Developmental Appearance of Phosphorylated Intermediate Filaments in Nuclei of Glioma and Neuroblastoma Cells

Thesis

Presented to the Graduate Council of

Southwest Texas State University

in Partial Fulfillment of the Requirements

for the degree of

Master of Science

by

Shannon E. Weigum, B.A.

San Marcos, Texas

May 2002

ACKNOWLEDGMENTS

I thank my mentor, Dr. Joseph Koke, for providing an academic environment in which your students are encouraged to think independently and develop their own ideas and interests. Thank you for giving me support when I needed it, while also giving me freedom to make my own mistakes in order to learn from them. I would also like to thank my additional committee members, Drs. Nick Christodoulides, Dana García, and Timothy Raabe, for sacrificing their time to take an active role in the development of my thesis. Dr. García, thank you for pushing me to do my best and never lowering your expectations. You are an excellent role model for the type of scientist I strive to become. Dr. Raabe and Dr. Christodoulides, thank you for making the long trips from San Antonio and Austin to be a part of my committee, I know it was not always easy on your schedules. Thank you all for your constructive criticism and invaluable advice.

I thank my husband, Kyle, for always encouraging me to do more than I ever believed I could. Your support and friendship is the best gift you could ever give me. I also thank my family for their help and guidance throughout my education.

This work has been supported in part by grants from the National Science Foundation (NSF DUE # 96-50654), National Institute of Health Bridges to the Baccalaureate (NIH GM58375-01A1), Texas Higher Education Coordinating Board (ATP003658-0193-1999 and ARP003658-0496-2001), and the Faculty Research Enhancement Program and Department of Biology of Southwest Texas State University.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

ABSTRACT

The structure and function of the proteinaceous skeleton in the nucleus of eukaryotic cells, termed the nucleoskeleton, is only vaguely known. Investigations into the role of intermediate filaments (IF) in the nucleus have primarily focused upon lamins, however some studies have suggested that there may be a role for non-lamin intermediate filaments in the nucleoskeleton. Recent studies of glioma and neuroblastoma cell lines have suggested that phosphorylated derivatives of IFs normally found in the cytoplasm can be demonstrated within nuclei. Additional reports indicate that IF phosphorylation may play an important role in regulating the dynamic organization of the intermediate filament network. In this study, the possibility that cytoplasmic phosphorylated IFs, recognized by J1-31 and SMI-31 mABs, can be found in the nuclei of F98 glioma and SH-SY5Y neuroblastoma cells, respectively, under conditions of cell growth or differentiation was investigated. I identified the growth stages of cells grown in culture by determining growth rates as a function of cell density, and I identified actively proliferating cells by tracking DNA synthesis using uptake of 5'-bromo 2'-deoxyuridine. Using immunocytochemistry, J1-31 and SMI-31 mAB nuclear labeling was exhibited primarily in proliferating cells with diminished nuclear labeling in quiescent cells found at high densities. SDS-PAGE and western blot analysis on whole cell lysates of F98 and SH-SY5Y cells were used to identify J1-31 and SMI-31 antigens. The J1-31 mAB appears to recognize a high molecular weight form of GFAP that contains phosphorylated serine residues in F98 glioma cells. SMI-31 mAB recognizes NF-H and NF-M peptide along with additional proteins in SH-SY5Y cells, but it was unclear if these proteins contained phosphorylated serine residues.

INTRODUCTION

The structure and function of the proteinaceous skeleton in the nucleus of eukaryotic cells, termed the nucleoskeleton, is only vaguely known. Investigations into the role of intermediate filaments (IF) in the nucleus have primarily focused upon lamins, which form a network or cortex inside the nuclear membrane and may extend inward to form functional domains deeper in the nucleus (Singh *et al.*, 1994). There they may play an important role in organization of the nuclear genome. Cytoplasmic IFs can interact with the nuclear lamina via nuclear pore complexes to anchor and support the nucleus within the cytoplasm (summarized by Singh *et al.,* 1994). Intermediate filaments other than lamin have not been found in the nucleus, but the possibility has not been excluded. Studies by Traub *et al.* (1983) demonstrated that vimentin can bind to single-stranded DNA suggesting a possible role for non-lamin intermediate filaments in nuclei. More recent studies of glioma (Herrera, 1995) and neuroblastoma (Glass *et al.*, 2002) cell lines have suggested that phosphorylated derivatives of IFs normally found in the cytoplasm may be found by immunocytochemistry in discrete locations within nuclei, suggesting binding to chromatin. The functional role of this binding, if it occurs, is unknown.

While using SMI-31 monoclonal antibody (mAB) to identify neurofilament proteins in neurites produced by SH-SY5Y neuroblastoma cells, Glass *et al.* (2002) unexpectedly found discrete SMI-31 labeling within nuclei. As characterized by Sternberger and Sternberger (1983), SMI-31 mAB is specific for phosphorylated epitopes on the highly phosphorylated heavy neurofilament peptide (NF-H) and to a lesser extent medium neurofilament peptide (NF-M) found in neurites. In mammals, neurofilaments are heteropolymers assembled from three peptides, light (NF-L), medium (NF-M), and heavy (NF-H) chain subunits that are classified based upon the size of their C-terminal tail

domain. These peptides are expressed differentially during development with NF-H and NF-M subunits present in axons and dendrites (Nixon and Shea, 1992). Phosphorylation of NF proteins regulates the polymerization and stability of the neurofilament network (Nixon and Sihag, 1991). Sternberger and Sternberger (1983) identified phosphorylated NFs (immunolabeled with SMI-31) in terminal axons and long projection fibers while non-phosphorylated NFs (immunolabeled with SMI-32) were found in cell bodies, dendrites and some proximal axons in rat cerebellar cortex.

The nuclear localization of SMI-31 mAB, observed by Glass *et al.* (2002), was surprising and could be the result of an association of phosphorylated NFs with nuclear components or recognition of non-neurofilament epitopes by SMI-31 mAB. Schilling *et al.* (1989) identified developmentally regulated nuclear antigens recognized by SMI-31 mAB in rat glial and neuronal nuclei *in vitro* and *in vivo* suggesting the presence non-neurofilament epitopes. These earlier studies prompted investigation of SMI-31 nuclear antigens in SH-SY5Y neuroblastoma cells and their dependence on the growth state or developmental state of the cell.

Herrera (1997) demonstrated nuclear localization patterns, similar to those obtained by Glass *et al.* (2002), using glioma cells (9L) immunolabeled with the J1-31 mAB directed against a cytoplasmic IF (Stevenson, 1996). This J1-31 mAB nuclear labeling was also identified in primary rat glial cultures by Singh *et al.* (1992) and suggested to be dependent on fixation. The J1-31 mAB was raised against human brain homogenates from multiple sclerosis plaques and was initially characterized as recognizing an intermediate filament associated protein because the antigenic determinants appeared distinct from GFAP (Malhotra *et al.*, 1984; Singh *et al.*,1986). Later work and sequence

analysis by Stevenson (1996) identified six discontinuous amino acid sequences of the J1-31 antigen that contained greater than 98% identity to human GFAP. However, there was approximately a 20,000 dalton discrepancy between the estimated molecular weight of the J1-31 antigen and GFAP. The molecular weight disparity suggested that the J1-31 mAB may be specific for a high molecular weight form of GFAP, possibly due to phosphorylation and/or oligomerization. GFAP contains five known phosphorylation sites in the N-terminal head domain and one in the C-terminal tail region (Inagaki *et al.,* 1996). Phosphorylation at four of these sites by protein kinase A and C in the head domain has been linked to disassembly of glial filaments *in vitro* (Inagaki *et al.,* 1990). Phosphorylation at all six GFAP sites would not account for the 20 kilodalton (kD) difference between the J1-31 antigen and GFAP, suggesting that phosphorylation alone is unlikely to distinguish the J1-31 antigen from GFAP.

The above studies indicate that there may be a role for non-lamin intermediate filaments in the nucleoskeleton and that phosphorylation plays an important role in regulating the dynamic organization of the intermediate filament network. The objective of this study was to utilize J1-31 and SMI-31 mAB immunolabeling in order to determine whether non-lamin phosphorylated IFs can be found in the nuclei of F98 glioma and SH-SY5Y neuroblastoma cells during periods of active cell growth.

F98 glioma and SH-SY5Y neuroblastoma cells were immunofluorescently labeled with J1-31 and SMI-31 mABs, respectively, and analyzed using confocal microscopy. Both antibodies exhibited discrete nuclear labeling and co-localized with a nucleic acid stain (TO-PRO-3) indicating fluorescent labels were within nuclei not just perinuclear. Non-phosphorylated IFs immunolabeled using anti-GFAP and SMI-32 did not

demonstrate any nuclear labeling suggesting that phosphorylation of IFs may be required for their appearance in nuclei.

I also examined the possibility that the nuclear labeling of J1-31 and SMI-31 mABs was an artifact of fixation by immunolabeling cells fixed in methanol and paraformaldehyde. Nuclear localization of both antibodies remained in sparsely populated cells indicating it was independent of fixation; however, nuclear localization diminished in densely populated cells fixed in paraformaldehyde suggesting that the nuclear epitopes were affected by the cell density and that paraformaldehyde fixation enhances these differences.

In order to determine a correlation between the growth state of the cell and J1-31 and SMI-31 nuclear localization, I identified actively proliferating cells by tracking DNA synthesis using uptake of 5'-bromo 2'-deoxyuridine, a thymidine analog. Quantitative measurement of the immunofluorescent label found in nuclei was used to identify significant changes in intensity as a function of increasing cell density. Nuclear localization of J1-31 and SMI-31 antigens diminished over time with increasing cell density.

SDS-PAGE and western blot analysis was used on whole cell lysates of F98 and SH-SY5Y cells to identify J1-31 and SMI-31 antigens, respectively. The J1-31 mAB appears to recognize a high molecular weight form of GFAP that contains phosphorylated serine residues in F98 glioma cells. SMI-31 mAB recognizes NF-H and NF-M peptides along with additional proteins in SH-SY5Y cells, but in this study it was unclear if these proteins contained phosphorylated serine residues.

MATERIALS AND METHODS

A. Preparation and Maintenance of Glioma and Neuroblastoma Cell Cultures

F98 glioma and SH-SY5Y neuroblastoma cells were seeded from frozen stock cultures in fetal bovine serum (FBS, Sigma, St. Louis, MO) with 10% dimethyl sulfoxide (DMSO, Sigma) stored in liquid nitrogen. Cells were rapidly thawed and DMSO was removed by centrifugation at 2500 rpm followed by resuspension of the cell pellet in minimum essential medium (MEM, Sigma, St. Louis, MO) containing 10% FBS, 2 mM L-glutamine and 10,000 U penicillin/10,000 mg/ml streptomyocin. The cell density was determined using a hemacytometer (Fisher Scientific, Pittsburgh, PA) and diluted appropriately to maintain an initial seeding density of $4.0x10^4$ cells/cm^2 of surface area. Cell cultures were seeded into 25 cm^2 culture flasks with 5 ml MEM or 150 cm^2 culture flasks with 25 ml MEM and maintained at 37°C in a humidified atmosphere containing 5% CO_2 for 24-96 hours. Cells cultured on glass coverslips were also seeded from frozen stock cultures, and plated into 100 x 15 mm petri dishes containing 6 (22 x 22 mm) glass coverslips and 10 ml MEM. The cells were allowed to grow in culture for 24-120 hours. (Note: all cells were grown for approximately 72 hours and fixed in cold methanol unless otherwise indicated)

B. Fixation and Staining for Immunofluorescence Microscopy

Cells grown on coverslips were washed briefly in a phosphate-buffered saline (PBS) solution (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) and then either fixed in 100% methanol at –20°C for 1 minute or at room temperature in a 4% formaldehyde (PFA) solution freshly made from paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) and buffered with PBS (or PBS with 0.2% Tween-20,

PBST) for 10 minutes followed by 3 washes in PBS/PBST for 10 minutes each. Next, the cell-bearing coverslips were incubated in 20% non-fat powdered milk in PBS to block non-specific protein-protein interactions for 2 hours at room temperature. The coverslips were then washed 3 times, 10 minutes each, and incubated with the desired primary antibody (Table 1) for 16-24 hours at 4°C. The cells were again washed three times in PBS (10 minutes each) and incubated with the appropriate secondary antibody (Table 2) for 2 hours at room temperature. During the final 30 minutes of secondary antibody incubation 40 µl of TO-PRO-3 (T-3605, Molecular Probes), a DNA specific probe was added for identification of nuclei (Table 1). Negative controls for all immuno-preparations were prepared by use of the appropriate normal serum in place of the primary antibody. The coverslips were then mounted in 90% glycerol with 1 mg/ml aqueous p-phenylenediamine and stored at –20°C until microscopic analysis.

C. Microscopy and Quantitative Analysis of Immunolabel Intensity

For fluorescence and confocal microscopy, an Olympus IX-70 fitted with a Bio-Rad MRC 1024 confocal scanhead via the Keller port was used. Image acquisition and initial processing were done using Bio-Rad LaserSharp software running on a Compaq PC; final processing and printing of images was done using Adobe Photoshop 6.0 software running on a G4-Macintosh and driving an Epson Stylus 980N color ink-jet printer.

Quantitation of fluorescent labeling in confocal images was performed on images collected using the same microscope channel settings for similarly labeled cells grown 1-5 days. Confocal stacks were imported into NIH Image 1.6 software and projected into a single image. Density slice filtering was used to select pixels representing staining above background, autofluorescence levels. The image software then averaged the pixel

intensities over the area selected, providing an intensity/unit area value that could be compared from preparation to preparation. Intensity measurements were collected from 8-15 random nuclei within each image field and imported into StatView software to permit comparison of staining intensity as a function of days in culture. Single-factor ANOVA tests were performed on the daily mean intensities followed by post-hoc Tukey/Kramer tests to identify statistical significance among the daily means. "P" values of less than 0.05 were judged to indicate significant differences.

D. Identification of Actively Growing Cells using 5-Bromo-2'-Deoxyuridine

F98 and SH-SY5Y cells were seeded onto glass coverslips at an initial density of $4.0x10^4$ cells/cm^2 and allowed to grow for 1-5 days. Twenty-four hours prior to fixation, the cells were incubated with 10μM 5-bromo-2'-deoxyuridine (BrdU, B-23151, Molecular Probes, Eugene, OR) in MEM. At the appropriate time, the cells were fixed in methanol at –20°C for 1-2 minutes, allowed to air dry, then stored at –20°C until all coverslips were ready for processing. The cells were rehydrated in PBS for 5 minutes followed by immersion in 2N HCl for 1 hour at room temperature. The cells were then placed in a 0.1M borate buffer (pH 8.5, 0.1M boric acid, 25 mM $Na_2B_4O_7$, and 75 mM NaCl) changing the solution once over a 10 minute period followed by three 10 minute washed in PBS. Next, cells were immunolabeled as described above using anti-BrdU monoclonal mouse IgG primary antibody at a 1:400 dilution (A-21300, Molecular Probes) followed by secondary antibody goat anti-mouse IgG conjugated to Alexa-Fluor 488 (1:200, A-11001, Molecular Probes). All antibody incubations were 1 hour at room temperature in reduced light. During the final 30 minutes of secondary antibody incubation TO-PRO-3 nucleic acid stain was added at a 1:1000 dilution. The coverslips were then washed and

mounted for microscopic analysis.

E. Cell Growth Analysis

Growth curves were developed for F98 glioma and SH-SY5Y neuroblastoma cells by culturing each cell line at an initial density of $4.0x10^4$ cells/cm^2 into four 25 cm^2 culture flasks (F98) or into five 25 cm^2 flasks (SH-SY5Y). Cells were harvested from one flask each day from both cultures using 5 ml Puck's saline solution (pH 7.3, 137 mM NaCl, 5.4 mM KCl, 0.7 mM Na_2HPO_4, 9.5 mM HEPES, 5.6 mM dextrose, and 0.8 mM EDTA). Detached cells were centrifuged at 2500 rpm for 5 minutes, and the cell pellet was resuspended in 1-2 ml of PBS. Representative samples from each culture were counted using a two-chambered hemacytometer over four to five consecutive days.

F. Preparation of Total Cell Lysates from Cultured Cell

F98 and SH-SY5Y cells were seeded at an initial density of $4.0x10^4$ cells/cm^2 and grown in 150 cm^2 flasks for 2 days. MEM was removed and cells were detached from the substrate using 10 ml Puck's saline solution and incubated at 37°C for 5 minutes. The detached cells were transferred to a centrifuge tube and spun at 2500 rpm for 5 minutes. The pellet was resuspended in 5 volumes of 2x sample buffer (60 mM Tris-HCl, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromphenol blue, and 1 mM EGTA) then diluted 1:1 in PBS. Samples were boiled at 100°C for 5 minutes followed by shearing of chromosomal DNA by passing samples through a 20-gauge and 26-gauge needle repeatedly. Cell lysates were centrifuged in a microcentrifuge at 4000 g for 15 minutes then aliquoted and stored at –20°C up to four weeks until sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Additional samples of isolated nuclei and cytoplasm were prepared using Sigma EZ

Prep Nuclei Isolation Kit (#NUC-101). Isolated nuclei were suspended in sample buffer, boiled at 100°C for 5 minutes and stored at –20°C up to four weeks until electrophoresis. Cytoplasmic fractions were concentrated by precipitation in 10% trichloroacetic acid and samples were prepared as above for SDS-PAGE and western analysis.

G. Electrophoresis and Western Blot Analysis

SDS-PAGE was performed according to the method originally described by Laemmli (1970). One volume of 5x sample buffer was added to 4 volumes of sample (except total cell lysates already in sample buffer); the resulting solution was boiled and then briefly centrifuged at 10,000g. Protein concentrations of the cell lysates and isolated nuclei samples were determined using the Bio-Rad Protein Assay (#500-0006, Hercules, CA). Bovine serum albumin standards were prepared exactly as experimental samples described above. Sample volumes were adjusted so that 5-10 μg of total protein were loaded into wells of a discontinuous (12% separating, 4% stacking) polyacrylamide gel. Gels were prepared in duplicate. Electrophoresis was carried out at 200 V until the dye front reached the bottom of the gel. One of each pair of gels was then stained and fixed in Coomassie blue dye (1.2 mM Coomassie blue R-250, 45% methanol, 10% acetic acid) for 10 minutes and destained in 10% acetic acid and 10% methanol in water for 12 hours. The duplicate gel was prepared for electrophoretic transfer onto a nitrocellulose membrane (#162-0115, Bio-Rad, Hercules, CA). The transfer was performed at 100 V for 2.5 hours at 4°C. The identities of the proteins bound to the nitrocellulose membrane were revealed by western blot analysis using the appropriate primary and secondary antibodies (Table 1 and 2). Rf ratios were calculated by measuring the distance from the bottom of the well to the protein band of interest divided by the distance from the bottom

of the well to the dye front. Standard curves were developed using log MW of protein standards versus Rf ratio of those standards. Apparent molecular weights of proteins identified by western blot analysis were determined by extrapolating MW from the standard curve using measured Rf ratios.

Table 1. Primary antibodies and dilutions used for immunofluorescence and western blot analysis.

Antibody (Catalog #) Specificity	**Species/ Isotype**	**Dilution for Fluorescence**	**Dilution for Western Analysis**	**Supplier**
J1-31 mAB from hybridoma line CRL-2253 Form of GFAP??	Mouse IgG	Undiluted culture supernatant	Undiluted culture supernatant	American Type Culture Collection, Rockville, MD
Anti-GFAP pAB (#G-9269)	Rabbit IgG	1:86 in PBS	1:400 in PBS	Sigma Chem. Co., St. Louis, MO
Anti-phospho-neurofilament, SMI-31 mAB	Mouse IgG1	1:1000 in PBS	1:2000 in PBS	Sternberger Monoclonals, Inc., Lutherville, MD
Anti-nonphospho-neurofilament, SMI-32 mAB	Mouse IgG1	1:1000 in PBS	1:1000 in PBS	Sternberger Monoclonals, Inc. Lutherville, MD
Anti-BrdU mAB (#A-21300)	Mouse IgG	1:50 in PBS	---	Molecular Probes, Eugene, OR
Anti-phosphoserine mAB (#P-3430)	Mouse IgG1	---	1:1000 in PBS	Sigma Chem. Co, St. Louis, MO

Table 2. Secondary antibodies and probes used for immunofluorescence and western blot analysis.

Antibody/Probe (Catalog #)	**Conjugate**	**Dilution**	**Supplier**
Goat anti-mouse IgG (#A-11001)	Alexa-fluor 488	1:200 in PBS	Molecular Probes, Eugene, OR
Goat anti-rabbit IgG (#T-6778)	TRITC	1:200 in PBS	Sigma Chem. Co, St. Louis, MO
Goat anti-mouse polyvalent (#A-0162)	Alkaline Phosphatase	1:4000 in PBS	Sigma Chem. Co, St. Louis, MO
Goat anti-rabbit IgG (#A-3812)	Alkaline Phosphatase	1:30,000 in PBS	Sigma Chem. Co., St. Louis, MO
Nucleic Acid binding probe; TO-PRO-3 iodine (#T-3605)	---	1:1000 in PBS	Molecular Probes, Eugene, OR

RESULTS

A. Using immunohistochemical analysis and confocal microscopy, can J1-31 and SMI-31 antigens be localized in the nuclei of cultured glioma and neuroblastoma cells, respectively?

In order to identify cytoplasmic and nuclear localization of the J1-31 antigen, F98 glioma cells were immunolabeled with various combinations of the following: J1-31 mouse IgG mAB, rabbit IgG anti-GFAP polyclonal antibody (pAB) for use as a cytoplasmic marker, and TO-PRO-3 nucleic acid stain for use as a nuclear marker. Intense labeling with J1-31 mAB was seen within nuclei and to a lesser extent in cytoplasm (Figure 1). Confocal micrographs from a single image plane of F98 glioma cells identified J1-31 mAB labeling in distinct ring formations within or around the nuclear membrane and in discrete locations within the nucleus (Figure 2A). A high degree of co-localization between J1-31 mAB and TO-PRO-3 can be seen on the micrograph in areas appearing light blue and by using a co-localization graph (Figure 2B). Co-localized pixels appear on the diagonal between the x-axis (blue signal intensity) and the y-axis (green signal intensity) because they contain both a green and blue intensity component. Pixels containing both green and blue signal intensities above 133 were selected from the co-localization graph and highlighted in white on the micrograph (Figure 2C). This confirms the presence of the J1-31 antigen within nuclei. The J1-31 antigen was also seen within the cytoplasm, where it can co-localize with GFAP (Figure 3B and 3C). Anti-GFAP labeling was found only in the cytoplasm (Figure 3A) (red).

To identify the cellular distribution of SMI-31 antigens, SH-SY5Y neuroblastoma cells were immunolabeled with various combinations of the following: SMI-31 mouse

IgG mAB, SMI-32 mouse IgG mAB (specific for non-phosphorylated neurofilaments) and TO-PRO-3 nucleic acid stain. SMI-31 mAB labeling was seen in both cytoplasm and select nuclei, while SMI-32 mAB labeling could only be found within the cytoplasm (Figure 4).

I also examined J1-31 mAB immunoreactivity in neuroblastoma cells and SMI-31 mAB labeling in glioma cells. J1-31 mAB did not demonstrate any specific labeling in SH-SY5Y cells (Figure 5C). It has previously been shown that the J1-31 mAB is specific to glial cells in the CNS, namely astrocytes, and does not react with other cell types (Singh *et al.,* 1986). On the other hand, F98 glioma cells stained with SMI-31 mAB exhibited intense labeling throughout the cell, nuclear and cytoplasmic (Figure 5B).

B. Is the nuclear localization of J1-31 and SMI-31 antigens dependent on fixation?

To determine the effects of various types of chemical fixation on the appearance of J1-31 and SMI-31 antigens within nuclei, I compared immunolabeling patterns from cells fixed in methanol versus those fixed in 4% paraformaldehyde. Nuclear localization of J1-31 antigen appeared to be affected by the type of chemical fixative as F98 cells fixed in methanol demonstrated J1-31 mAB labeling primarily within nuclei and to a lesser extent in the cytoplasm (Figure 6A) while the same cells fixed in paraformaldehyde showed diminished nuclear labeling but retained equivalent or possibly increased cytoplasmic labeling (Figure 6C). However, this apparent dependence on fixation disappeared in sparsely populated cultures (Figure 6B, D). Small nuclear "dots" seen in newly cultured cells (12-24 hours growth) labeled with J1-31 mAB were independent of fixation, but ring formations created around the nuclei were only seen in methanol fixed cells. SMI-31

mAB labeling in SH-SY5Y nuclei also exhibited an apparent dependence on fixation that disappeared in sparsely populated cultures (Figure 7).

The possibility that diminished nuclear labeling in paraformaldehyde fixed cells was caused by failure of the antibodies to penetrate cell membranes was also investigated. F98 and SH-SY5Y cells were fixed in paraformaldehyde, permeabilized using PBS with 0.2% Tween-20, and immunolabeled as described above replacing PBS with PBST. There was no difference in immunolabeling patterns of J1-31 and SMI-31 mABs in permeabilized versus non-permeabilized cells fixed in paraformaldehyde (data not shown).

C. Growth curves and identification of proliferating cells using 5-bromo-2'-deoxyuridine

Growth curves for F98 glioma and SH-SY5Y neuroblastoma cells were developed to help identify the growth phase of cells seeded at $4.0x10^4$ cells/cm^2 and grown for various lengths of time. F98 cells exhibited a characteristic lag phase after initial seeding followed by a steady growth phase (Figure 8A). SH-SY5Y growth curve showed a decrease in cell density following initial seeding, possibly due to low cell viability, followed by a brief growth phase. The SH-SY5Y overall growth phase was not steady but seemed to exhibit a twenty-four hour growth delay followed by rapid growth periods (Figure 8B).

In addition to the growth curves, the portion of cells that were engaged in DNA synthesis were identified using the thymidine analog, 5-bromo-2'-deoxyuridine. Incorporation of BrdU in newly synthesized DNA marks cells that have progressed through the S-phase of the cell cycle. F98 cells incubated in BrdU and grown for 1-5

days demonstrated a decrease in anti-BrdU labeling with increasing confluency and in densely clustered cells (Figure 9A-E). Following the initial lag phase with little growth (day 1), anti-BrdU labeling could be seen in almost all non-confluent cells (day 2 and 3). Cells located within clusters (day 4 and 5) show diminished anti-BrdU labeling as seen by the cells lacking anti-BrdU signal (green) but retain TO-PRO-3 labeling (blue). Quantitative analysis of the anti-BrdU fluorescent label intensity found in these F98 cells confirmed this result, indicating a significant decrease in mean pixel intensity over time (Figure 9F). SH-SY5Y cells incubated in BrdU also exhibited decreases in BrdU labeling within clustered cells and at higher cell densities found on day 5 (Figure 10A-E). Quantitative analysis of the BrdU fluorescent label identified a low initial intensity found on day 1, possibly due to a lag in growth found after seeding, with a sharp increase on day 2 followed by a steady decline through day 5 (Figure 10F). The mean pixel intensity found on days four and five were not significant from day 1; however, they were significant when compared to day 2 intensity.

D. Correlation between nuclear localization of J1-31 and SMI-31mAB labeling and growth time in culture (cell density) of F98 glioma and SH-SY5Y neuroblastoma cells

In order to identify the relationship between J1-31 mAB nuclear labeling and cell growth in culture, I immunolabeled cells grown for various lengths of time and analyzed them using confocal microscopy. Nuclear labeling of the J1-31 antigen was more intense in low density cells cultured 1-2 days and decreased over time with increasing cell density as seen on days 3 and 4 (Figure 11A-D). Analysis of mean pixel intensity (on a scale of 0-256) indicated a significant decrease from cells grown 1 day (83.16) to those grown 4

days (49.90) (Figure 11E). Anti-GFAP labeling was only found in the cytoplasm and appeared most intense on day 2 (Figure 12). Analysis of pixel intensity for anti-GFAP was not completed due to the absence of anti-GFAP nuclear localization.

An association between the appearance/disappearance of SMI-31 antigens, phosphorylated NFs, and growth time in culture or cell density was determined. SH-SY5Y cells immunofluorescently labeled with SMI-31 mAB exhibited a gradual decline in SMI-31 fluorescent intensity in cells grown from 1-3 days (mean pixel intensity 60.04, 54.39, and 49.99) but drastically declined in cells grown for 4 days (23.90) (Figure 13A-D). At the same time (day 4), SMI-31 labeling appeared within neurites (Figure 13D). Staining with SMI-32 mAB, specific for non-phosphorylated NFs (Sternberger and Sternberger, 1983), did not result in any nuclear labeling; however, the cytoplasmic labeling appeared much stronger on days 4 and 5 (Figure 14). Analysis of pixel intensity was not completed for SMI-32 mAB label due to the lack of nuclear localization.

E. SDS-PAGE and western blot analysis of F98 glioma and SH-SY5Y neuroblastoma cell lysates

To identify the molecular weights of proteins labeled by J1-31 and SMI-31 mABs, I analyzed F98 and SH-SY5Y cells using SDS-PAGE and western blotting techniques. Initial antibody dilutions were determined from previous studies (Schilling *et al.*, 1989) and supplier recommendations. These dilutions were adjusted experimentally until non-specific background labeling was minimized with specific protein bands still visible. Purified bovine GFAP (#GF-01 Cytoskeleton Inc., Denver, CO), served as a positive control for anti-GFAP western blots. The J1-31 mAB labeled five protein bands at estimated MW of 207 kD, 100 kD, 74 kD, 71 kD, and 36 kD in F98 cell lysates (Figure

15c). Two of these bands (100 kD and 36 kD) were also recognized by anti-GFAP antibody. Anti-GFAP antibody labeled additional bands at 160 kD, 155 kD, 81 kD, and 51 kD (Figure 15d). Western blots using anti-phosphoserine mAB were also used to determine whether the J1-31 antigens contained phosphorylated serine residues. Anti-phosphoserine mAB recognized two proteins at an estimated MW of 100 kD and 71 kD (also labeled by J1-31 mAB) as well as additional proteins at 162 kD, 140 kD, and 60 kD (Figure 15e). One particularly notable band is the 100 kD protein recognized by anti-GFAP, J1-31 mAB, and anti-phosphoserine mAB. Western blot analysis of isolated nuclei did not identify any difference between antigens in nuclei from those in whole cell lysates. No proteins were identified in cytoplasmic fractions on western blots (data not shown).

Purified bovine GFAP was separated using SDS-PAGE followed by western blot analysis using J1-31 mAB and anti-GFAP antibody as a positive control. Purified GFAP protein was labeled by the J1-31 mAB at 51 kD (Figure 16d), the expected molecular weight of GFAP. This 51 kD band was also labeled by anti-GFAP antibody; however, anti-GFAP also identified numerous proteins above and below this mass (Figure 16c).

SDS-PAGE and western blots using SH-SY5Y cell lysates labeled with SMI-31 mAB identified protein bands at an apparent Mr of 210 kD, 177, 150, 132, and 91 (Figure 17c). Two of these protein bands at Mr of 210 and 150 kD were also labeled with the SMI-32 mAB. Western blots using anti-phosphoserine mAB did not identify any noticeable bands (data not shown).

Figure 1

Confocal micrograph prepared by digital projection of F98 glioma cells grown on coverslips for 72 hours and fixed in cold methanol. Cells were immunolabeled with J1-31 mAB with goat anti-mouse IgG secondary conjugated to Alexa-fluor 488. Labeling can be seen within cytoplasmic extensions or processes (small arrow) and diffusely in the cytoplasm. Intense labeling can be seen in discrete locations within select nuclei (large arrow) and appear as small nuclear "dots" or ring formations.

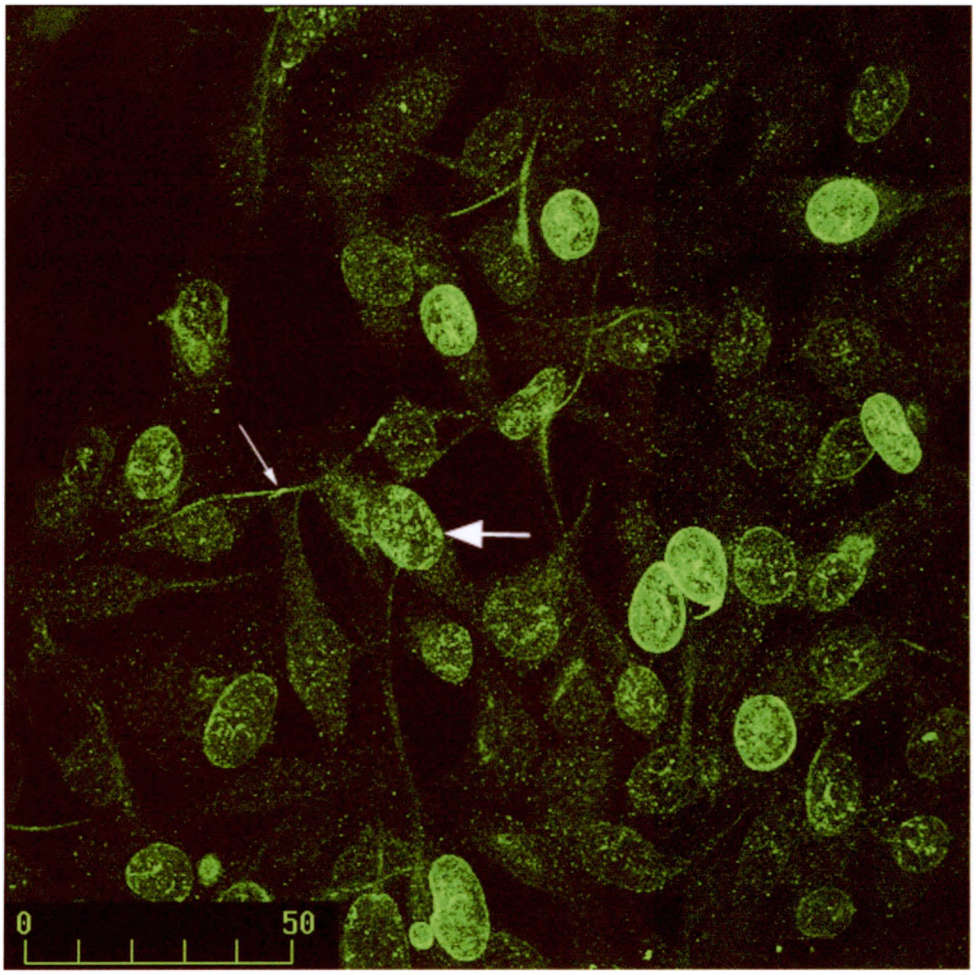

0
50

Figure 2

A confocal micrograph of a single image plane showing F98 nuclei immunolabeled with J1-31 mAB, goat anti-mouse secondary conjugated to Alexa-fluor 488, (green) and stained with TO-PRO-3 (blue) (panel A). Notice the discrete localizations of the J1-31 antigen within nuclei. This punctate staining pattern suggests that the J1-31 mAB is labeling an antigen possibly complexed with chromatin. A co-localization plot identifies a high degree of co-localization between J1-31 mAB and TO-PRO-3 stain (panel B). Pixels containing green and blue intensities above 133 were selected from the co-localization graph and highlighted in white on the micrograph (panel C). This confirms the presence of the J1-31 antigen within nuclei of F98 glioma cells.

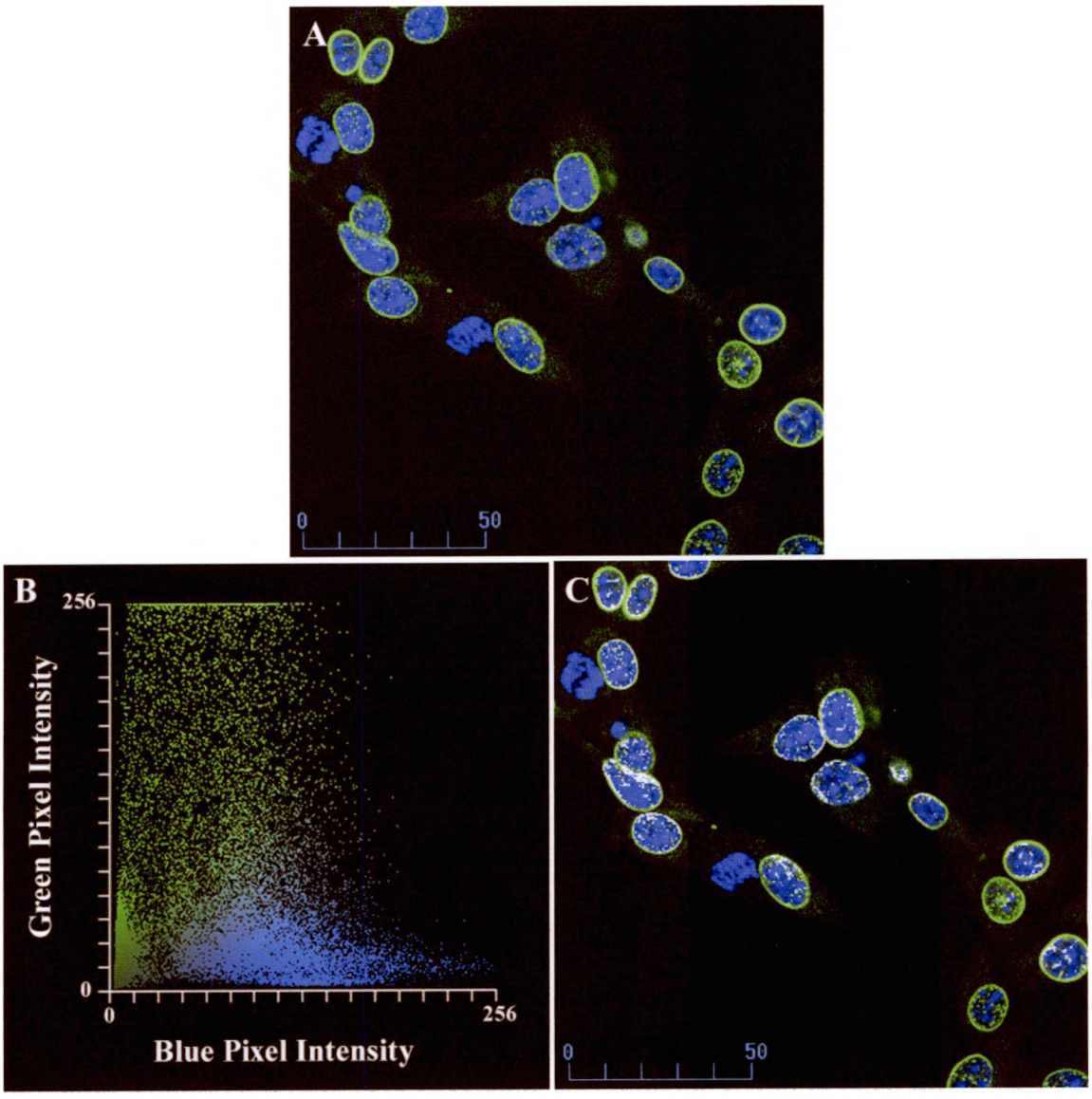

A
0
50
B
256
Green Pixel Intensity
0
0
256
Blue Pixel Intensity
C
0
50

Figure 3

A confocal micrograph from a single image plane of F98 cells grown on coverslips and double labeled with the J1-31 mAB and anti-GFAP antibody. The J1-31 antigen was stained with a primary J1-31 mAB followed by an Alexa-Fluor-488 conjugated secondary antibody (green) while GFAP was labeled using anti-GFAP primary antibody followed by a TRITC conjugated secondary (red) (panel A). Co-localization plot (panel B) identifies a low degree of co-localization between the J1-31 antigen and GFAP. Pixels containing both red and green signal intensities above 133 were selected from the co-localization graph and highlighted in white on the micrograph (panel C). This demonstrates that the J1-31 antigen and GFAP co-localization is only found in the cytoplasm.

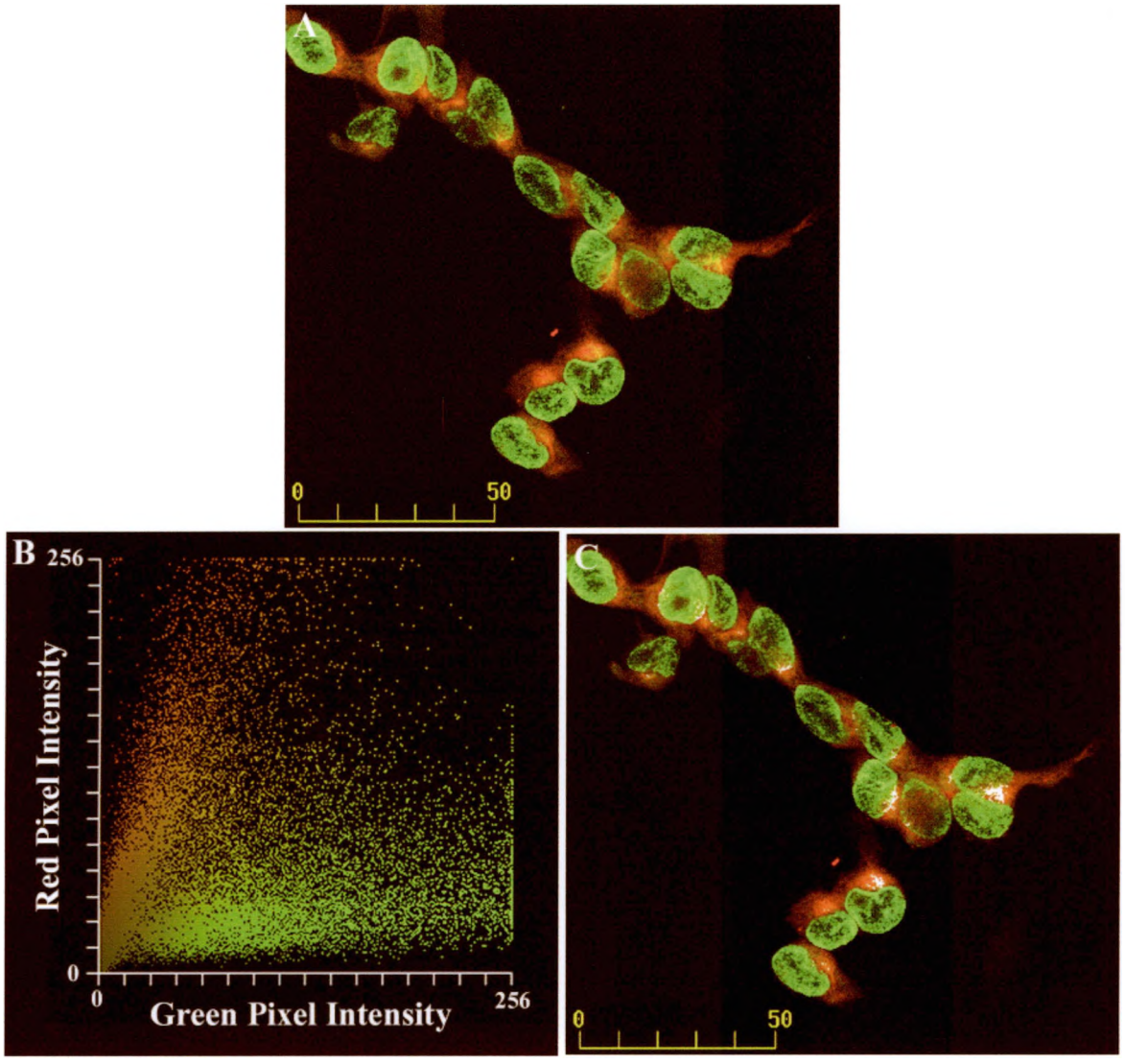

A
0
50
B
256
Red Pixel Intensity
0
0
256
Green Pixel Intensity
C
0
50

Figure 4

Confocal micrographs prepared from single image planes of SH-SY5Y cells grown on glass coverslips and labeled with the SMI-31 (panel A) and SMI-32 mABs (panel B) followed by an Alexa-Fluor-488 conjugated secondary antibody (green). Nuclei were revealed by TO-PRO-3 probe (blue). Detection of SMI-31 mAB labeling is observed in select nuclei (large arrow) and in filamentous structures seen in neuronal processes (small arrow). SMI-32 mAB labeling is found only in cytoplasmic areas.

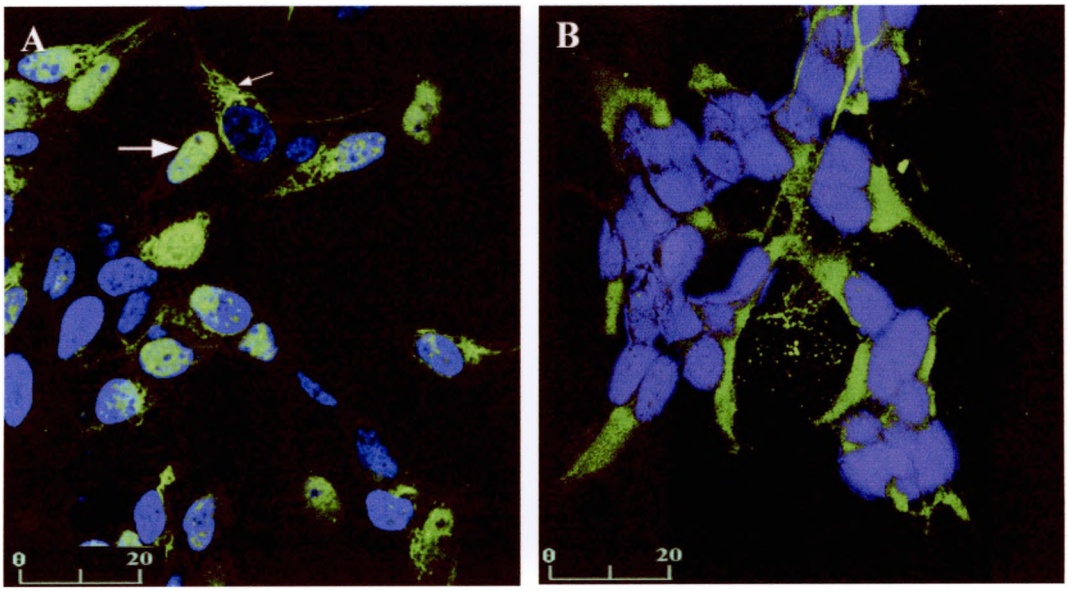

A
0
20
B
0
20

Figure 5

Matrix of confocal projections identifying the various immunolabeling patterns of J1-31 and SMI-31 mABs found in F98 and SH-SY5Y cell lines. All primary antibodies were followed by goat-anti-mouse secondary antibody conjugated to Alexa-Fluor 488 (green) and nuclei were revealed with TO-PRO-3 probe (blue). F98 cells immunolabeled with J1-31 mAB demonstrated intense nuclear labeling and diffuse cytoplasmic labeling (panel A) while the same cells labeled with SMI-31 mAB demonstrated high levels of labeling throughout the cells (panel B). SH-SY5Y cells immunolabeled with SMI-31 mAB demonstrated labeling within neuronal processes and in select nuclei (panel D); however, J1-31 mAB did not demonstrate any specific labeling in neuroblastoma cells (panel C).

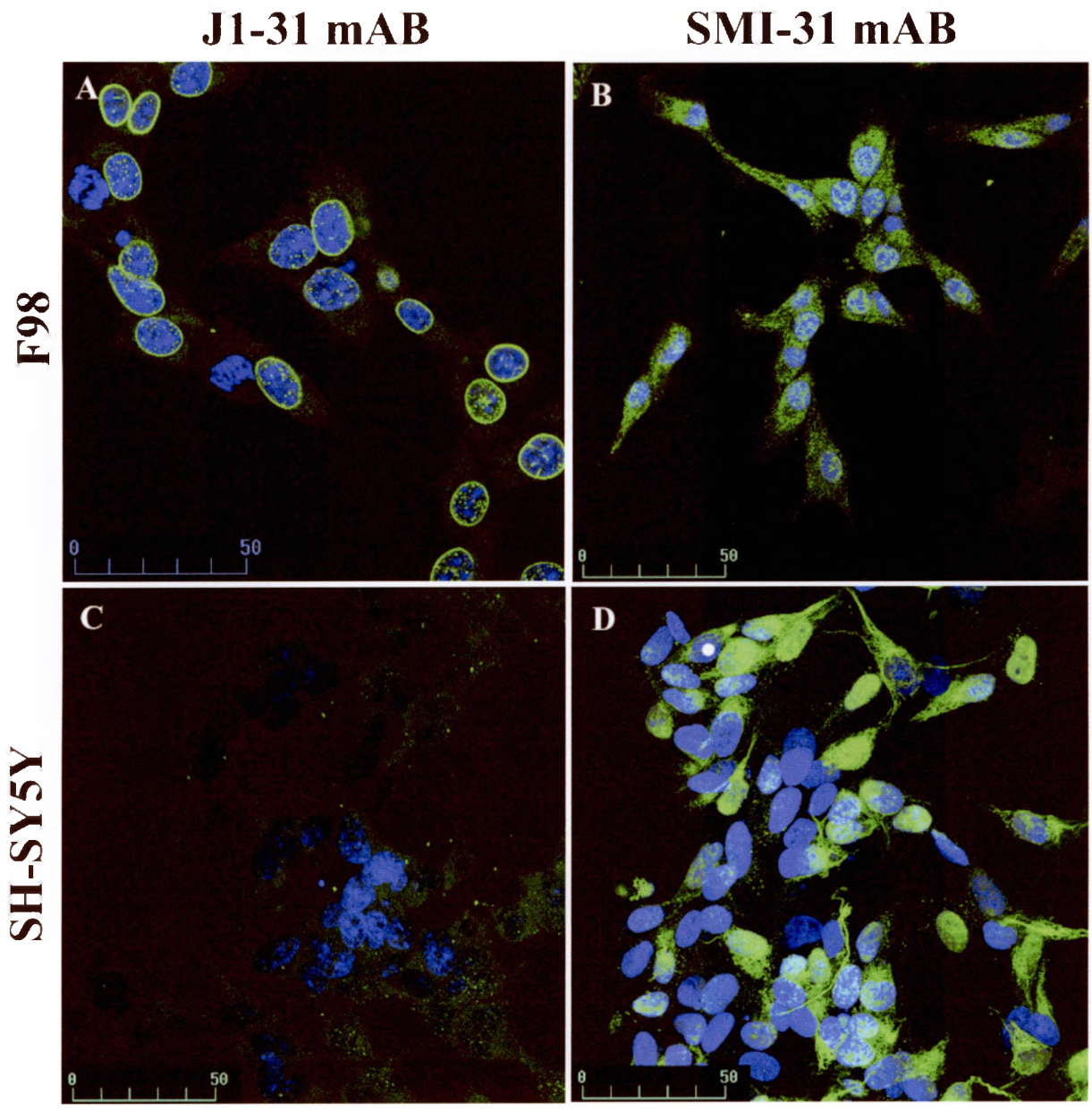

J1-31 mAB
SMI-31 mAB
F98
SH-SY5Y
A
B
C
D
0
50

Figure 6

Confocal micrographs prepared from a single focal plane of F98 glioma cells fixed in methanol (panel A, B) and 4% paraformaldehyde (panel C, D) immunolabeled with J1-31 mAB with secondary antibody conjugated to Alexa-Fluor-488 (green). Nuclei are revealed using TO-PRO-3 stain (blue). The cytoplasmic labeling intensity is altered, but remains in both fixation types while the nuclear localization of J1-31 mAB label is absent in moderately dense cells fixed in paraformaldehyde (panel C). However, in very sparse cell populations (panel B, D) small discrete "dots" of J1-31 mAB label can be seen in nuclei regardless of fixation method.

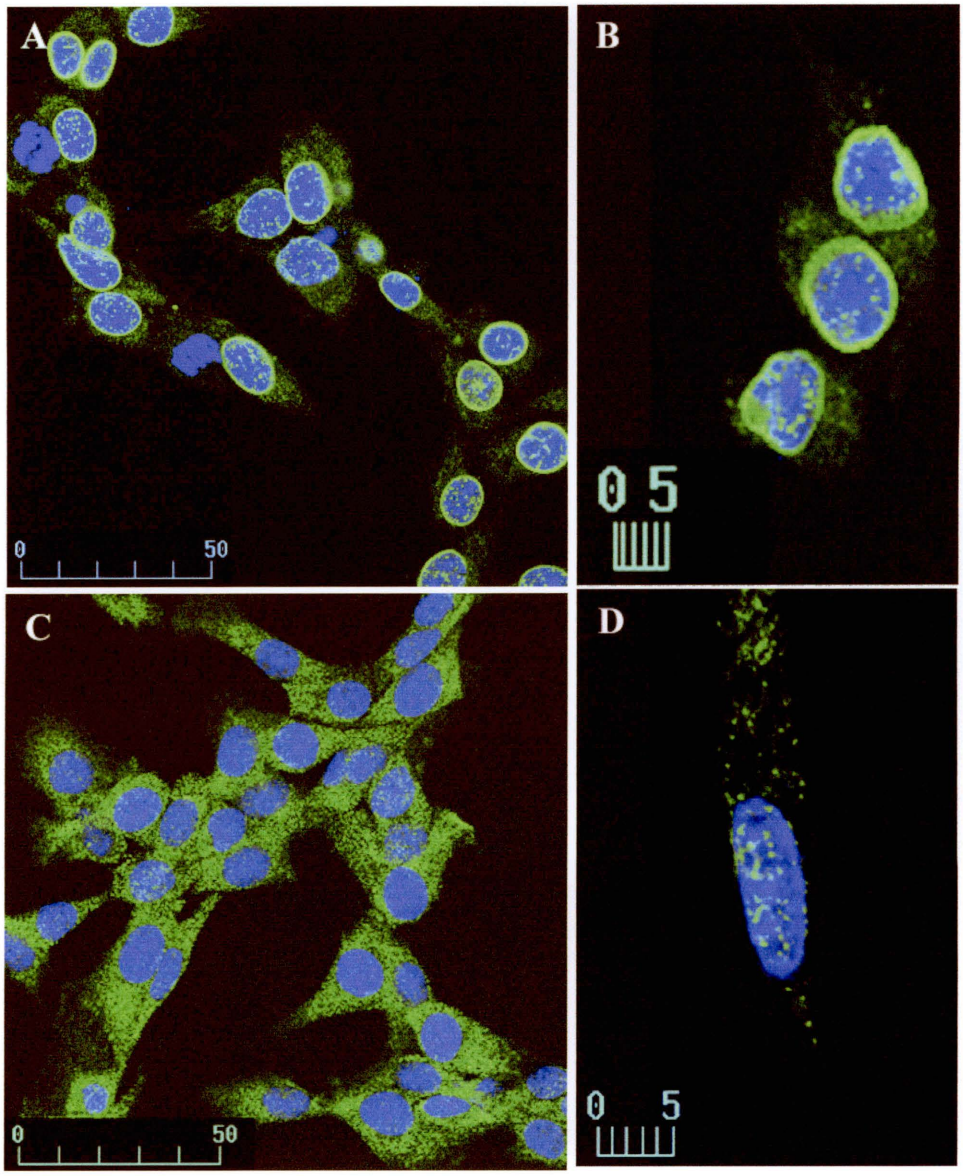

A
0
50
B
0 5
C
0
50
D
0 5

Figure 7

Confocal micrographs from a single image plane of SH-SY5Y neuroblastoma cells grown on glass coverslips followed by fixation in methanol (panel A, B) and 4% paraformaldehyde (panel C, D). Cells were immunolabeled with SMI-31 mAB, secondary conjugated to Alexa-Fluor-488 (green), and stained with TO-PRO-3 nucleic acid probe (blue). Nuclear localization of SMI-31 antigens appears to be fixation dependent (panel A, C); however, when sparsely populated cells are analyzed this dependence disappears (panel B, D).

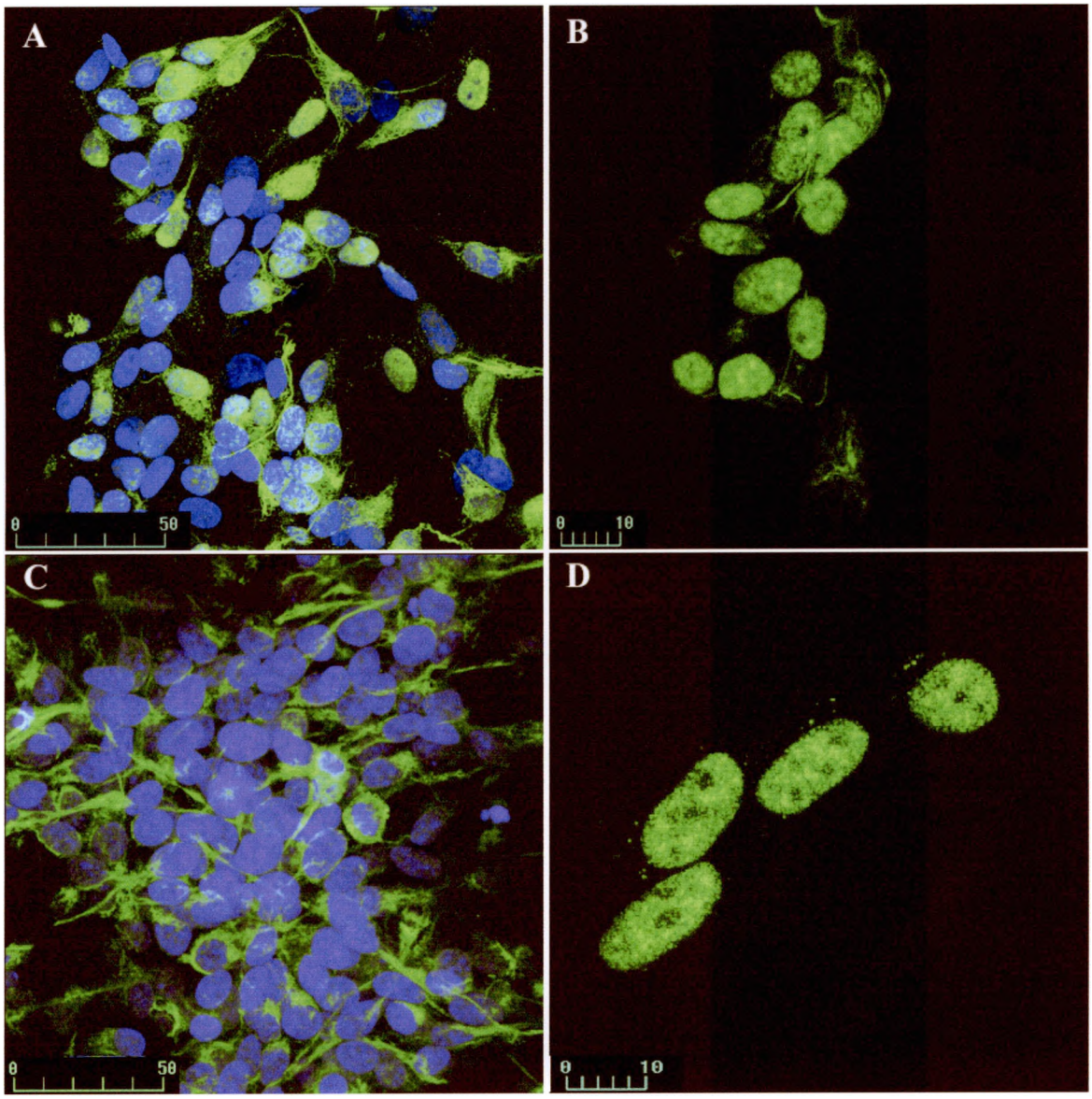

A
0
50
B
0
10
C
0
50
D
0
10

Figure 8

Growth curves for F98 glioma and SH-SY5Y neuroblastoma cells grown in culture. Growth rates were plotted for cells cultured from frozen stock and grown for 24-120 hours. F98 glioma cells demonstrated a characteristic lag phase after initial seeding followed by a steady growth phase. SH-SY5Y neuroblastoma cells showed a decline in cell density after initial seeding followed by a slow growth phase.

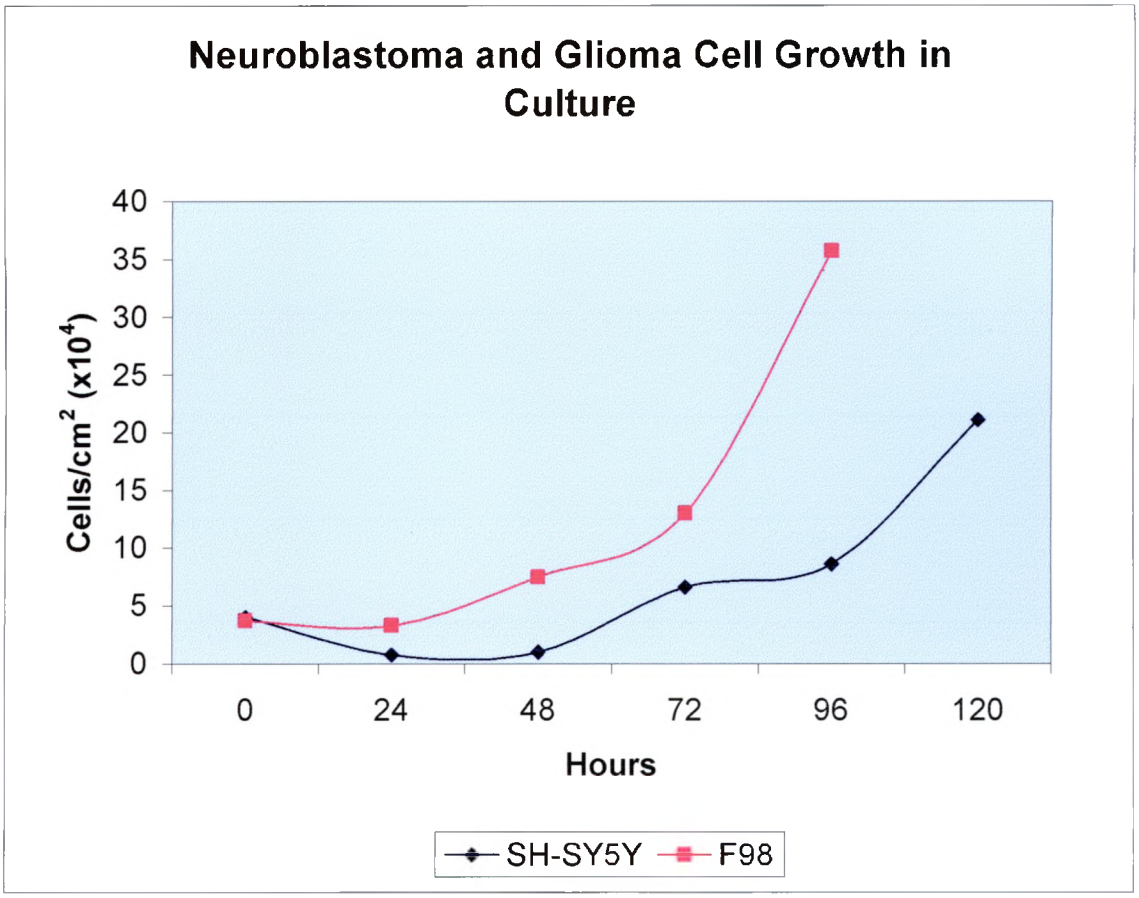

Neuroblastoma and Glioma Cell Growth in Culture
Cells/cm2 (x104)
40
35
30
25
20
15
10
5
0
0
24
48
72
96
120
Hours
SH-SY5Y
F98

Figure 9

Confocal projections of F98 cells grown on coverslips for 1-5 days (panel A-E) and incubated 24 hours prior to fixation in 10μM BrdU followed by immunofluorescent labeling. Anti-BrdU mAB was used for primary incubation followed by goat anti-mouse IgG conjugated to Alexa-Fluor 488 (green). Nuclei were revealed using TO-PRO-3 nucleic acid stain (blue). BrdU fluorescent labeling is high on days 1-3, but decreases on days 4 and 5. Thick clusters of cells, as seen in the upper right corner on day five, contain very low green fluorescent signal intensity indicating these cells are non-proliferating cells. Panel F identifies the relationship between anti-BRDU fluorescent signal intensity and days of F98 cell growth in culture. Intensity measurements within nuclei were collected in a random fashion from confocal projections (green channel only) using NIH Image software. Single-factor ANOVA and post hoc Tukey/Kramer tests were performed to identify statistical significance between daily mean pixel intensities as compared to the intensity found on day 1(*). In F98 glioma cells the BrdU mean pixel intensity decreases over time and with increasing confluency.

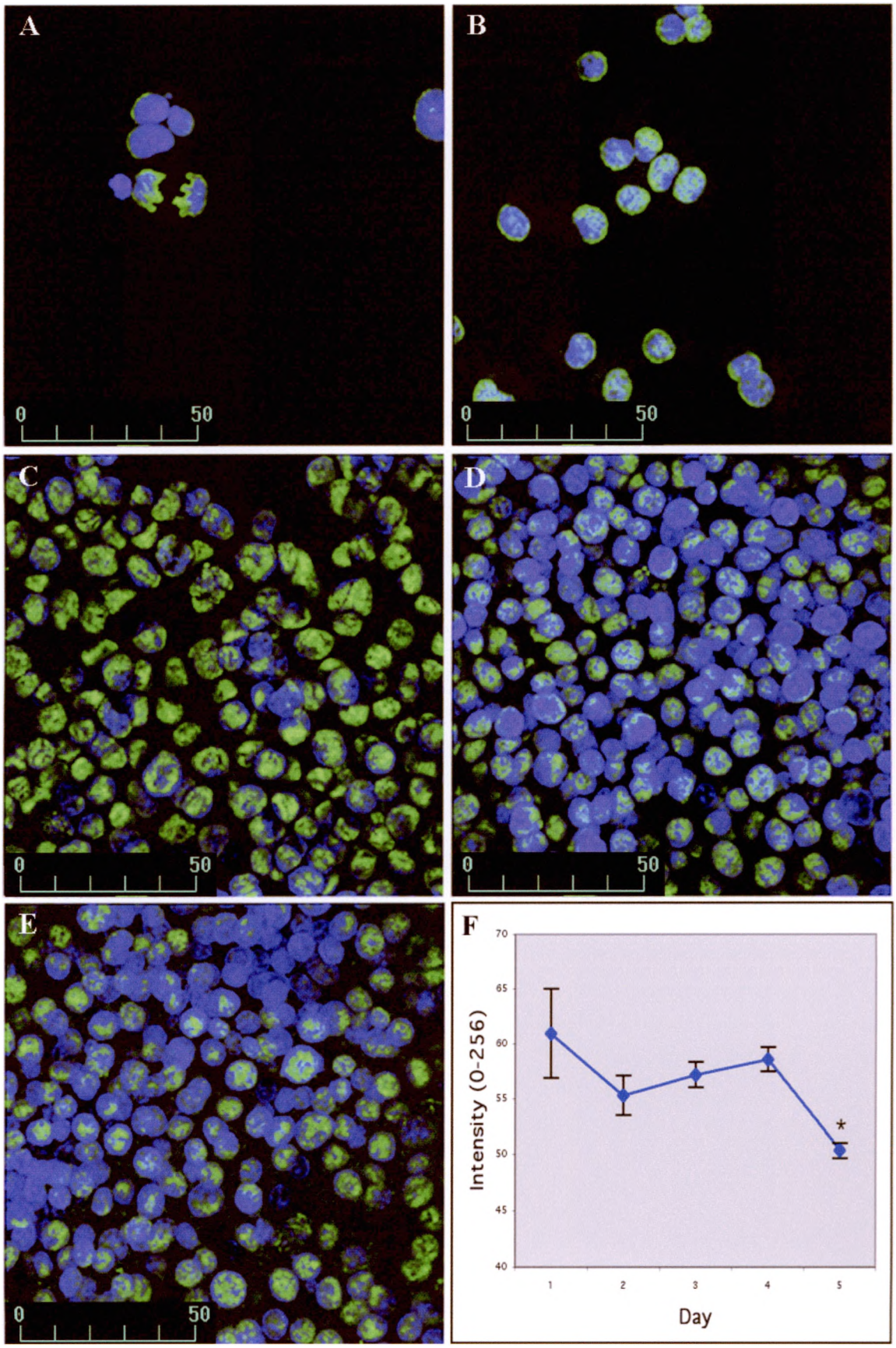

A
0
50
B
0
50
C
0
50
D
0
50
E
0
50
F
Intensity (0-256)
70
65
60
55
50
45
40
1
2
3
4
5
Day
*

Figure 10

Confocal micrographs of multiple image planes of SH-SY5Y cells grown on coverslips for 1-5 days (panels A-E) and incubated 24 hours prior to fixation in 10μM BrdU followed by immunofluorescent labeling. Primary antibody incubation was done using anti-BrdU mAB followed by goat anti-mouse conjugated to Alexa-Fluor 488 (green). Nuclei were revealed by TO-PRO-3 nucleic acid stain (blue). Cells grown for longer periods of time seemed to contain fewer proliferating cells as seen by an increased number of nuclei without BrdU fluorescent label. Notice cells located in thick clusters found on days 4 and 5 exhibit diminished BrdU label indicating that confluent cells (cells touching other cells) are not proliferating. Panel F identifies the relationship between anti-BRDU immunofluorescent signal intensity and days of SH-SY5Y cell growth in culture. Intensity measurements within nuclei were collected in a random fashion from confocal projections (green channel only) using NIH Image software. ANOVA and post hoc Tukey/Kramer tests were performed to identify statistical significance between daily mean pixel intensities as compared to the intensity found on day 2(*). BrdU intensity is low initially on day 1, but increases sharply on day 2 followed by a steady decline through day 5.

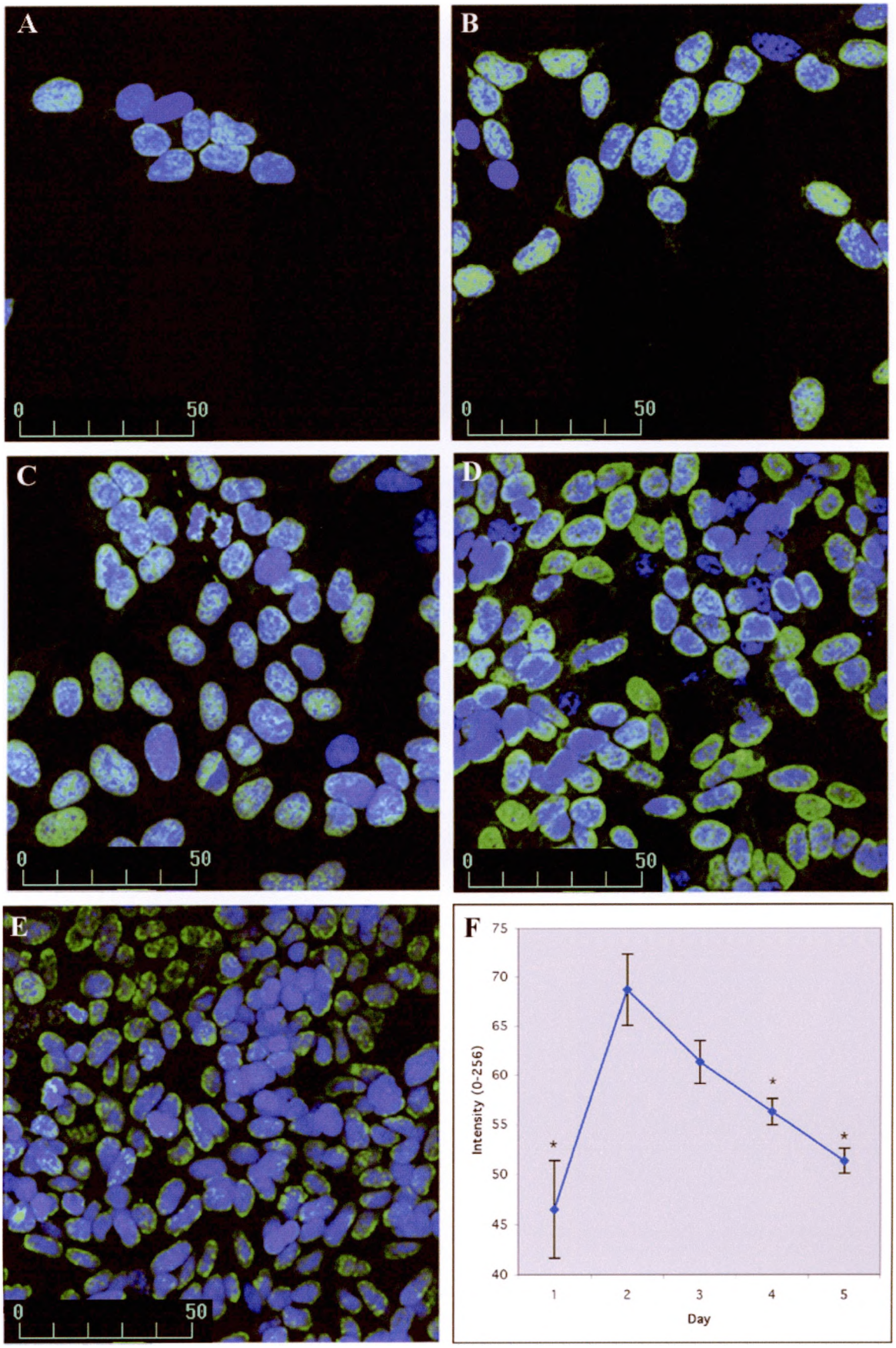

A
0
50
B
0
50
C
0
50
D
0
50
E
0
50
F
75
70
65
60
55
50
45
40
Intensity (0-256)
1
2
3
4
5
Day
*
*
*

Figure 11

Confocal micrographs of F98 glioma cells grown on coverslips for 1-4 days (panel A-D) immunolabeled with J1-31 mAB using a goat anti-mouse secondary antibody conjugated to Alexa-Fluor 488 (green), and stained with TO-PRO-3 nucleic acid probe (blue). Nuclear localization of J1-31 mAB label appears highest on day one and day two followed by a decline in signal intensity on days three and four when cell layers become increasingly dense. Panel E identifies the relationship between J1-31 immunofluorescent signal intensity and days of F98 cell growth in culture. Intensity measurements within nuclei were collected in a random fashion from confocal projections (green channel only) using NIH Image software. ANOVA and post hoc Tukey/Kramer tests were performed to identify statistical significance between daily mean pixel intensities as compared to day 1 intensity (*). There was a significant decline in pixel intensity found on days 3 and 4.

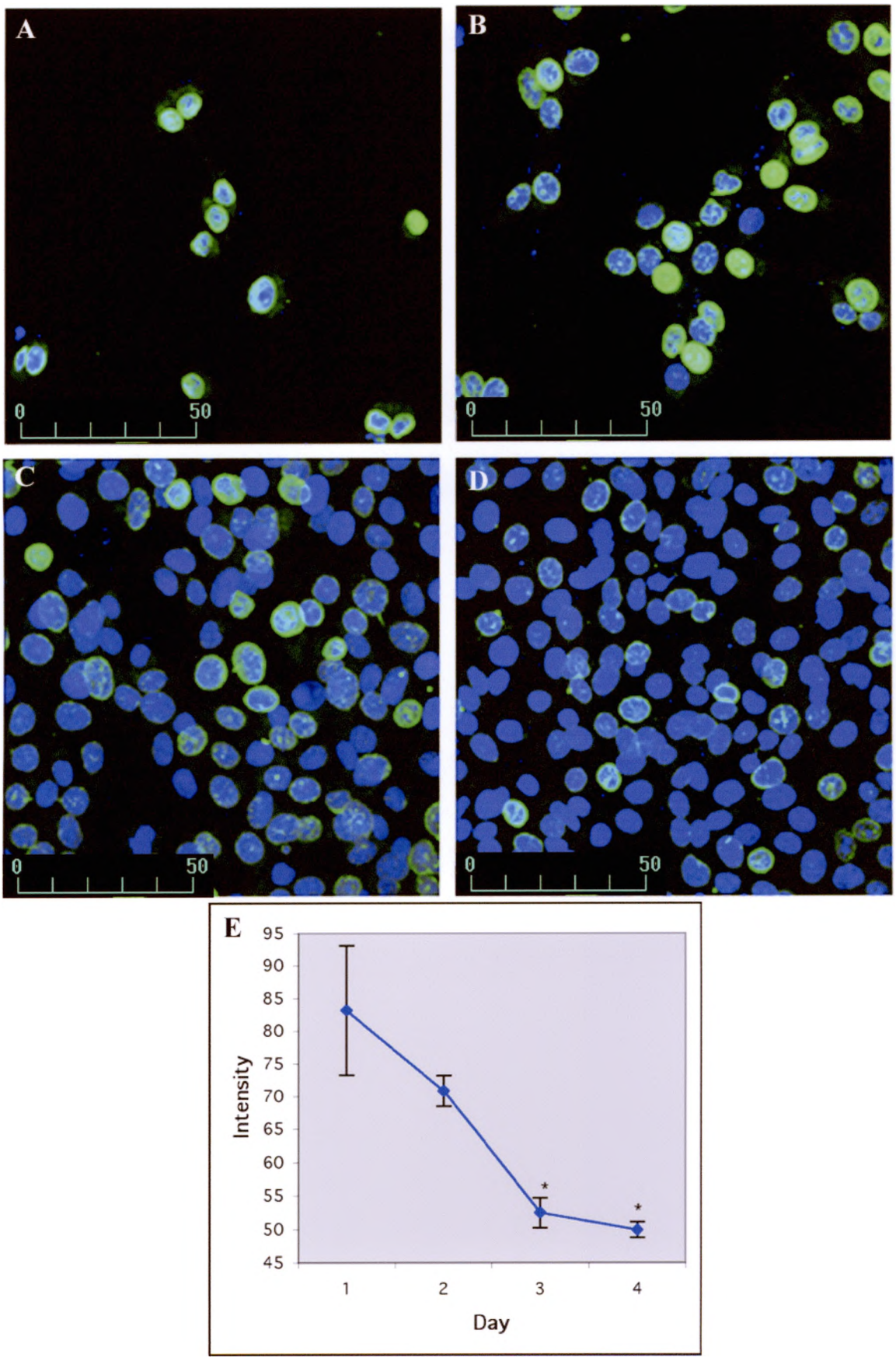

A
0
50
B
0
50
C
0
50
D
0
50
E
Intensity
95
90
85
80
75
70
65
60
55
50
45
1
2
3
4
Day
*
*

Figure 12

Confocal micrographs of F98 glioma cells grown in culture 1-4 days (panel A-D) immunolabeled with anti-GFAP antibody using a goat anti-mouse secondary antibody conjugated to Alexa-Fluor 488 (green) and stained with TO-PRO-3 nucleic acid stain (blue). Anti-GFAP labeling was found only in the cytoplasm. GFAP label intensity appeared highest on day 2 but was not quantitatively analyzed for a correlation to cell growth.

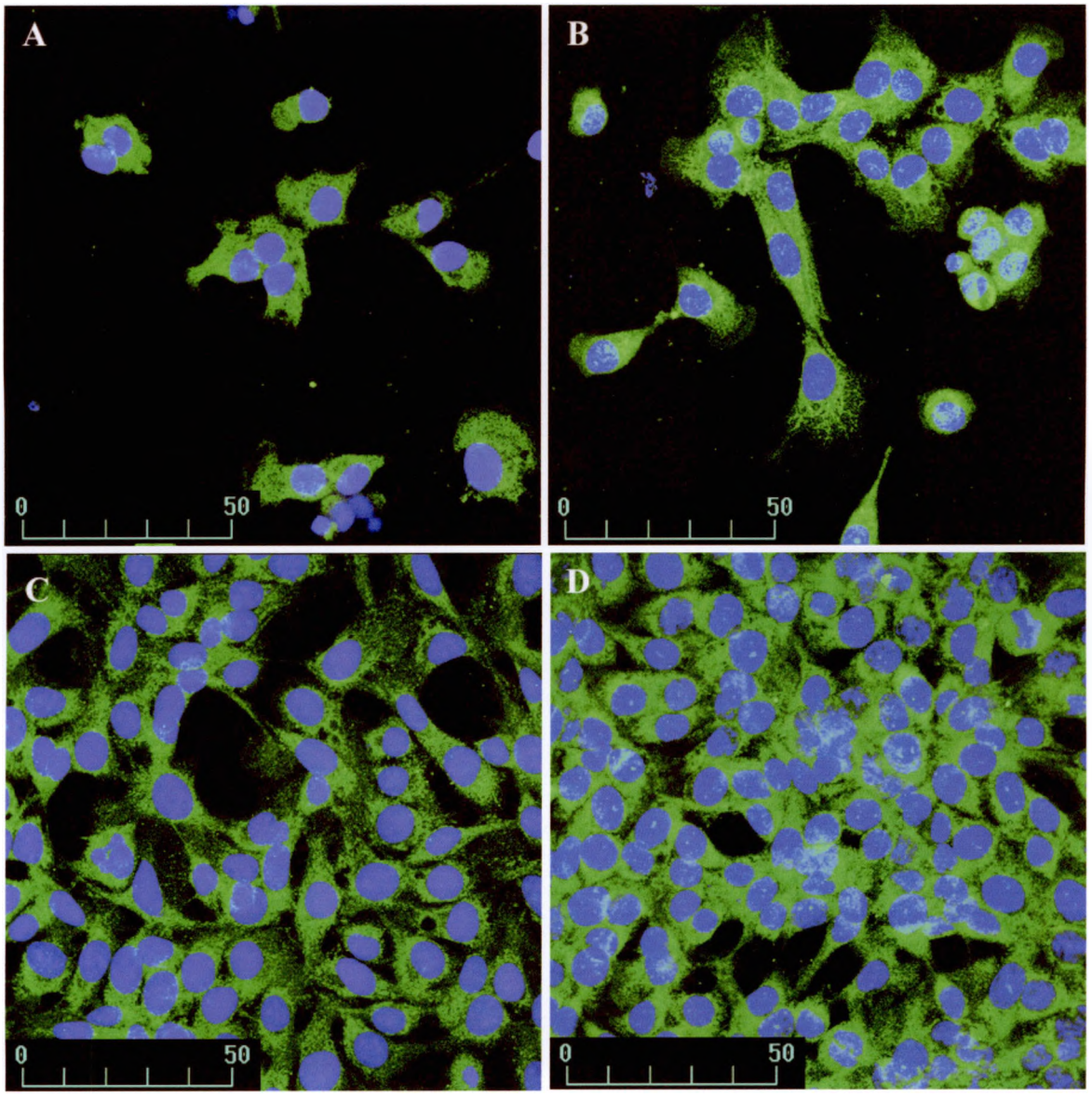

A
0
50
B
0
50
C
0
50
D
0
50

Figure 13

Confocal micrographs of SH-SY5Y neuroblastoma cells grown in culture 1-4 days (panel A-D) immunolabeled with SMI-31 monoclonal antibody using a goat anti-mouse secondary antibody conjugated to Alexa-Fluor 488 (green) and stained with TO-PRO-3 nucleic acid stain (blue). Nuclear localization of SMI-31 mAB label was seen primarily in cells cultured 1-3 days and drastically diminished on day 4. This decrease in nuclear localization may correlate to the appearance of SMI-31 labeling in mature neurites found on day four. Panel E identifies the relationship between SMI-31 immunofluorescent signal intensity and days of SH-SY5Y cell growth in culture. Intensity measurements within nuclei were collected in a random fashion from confocal projections (green channel only) using NIH Image software. ANOVA and post hoc Tukey/Kramer tests were performed to identify statistical significance between daily mean pixel intensities as compared to day 1 intensity (*). There was a significant decline in pixel intensity found on days 3 and 4.

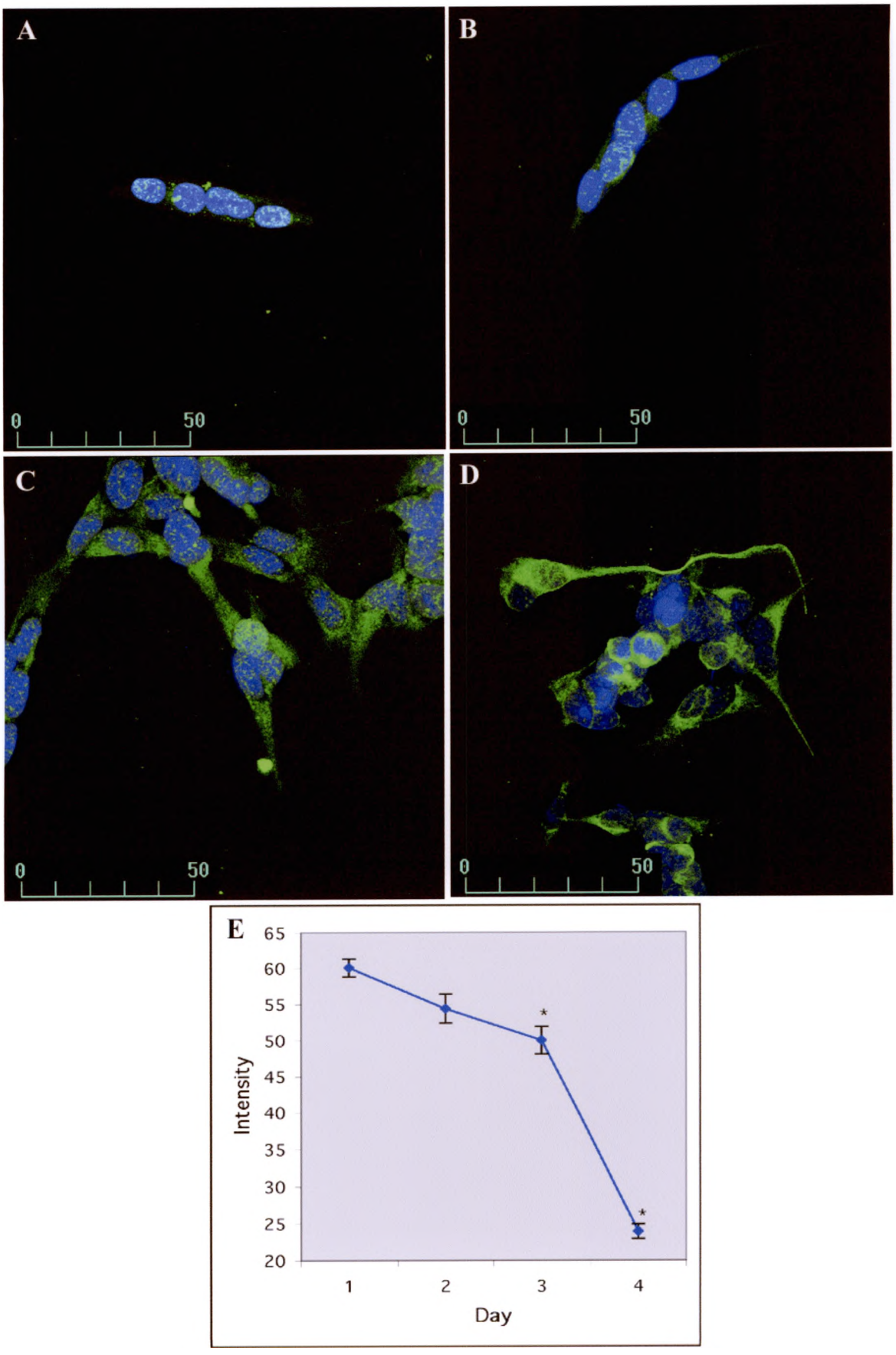

A
0
50
B
0
50
C
0
50
D
0
50
E
Intensity
65
60
55
50
45
40
35
30
25
20
1
2
3
4
Day
*
*

Figure 14

Confocal micrographs of SH-SY5Y neuroblastoma cells grown in culture 1-5 days (panel A-D) immunolabeled with SMI-32 monoclonal antibody using a goat anti-mouse secondary antibody conjugated to Alexa-Fluor 488 (green) and stained with TO-PRO-3 nucleic acid stain (blue). SMI-32 mAB labeling was found only in the cytoplasm and appeared very faintly on days one through three but increased on days four and five. This altered labeling pattern may also correlate to the appearance of neurites found on days four and five.

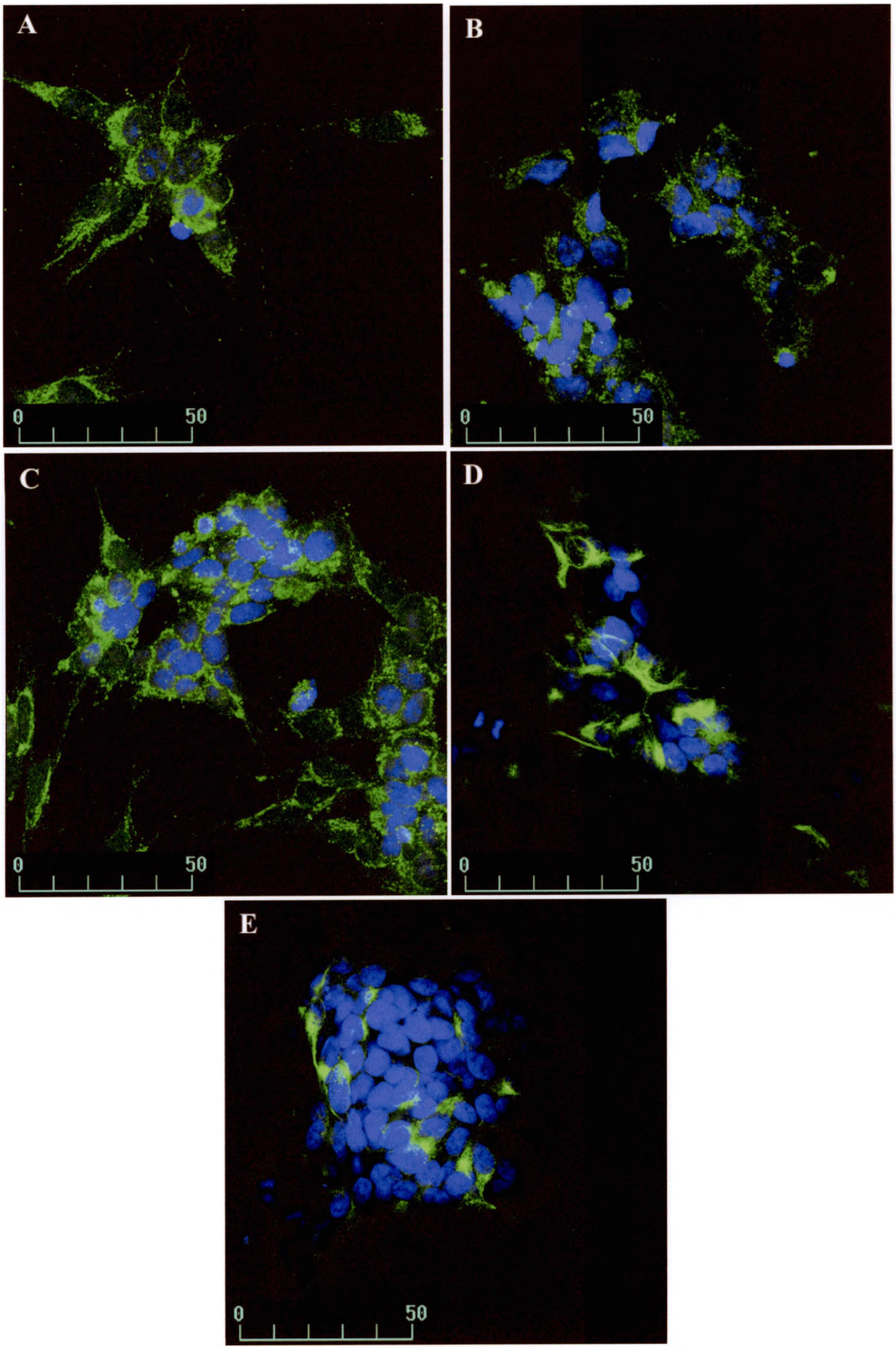

A
0
50
B
0
50
C
0
50
D
0
50
E
0
50

Figure 15

SDS-PAGE and western blot analysis of F98 glioma cell lysates. MW standards (a); coomassie stained for total protein (b); immunoblot with J1-31 mAB (c); immunoblot with anti-GFAP (d); immunoblot with anti-phosphoserine mAB (e).

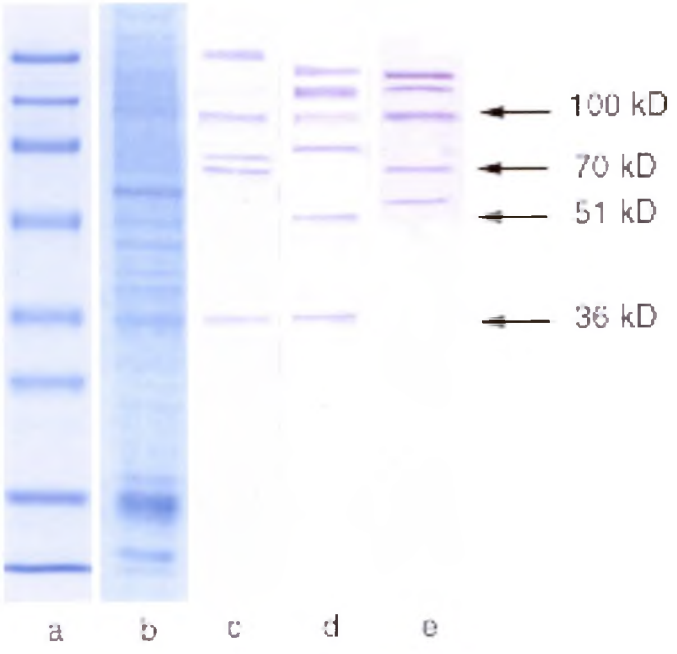

100 kD
70 kD
51 kD
36 kD
a
b
c
d
e

Figure 16

SDS-PAGE and western blot analysis of purified GFAP (unpolymerized). MW standards (a); coomassie stain for total protein (b); immunoblot using anti-GFAP (c); immunoblot using J1-31 mAB (d)

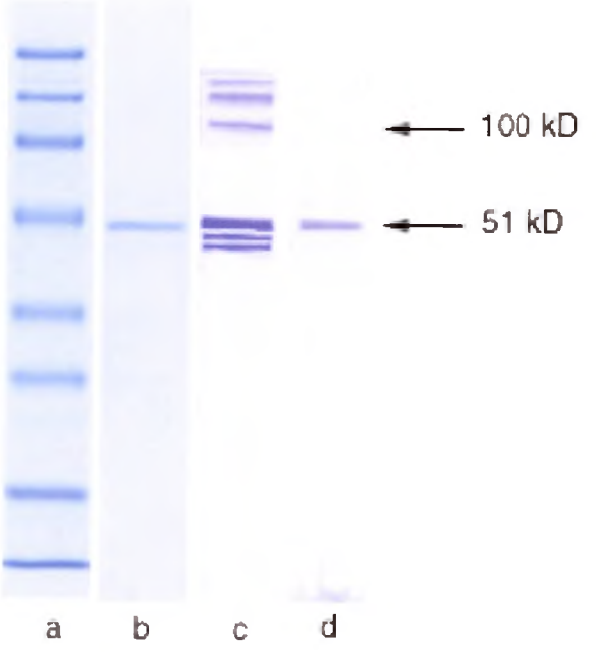
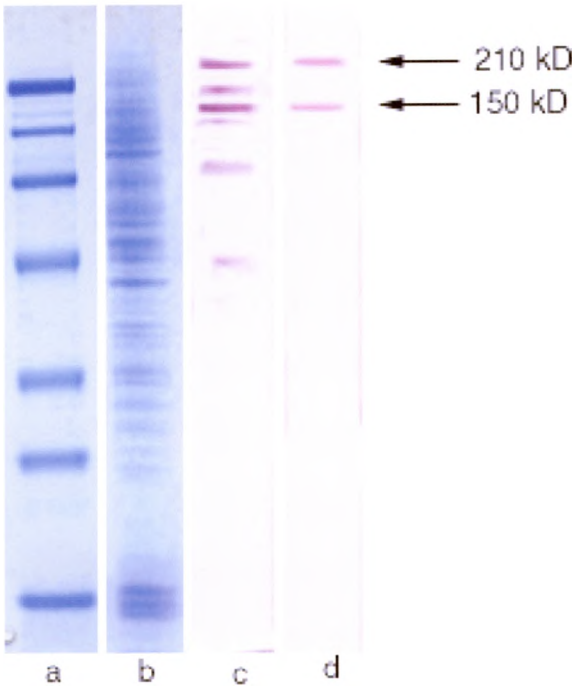

100 kD
51 kD
a
b
c
d

Figure 17

SDS-PAGE and western analysis of SH-SY5Y neuroblastoma cell lysates. MW standards (a); coomassie stain for total protein (b); immunoblot using SMI-31 mAB (c); immunoblot using SMI-32 mAB (d)


210 kD
150 kD
a
b
c
d

DISCUSSION

A. Nuclear and cytoplasmic localization of J1-31 and SMI-31 antigens in F98 glioma and SH-SY5Y neuroblastoma cells, respectively.

F98 glioma cells immunolabeled with J1-31 mAB demonstrated a punctate staining pattern within nuclei and, in some cases, a ring of staining surrounding the nuclear area. These discrete nuclear localizations suggest that the J1-31 antigen could be complexed with chromatin in the nuclear interior and/or other proteins in the nuclear lamina, possibly serving as structural or organization sites. Because the appearance of J1-31 epitopes in the nucleus was related to cell growth – actively growing cells, as identified by BrdU uptake, demonstrated higher intensities of J1-31 nuclear labeling (discussed below) – then it seems likely that the J1-31 antigen may be serving a role related to changing patterns of chromatin organization.

Diffuse J1-31 mAB labeling was also found in the cell body and cytoplasmic extensions or processes. Anti-GFAP immunolabeling, for use as an IF control, was found only in the cytoplasm where it co-localized with the J1-31 mAB label. These results support the idea that the J1-31 antigen could either be a form of GFAP (Stevenson, 1996), possibly phosphorylated or a GFAP associated protein (Singh *et al.,* 1992; Price *et al.,* 1993).

SH-SY5Y cells immunolabeled with SMI-31 mAB demonstrated discrete nuclear localizations in select nuclei with pronounced staining in neuronal processes and diffuse staining within cell bodies. According to Sternberger and Sternberger (1983), SMI-31 mAB is specific for phosphorylated NF-H and NF-M found primarily in axons and dendrites. Thus, nuclear localization of this antibody was unexpected, but could be the

result of two possible scenarios: phosphorylated NF-H and NF-M can be found in neuronal nuclei, or SMI-31 mAB is recognizing epitopes on non-neurofilament proteins. This second scenario is supported by the observed SMI-31 mAB immunoreaction in nuclei and cytoplasm of glioma cells found in this study and previously reported by Schilling *et al.* (1983). While this does not invalidate the specificity of SMI-31 mAB, it does suggest that the phosphorylated epitopes of SMI-31 have broader distribution among protein motifs, and that the nuclear epitopes recognized by J1-31 and SMI-31 have phosphorylation and some IF domain in common.

B. Effect of fixation on nuclear localization of J1-31 and SMI-31 mABs.

In previous studies (Singh *et al.*, 1994) and preliminary work here, it was found that methanol fixation was required to visualize the discrete J1-31 nuclear epitopes. However, when fixation analysis was performed on new, low density cultures, this dependence on fixation disappeared. This result suggests that the nuclear antigens may be developmentally regulated and that detection of the developmental differences is enhanced by paraformaldehyde fixation. Although, some of the differences in the appearance of J1-31 mAB labeling was fixation dependent, such as the nuclear ring formations found only in methanol fixed cells. These differences can be explained by the coagulation and collapse of the protein structures known to be associated with methanol fixation (Matsumoto, 1993). In comparison, paraformaldehyde is a gel-forming fixative that maintains the protein structure allowing J1-31 mAB label to be distinguished in the cytoplasm. Since the J1-31 mAB nuclear "dots" can be found regardless, this indicates that the appearance of J1-31 mAB in nuclei is not an artifact of fixation. Nuclear labeling of SMI-31 mAB in SH-SY5Y neuroblastoma cells demonstrated similar developmental

differences in fixation type suggesting that SMI-31 antigens may also be developmentally regulated. In addition, SMI-31 mAB labeling was found in neuroblastoma nuclei regardless of fixation indicating that SMI-31 appearance in nuclei is not an artifact of fixation.

C. Association between the appearance of J1-31 and SMI-31 antigens in glioma and neuroblastoma nuclei and the growth state of the cell.

As shown in Figure 11, J1-31 mAB nuclear labeling in F98 glioma cells decreased over time with increasing cell density. At higher cell densities, fewer cells were actively growing, as shown by BrdU uptake indicating that F98 cells exhibit contact inhibition in culture. The J1-31 antigen appears to be preferentially found within nuclei of proliferating F98 cells. Cells found in clusters or at high cell densities, showed diminished nuclear localization of J1-31 antigen, indicating quiescence and a correlation between J1-31 antigen expression and the growth state of the cell. Previous studies have identified a relationship between the J1-31 nuclear epitope and cellular transformation of astrocytes into the reactive state (Malhotra *et al.,* 1995). Reactive astrocytes are found following trauma and can be associated with neurological diseases such as Multiple Sclerosis, Alzheimer's and HIV-1 Encephalitis; they are characterized by hypertrophy, hyperplasia and extension of cytoplasmic processes (reviewed by Malhotra *et al.,* 1993). In this study, actively growing cells exhibit similar characteristics to reactive astrocytes.

SMI-31 mAB nuclear labeling in SH-SY5Y neuroblastoma cells was found in sparsely populated cells and diminished over time with increasing cell density. At these higher densities, BrdU uptake indicated fewer cells were actively growing. It appears that there was a correlation between nuclear localization of SMI-31 antigens and the growth

state or developmental state of the cell. It was also apparent that the decreased nuclear localization coincided with the appearance of SMI-31 labeling within neuronal processes, indicating maturation of the cell (Nixon and Shea, 1992). These results suggest that SMI-31 antigens preferentially appeared in nuclei of immature, actively growing SH-SY5Y cells *in vitro*. Previous studies by Schilling *et al.* (1989) have also indicated developmental regulation of SMI-31 nuclear antigens *in vivo* using fetal and adult rat cortex. Nuclei in fetal tissue were positively labeled with SMI-31 mAB, but adult rat cortex showed no nuclear labeling in neuronal nuclei. On the other hand, Glass *et al.* (2002) demonstrated nuclear localization of SMI-31 mAB label within adult rat brain; however, given the findings presented here, it is possible that this nuclear labeling was due to an immunoreaction between SMI-31 mAB and glial nuclei present in brain tissue.

D. J1-31 and SMI-31 mABs recognize intermediate filaments, possibly phosphorylated, along with additional antigens.

I attempted to determine if the antigens recognized by J1-31 and SMI-31 mABs in the cytoplasm were the same as those recognized within the nuclei of glioma and neuroblastoma cells using SDS-PAGE and western analysis of isolated nuclei and cytoplasm. No differences were found among isolated nuclear fractions or whole cell lysates from either F98 and SH-SY5Y cells with no identifiable protein bands located in cytoplasmic fractions. It is possible that the isolated nuclei were contaminated with cytoplasmic proteins, for example, an interaction between cytoplasmic intermediate filaments and the nucleoskeleton via nuclear pore complexes may have caused these cytoplasmic IFs to fractionate with the nuclei during centrifugation. Therefore, it was not possible to verify that the nuclear and cytoplasmic epitopes were identical; however, I

was able to use SDS-PAGE and western blot analysis to verify that J1-31 and SMI-31 mABs recognize proteins at the same molecular weights as known intermediate filaments.

SDS-PAGE and western blot analysis of F98 glioma cell lysates identified several proteins recognized by the J1-31 mAB. One particularly notable band was a 100 kD protein recognized by anti-GFAP pAB, J1-31 mAB, and anti-phosphoserine mAB. On the basis of this labeling and apparent MW, this band likely represents a GFAP dimer that is phosphorylated on serine residues. An additional band at 36 kD was labeled with J1-31 mAB and anti-GFAP. It has previously been reported that this protein band is a degradation product of the J1-31 antigen and can be immunolabeled with anti-GFAP (Stevenson, 1996). The J1-31 mAB was able to recognize GFAP (51 kD) in western blots using purified bovine GFAP but not in F98 cell lysates. This result suggests that the J1-31 mAB has weak avidity for epitope(s) on non-phosphorylated GFAP, but preferentially binds to the higher weight, phosphorylated forms of GFAP. Previous western blots of the J1-31 antigen have demonstrated two close protein bands at approximately 70 kD (Malhotra *et al.,* 1993; Malhotra *et al,* 1995; Stevenson, 1996) and these were identified in this study in F98 glioma cell lysates immunolabeled with J1-31 mAB (71 kD and 74 kD). Neither peptide was recognized using anti-GFAP antibody, but the protein at 71 kD was labeled using anti-phosphoserine mAB. It is possible that steric or conformational changes due to various phophorylation states of this protein reveals epitopes recognized by J1-31 mAB while masking epitopes recognized by anti-GFAP.

Western blot analysis of SH-SY5Y cell lysates using SMI-31 mAB indicated that SMI-31 was indeed labeling NF-H (210 kD) and NF-M (150 kD) as well as additional proteins with high molecular weights; these additional bands could be due to various

neurofilament phosphorylation states (Raabe *et al.,* 1996). Western blots using SMI-32 identified two proteins: NF-H (210 kD) and NF-M (150 kD). According to Sternberger and Sternberger (1983), SMI-31 recognizes phosphorylated epitopes on NF-H and NF-M while SMI-32 recognizes non-phosphorylated epitopes on the same proteins. I was unable to identify if any of these protein bands contained phosphorylated serine residues using anti-phosphoserine antibody on western blots due to extremely high background staining.

E. Conclusions and Further Experiments

Based on these results, I conclude that the J1-31 mAB can recognize a phosphorylated, high molecular mass form of GFAP that may be localized within nuclei of proliferating F98 glioma cells *in vitro* using immunocytochemistry and confocal microscopy. In addition, SMI-31 mAB can be demonstrated in nuclei of actively growing SH-SY5Y neuroblastoma cells as shown by immunocytochemistry and confocal microscopy. It remains unclear exactly which antigens recognized by SMI-31 and J1-31 mAB are found in nuclei and if they are indeed cytoskeletal intermediate filaments. However, these circumstantial findings suggest that phosphorylated intermediate filaments, usually found in the cytoplasm, may visit the nuclear interior during periods of active cell growth. This study also suggests that there could be a developmental role for non-lamin intermediate filaments in the nucleoskeleton of neural cells possibly functioning as structural or organization sites.

Future experiments should be aimed at identifying exactly which antigens recognized by J1-31 and SMI-31 mABs are present in nuclei. This could be accomplished by optimization of nuclear isolations free of cytoplasmic contaminants; however, this could

be difficult due to the interaction of various cytoskeletal proteins with lamins. In addition, developing an expression system for the J1-31 antigen and complete sequencing would allow identification of amino acid variations from GFAP, the location(s) of phosphorylation consensus sequences and the presence of any nuclear sorting sequences.

REFERENCES

Glass, T.L., Raabe, T.D., García, D.M., and Koke, J.R. 2002. Phosphorylated neurofilaments and SNAP-25 in cultured SH-SY5Y neuroblastoma cells. *Brain Research.* (in press)

Herrera J. J., Taylor T. A., Malhotra S. K. and Koke J. R. 1995. *In vitro* astrogliosis: chronology of J1-31 antigen expression in reactive 9L astrocytes. *Molecular Biology of the Cell,* **6**(suppl.): 748a

Herrera, J.J. 1997. Characterization of the Expression of Intermediate Filament Proteins During Astrogliosis, Thesis, Southwest Texas State University.

Inagaki, M., Gonda, Y., Mishizawa, K., Kitamura, S., Sato, C., Ando, S., Tanabe, K., Kikuchi, K., Tsuiki, S. and Nishi, Y. 1990. Phosphorylation sites linked to glial filament disassembly *in vitro* locate in a non-α-helical head domain. *Journal of Biological Chemistry.* **265** (8): 4722-4729.

Inagaki, N., Tsujimura, K., Tanaka, J., Sekimata, M., Kamei, Y., and Inagaki, M. 1996. Visualization of protein kinase activities in single cells by antibodies against phosphorylated vimentin and GFAP. *Neurochemical Research.* **21** (7):795-800.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* **227**: 680-685.

Malhotra, S.K., Bhatnagar, R., Shnitka, T.K., Herrera, J.J., Koke, J.R., and Singh, M.V. (1995). Rat glioma cell line as a model for astrogliosis. *Cytobios* **82**:39-51.

Malhotra, S.K., Svensson, M., Aldskogius, H., Bhatnagar, R., Das, G.D. and Shnitka, T.K. (1993). Diversity among reactive astrocytes: proximal reactive astrocytes in lacerated spinal cord preferentially react with monoclonal antibody J1-31. *Brain Research Bulletin* **30**: 395-404.

Malhotra, S.K., Wong, F., Cumming, P., Ross, S.D., Shnitka, T.K., Manickavel, V., Warren, K.G. and Jeffrey, V. (1984). A monoclonal antibody for cytoskeletal antigenic determinant(s) distinguishable from glial fibrillary acidic protein in astrocytes. *Microbios Lett.* **26**:151-157.

Matsumoto, B. (1993). Cell Biological Applications of Confocal Microscopy. *Methods in Cell Biology*. **38**:242-247.

McNulty, A.K. and Saunders, M.J. 1992. Purification and immunological detection of pea nuclear intermediate filaments: evidence for plant nuclear lamins. *J. Cell Sci.* **103**:407-414.

Nixon, R.A., and Shea, T.B. (1992). Dynamics of neuronal intermediate filaments: a developmental perspective. *Cell Motil Cytoskeleton* **22**: 81-91.

Nixon, R.A. and Sihag, R.K. (1991). Neurofilament phosphorylation: a new look at regulation and function. *TINS* **14** (11): 501-506.

Predy, R. and Malhotra, S.K. (1989). Reactive astrocytes in lesioned rat spinal cord: effect of neural transplants. *Brain Research Bulletin* **22**:81-87.

Price, K.A.S., Malhotra, S.K., and Koke, J.R. (1993). Localization and characterization of an intermediate filament-associated protein. *Cytobios* **76**: 157-173.

Raabe, T.D., Nguyen, T., Archer, C. and Bittner, G.D. (1996). Mechanisms for the maintenance and eventual degradation of neurofilament proteins in the distal segments of severed goldfish mauthner axons. *J Neuroscience* **16** (5): 1605-1613

Schilling, K., Duvernoy, C., Keck, S., and Pilgrim, C. (1989). Detection and partial characterization of a developmentally regulated nuclear antigen in neural cells in vitro and in vivo. *J Histochem Cytochem* **37**: 241-247.

Shea, T.B., Majocha, R.E., Marotta, C.A., and Nixon, R.A. (1988). Soluble, phosphorylated forms of the high molecular weight neurofilament protein in perikarya of cultured neuronal cells. *Neurosci Letters* **92**: 291-297.

Singh, M.V., Price, K.J., Bhatnagar, R., Johnson, E.S. and Malhotra, S.K. (1992). J1-31

antigen of astrocytes: cytoplasmic and nuclear localization. *Dendron* **1**:91-108.

Singh, M.V., Price, K.J., Bhatnagar, R. and Malhotra, S.K. (1994). Novel rod-shaped structures identified in glioma cell nuclei by immunolabeling and confocal laser fluorescence microscopy. *Biomedical Letters* **50**: 163-172.

Singh, S., Koke, J.R., Gupta, P.D. and Malhotra, S.K. (1994). Multiple roles of intermediate filaments. *Cytobios* **77** (308): 41-57.

Singh, R., Singh, B. and Malhotra, S.K. (1986). A new "marker" protein for astrocytes. *Bioscience Reports* **6** (1): 73-79.

Sternberger, L.A., and Sternberger, N.H. (1983). Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments *in situ*. *Proc Natl Acad Sci* **80**: 6126-6130.

Stevenson, P. 1996. Biochemical Characterization of a Possible Precursor to Glial Fibrillary Acidic Protein in Reactive Astrocytes, Thesis, Southwest Texas State University.

Traub, P., Nelson, W. J., Kuhn, S. and Vorgias, C. E. 1983. The interaction *in vitro* of the intermediate filament protein vimentin with naturally occurring RNAs and DNAs. *J. Biol. Chem.* **258**:1456.