct cell abscission.

¹Abbreviations: TPD52, Tumor Protein D52; VAMP 8, vesicle associated membrane protein 8; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; LAMP1, lysosome associated membrane protein; HA, hemagglutinin; pfu, plaque forming units;

Materials and Methods

Reagents

Anti-D52 polyclonal antibodies were previously described [20]. VAMP 8 antibodies were previously described [25]. Hemagglutinin¹ (HA) mouse monoclonal antibody (cat. no. 2367) was purchased from Cell Signaling Technology (Danvers, MA). Goat serum, Triton X-100 and cold-water fish skin gelatin were purchased from Sigma-Aldrich (St. Louis, MO). ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI), fetal bovine serum, DMEM and TrypLE Express were purchased from Invitrogen (Carlsbad, CA). HAMS F12K and proline were purchased from Mediatech, Cellgro (Manassas, VA). Bovine serum albumin was purchased from Roche Diagnostics GmbH (Switzerland). TransIT-CHO-K1 and Endotoxin removal kits were purchased from Mirus Biotechnology (Madison, WI). DNA Maxi-prep kit was purchased from Promega (Madison, WI). Formaldehyde and glutaraldehyde were purchased from Electron Microscopy Sciences (Hatfield, PA). The AdEasy kit for adenoviral production was purchased from Agilent (Stratagene) (Santa Clara, CA). Fast Link DNA-ligation kit was purchased from Epicentre Biotechnologies (Madison, WI). Chinese hamster ovary (CHO-K1) cells were obtained from American Type Culture Collection (Manassas, VA). CHO-CAR cells were a generous gift from Dr. JM Bergelson at Children's Hospital of Philadelphia [26].

Tissue Culture

CHO-K1 cells were grown, transfected and processed for immunofluorescence as previously described [22]. CHO-CAR cells were maintained in DMEM supplemented with 10% FBS. AD-293 cells were grown in DMEM supplemented with 10% FBS. All media contained penicillin, streptomycin, and gentamicin. Stock cultures were maintained in a 37°C and 5% CO₂ humidified atmosphere and were passaged by using TrypLE Express. Media was changed the day after seeding and every 3–4 days thereafter. Experiments were conducted on confluent cell monolayers. All cell types were used between passages 4 and 27. CHO-CAR cells were seeded in six-well plates at 70% confluency and transfected with 10⁶ or 10⁷ pfu/ml adenoviral D52wt or GFP control for 1 h. Adenovirus was washed out and cells were incubated in a 37°C and 5% CO₂ humidified atmosphere for 48 h before treatment.

Subcloning

For adenoviral expression studies in CHO-CAR cells, the coding region of human D52wt was subcloned into the pShuttle-CMV vector (Stratagene) containing an NH₂-terminal HA-tag with SalI and EcoRV restriction sites using 5'-GTCGGTCGACCCACCATGGGCTACCCATACGACGTCC-3' (sense) and 5'-GCGATATCGATGATGATGCACGTGTAG-3' (antisense). Virus production of the HA-tagged human D52 was performed via the manufacturer's instructions for the Ad-Easy kit.

Quantification of immunofluorescence images

Analysis of multinucleated cells was conducted by counting the number of CHO-K1 cells transfected with D52wt, S136/A, S136/D or non-transfected with ≥ 2 nuclei in 10 individual optical fields obtained with a 60× objective for each treatment group. Data were quantified from three independent experiments.

Electron microscopy

CHO-CAR cells virally transfected with D52wt were fixed for 1 h at RT in 2% formaldehyde and 2.5% glutaraldehyde in 1× phosphate buffer (PB), pH 7.4. Tissue was treated with 4% osmium tetroxide in 1× PB, taken through an ethanol series dehydration and embedded in durcupan. Sections (60–90 µm) were placed on 200 mesh thin bar grids and

tissue was stained in Reynolds lead citrate and uranyl acetate. Sections were evaluated with a Philips CM 120 electron microscope. Captured images were converted to TIFF files and edited for publication in Adobe Photoshop.

Results and Discussion

D52 phosphorylation modulates cytokinesis in CHO-K1 cells

Boutros and Byrne [12] previously reported that the TPD52 family member, D53, is differentially expressed throughout the cell cycle with highest levels detected at the G2/M transition. Moreover, inducing the constitutive expression of wild type D52 throughout mitosis disrupted cell division resulting in a multinucleated phenotype in MDA-MB-231 breast cancer cells. Given recent evidence that D52 undergoes Ca^{2+} -regulated phosphorylation at serine 136 [21,22] and further that D52 phosphorylation modulates membrane trafficking within a lysosome- and endosome-related secretory pathway in CHO-K1 cells [22], we investigated a potential role for D52 phosphorylation in the membrane trafficking events that mediate the final stages of cytokinesis.

Transient expression of HA-tagged D52 phospho-null mutant serine 136/alanine (S136/A) in CHO-K1 cells resulted in a marked accumulation of multinucleated cells (Fig 1A). As seen for endogenous D52 in differentiated polarized epithelia [27,28], when expressed in CHO-K1 cells, D52 and D52 phosphomutants were abundant on perinuclear vesicular structures. Further, in multinucleated cells, D52 was clearly concentrated at the mid-line between the juxtaposed nuclei (Fig 1A). We previously reported that CHO-K1 cells express low levels of endogenous D52 [22]. Thus, for comparison, D52 localization was analyzed in HeLa cells which, consistent with multiple cancer cell lines, normally express high levels of D52 (Fig 1B). Endogenous D52 localization in multinucleated cells was essentially identical to that seen for overexpressed D52wt exhibiting significant immunoreactivity on vesicles that are accumulated along the mid-line between nuclei (Fig 1B).

The number of multinucleated cells expressing wild type D52 (D52wt), S136/A or serine 136/glutamate (S136/E) phosphomutants was quantified at 24 and 48 h post-transfection and compared with control cells expressing either empty vector or treated with transfection reagents alone (non-transfected). Following D52wt expression, 17% of cells were multinucleated at 24 h with a reduction to 9% at 48 h (Fig 2). These levels were greater than 2 fold higher than vector-transfected and nontransfected control cells which remained below 4% at 24 and 48 h. An explanation for the reduction in multinucleation at 48 h in D52wt cells is unclear but may be related to enhanced rates of apoptosis by 48 h. This is uncertain however, as expression of the S136/E phosphomimetic mutant induced approximately 10% multinucleation at both 24 and 48 h. In contrast, S136/A mutants exhibited a greater than 9 fold increase in multinucleation compared to controls at 24 and 48 h, respectively. The enhanced multinucleation of cells expressing S136/A was not due to an alteration of D52 expression as protein levels remained relatively constant following expression D52wt or the phospho-mutants (Fig 2B).

D52 localizes to VAMP 8-positive endosomal vesicles during cytokinesis

Cell abscission during the final stages of cytokinesis is dependent on the polarized delivery of endocytic vesicles to the central mid-body region between the nuclei [29,30]. D52 is known to regulate endosome- and lysosome-related membrane trafficking [22,28], and further its effects on the exocytic process are dependent on its Ca^{2+} -dependent phosphorylation at S136 [1,22]. Thus, results that the S136/A phospho-null mutant strongly inhibited cytokinesis suggested that D52 phosphorylation acutely regulates endosomal vesicle trafficking to complete cell abscission. To further examine the effects of D52wt

expression on cell structure, the protein was adenovirally expressed in CHO-CAR cells which provided a greater than 95% infection efficiency (Fig 3). Electron micrographs of binucleated cells revealed that D52 expression caused a large accumulation of tubule-vesicular structures along the mid-line between the nuclei. These results are consistent with previous studies indicating that inhibition of the late stages of cytokinesis results in the accumulation of endosome-derived vesicles at the central mid-body region and the retraction of the previously-formed cleavage furrows that originate at the plasma membrane [29–31].

The membrane fusion events that complete the final stages of cell abscission are regulated by the soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) proteins in both mammalian cells and developing zebra fish embryos [32,33]. Moreover, membrane fusion during cytokinesis was shown to be triggered by the acute elevation of intracellular Ca^{2+} [24]. SNARE proteins are known to specifically regulate the terminal steps of cytokinesis along the mid-body but are not essential for the initial cleavage furrow invagination [29,30,32,33]. In particular, a dominant negative construct of vesicle associated membrane protein 8 (VAMP 8) was shown to inhibit completion of cytokinesis leading to increased binucleation in NRK cells [32]. Because VAMP 8, like D52, is known to localize to the endo/lysosomal compartment, we investigated a potential colocalization of these molecules in dividing cells expressing D52wt (Fig 4). D52 and VAMP 8 immunoreactivity significantly colocalized on vesicular structures throughout the cytoplasm and were especially abundant between juxtaposed nuclei along the mid-line in multinucleated cells. Quantitative analysis of multiple reconstructed z-series images indicated 50% of D52-positive voxels colocalized with VAMP 8 under basal conditions (Fig 4B). Because it was not possible to synchronize a population of cells during cytokinesis to evaluate the effects of changing intracellular Ca^{2+} concentrations, we treated cells with Ca^{2+} ionophore. Acute elevation of cellular Ca^{2+} significantly increased the colocalization of D52 and VAMP8 by 25% at 5 min.

Results that the D52 S136/A phospho-null mutation strongly induced multinucleation suggests that the Ca^{2+} -stimulated phosphorylation of D52 is a key event in regulating VAMP 8-positive endomembrane trafficking during cytokinesis. The enhanced multinucleation of cells overexpressing the D52wt or S136/E mutant are similar to the effects of D52 on LAMP1 exocytosis where high expression of D52wt or the phosphomutants begin to function independently of elevated cellular Ca^{2+} [22]. The loss of Ca^{2+} - and phospho-regulation seen with high levels of expression suggest that phosphorylation/dephosphorylation alters the affinity of D52 for interacting with additional cellular regulatory proteins. D52 and other TPD52 family members have been shown to interact with a number of proteins involved in membrane trafficking (see Introduction) however the impact of phosphorylation on these interactions or their significance in cytokinesis will require further investigation.

Conclusions

D52 expression is well known to be enhanced in multiple cancers and cancer cell lines [2–15]. The present data indicate that these enhanced levels of D52 likely act to support the high rates of cell division in cancers. Furthermore, these findings support that the Ca^{2+} -dependent phosphorylation of D52 at serine 136 plays a mechanistic role in regulating VAMP 8-positive endosomal vesicle trafficking necessary for cell abscission during cytokinesis.

Research Highlights

1. D52 localizes to vesicles at the mid-line in multinucleated cells.

2. Expression of a D52 serine136/alanine mutant induced multinucleation of cells.
3. D52 localizes to VAMP 8 positive endosomes necessary for cytokinesis.
4. The Ca²⁺-dependent phosphorylation of D52 regulates cytokinesis.

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Figure 1. D52 localizes to vesicles accumulated at the mid-line in binucleated cells

(A) CHO-K1 cells transfected with S136/A were fixed in 2% formaldehyde and labeled with antibodies for D52 or HA tag, both at 1:100. Immunoreactivities were detected by using Alexa Fluor 488-conjugated- anti-rabbit IgG (1:500) or –anti-mouse IgG (1:250) respectively. (B) HeLa cells were fixed in 2% formaldehyde and labeled with antibodies for D52 as in (A). Nuclei were labeled with DAPI. Each image is a reconstructed z-series obtained by brightfield microscopy. All images are representative of multiple determinations performed on 4 separate tissue preparations. Bars, 13 μ M.

Figure 2. The D52 phospho-null mutant strongly induces multinucleation of cells

(A) Cells expressing D52wt, S136/A or S136/E were compared at 24 and 48 h post-transfection (T) with control cells expressing empty vector (control plasmid) or treated with transfection reagents alone (NT). Using a 60 $\times$ objective, a total of 10 fields of view in 4 separate tissue preparations from different days were scored. (B) Immunoblot of D52 (1:1000) in lysates (40 μ g) from transfected CHO-K1 cells showing equal expression levels of D52 mutants. Note in all cases D52 and D52 mutant expressing cells had significantly ($p > 0.04$) higher levels of multinucleated cells then respective controls.

Figure 3. Electron microscopic analysis of binucleated cells expressing D52

CHO-CAR cells were infected with D52wt adenovirus. Cells were embedded in durcupan, sectioned, counterstained and evaluated using a Philips CM 120 electron microscope. Note the large accumulation of tubulo-vesicular structures at the mid-line between nuclei. Image is representative of multiple determinations performed on 2 separate tissue preparations. Bar, 2 μ M.

Figure 4. D52 strongly colocalizes with VAMP 8-positive endosomes which mediate plasma membrane remodeling during cytokinesis

(A) CHO-CAR cells transfected with D52wt were treated as control or with [2 μ M] ionomycin and then fixed in 2% formaldehyde and labeled with antibodies for HA tag or VAMP 8, both at 1:20. Immunoreactivities were detected using Alexa Fluor 488-conjugated- anti-rabbit IgG (1:100) and Alexa Fluor 546-conjugated –anti-mouse IgG (1:100) respectively. Nuclei were labeled with DAPI. Each image is a reconstructed z-series obtained by brightfield microscopy. (B) Quantitative analysis of D52 voxel colocalization with VAMP 8 acquired from multiple z-series images from three separate acinar cell

preparations. Data are mean and S.E. ($n = 17$). Bars, $7\mu\text{m}$. All images are representative of multiple determinations performed on 3 separate tissue preparations.