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Phosphorylation State of Ser¹⁶⁵ in α -Tubulin is a Toggle Switch That Controls Proliferating Human Breast Tumors

Ela Markovskiy³, Elisa de Stanchina⁴, Aryeh Itzkowitz¹, Adriana Haimovitz-Friedman³, Susan A. Rotenberg^{1,2,*}

¹Department of Chemistry & Biochemistry, Queens College of The City University of New York

²Graduate Center of The City University of New York

³Department of Radiation Oncology of Memorial Sloan-Kettering Cancer Institute

⁴Antitumor Assessment Core Facility of Memorial Sloan-Kettering Cancer Institute.

Abstract

Engineered overexpression of protein kinase C α (PKC α) is known to phosphorylate Ser¹⁶⁵ in α -tubulin resulting in stimulated microtubule dynamics and cell motility, and activation of an epithelial-mesenchymal transition (EMT) in non-transformed human breast cells. Here it is shown that endogenous phosphorylation of native α -tubulin in two metastatic breast cell lines, MDA-MB-231-LM2–4175 and MDA-MB-468 is detected at PKC phosphorylation sites. α -Tubulin mutants that simulated phosphorylated (S165D) or non-phosphorylated (S165N) states were stably expressed in MDA-MB-231-LM2–4175 cells. The S165D- α -tubulin mutant engendered expression of the EMT biomarker N-cadherin, whereas S165N- α -tubulin suppressed N-cadherin and induced E-cadherin expression, revealing a ‘cadherin switch’. S165N- α -tubulin engendered more rapid passage through the cell cycle, induced shorter spindle fibers and exhibited more rapid proliferation. In nude mice injected with MDA-MB-231-LM2–4175 cells, cells expressing S165N- α -tubulin (but not the S165D mutant) produced hyper-proliferative lung tumors with increased tumor incidence and higher Ki67 expression. These results implicate the phosphorylation state of Ser¹⁶⁵ in α -tubulin as a PKC-regulated molecular switch that causes breast cells to exhibit either EMT characteristics or hyper-proliferation. Evaluation of genomic databases of human tumors strengthens the clinical significance of these findings.

Keywords

Protein kinase C; EMT; cadherin switch; cell cycle; spindle fibers; biomarker

*To whom correspondence should be addressed: Susan.Rotenberg@qc.cuny.edu.

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1. INTRODUCTION

Protein kinase C (PKC) is activated by growth factor- and chemokine-stimulated pathways and, in advanced human breast cancer, by the erbB2 and interleukin-8 pathways to produce cancer-related phenotypes such as invasion and metastasis (1, 2). The conventional isoform PKC α serves as a major signaling node in breast cancer tumor-initiating stem cells that drives epithelial-mesenchymal transition (EMT) and the acquisition of metastatic potential (3, 4). Furthermore, inhibition of PKC α decreases intravasation and lung seeding of metastatic breast cells in an animal model (5). These findings reinforce the ongoing status of PKC α as a powerful therapeutic target for controlling metastatic breast cancer (6–9).

The notion of a toggle switch that reciprocally controls cell migration and proliferation is emerging as a general principle in developmental biology, as shown with the multicellular organism *C. elegans* where parallels have been drawn to various cancer cell models (10, 11). Switching between migration/invasion and proliferation can occur as the result of intracellular signaling pathways as well as environmental cues (12–16). Earlier studies suggested that a signaling pathway mediated by PKC α is instrumental in this phenomenon. Thus, overexpression of PKC α in non-transformed, non-motile MCF-10A human breast cells (17) led to the acquisition of motile behavior, absence of cell-cell contacts, and loss of E-cadherin protein to undetectable levels. These EMT-associated events were accompanied by suppressed proliferation and attenuated passage through the cell cycle. A more detailed mechanism by which PKC α causes these opposing effects on motility and proliferation remained undetermined until identification could be made of a relevant PKC substrate.

By using the traceable kinase method, we identified α -tubulin as a substrate of PKC α of MCF-10A cells (18, 19). To determine the site of phosphorylation in α -tubulin, inspection of its primary sequence revealed four potential PKC consensus sites. Mutation of each site to an Asp (D) residue was performed to simulate phosphorylation. A Ser $\rightarrow$ Asp mutation at Ser¹⁶⁵ was the only site found to engender motility in normally non-motile MCF-10A cells and therefore recapitulated a known effect of PKC. Under conditions of PKC activation, endogenous α -tubulin underwent intracellular phosphorylation that could be blocked either by expression of the phosphorylation-resistant mutant S165N- α -tubulin, or PKC inhibitor *bis*-indolylmaleimide. Therefore, Ser¹⁶⁵ in α -tubulin was judged to be a major PKC target site having functional significance. Subsequent studies with MCF-10A cells demonstrated that expression of S165D- α 6-tubulin prolonged the microtubule growth phase by 3-fold, whereas the corresponding S165N mutant did not (20). Therefore, the phosphorylation state of Ser¹⁶⁵ in α -tubulin serves as a means to control microtubule dynamics. Post-translational modifications of α -tubulin are known to have pronounced effects on microtubule dynamics that give rise to disease states, and can therefore suggest meaningful therapeutic approaches for controlling cancer (21–23).

In human breast cancer cells, such as MDA-MB-231 cells, that are intrinsically metastatic and thereby motile in vitro, expression of phosphorylation-resistant S165N- α -tubulin blocked cell motility (19) and dramatically suppressed microtubule dynamics (20), whereas the S165D mutant had little or no effect on these events presumably due to pre-existing phospho- α -tubulin. These findings suggested a model in which phosphorylation of Ser¹⁶⁵

and its blockade of phosphorylation counteract each other with respect to microtubule dynamics and cell movement of human breast cells. To further develop this model in the present study, Ser¹⁶⁵ phosphorylation of α -tubulin is investigated for its impact on expression of molecular markers associated with EMT, and on events related to proliferation of breast cancer cells in monolayer and *in vivo*. Our findings implicate Ser¹⁶⁵ phosphorylation as a switch that controls expression of EMT markers and rate of proliferation.

2. MATERIALS AND METHODS

2.1 Materials

Cell culture reagents, antibiotics, and Lipofectamine 3000 were purchased from Life Technologies (Carlsbad, CA) except where noted. Immunochemical reagents (PKC substrates antibody, erbB2, E-cadherin, N-cadherin, vimentin, SLUG, ZEB1, myc-tag and β -actin) were purchased from Cell Signaling Technologies, Sigma-Aldrich (α -tubulin antibody, DM1A), Abcam (Ki67), and Santa Cruz Biotechnology (anti-rabbit secondary antibodies, anti-mouse IgG-agarose beads, A/G-agarose beads) (Santa Cruz, CA). Protease inhibitor and phosphatase inhibitor cocktails were obtained from Bimake.com (Houston, TX). Hematoxylin and eosin counterstains and the DAB detection system were purchased from Ventana Medical Systems. Premo™ FUCCI Cell Cycle Sensor, Tubulin Tracker, and bis-indolylmaleimide were obtained from Thermo-Fisher (Waltham, MA).

2.2 Methods

2.2.1 Cell culture.—MDA-MB-468 cells and Luc-MDA-MB-231-LM2–4175 cells were kindly provided by Dr. J. Massague (Memorial Sloan-Kettering Cancer Institute).

Authentication of each cell line showed a 100% match with cells in the ATCC database by the human-specific PowerPlex16HS method (Genetica, Burlington, NC). Cells were cultured on 10-cm Falcon dishes in DMEM (ATCC) containing 10% fetal bovine serum, 1% penicillin/streptomycin, and fungizone (0.25 μ g/ml). Cells were incubated at 37°C and 5% CO₂ and maintained in culture for 2–3 months. Sub-lines established by stable transfection of MDA-MB-231-LM2–4175 cells were tested for *Mycoplasma* within one month prior to injection into mice. The test was performed with MycoAlert Plus (Lonza) at the Antibody and Bioresource Core Facility of MSKCC.

2.2.2 Stable transfection with α -tubulin single-site mutants.—Construction of S165D and S165N mutants of α -tubulin was previously described (19), and the mutations were confirmed by DNA sequencing (Macrogen, Inc.). MDA-MB-231-LM2–4175 cells stably expressing the luciferase gene and neomycin resistance were transfected with a plasmid encoding the phospho-mimetic of S165 (S165D) in α 6-tubulin (TUBA1C) or phosphorylation-resistant mutant (S165N) along with a hygromycin-resistant marker (Clontech) using Lipofectamine 3000. Stable transfectants were isolated under selection with 500 ng/ml hygromycin (Life Technologies). Resulting clones were maintained at a hygromycin concentration of 200 ng/ml, and G418 (300 ng/ml) (Sigma-Aldrich).

2.2.3 Immunoprecipitation and detection of native phospho- α -tubulin.—Cells were lysed in 0.3 ml ice-cold IP lysis buffer (50 mM TRIS-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.5% NP-40) supplemented with 10 μ M *bis*-indolylmaleimide, 1 mM dithiothreitol, 1X protease inhibitors and 1X phosphatase inhibitors (Bimake.com). Samples were sonicated (3×10 sec) and centrifuged at $5653 \times g$ for 10 min, and the resulting supernatant (lysate) was transferred and analyzed for total protein content using bovine serum albumin as a standard. Samples were normalized for total protein content (0.7 – 0.8 mg), and the sample volume was increased to 1 ml with buffer that contained no detergent. α -Tubulin (DM1A) antibody (1 μ g) was added followed by rotation for 2 h at 8°C. To collect the immunocomplexes, 50 μ l A/G-agarose beads was added and the samples were rotated for an additional 1 h at 8°C. Following centrifugation, the pellets were washed three times with 0.5 ml non-detergent buffer, resuspended in sample buffer and heated for 5 min at 95°C. Samples were resolved by 7% SDS-PAGE, transferred to a PDVF membrane, and probed overnight with either rabbit PKC substrates antibody (1:1000) or for 2 h with rabbit α -tubulin antibody (1:50000) to establish equivalent loading, and followed with a HRP-conjugated anti-rabbit secondary antibody (1:5000). Detection of chemiluminescence was performed with West Pico Super Signal reagents (Pierce Biotechnology). Band intensities were measured with ImageJ software.

2.2.4 Preparation and analysis of mutant α -tubulin in whole cell lysates.—After washing twice with 10 ml 1X PBS, cells from one 10-cm plate were collected in 1 ml PBS by scraping, and transferred to Eppendorf tubes. The tubes were centrifuged at $200 \times g$ for 5 min and the supernatant was discarded. Pellets were suspended in ice-cold 0.20 ml lysis buffer (Cell Signaling Technologies) containing protease inhibitors (Bitake). The tubes were vortexed for 10 sec, subjected to sonication for 10 sec, and the process was repeated for a total of three rounds. Following centrifugation at $5653 \times g$ for 10 min, the supernatants (whole cell lysates) were transferred to new Eppendorf tubes and kept on ice. Protein assays were performed with protein dye reagent (Bio-Rad) using bovine serum albumin as a standard. Sample buffer (5X) was added to a final concentration of 1X (50 mM TRIS, pH 6.8, 1 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The samples were heated for 5 min at 95°C, followed by storage at –20°C. For Western blot analysis, whole cell lysates (550 μ g/well) were resolved by 8% SDS-PAGE and transferred to a PVDF membrane. To detect the myc-tagged mutant α -tubulin proteins, the membrane was rotated at 4–6 °C overnight with anti-myc (1:1000), followed by anti-rabbit HRP-conjugated secondary antibody (1:5000) for 1 h at room temperature. Detection was performed with chemiluminescent substrates.

2.2.5 Proliferation Assay—Cells were plated in 24-well plates at a concentration of 10,000 cells/well and cell number was recorded every 24 hours using Cytation5 Image Reader (Biotek, USA). The entire well area was imaged at 4 $\times$ resolution and combined into a single image using the image montage feature. Cell number was measured using an automated processing method in the instrument software with threshold set to 2500.

2.2.6 Cell cycle analysis.—Cells were plated in 4-well chamber slides at a concentration of 20,000 cells/well and were evaluated for the number of cells that reached

mitosis. Cells were treated with Premo™ FUCCI Cell Cycle Sensor reagent (Thermo Fisher) according to the manufacturer's instructions. This reagent fluoresces green only during the S/G2/M phase of the cell cycle. Live cell time-lapse microscopy was performed on an Axio Observer Z1 fluorescence microscope (Carl Zeiss, Germany) using a 10×/0.45NA objective. Brightfield and FITC channels were used for cell imaging for 65 h at 10 different pre-programmed positions per well either every 15 minutes (proliferation) or every 2 minutes (duration of S/G2→M). For proliferation experiments, bright field images were used to count the total number of cells at each time point in all 10 positions in each well using an automated analysis method in ImageJ software (NIH). For cell cycle studies, the number of dividing vs. non-dividing cells was measured for only fluorescent cells. In three independent experiments, the total numbers of fluorescent cells visualized were approximately 700 S165D-expressing cells, 1500 S165N-expressing cells, and 800 VC cells.

2.2.7 Confocal imaging of spindle fibers—Cells were plated in 4-well chamber slides at a concentration of 50,000 cells/well. Cells were stained with a solution of Tubulin Tracker Green (250 nM) (Thermo Fisher) and Hoechst 33342 (5 µg/ml) in PBS 30 min prior to imaging. Images were taken no later than 2 hours after staining. Image data were acquired on a Leica TCS SP8 confocal microscope under optimal Nyquist sampling conditions and a 40× NA 1.1 Water objective. Images were deconvolved in AutoQuant deconvolution software (Media Cybernetics) with the experimentally obtained Point Spread Function (PSF) using 175 nm TetraSpeck fluorescent beads (Molecular Probes). The length of 10 tubulin spindle fibers per cell was analyzed using ImageJ software. In three experiments, this procedure was carried out in a total of 7 S165D-expressing cells (70 fibers), 7 S165N-expressing cells (70 fibers), and 5 VC cells (50 fibers) and displayed as individual data points.

2.2.8 Xenograft studies.—Nude mice (*nu/nu*; Envigo Laboratories) were used for *in vivo* studies and were cared for in accordance with guidelines approved by MSKCC Institutional Animal Care and Use Committee and Research Animal Resource Center. Eight week-old female mice were injected intravenously with 1.5×10^6 MDA-MB-231-LM2–4175 stably transfected cells, which preferentially home to the lungs (24). Mice were observed daily for signs of morbidity, and body weights were assessed twice weekly. Mice were imaged once per week with an In Vivo Imaging System (IVIS, Xenogen) with a collection time of 10 sec. Tumor bioluminescence was quantified by integrating the photonic flux (photons per second) through a region encircling each tumor as determined by the LIVING IMAGES software package (Xenogen). At the end of the study, lungs were removed and fixed in 10% formalin for 24 h and stored in 70% ethanol prior to immunohistochemistry.

2.2.9 Immunohistochemistry.—Tissue sections were blocked with avidin/biotin block for 8 min, followed by incubation with Ki67 antibody (Abeam) for 5 h, followed by 1 h incubation with biotinylated goat anti-rabbit IgG (Vector Laboratories) at a dilution of 1:200. The detection was performed with DAB detection kit according to the manufacturer's instructions, followed by counterstaining with hematoxylin and eosin (H&E), and cover-slipped and sealed with Permount (Fisher Scientific). Slides were imaged on a Panoramic

250 slide scanner (3DHISTECH) at 40× resolution. The threshold was adjusted to exclude background and the stained area was quantified using ImageJ software.

3. RESULTS

3.1 Phosphorylation of native α -tubulin in metastatic breast cells.

To establish that native α -tubulin is phosphorylated by endogenous PKC in MDA-MB-231-LM2–4175 and MDA-MB-468 cells, immunoprecipitation of α -tubulin followed by western blot analysis was performed with an antibody that detects the phosphorylated PKC recognition motif. PKC phosphorylation sites were detected in α -tubulin immunoprecipitated from both cell lines (Figure 1A). The results for a representative experiment are given along with quantitation of the signals (Figure 1B).

3.2 Expression of α -tubulin mutants in stable transfectants of MDA-MB-231-LM2–4175 cells.

We demonstrated in an earlier study (19) that phosphorylation of α -tubulin occurred predominantly at Ser¹⁶⁵ since its replacement with Asn (S165N- α -tubulin) blocked phosphorylation of this mutant in MDA-MB-468 cells, as demonstrated with the PKC substrates antibody. In parallel it was shown that a PKC inhibitor (*bis*-indolylmaleimide) also blocked phosphorylation of a wildtype construct of α -tubulin in these cells, confirming PKC as the active kinase. As shown in Figure 2A, Ser¹⁶⁵ is embedded in a PKC recognition motif (underlined) consisting of flanking lysine residues that is required for interaction with PKC (25). Although the α 6-isotype of α -tubulin (TUBA1C) was initially discovered as the PKC target, it is noted that the recognition sequence centered at Ser¹⁶⁵ is identical and present in all isotypes of α -tubulin.

To further explore the significance of Ser¹⁶⁵ phosphorylation in α -tubulin to characteristics of human breast cancer cells, we performed stable transfection of MDA-MB-231-LM2–4175 cells with a plasmid that encoded myc-tagged α 6-tubulin mutated at Ser¹⁶⁵. Micrographs of stable cell transfectants (Figure 2B) revealed some slight morphological differences. Cells expressing the S165D mutant were rounder than VC cells, whereas cells expressing the S165N mutant appeared smaller. However, measurement of cell perimeters using Motic Image software did not reveal a statistically significant difference in size (data not shown). To demonstrate stable expression, myc-tagged α 6-tubulin was analyzed in whole cell lysates by Western blot using anti-myc (Figure 2C). Both mutants of α -tubulin (S165D, S165N) gave detectable myc-tag signals at 50–55 kDa, whereas the signal was absent with cells stably transfected with the empty vector control (VC). Quantification of these signals and native α -tubulin as a loading control are shown (Figure 2D).

3.3 Impact of α -tubulin mutants on EMT marker expression.

Our previous studies showed that expression of the phospho-mimetic construct, S165D- α 6-tubulin, produced highly motile cell behavior of MCF-10A cells, which are normally non-motile (19). Acquisition of cell motility is an indication that a transition from an epithelial cell to a mesenchymal cell (EMT) had occurred. Therefore, in MDA-MB-231-LM2–4175 transfectant cells (which have mesenchymal morphology), we examined the expression of

certain molecular markers known to be associated with EMT to determine whether the phosphorylation state of α -tubulin impacts this transition (Figure 3). Consistent with the triple-negative status of these cells (ER-, PR-, and amplified erbB2), we observed that erbB2, a marker for EMT, was highly expressed in VC cells. Compared with control cells, ErbB2 expression remained high in S165D-expressing cells but was dramatically decreased by 80% in S165N-expressing cells. Decreased expression of E-cadherin is another characteristic of mesenchymal cells with high metastatic potential that is coincident with increased expression of N-cadherin (26). Recapitulating the effect of PKC α overexpression in MCF-10A cells in which E-cadherin expression fell below detectable limits (17), E-cadherin (E-cad) was undetectable in VC cells and was only slightly detectable in cells expressing S165D- α -tubulin. However, cells expressing the S165N mutant displayed a E-cadherin signal that was almost 4-fold higher than control cells, thus reflecting the acquisition of epithelial character. A complementary expression profile was found for N-cadherin in that the S165D mutant produced a 10-fold higher N-cadherin signal, a characteristic of EMT that was not detectable in cells expressing the S165N mutant. It is noted that endogenous levels of N-cadherin was not observed in control cells, suggesting that there is a requirement for higher PKC activity that can be met by expressing the S165D mutant. Taken together, these results are consistent with a 'cadherin switch' that is controlled by the phosphorylation state of Ser¹⁶⁵ in α -tubulin.

Further analysis was carried out to examine the expression levels of vimentin, SLUG, and ZEB1, all known EMT molecular markers whose elevated expression is associated with a poor prognosis in breast adenocarcinoma (27, 28). It was found that SLUG and ZEB1 levels were markedly decreased by over 60% in cells expressing the S165N mutant as compared with cells expressing the S165D mutant or empty vector. In contrast, vimentin was expressed at a high and comparable level in all three conditions (VC, S165D, and S165N). That the S165N mutant of α -tubulin eliminated the expression of EMT-inducers ZEB1 and SLUG is again consistent with the acquisition of epithelial cell character and the reversal of EMT. Signals for β -actin confirmed that loaded protein amounts were within 10%. Overall, these expression patterns suggest that the S165N mutant reverses EMT thereby favoring a state of lower metastatic potential.

3.4 Effect of α -tubulin mutants on proliferation and cell division.

Rates of cell proliferation were measured with monolayers of cells expressing each α -tubulin mutant. Cells expressing S165N- α -tubulin proliferated at a higher rate than cells expressing the S165D mutant (Figure 4A). No significant difference was noted between the S165N mutant and control cells (VC). The slower rate found with cells expressing the S165D mutant supports a functional link between phosphorylation of α -tubulin and a slower rate of cell division and recapitulates the suppressive effect on proliferation previously found with PKC α over-expression in MCF-10A cells (17).

Analysis of the cell cycle was explored by transfecting MDA-MB-231-LM2-4175 cells with a plasmid encoding a fluorescent cell cycle indicator (FUCCI) (Fisher Scientific) that detects temporal expression of the cell cycle regulator gemenin tagged with GFP, making it possible to distinguish cells in the S/G2/M phase (29). Cells expressing a α -tubulin mutant were

assessed for their mitotic activity by quantifying the number of fluorescent cells proceeding to cell division. While S165N-expressing cells and control cells showed comparable activity, the number of non-dividing fluorescent cells was found to be 4- to 5-fold higher with S165D-expressing cells over a 65h period (Figure 4B). With $t=0$ designated as the moment a cell rounded up and turned green, the time interval required for fluorescent cells to transit from S/G2 to mitosis was determined (Figure 4C). S165D-expressing cells on average required a two-fold longer period to reach mitosis (38.3 min) than did S165N cells (19.1 min) and VC cells (21.4 min).

To further evaluate the difference in proliferative rate by S165D- and S165N-expressing cells, live cells were treated with fluorescein-labeled tubulin-tracker (green) plus Hoechst 33342 to detect chromosomal DNA (depicted in blue). Experiments examined the impact of each α -tubulin mutant on the length of kinetochore microtubules at metaphase (Figure 5A). The average kinetochore fiber length ($\pm$ SEM) in S165D-expressing cells was longer ($5.77 \pm 0.15 \mu\text{m}$) than those in VC cells ($4.42 \pm 0.14 \mu\text{m}$), whereas the fibers in S165N-expressing cells ($3.37 \pm 0.1 \mu\text{m}$) were shorter than those in VC cells (Figure 5B). The feature of shortened spindle fibers during metaphase was previously observed to coincide with increased proliferative rate (30, 31), whereas longer metaphase spindle fibers were observed in cell migration (32). Thus, α -tubulin mutants induced differences in kinetochore fiber length that were consistent with whether the prevailing phenotype was proliferation or motility.

3.5 Effect of α -tubulin mutants on tumor growth in nude mice.

The human breast cell line (MDA-MB-231-LM2-4175) being used for these studies is a sub-population of parental MDA-MB-231 cells originally isolated from a pleural effusion. Once injected into the animal, these cells preferentially home to the lungs (24). Their stable expression of luciferase makes their detection possible by whole animal imaging following injection of luciferin. Stable transfectants expressing a α -tubulin mutant (S165D or S165N) or the control vector (VC) were introduced into nude mice by tail vein injection. Immediately following injection of 8 mice per condition (Figure 6A; day 1) luciferase-generated signals of equivalent intensity could be observed exclusively in the lungs. Subsequently, signals for the animals were recorded similarly once each week for 5 consecutive weeks. During this period, signals in animals harboring the S165N- α -tubulin expressing cells became increasingly stronger, whereas signals for cells expressing the S165D- α -tubulin or VC grew progressively weaker. By day 35 (Figure 6A), signals in 5 out of 8 S165N-expressing mice encompassed the entire thoracic region, whereas signals for cells expressing either the S165D mutant or VC were unchanged, decreased or undetectable. Normalization of the intensities (Figure 6B) revealed that the S165N- α -tubulin mutant boosted the strength of the luciferase-generated signal by almost 500-fold, an indication of strong proliferative activity by these cells. Since the high incidence of tumors was confined to the lung region, as predicted by the route of cell injection, it is consistent with proliferation rather than metastasis.

It is noted that signals near the tail base of two animals injected with S165N-expressing cells (day 35) may have been the result of cells that did not enter the bloodstream after tail vein

injection. None of the VC animals displayed similar activity by these MDA-MB-231-LM2–4175 cells that are intrinsically metastatic. It is possible that sustained incubation of the cells with antibiotics (G418, hygromycin) may have diminished their intrinsic metastatic potential, and that incubation for longer than 35 days was required for these human cells to metastasize in a murine system.

Following sacrifice of the animals, the lungs were excised and formalin-fixed for subsequent analysis. H&E staining of lung specimens and counting of individual tumors (Figure 7A, 7B) revealed that the average number of tumors was 18.5 ± 6.3 in the VC group, 9.6 ± 2.9 in the S165D group, and 61.8 ± 10.8 in the S165N group. Furthermore, immunostaining of lung specimens for the proliferation marker Ki67 (Figure 7C, 7D) showed that the average percent of stained area in the VC group was 3.7 ± 1.3 and was similar to the S165D group for which the average percent of stained area was 5.4 ± 2.4 . In contrast, the average percent of stained area for the S165N group was 13.7 ± 2.4 . The difference in Ki67 expression for the two mutants is consistent with the hyper-proliferation observed for the S165N mutant *in vivo* and suppressed tumor growth associated with the S165D mutant.

4. DISCUSSION

An earlier investigation of PKC substrates identified α -tubulin as a key substrate that promoted microtubule dynamics and motility of human breast cells (19, 20). In this study, we demonstrated that the state of phosphorylation of Ser¹⁶⁵, as exemplified by site-specific mutants, appears to dictate expression of EMT markers as well as proliferative activity of human breast tumor cells. By use of mutants that simulate (S165D) or block (S165N) phosphorylation of α -tubulin at this site, we showed that the phosphorylation-resistant mutant engendered rapid proliferation of breast cancer cells in monolayer and *in vivo* settings (Figures 4, 6), whereas expression of the phospho-mimetic α -tubulin (S165D) produced EMT-related hallmarks and suppressed proliferative growth. Blockade of phosphorylation at this site using the S165N mutant was previously shown to inhibit motility *in vitro* of two transfected human breast cell lines (MDA-MB-231 and MDA-MB-468) by 60–75% (19). Building on these previous studies, our present findings suggest that the state of phosphorylation of Ser¹⁶⁵ as exemplified by site-specific mutants, serves as an ‘on-off’ switch that controls EMT marker expression, rate of transit through the cell cycle, kinetochore microtubule length, and proliferation *in vivo*.

A prominent response to these α -tubulin mutants was the expression of E-cadherin, an EMT-related protein and a major adhesion molecule that participates in cell-cell junctions (26). Our earlier work (17) showed that engineered overexpression of PKC α in MCF-10A cells leads to elimination of detectable E-cadherin protein that is accompanied by loss of cell-cell contacts and increased cell movement (17), and revealed the PKC signaling pathway as promoting EMT. The present work with triple negative breast cancer cells showed that there is a functional link between PKC-mediated phosphorylation of α -tubulin and loss of E-cadherin protein accompanied by increased expression of N-cadherin. Expression of the S165D- α -tubulin mutant (simulating PKC phosphorylation) promotes high expression of N-cadherin while suppressing E-cadherin protein. However, when S165N- α -tubulin is expressed, thus blocking phosphorylation of Ser¹⁶⁵, E-cadherin remains high, whereas N-

cadherin is decreased to undetectable levels (Figure 3). Regulated expression of transcriptional activators such as SLUG and ZEB1 that are known to repress the E-cadherin promoter (28, 33) is the apparent cause of this 'cadherin switch'. Thus, decreased expression of these factors during S165N mutant expression is consistent with the observed high-level expression of E-cadherin protein. Our findings suggest that the phosphorylation state of Ser¹⁶⁵ in α -tubulin plays a critical role in cadherin switching and provides an avenue for further inquiry.

An intriguing finding was that the increased stability afforded to microtubules by the S165D mutant (20), coincided with a longer S/G2→M phase of the cell cycle, increased length of kinetochore fibers, and a slower rate of cell division. As shown by others with various cancer cells (32), metaphase kinetochore microtubules that are longer correspond to an elevated rate of polymerization along with cell migration and metastatic potential, echoing our findings with the S165D mutant in breast cancer cells (19, 20). Conversely, kinetochore spindle fibers are shorter when rates of polymerization are decreased either by mutation or depletion of proteins that modulate microtubule dynamics (34). In support of this idea, our findings with cells expressing the S165N- α -tubulin show that this mutant causes both shorter kinetochore length (Figure 5) and dramatically weaker microtubule polymerization as compared with control MDA-MB-231 cells (20).

In view of the fundamental role that microtubules play in cell movement and cell division, the regulation of microtubule dynamics by a phosphorylation switch at Ser¹⁶⁵ in α -tubulin apparently provides a robust mechanism for coordinating these cellular phenotypes. It remains to be determined how this switch causes cells to proliferate at different rates. It has been suggested that unknown regulators of the cell cycle are released from disassembling microtubules (35, 36), and that a shorter cell cycle length correlates with accelerated cell proliferation (30). However, these ideas have yet to be fully explored.

The clinical significance of Ser¹⁶⁵- α -tubulin was explored at cBioportal.org, a genomic database of primary human breast tumors and other cancers. We looked for alteration of the PKC recognition sequence at Ser¹⁶⁵ of multiple α -tubulin isoforms by a substitution that would prevent or weaken the ability of PKC to recognize Ser¹⁶⁵ as a substrate. We found that the primary sequence of α -tubulin can indeed exhibit a defective PKC recognition motif at Ser¹⁶⁵. In various tumors (invasive breast carcinoma, stomach/esophageal cancer, non-small cell lung cancer), several α -tubulin isoforms (TUBA1A, TUBA3C, TUBA4A, TUBA8) were mutated in the flanking cationic residues to uncharged residues within the PKC consensus site (aa 163–166) (Figure 2A). Such derangements could cause partial or complete impairment of phosphorylation by PKC, a condition that would promote a proliferative tumor, as implied by the findings presented here. Other tumors (non-small cell lung cancer, cutaneous melanoma) exhibited a Ser→Phe substitution at Ser¹⁶⁵, thereby fully blocking phosphorylation. One mutant of α -tubulin (TUBA1 A) was observed in endometrial carcinoma in which a Glu residue had been substituted for Gly¹⁶², which is immediately adjacent to the PKC recognition motif centered at Ser¹⁶⁵ and could serve as a phospho-mimetic since it introduces a negative charge near the actual phosphorylation site. Based on 177 studies/44613 patients with 30660 sequenced cases/patients consisting of a wide range of cancers, we estimate that mutations arise in the region of aa155–175 of α -

tubulin with a frequency of 0.00 – 0.04% depending on the isotype. Of seven isotypes evaluated, TUBA3C exhibited the highest mutational frequency: TUBA1A 0.024%; TUBA1B 0.003%; TUBA1C 0.006%; TUBA3C 0.041%; TUBA4A 0.010%; TUBA4B 0.000%; TUBA8 0.015%. With cancer genomics as a diagnostic tool, the immediate region surrounding Ser¹⁶⁵ in α -tubulin should be further pursued as a potential biomarker site when evaluating tumor biopsies for their metastatic potential.

In a previous study of 46 breast tumors having known clinical pathology, we found that PKC expression was substantially down-regulated when compared with 25 specimens of normal adjacent tissue (37). No staining was observed for 67% of tumor specimens, and only 4% showed intensities greater than the median observed in normal tissue. Similarly, analysis of >5000 primary breast tumors in the cBioportal.org database showed that gene amplification of PKC α occurred in no more than 10% of the specimens and was the highest among all PKC isoforms. On the basis these studies, we propose that primary tumors show a diminished role for PKC in cells apparently because they are proliferative rather than metastatic. Those few tumors found to express high levels of PKC α would be predicted to exhibit a more aggressive phenotype (3–5) and may be smaller in size due to suppressed proliferation (17). Taken together, our findings lend further support to a model in which inhibition of PKC activity or blockade of Ser¹⁶⁵ in α -tubulin reverses metastatic potential and promotes proliferation of breast tumors.

5. CONCLUSIONS

In the primary structure of α -tubulin, Ser¹⁶⁵ is embedded in a PKC recognition site that undergoes phosphorylation in triple negative breast cells. In view of their EMT marker expression profile, these cells already exhibit metastatic potential that can be augmented by the phospho-mimetic form of Ser¹⁶⁵ (S165D- α -tubulin) thereby activating a ‘cadherin switch’ and a slower rate of proliferation. Conversely, blocked phosphorylation of Ser¹⁶⁵ (S165N- α -tubulin) causes a reversal of EMT marker expression as well as increased proliferation in vitro and formation of hyper-proliferative tumors in an animal model. An apparent mechanism for these observations is that the two α -tubulin mutants have opposing effects on microtubule structure leading to altered spindle fibers and characteristics of the cell cycle. While giving a more complete understanding of a PKC signaling pathway in breast cells, these findings prompt the conclusion that the phosphorylation state of Ser¹⁶⁵ in α -tubulin is a switch that determines either the metastatic potential (EMT) of human breast cells or their prospects for tumor formation. That human tumors are found to exhibit blocking mutations to Ser¹⁶⁵ or the surrounding PKC recognition motif warrants further study of this site as a means to judge the metastatic potential of tumor specimens.

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HIGHLIGHTS

- α - and β -tubulin are building blocks of microtubules of the cell cytoskeleton.
- Phosphorylation of α -tubulin at PKC sites is found in human breast cells.
- Phosphorylation at Ser¹⁶⁵ slows cell division and increases metastatic potential.
- Blocked phosphorylation of Ser¹⁶⁵ increases cell division and tumor growth in mice.
- State of phosphorylation of Ser¹⁶⁵ switches cells between mitosis and metastasis.

Figure 1.

Analysis of native and mutant forms of α -tubulin in human breast cells. A. PKC-mediated phosphorylation of native α -tubulin in human breast cells. Parental MDA-MB-231-LM2-4175 or MDA-MB-468 cells were analyzed by Western blot with PKC substrates antibody (1:1000) or α -tubulin antibody (1:50,000). The blot is representative of three independent experiments. B. Signals of the blot were quantified by using ImageJ. Additional details are given in the 'Methods'.

Figure 2.

Analysis of α -tubulin mutants in MDA-MB-231-LM2-4175 cells. A. PKC recognition motif centered at Ser¹⁶⁵ (underlined) in human α 6-tubulin. B. MDA-MB-231-LM2-4175 cells were stably transfected with a cDNA plasmid encoding a mutant of α 6-tubulin (S165D or S165N) or the empty vector (VC) and maintained under selection pressure with hygromycin and G418. Transfectants were imaged at 100X magnification with a Nikon phase contrast microscope equipped with a Motic Image camera and Motic Image 2.0 software. C. Western blot analysis of myc-tagged α 6-tubulin proteins in whole cell lysates of MDA-MB-231-LM2-4175 cells. D. Quantitation of Western blot signals with ImageJ depicts myc- α 6-tubulin (light grey) and total α -tubulin signals (dark grey). The presented data are representative of three independent experiments.

Figure 3.

Analysis of EMT marker expression in whole lysates of MDA-MB-231-LM2-4175 transfectants. A. Protein samples (150 µg/lane) were resolved by 8% SDS-PAGE, and the resulting blots were probed with rabbit primary antibodies corresponding to the indicated EMT markers (1:1000) or β -actin (1:20,000) as a loading control. The loading control signals were equivalent for all samples evaluated. The results for each protein isolated from cells expressing S165D- α 6-tubulin, S165N- α 6-tubulin or the empty vector (VC) were from a single gel and are representative of three independent experiments. B. Densitometric analysis of western blot signals shown in (A) using ImageJ.

Figure 4.

Measurement of proliferation and cell cycle rates in monolayer cultures of MDA-MB-231-LM2-4175 transfectants. A. For proliferation experiments, cells were plated in 24-well plates at a concentration of 10,000 cells/well and cell number was recorded every 24 h using Cytation5 Image Reader (Biotek, USA). B. Cells were transfected with a cell cycle sensor that fluoresces only during the S/G2/M phase of the cell cycle. The number of fluorescent dividing and non-dividing cells was measured over the course of 65 h using live cell time-lapse microscopy on an Axio Observer Z1 fluorescence microscope. Images were taken every 15 min. and 10 positions were imaged in each well. C. Time period required for fluorescent cells to transit from S/G2 to mitosis. Cells were transfected with the fluorescent sensor and imaged as described in (B) except that images were taken every 2 min and 6 positions were imaged in each well. Transit time began when a cell acquired green fluorescence, and ended when the cell divided. The results are representative of three independent experiments. (****, $p < 0.0001$)

Figure 5.

Measurement of kinetochore fiber length at metaphase of in MDA-MB-231-LM2-4175 cells expressing α -tubulin mutants. A. Representative images of live cells that had been stained with Tubulin Tracker Green and Hoechst 33342 followed by recording of confocal images, as described in 'Methods'. B. Length of spindle fibers was measured by ImageJ. For each cell line, data for 10 fiber lengths (in μm) were measured per cell and reported for 5–7 cells. The average length is given by the horizontal bar. Numerical values $\pm$ S.E.M. are given in the text. (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$)

Figure 6.

In vivo studies of MDA-MB-231-LM2-4175 transfectants. A. Tumor growth in nude mice associated with MDA-MB-231-LM2-4175 transfectants that stably express luciferase. Following injection of cells into 8–10 mice per condition, analysis was performed each week by injecting luciferin and detecting luciferase-generated signals by whole animal imaging (IVIS). After five weeks, the animals were sacrificed. (See ‘Methods’.) B. Tumor bioluminescence was quantified by integrating the photons per second (p/s) by the LIVING IMAGES software package.

Figure 7.

Analysis of tumors resulting from MDA-MB-231-LM2-4175 transfectants. A.

Counterstaining of representative tumor sections was performed with hematoxylin and eosin (H&E) and the samples were cover slipped and sealed. Slides were imaged on a Panoramic 250 slide scanner (3DHISTECH) at 40 $\times$ resolution. B. Quantification of the number of tumors formed for all animals per condition ($\pm$ S.E.M.). (***) $p < 0.001$ for S165D vs. S165N and (**) $p < 0.01$ for S165N vs. VC. C. Tissue sections were blocked with avidin/biotin block followed by incubation with Ki67 antibody for 5 h, a 1-h incubation with biotinylated goat anti-rabbit IgG (1:200 dilution), and detected with DAB, as described in the 'Methods'. Representative images are shown. D. Threshold values were adjusted to exclude background and the stained areas were quantified for the percentage of stained area ($\pm$ S.E.M.) (*) $p < 0.05$ for S165D vs. S165N, and (**) $p < 0.01$ for S165N vs. VC.