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The β isotypes of tubulin in neuronal differentiation

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Abstract

The differences among the vertebrate β isotypes of tubulin are highly conserved in evolution, suggesting that they have functional significance. To address this, we have used differentiating neuroblastoma cells as a model system. These cells express the β I, β II, and β III isotypes. Although there is no difference prior to differentiation, a striking difference is seen after differentiation. Both β I and β III occur in cell bodies and neurites, while β II occurs mostly in neurites. Knocking down β I causes a large decrease in cell viability while silencing β II and β III does not. Knocking down β II causes a large decrease in neurite outgrowth without affecting viability. Knocking down β III has little effect on neurite outgrowth and only decreases viability if cells are treated with glutamate and glycine, a combination known to generate free radicals and reactive oxygen species. It appears, therefore, that β I is required for cell viability, β II for neurite outgrowth and β III for protection against free radicals and reactive oxygen species.

Keywords

tubulin; neuroblastoma; microtubules; β I-tubulin; β II-tubulin; β III-tubulin

Introduction

Microtubules are involved in many cellular functions, including mitosis, intracellular transport, determination of cell morphology, and differentiation (Desai and Mitchison 1997; Howard and Hyman 2003). Tubulin, the subunit protein of microtubules, is an α/β heterodimer, with both α - and β -tubulin existing as multiple isotypes, differing from each other in amino acid sequence and tissue distribution. In mammals, there are seven well-established β -tubulin isotypes, referred to as β I, β II, β III, β IVa, β IVb, β V, and β VI (Ludueña 1998). The differences among vertebrate β -tubulin isotypes have been highly conserved in evolution, implying that these differences are functionally significant. The precise functions of all of the isotypes have yet to be determined.

Early studies of isotype function, focusing on cultured and undifferentiated cells, suggested that any of the isotypes could carry out the basic functions such as forming the mitotic spindle and the interphase network (Joshi et al. 1987; Lewis and Cowan 1988). However, studies in intact tissues indicated that ciliated or flagellated cells were highly enriched for the β IV and β I isotypes in their axonemal microtubules, suggesting the possibility that the isotypes could be functionally specialized within certain cells (Jensen-Smith et al. 2003;

Renthal et al. 1993). For the purpose of addressing isotype function by manipulating isotype expression levels, intact tissues are not satisfactory. Therefore, we have focused on neuroblastoma cells which can be cultured *in vitro* and induced to differentiate, since retinoic acid (RA) will induce them to generate neurites (Gaitonde et al. 2001; Lombet et al. 2001). Neuronal cells are well-adapted to this purpose, since microtubules are a major component of their cytoskeleton and are involved in neurite outgrowth (Baas et al. 1988).

We have examined the intracellular distributions of the β I, β II and β III isotypes in cultured SK-N-SH neuroblastoma cells that have been induced to differentiate and we have used specific siRNAs to decrease the expression of these isotypes in these cells. Our results suggest that β I plays a major role in cell viability and that β II is required for neurite outgrowth, while β III may protect neurons against free radicals and reactive oxygen species.

Materials and methods

Antibodies

The monoclonal antibodies specific to the β isotypes of tubulin (β I, β II, β III) were prepared as previously described (Banerjee et al. 1990, 1992, 1988; Roach et al. 1998). Hybridoma supernatants containing antibodies to β I (SAP.4G5), β II (JDR.3B8), β III (SDL.3D10), and β IV (ONS.1A6) were passed through a protein A-agarose column and washed with PBS. Bound antibody was eluted with 0.1 M glycine-HCl (pH 2.3) and fractions were collected. Based on A280 readings, fractions were pooled and dialyzed overnight at 4°C with PBS containing 0.02% NaN_3 . The antibodies were stored at -20°C until use. The human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody was purchased from Invitrogen.

Cell culture

Human SK-N-SH neuroblastoma cells were plated and grown at 37°C in a humidified atmosphere of 5% CO_2 , 95% O_2 in Minimum Essential Medium with Earle's salts and L-glutamine (MEM, Cellgro) supplemented with 10% fetal bovine serum, 1mM sodium pyruvate, 1% non-essential amino acids, penicillin-streptomycin-fungisone antibiotics and 1.5 g/l sodium bicarbonate (Lombet et al. 2001). For cell differentiation, cells were plated in normal medium for 24 hours. Then cells were differentiated by adding retinoic acid (RA, Sigma) to the culture medium to a final concentration of 30 μM . The medium was changed on alternate days, and cultures were allowed to differentiate for 1 week.

Quantitative analysis of β tubulin isotypes in SK-N-SH cells

To determine accurately the amount of each β tubulin isotype present in SK-N-SH cells, cell extracts were electrophoresed on a gel along with either purified $\alpha\beta$ II- and $\alpha\beta$ III-tubulin from bovine brain as standards. Bands were transferred to nitrocellulose membranes and treated with isotype-specific monoclonal antibodies followed by horseradish peroxidase as described by Joe et al. (2009). Bands were quantitated by image analysis using the Odyssey software (LI-COR Biosciences). Since purified β I is not available, phosphocellulose-purified bovine brain tubulin (PC-tubulin), whose β -tubulin isotype composition is known (Banerjee et al. 1988), was used as a standard for β I. $\alpha\beta$ II- and $\alpha\beta$ III-tubulin were purified as described previously (Banerjee et al. 1992). PC-tubulin was prepared as described by Fellous et al. (1977). Different amounts of each standard were run to obtain a standard curve for calibration. The size of the corresponding band in the neuroblastoma cell extract was measured and was interpolated into the standard curve to calculate the actual amount of the isotype in the cell extract. This was compared to the total amount of protein in the cell extract aliquot that was run on the gel to calculate the percentage of that isotype in the proteins of the neuroblastoma cells.

Indirect immunofluorescence microscopy

All cells were grown on glass coverslips at 37°C and 5% CO₂. Cells were then washed twice with PBS, fixed for 15 minutes with 3.7% paraformaldehyde at room temperature, and permeabilized for 1 minute with 0.5% Triton X-100 in PBS. Cells were then incubated at 4°C overnight with the respective isotype-specific monoclonal IgG mouse antibody diluted in PBS containing 10% normal goat serum (Jackson ImmunoResearch). Cells were rinsed in PBS and labeled with Cy3-conjugated goat anti-mouse antibody or FITC-conjugated AffiniPure goat anti-mouse antibody (1:100; Jackson ImmunoResearch) for 2 hours at room temperature. Cells were then rinsed with PBS. For nuclear staining, cells were stained with DAPI (6'-diamidino-2-phenylindole, Molecular Probes) during the last wash with PBS after incubation with the secondary antibody. Coverslips were mounted on glass slides. All images were collected with an Olympus FV1000 confocal scanner mounted on an IX-81 microscope using an UPlanApo 60×, NA 1.42 lens. For double immunofluorescence, Bodipy FL phalloidin (Sigma) was used as a marker for actin filaments. Cells were treated as described above and then incubated with Bodipy phalloidin (1:50) for 1 hour at room temperature. Cells were then rinsed and fixed for visualization.

Western blot analysis

Cells were harvested in lysis buffer [50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP-40, 0.2 mM sodium orthovanadate, 100 mM sodium fluoride, 1% 200 mM phenylmethylsulfonyl fluoride [PMSF, dissolved in dimethyl sulfoxide (DMSO)], 1% Protease Inhibitor Cocktail]. Supernatants were collected and total protein concentration was measured as described by Lowry et al. (1951). Supernatants were solubilized in Laemmli loading buffer and separated on 7.5% SDS-PAGE gels. Proteins were transferred onto a Bio-Dot Blotting Media Nitrocellulose Membrane (Bio-Rad Laboratories). The membranes were blocked with 5% non-fat dry milk and 0.1% bovine serum albumin for 1.5 hour at room temperature. Immunodetection was performed by incubating the membrane for 1 hour at room temperature with the primer antibodies and GAPDH (loading control). Then the membranes were incubated with the secondary antibody (peroxidase-conjugated AffiniPure Goat anti-mouse antibody, Jackson ImmunoResearch Lab) for 1 hour. Finally, the membranes were developed using SuperSignal West Pico stable peroxide solution and enhancer solution (1:1) (Pierce). Western blots were scanned by MICROTEK ScanMaker 9800XL and quantitatively analyzed by the Odyssey program.

Trypan blue exclusion assay

Cellular viability was determined by the Trypan blue exclusion method (Das et al. 2005), in which damaged neurons leak and allow the Trypan blue to enter the cells. The cultures were incubated with a 0.4% solution of the Trypan blue (1:1) for 3-5 minutes at room temperature. Cells were counted in four separate areas with a haemocytometer. The average numbers of live and total cells were used to calculate the percentage of cell viability.

MTT cell proliferation assay

The MTT cell proliferation assay has been previously described (Holst-Hansen C 1998). The method is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and form dark blue crystals which are largely impermeable to cell membranes, thus resulting in their accumulation within healthy cells. The number of cells surviving is directly proportional to the quantity of the formazan produced. The cell culture medium was aspirated and 0.5mg/ml MTT in MEM (supplemented) was added to each well and incubated for approximately 3 hours at 37°C. The MTT medium formulation was aspirated and then isopropanol was added. Plates were left at 37°C for approximately

1.5 hours to ensure purple crystals were fully dissolved. After the crystals were solubilized with isopropanol, absorbance was measured at 570nm with an ELISA reader.

RNA interference

The β I small interfering RNA (siRNA) was obtained from Ambion (Applied Biosystems). Both the β II and β III siRNA siGENOME SMARTpool reagents were obtained from Dharmacon Research, Inc. The negative control siRNA (Ambion #1), which has no sequence homology to any known human gene sequence, was used in all experiments as a control; this strategy was intended to minimize non-specific effects. Cells were transfected with β isotype-specific siRNA at a final concentration of 100nM using lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Analysis of neurite outgrowth in SK-N-SH cells

Neurites are defined as protrusions with lengths that are at least 2-fold longer than cell bodies (Motegi et al. 2004). Areas (0.9mm $\times$ 0.7mm) were randomly selected from cell slides. siRNA transfected cells and their neurites were counted, and the numbers of neurites per cells were plotted using Microsoft Excel (Al Chawaf et al. 2007). Cell edges were outlined by fluorescence of actin stained by Bodipy phalloidin. Three independent experiments with at least 100-200 cells per condition were evaluated.

Measurement of Reactive Oxygen Species (ROS)

The generation of reactive oxygen species was monitored using 2', 7'-dichlorodihydrofluorescein-diacetate (DCFH-DA) (Invitrogen Molecular Probes), which is capable of detecting a variety of reactive oxygen species. This compound easily enters cells and hydrolyzes to 7'-dichlorodihydrofluorescein, which reacts with H₂O₂ and other reactive oxygen species to form the fluorescent derivative dichlorofluorescein (McArdle et al. 2005). In this procedure, cells were harvested and washed with PBS three times. Then the cells were resuspended in 1 mL PBS and incubated with 10 μ M DCFH-DA at 37°C for 30 minutes. After incubation, the cells were washed with PBS, then pelleted and lysed in 100 μ l of lysis buffer (50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP-40, 0.2 mM sodium orthovanadate, 100 mM sodium fluoride, 1% 200 mM PMSF, dissolved in dimethylsulfoxide, 1% Protease Inhibitor Cocktail). The lysed cells were centrifuged at 15,000 $\times$ g for 30 minutes at 4°C. Fluorescence intensities of the supernatants were measured at an excitation wavelength of 450 nm and an emission wavelength of 510 nm. Fluorescence intensities are given as arbitrary units (F.I.U.).

Statistical analysis

All statistics were performed by Student's *t* test compared with a control within each group. In all the figures, * indicates $p < 0.05$; ** indicates $p < 0.001$; and *** indicates $p < 0.0001$. All data are presented as mean $\pm$ S.D., $n=3$.

Results

Expression and localization of β isotypes of tubulin

Before examining tubulin isotype utilization in SK-N-SH cells, we sought to identify which β tubulin isotypes are expressed in both undifferentiated and differentiated cells. We observed that the β I, β II, and β III tubulin isotypes were expressed in undifferentiated SK-N-SH cells (Fig. 1), as well as in cells that had been induced to differentiate with retinoic acid (Fig. 2), while β IV tubulin was not detected in either (not shown). In the undifferentiated cells, the β I, β II, and β III tubulin isotypes formed microtubules in the cell body, which is flat and lacks neurites (Fig. 1). After differentiation, most cells acquired neuronal morphology:

that is, they had developed neurites, as has been observed by others (Lombet et al. 2001). Immunofluorescence staining images of differentiated SK-N-SH cells showed that the β I and β III tubulin isotypes were present in both the cell body and neurites (Fig. 2A,C). The β II tubulin isotype, however, was predominantly present in the neurites (Fig. 2B).

Quantitation of individual β isotypes of tubulin in SK-N-SH cells

The relative amounts of β tubulin isotypes in different tissues are very variable. It has been reported that the β I, β II, and β III tubulin isotypes constitute respectively about 3%, 58%, and 25% of the total β tubulin isotypes in cow brains (Banerjee et al. 1988). To determine the levels of individual β tubulin isotypes, we performed quantitative immunoblot analysis with each of three isotype-specific monoclonal antibodies. Quantitative standards for β I, β II, and β III tubulin isotypes were PC-tubulin, as well as purified β II-tubulin and purified- β III tubulin, all prepared from bovine cerebra. PC-tubulin was purified from bovine cerebra by cycling and chromatography on phosphocellulose (Fellous et al. 1977). PC-tubulin consists of α/β heterodimers, which was the starting material for all further tubulin purification. Samples (2.5 μ g -15 μ g) of PC-tubulin were loaded on a SDS-PAGE gel as standards together with undifferentiated and differentiated SK-N-SH cell extracts (Fig. 3A). We found that β I tubulin constitutes 0.80% of the total protein in undifferentiated cells and 0.91% of the total protein in differentiated cells. Similarly, we found that β II tubulin accounts for 1.3% of the total protein in undifferentiated cells and 1.2% in differentiated cells and that β III accounts for 0.75% and 0.80%, respectively, of the total protein in undifferentiated and differentiated cells (Fig. 3B).

Silencing of β isotypes of tubulin during neuronal differentiation

To examine the effects of β tubulin isotypes on neuronal differentiation, β tubulin isotype-specific siRNAs were used. Western blots were performed to examine silencing of protein expression. Cells were treated with siRNA on day 1, then retinoic acid (RA) on day 2 and subsequently every 48 hours. To enhance the silencing of β tubulin isotype expression during neuronal differentiation, siRNAs were applied a second time on day 5. Cells treated with siRNA and RA were collected on day 9 for western blot and immunofluorescence staining (Figs. 4, 5). Treatment with β I siRNA resulted in significant knockdown of the β I isotype compared to that in the cells transfected with the negative control siRNA (Fig. 4A). The β I siRNA specifically targeted the β I tubulin isotype and had almost no cross-reactivity with other β tubulin isotypes examined by Western blots (Fig. 4A). Very similar results were obtained with siRNAs for β II and β III: in each case the siRNA caused a large decrease in the expression of the corresponding isotype (Fig. 4C,D). In addition, silencing of one β tubulin isotype did not cause obvious compensatory changes in the other isotypes examined.

β I tubulin is necessary for cell viability during neuronal differentiation

To address whether β tubulin isotypes play a role in cell proliferation during neuronal differentiation, we examined cell viability in differentiated cells after knocking down a specific β isotype. For this purpose, cells were transfected with each β isotype-specific siRNA. Cells were then treated with or without RA to induce differentiation. Both trypan blue exclusion and the MTT method were used to determine cell viability. The effects of the siRNAs on cell viability are shown in Fig. 6. The results showed that, in undifferentiated cells, knocking down any single isotype had no effect on viability. In differentiated cells, in contrast, the viability of cells in which β I was knocked down decreased by 53% compared to the control cells, which were transfected with negative control siRNA, while silencing β II or β III had no effect on viability as shown by the trypan blue assay. Similarly, in the MTT assay, the major effect on viability was obtained by silencing β I, although a small but significant decrease was observed by silencing β II.

The roles of β I, β II and β III tubulin in neurite outgrowth

We examined the morphology and isotype distribution of cells treated with the various siRNAs. As shown in Fig. 5A1-A4, knocking down expression of β I had relatively little effect on these parameters. Neurites were still visible although they lacked β I. Although there were fewer viable cells, those that remained viable appeared to have normal morphology, although there was a small but not significant decrease in the number ratio of neurites to cell bodies (Fig. 7). However, reducing expression of β III, although it had no effect on viability, causes a 40% decrease in the ratio of neurites to cell bodies; some cells retain neurites and some do not (Fig. 5C1-C4). This is consistent with the results of Ferreira and Caceres (1992). The results observed with silencing β II, however, were dramatically different. Neurites were almost entirely absent from differentiated cells (Fig. 5B1,B2) and the number ratio of neurites to cell bodies diminished by over 90% (Fig. 7).

β III protects SK-N-SH cells treated with glutamate/glycine

Glutamate is a neurotransmitter that, together with glycine, interacts with the N-methyl D-aspartate (NMDA) receptor in neurons; a side effect of this interaction is production of free radicals and reactive oxygen species, including nitric oxide, superoxide anion, and peroxynitrite (Lipton 2006). At high levels, some of these can be toxic. Interestingly, tubulin is highly susceptible to reaction with peroxynitrite, which reacts with an as yet unidentified cysteine residue to form an intra-dimer disulfide that inhibits microtubule assembly (Landino et al. 2002). The β I, β II and β IV isotypes of tubulin contain cys239 whose sulfhydryl group is readily oxidized and whose oxidation inhibits microtubule assembly (Bai et al. 1989). β III has ser239 instead of cys239 and one could argue that it would be more resistant to reactive oxygen species and, hence, that β III may protect neuronal cells against the side effects of glutamate. In order to test this hypothesis, differentiated SK-N-SH cells were treated, separately, with siRNA's against β I, β II and β III and then with glutamate and glycine for 24 hours, with cell viability being measured at different times (Fig. 8). In cells treated with the negative control siRNA, there was no effect on viability. However, knocking down the levels of the different isotypes had significant effects. Cells that had β I knocked down showed an immediate 44% decrease in viability but gradually recovered over time. This is essentially the result observed earlier with knocking down β I (Fig. 6) and may have nothing to do with the reactive oxygen species generated by treatment with glutamate and glycine. Cells with β II knocked down exhibited an increasing loss of viability resulting in one hour in a small but significant decrease of 18%, at which point viability recovered over time. However, the results of knocking down β III were much more dramatic. Viability decreased by 44% at one hour and then gradually recovered. When the same experiment was done in the presence of the NMDA (N-methyl-D-aspartate) receptor inhibitor MK 801 (Pizzi et al. 2002) no loss of viability was observed (results not shown). Similarly, treatment of undifferentiated SK-N-SH cells, which lack NMDA receptor, with glutamate/glycine had no effect on viability (not shown). These observations suggest that the loss of viability caused by knocking down the β II and β III isotypes requires an active NMDA receptor.

The complexity of the results of the experiment shown in Figure 8 can be illuminated by measurement of the levels of reactive oxygen species (Fig. 9). When differentiated SK-N-SH cells were treated with glutamate and glycine, the result was a large initial rise in the level of reactive oxygen species peaking at 30 minutes and one hour and then declining after one hour to lower than the initial level. It is not surprising, therefore, that the maximal loss in viability in cells with either β II or β III knocked down also occurred at one hour and that this loss in viability was followed by recovery (Fig. 8). As one would expect, hardly any change in the levels of reactive oxygen species was observed when undifferentiated SK-N-SH cells were treated with glutamate and glycine (not shown).

Discussion

The strong evolutionary conservation of the differences among the vertebrate β -tubulin isotypes has raised the possibility that the different isotypes have different functions. For example, the constant association of the β IV isotype with axonemal microtubules strongly suggests a particular role for this isotype in forming these specialized microtubules (Lu et al. 1998; Renthal et al. 1993). It also appears that the β VI isotype has unique properties that allow it to polymerize into the curved microtubules of platelets (Italiano et al. 2003; Schwer et al. 2001). In addition, recent findings suggest that specific tubulin isotypes may play roles that are highly unique and may not even involve being present in microtubules. For example, β III is present as the $\alpha\beta$ III dimer in the outer mitochondrial membranes of certain cancer cells (Carre et al. 2002). An unusual isotype--- β V---has been found to actually decrease microtubule assembly in cells where it is over-expressed, hinting at a highly unique and still undetermined function (Bhattacharya and Cabral 2004).

In this work, we have approached the question of isotype function by knocking down expression of individual isotypes in differentiating human neuroblastoma cells. It appears that knocking down any one of the three isotypes--- β I, β II, and β III---we see expressed in these cells causes a unique outcome.

β I is the most widespread of the vertebrate β isotypes, occurring in almost all tissues (Roach et al. 1998); our results presented here suggest that β I is vital for cell viability. Hitherto the only clues to specific functions for β I are that it appears not to interact with actin filaments (Lezama et al. 2001) and that, like β IV, it occurs in axonemes, although not as prominently as does β IV (Jensen-Smith et al. 2003). The results presented here suggest that β I may play a role in viability, or perhaps better put, that its role in viability cannot be assumed by β II or β III. It is perhaps not coincidental that cutting β I expression by 55% results in a loss of viability of 53%. This is not likely to be due to a decrease in total tubulin expression, since β I is not the major isotype expressed in these cells, and a larger degree of suppression of either β II or β III does not result in any significant loss of viability. The fact that β I is so widespread in cells and tissues (Roach et al. 1998) suggests that it is capable of carrying out the canonical microtubule functions of mitosis and intracellular transport. The fact that β I occurs in both cell bodies and neurites is consistent with these roles and may account for its being required for cell viability, especially if β II and β III have evolved to have more specialized roles. On the other hand, both β II and β III are known to occur in mitotic spindles and interphase microtubule networks, implying that they also can carry out these functions. Obviously, for any isotype to perform these functions, they need to interact with a variety of other proteins. However, it may be that in SK-N-SH neuroblastoma cells, and perhaps in developing neurons as well, the particular microtubule-interacting proteins present in these cells that are required for the function of the interphase microtubule network interact preferentially with β I but less so with β II or β III.

β II is significantly less widespread in tissues than is β I; it is probably most abundant in the nervous system (Luduena 1998). The fact that silencing β II, even incompletely, causes such a great decrease in neurite outgrowth, suggests that β II plays a critical role in this process. Since silencing β I has very little effect on this process and silencing β III causes only a small inhibition, our results strongly suggest that β II has a specific function in neurite formation. The connection of β II to neurite formation is supported by the observation that in differentiated neuroblastoma cells β II occurs mostly in the neurites with apparently very little in the cell body (Fig. 2B). Furthermore, it has been reported that only the β II and β III tubulin isotypes accumulate significantly as a consequence of nerve growth factor-stimulated neurite outgrowth in rat PC-12 cells, which express five β tubulin isotypes (Joshi and Cleveland 1989). β II tubulin was recently found to be required for neuronal migration

(Jaglin XH 2009). Mutations of four residues in the β II tubulin gene result in asymmetrical polymicrogyria (Jaglin XH 2009), indicating a possible role for β II in determining cell morphology, consistent with the results presented here. The specific functions of the β II isotype have hitherto remained mysterious. Interestingly, although β II is present in a number of tissues, it is most abundant in the brain where it constitutes 58% of total β -tubulin (Banerjee et al. 1988). We have previously reported that β II occurs in the nuclei of certain cultured cells, such as rat kidney mesangial cells as well as placental cells and a wide variety of cancer cells (Yeh and Luduena 2004). Nuclear β II does not appear to be in microtubule form (Walss et al. 1999), nor does it appear to be transported into intact nuclei; rather the re-forming nuclear envelope appears to trap β II inside the nucleus (Walss-Bass et al. 2001). In addition, Kourmouli et al. (2001) have found that β II may have a specific interaction with a nuclear protein and they speculate that β II may play a role in re-formation of the nuclear envelope after cell division (Kourmouli et al. 2001). However, this is not likely to be its role in brain, since neurons rarely undergo mitosis. Hence, β II probably has some other role and our observation that β II is critical for neurite outgrowth may point to such a role. All the observed or postulated roles of β II---nuclear envelope reassembly, neurite outgrowth, and neuronal migration---involve substantial changes in membrane structure. Conceivably β II may interact directly or indirectly with a membrane protein in all of these roles; in the case of the nuclear envelope, β II binds to heterochromatin protein 1 (Kourmouli et al. 2001); perhaps there is an analogous protein in neurites.

It is striking that β I and β II are very similar in sequence but yet the effects of silencing each one, are very different. Certainly the sequence differences between β I and β II are very few, but these differences, which are highly conserved in evolution, are clustered in one region of the protein, namely, the C-terminal end. Although the C-terminal regions of β I and β II are both highly negatively charged (with many aspartate and glutamate residues) the C-terminal 14 amino acids of β I differ at 9 positions from those of β II. The C-terminal region is also where microtubule-associated proteins (MAPs) are thought to bind (Littauer et al. 1986). It is quite likely that specific MAPs, such as tau and MAP2, and perhaps others, may play determining roles in neurite formation. For example, a putative MAP required for neurite formation could conceivably bind better to the C-terminal region of β II than to the C-terminal region of β I. That MAP could therefore allow β II to promote neurite formation without conveying that same ability to β I. A comparable argument could be made about silencing β I, which causes a major loss of viability. If β I is necessary for viability, it is reasonable to suppose that microtubules containing β I are involved in intra-cellular transport. Conceivably, such transport may require a motor protein that binds to the C-terminal region and is capable of distinguishing between β I and β II; there is considerable evidence that both of the motor proteins dynein and kinesin bind to the C-terminal region of either α - or β -tubulin (Mizuno et al. 2004; Skiniotis et al. 2004); in the case of dynein, that region appears to be the C-terminus of the β isotype (Vent et al. 2005).

It is interesting that silencing β II causes a small but significant decrease in cell viability according to the MTT assay, but not in the trypan blue assay. The fact that knocking down β II causes a major decrease in neurite outgrowth may explain this result. Since trypan blue reports only on cell viability and MTT reports on metabolism, as well as viability, it is possible that a major decrease in neurite outgrowth would cause some decline in metabolism since presumably some metabolism would be required to fuel neurite outgrowth.

It is interesting that, although not as strikingly as is the case with β II, knocking down β III also diminishes neurite outgrowth. β III tubulin is believed not to be a primary factor involved in the regulation of microtubule assembly during early neurite outgrowth, but to be important for maintaining further neurite elongation (Ferreira and Caceres 1992). It is

possible that β III may assist in neurite outgrowth but is not absolutely critical for the process. Our results, however, suggest a more important role for β III.

β III normally has a very narrow distribution, occurring largely in neurons and the testis (Ludueno 1998). As mentioned above, β III lacks the highly reactive cys239, so one might imagine that it could be more resistant to the free radicals and reactive oxygen species produced in neuroblastoma cells by treatment with glutamate and glycine. Interestingly, β III contains a cys124, missing in the β I and β II isotypes. This cysteine is very close to the highly conserved cys127 and cys129 so it is conceivable that this cysteine cluster could conceivably act as a sink for these free radicals and reactive oxygen species. One could perhaps invoke the same function for cys239 in β I and β II. However, there is one observation that suggests that this is more likely to be the function of β III, namely, that knocking down β III is much more deleterious to cell viability upon glutamate/glycine treatment than is knocking down β II. This therefore raises the possibility that β III evolved to play some kind of protective role when the NMDA receptor is activated.

Of course, one cannot be certain that the protective role of β III is against the free radicals and reactive oxygen species produced by activation of the NMDA receptor by glutamate/glycine treatment. There could conceivably be some other phenomenon occurring as a consequence of the receptor being activated that could be deleterious to the cell when β III is knocked down. Interestingly, however, an experiment done on non-small cell lung cancer cells by Gan et al (2007) gives results somewhat parallel to ours and point more directly to the protective role being played by β III (Gan et al. 2007). silenced β I, β II and β III in these cells and then treated them with anti-tumor drugs known to interact with tubulin, namely, paclitaxel, vincristine and vinorelbine and also with drugs that do not interact with tubulin, namely, cisplatin, doxorubicin and etoposide. Silencing either β I or β II had no effect on viability of the cells in the presence of these drugs. However, silencing β III greatly increased susceptibility to all of the drugs. It is not surprising that silencing β III would sensitize the cells to taxanes and Vinca alkaloids since, of all the β isotypes, β III is known to interact least well with paclitaxel and vinblastine (Khan and Ludueno 2003). However, the only reason that silencing β III would enhance susceptibility to cisplatin, doxorubicin and etoposide, three drugs with three different mechanisms of action, is if β III exerts some kind of general protective effect on cells that express it; such a general effect could well involve protecting cells against reactive oxygen species. In this context, it is interesting that doxorubicin strongly promotes production of free radicals and reactive oxygen species in cells (Minotti et al. 1999).

Finally, it is striking that silencing any of the isotypes in undifferentiated cells had no effect on viability in contrast to differentiated cells where silencing β I had a major effect (Fig. 5). It is likely that in undifferentiated cells in general, each isotype is capable of performing the canonical functions of mitotic spindle formation and intracellular transport, but that, once the cell has differentiated, the isotype functions differentiate as well and each isotype is now required to carry out the specific function for which it evolved.

Conclusions

The small differences among the vertebrate β -tubulin isotypes have been highly conserved in evolution suggesting that they have functional significance, although, up to this point, relatively little functional significance has been assigned to specific isotypes. Since all the isotypes are capable of participating in the canonical functions of forming the interphase microtubule network and the mitotic spindle, it is likely that putative functional differences will be revealed in differentiated cells. Using differentiating human neuroblastoma cells as a model system and immunofluorescence microscopy and mRNA knockdown as tools, we

have obtained evidence suggesting that of the three β isotypes we have studied, β I is vital for cell survival, β II is not required for cell survival but is crucial for differentiation, and β III provides protection for the cell against free radicals and/or reactive oxygen species.

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Fig.1. Immunofluorescence micrographs of the β isotypes in undifferentiated cells
A) SK-N-SH neuroblastoma cells were labeled with an antibody to the β I-tubulin isotype; **B)** Cells were labeled with an antibody to the β II-tubulin isotype; **C)** Cells were labeled with an antibody to the β III-tubulin isotype. All antibodies are green. Nuclei are labeled with DAPI (blue). Bar (in panel C) = 20 μ m.

Fig.2. Immunofluorescence micrographs of the β isotypes in differentiated cells
SK-N-SH neuroblastoma cells were made to differentiate by treatment with retinoic acid. **A)** Cells were labeled with an antibody to the β I-tubulin isotype; **B)** Cells were labeled with an antibody to the β II-tubulin isotype; **C)** Cells were labeled with an antibody to the β III-tubulin isotype. All antibodies are green. Nuclei are labeled with DAPI (blue). Bar (in panel C) = 40 μ m.

Fig. 3. Quantitation of individual β tubulin isotypes in SK-N-SH cells

(A) Standard quantitative immunoblot for the β I, β II, and β III tubulin isotypes. Phosphocellulose-purified tubulin (PC-tubulin) (2.5 μ g, 5 μ g, 10 μ g, and 15 μ g) was used as standards for the β I isotype, and 10 μ g of undifferentiated & differentiated SK-N-SH cell extract were loaded to compare to the standards. Purified β II (1 μ g, 2 μ g) and β III tubulin (1 μ g, 2 μ g) from cow brain were used as standards for the β II and β III isotypes, respectively, and 20 μ g of undifferentiated and differentiated SK-N-SH cell extract were loaded for each blot. Immunoblots were labeled by β I, β II, and β III tubulin antibody, respectively. (B) Quantitation of β I, β II, and β III tubulin isotypes in undifferentiated and differentiated SK-N-SH cells. Each measurement was made in triplicate.

Fig. 4. Silencing of β tubulin isotypes in differentiated SK-N-SH cells

(A) Time schedule for RA treatment of siRNA-transfected SK-N-SH cells. Cells were treated with β isotype-specific siRNAs on day 1, then RA on day 2 and day 4. Further siRNA treatment was applied on day 5, and RA again on day 6 and day 8. Cells were collected on day 9 for Western blotting and indirect immunofluorescence assay. (B) Analysis of β I tubulin expression by Western blotting following 8 days of β I siRNA transfection and RA-induced neuronal differentiation. GAPDH was used as a loading control. No significant changes were observed for the β II and β III tubulin isotypes. (C)

Analysis of β II tubulin expression by Western blotting under identical conditions. **(D)**
Analysis of β III tubulin expression by Western blotting under identical conditions. Each measurement was made in triplicate.

Fig. 5. The effect of β isotype-specific siRNAs on cell morphology during neuronal differentiation in SK-N-SH cells

(A) A1-A2: Differentiated SK-N-SH cells transfected with the negative control siRNA were double-labeled with the β I-tubulin antibody (red) (A1) and Bodipy FL phalloidin (actin, green) (A2). A3-A4: Differentiated cells transfected with siRNA to β I were stained for β I (A3) and actin (A4). (B) B1-B2: Differentiated SK-N-SH cells transfected with the negative control siRNA were double-labeled with the β II-tubulin antibody (B1) and Bodipy FL phalloidin (B2). B3-B4: Differentiated cells treated with siRNA to β II were stained for β II (B3) and actin (B4). Note a significant decrease in neurites in cells lacking β II. (C) C1-C2: Differentiated SK-N-SH cells transfected with the negative control siRNA were double-labeled with the β III-tubulin antibody (C1) and Bodipy FL phalloidin (C2). C3-C4: Differentiated cells treated with siRNA to β III were stained for β III (C3) and actin (C4). Nuclei are labeled with DAPI (blue). Bar = 20 μ m.

Fig. 6. Cell viability during neuronal differentiation after siRNA transfection

Cells were transfected with each β isotype-specific siRNA, then with or without RA for differentiation. **A:** Cells were evaluated by Trypan blue exclusion method two days later after RA-induced differentiation or without differentiation. The ratio of live cells and total cells was shown as cell viability. **B:** The MTT method was used to evaluate cell proliferation under the same conditions. β I tubulin siRNA induced a significant decrease of cell viability during cell differentiation compared to the cells without RA treatment. Control: negative control siRNA was transfected. Each measurement was made in triplicate. *, $p < 0.05$; ***, $p < 0.0001$.

Fig. 7. Changes in neurite outgrowth in isotype-specific siRNA transfected & RA-induced differentiated SK-N-SH cells

Cells were treated with siRNA and RA following the 9-day protocol (see Fig.3.A). Cells with specified silenced β tubulin isotypes (siRNA knockdown) were selected for quantification of neurite outgrowth. The ratio of neurite number to total cell number was determined. At least 100 cells per condition from three dependent groups were evaluated. *, $p < 0.05$; ***, $p < 0.0001$.

Fig. 8. Effect of β -tubulin isotype knock-down on the viability of SK-N-SH cells treated with glutamate/glycine

Differentiated SK-N-SH cells were treated with isotype-specific siRNA, as indicated, then treated with 500 μ M glutamate, 100 μ M glycine, and 2 mM CaCl_2 for the indicated times. Cell viability was measured by the MTT method. Negative control siRNA was used as a control. Each measurement was made in triplicate. *, $p < 0.05$; **, $p < 0.001$.

Fig. 9. Effect of glutamate/glycine treatment on levels of reactive oxygen species

Differentiated SK-N-SH cells were treated with 500 μ M glutamate, 100 μ M glycine, and 2 mM CaCl_2 for the indicated times and the levels of reactive oxygen species were measured by the dichlorofluorescein method. Each measurement was made in triplicate. *, $p < 0.05$; **, $p < 0.001$.