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A role for tumor protein TPD52 phosphorylation in endo-membrane 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 Ca^{2+}-dependent protein secretion; however, D52 expression is also highly induced in multiple cancers. The present study investigated a role for the Ca^{2+}-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 8 (VAMP 8) and this colocalization significantly increased with elevations in cellular Ca^{2+}. 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 Ca^{2+}-dependent membrane trafficking events necessary for cytokinesis in rapidly proliferating cancer cells.

1. Introduction

Tumor protein D52 (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 Ca^{2+}-regulated phospholipid binding protein [17], synaptobrevin 2 and syntaxin 1, members of the soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (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 Ca^{2+}-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 1 (LAMP1) [22]. D52 was also shown to undergo Ca^{2+}-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 Ca^{2+}-stimulated accumulation of LAMP1 at the plasma membrane whereas phosphomimetic mutants constitutively induced LAMP1 plasma membrane accumulation independent of elevated intra-cellular Ca^{2+} [22].

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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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. Boutros 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 Ca^{2+} [24], these findings suggest that D52 phosphorylation plays an important role in the vesicle trafficking events that direct cell abscission.

2. Materials and methods

2.1. Reagents

Anti-D52 polyclonal antibodies were previously described [20]. VAMP 8 antibodies were previously described [25]. Hemagglutinin (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 Ad-Easy kit for adenoviral production was purchased from Agilent (Strategene) (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].

2.2. 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% CO_2 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 10^6 or 10^7 pfu/ml adenoviral D52wt or GFP control for 1 h. Adenovirus was washed out and cells were incubated in a 37 °C and 5% CO_2 humidified atmosphere for 48 h before treatment.

2.3. Subcloning

For adenoviral expression studies in CHO-CAR cells, the coding region of human D52wt was subcloned into the pShuttle-CMV vector (Strategene) containing an NH_2-terminal HA-tag with SalI and EcoRV restriction sites using 5′-GTCGGTCGACCCACCATGGGCTAC CCATACGACGTCC-3′ (sense) and 5′-GCGATATCGATGATGATGCACG TGTAG-3′ (antisense). Virus production of the HA-tagged human D52 was performed via the manufacturer's instructions for the Ad-Easy kit.

2.4. 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 in10 individual optical fields obtained with a 60× objective for each treatment group. Data were quantified from three independent experiments.

2.5. 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 durcapan. 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.

3. Results and discussion

3.1. 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