PHOSPHORYLATION OF GFAP AND LAMIN B AND ASTROCYTE ACTIVATION AS REVEALED BY MONOCLONAL ANTIBODY J1-31

THESIS

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San Marcos, Texas

May 2005

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ABSTRACT

PHOSPHORYLATION OF GFAP AND LAMIN B AND ASTROCYTE ACTIVATION AS REVEALED BY MONOCLONAL ANTIBODY J1-31

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SUPERVISING PROFESSOR: JOSEPH KOKE

Monoclonal antibody J1-31 has been shown to recognize cytoplasmic and nuclear antigens found in F98 glioblastoma cells *in vitro* and in astrocytes of the central nervous system near sites of injury. J1-31 antigen(s) labeling increases in astrocytes and F98 cells when these cells are in a reactive state. Recent reports suggest that mAB J1-31 specifically recognizes an epitope found on both GFAP and lamin B that contains a phosphorylated serine residue, and consensus sequences for appropriate protein kinases are present in GFAP and lamin B. To provide additional clues to the nature of the epitope recognized by mAB J1-31 and to provide insight into signaling pathways involved in cytoskeletal remodeling associated with reactive astrocytes, F98 cells were

activated in culture with forskolin and the resulting changes in intermediate filaments analyzed by immunocytochemical methods. Activation of adenylyl cyclase in F98 cells with forskolin resulted in a significant ($p < 0.001$) increase in mAB J1-31 labeling of nuclear and cytoplasmic structures consistent with the known locations of lamins and GFAP. A similar increase in labeling intensity was not observed using antibodies specific for lamin B or GFAP.

Further analysis included analysis of the J1-31 antigens using immuno-magnetic bead precipitation from F98 cell lysates, and characterization of intracellular pathways involved in the forskolin induced increase in J1-31 labeling intensity. Downstream targets of cAMP including PKA and Ca^{+2} channels were inhibited in an attempt to elucidate the kinase responsible for phosphorylating the J1-31 antigen. Inhibitors of PKA had no effect on the forskolin induced increase in J1-31 labeling, but treating F98 cells with verapamil, a known inhibitor of Ca^{+2} channels, caused strong attenuation of the forskolin-induced increase in mAB J1-31 labeling. These results support the notion that the J1-31 mAB recognizes an epitope on GFAP and lamin B when that epitope contains a phosphorylated amino acid, most likely serine. Furthermore, that residue may be phosphorylated in a Ca+2 -dependent manner as a result of adenylyl cyclase activation. Immunoprecipitation of F98 cell extracts using magnetic beads with covalently bound mAB J1-31 produced ambiguous results. Western analysis of proteins released from the beads identified GFAP and lamin B among others.

INTRODUCTION

In 1984, S. K. Malhotra produced a hybridoma cell line using BALB-C mice which produced a monoclonal antibody (mAB) designated J1-31 (Malhotra *et al.* 1984). Plaque material from brains of patients who had died from multiple sclerosis was used as the immunogen that yielded mAB J1-31. Initially, mAB J1-31 was thought to recognize an intermediate filament-associated protein with a molecular weight of 68-70 kDa associated with glial fibrillary acidic protein (Singh *et al.* 1992). Later reports indicated that mAB J1-31 labeled structures in tissue surrounding destructive lesions in the CNS (Singh *et al.* 1992). Similar labeling patterns were seen in human astrocytomas and subsequently in astrocytoma (9L cells) cultures that were activated by scratch wound (Singh *et al.* 1992, Malhotra et al 1995). Singh *et al.* (1992) also used immunogold electron microscopy to show co-localization between J1-31 antigen and GFAP, which supported the theory that mAB J1-31 recognized an intermediate filament associated protein. However, Stevenson (1996), using preparative two dimensional electrophoresis, isolated a single J1-31 positive protein from human brain MS plaques which, upon amino acid sequence analysis of 6 different proteolytic fragments, was identified as GFAP. This suggested that the J1-31 antigen was not an intermediate filament associated protein but perhaps a "pre-form" of GFAP. More recent investigations have suggested that the J1-31 mAB recognizes a phosphoepitope that, in addition to being present on GFAP, also occurs on lamin intermediate filaments, specifically lamin B (García *et al.* 2003). This

finding may explain the labeling of nuclear dots as well as the labeling of cytoplasmic filaments described earlier (Singh *et al.* 1992). Additionally, investigation of F98 cells in culture by García *et al.* (2003) correlated DNA replication using BrdU uptake to the appearance of J1-31 labeling in both the cytoplasm and nucleus. It is well known that phosphorylation of IFs can regulate polymerization and depolymerization (Herrmann *et al.* 2000); thus, the correlation of J1-31 labeling to rapid proliferation of cells in culture suggested that mAB J1-31 might be recognizing a phospho-epitope on both GFAP and lamin B. Weigum further showed (in Weigum *et al.* 2003 and García *et al.* 2003) by western analysis of lysates prepared from rapidly growing F98 cells, antibodies against GFAP, or lamin B, or phosphorylated serine residues, or mAB J1-31 all labeled proteins with common molecular masses. These results raised the possibility that mAB J1-31 recognizes an epitope which includes a phosphorylated serine residue.

As mentioned above, two-dimensional preparative electrophoresis of human cerebral hemispheres containing multiple sclerosis provided J1-31 antigen for sequence analysis. Of the resulting six fragments of sequence, five had 100% identity with GFAP and the fifth 88% (one amino acid difference; García *et al.* 2003). Additionally, the predicted cyanogen bromide cleavage products of GFAP included the actual sequences of purified J1-31 antigen, and the products of J1-31 antigen yielded by CNBr-cleavage were of similar molecular weight to those expected for CNBr cleaved GFAP. When GFAP and lamin B sequences were aligned, there were two distinct sections that had a high degree of sequence homology and two corresponding serine residues that were predicted by NetPhos (García *et al.* 2003; http://www.cbs.dtu.dk/services/NetPhos/) to have a high (>94%) degree of probability of being phosphorylated (García *et al.* 2003). These

findings in addition to the western analyses described above suggested that mAB J1-31 recognizes a phosphorylated epitope (García *et al.* 2003).

Astrocytes are the most abundant cell type in the CNS and perform many tasks associated with local homeostasis (Penky *et al.* 2004). Astrocytes have been implicated in a wide range of CNS pathologies including but not limited to trauma, ischemia, and neurodegeneration. During such pathologies astrocytes undergo both morphological and protein expression changes in what has been termed reactive astrogliosis. Morphological changes include hypertrophy and hyperplasia (Malhotra *et al.* 1990). Hypertrophy of the cell body and astrocytic processes have been attributed to the increased expression or reorganization of the intermediate filament glial fibrillary acidic protein (GFAP) (Penky *et al.* 2004; Wilhelmson *et al.* 2004). To date the major indicator of reactive astrocytes is increased immunocytochemical labeling for GFAP. Reactive astrogliosis has been implicated in glial scar formation in what has been postulated as an attempt to quarantine the damaged region (Malhotra *et al.* 1990)

In vitro models of reactive astrogliosis have been developed using mechanical or chemical stimulus to a monolayer of glioblastoma cells (e.g., the scratch wound study described above) to stimulate quiescent cells to "react" by re-entering the cell cycle and proliferating to repair the insult (Malhotra *et al.* 1995). Such *in vitro* models take advantage of similarities between glioblastoma cells and primary cultures of astrocytes in that both express GFAP, which is a glial cell specific intermediate filament, and both respond to activation in a predictable and similar manner.

Activation of cells, including astrocytes, involves cytoskeletal remodeling in the cytoplasm and nucleus (Singh *et al.* 1994). Intermediate filaments (IFs) form the most

flexible element of the cytoskeleton in eukaryotic cells of virtually all types. Although much variability has been described in the sequence homology of the head domain, a feature all IFs share is the alpha helical rod domain, which is capable of forming a coiled-coil when the intermediate filament proteins dimerize. Roughly 310-360 amino acids of sequence form the highly conserved rod domain (Herrmann *et al.* 2000)

As indicated above, phosphorylation can regulate the solubility of IFs. When head domains are phosphorylated by protein kinases on serine or threonine residues the filaments depolymerize to their constitutive tetramers (Herrmann *et al.* 2000; Inagaki *et al.* 1990). Although the direct mechanism remains the focus of debate, current results imply that residue phosphorylation does in fact occur within the head domain, and that this phosphorylation is followed by depolymerization of high order, insoluble IF polymers to the soluble, coiled-coil dimer (Herrmann *et al.* 2000). Given that cytoskeleton reorganization occurs during activation, it seems likely that the phosphorylation of IF polymers may provide a mechanism for reorganization during reactive astrogliosis.

Nine isoforms of adenylyl cyclase have been described since its original discovery in the 1950s (Kuznetsova *et al.* 2002). Structurally, these isoforms have in common twelve transmembrane domains. Both N- and C-termini reside intracellularly and all forms of adenylyl cyclase have a catalytic center. Cyclic adenosine monophosphate is synthesized by the membrane bound adenylyl cyclase. Activation of adenylyl cyclase results from binding of $G_{S\alpha}$, a subunit of the heterotrimeric G-stimulatory protein. When a stimulatory G-protein is activated by a G-protein coupled receptor, the $G_{S\alpha}$ subunit binds to and activates adenylyl cyclase. Adenylyl cylase then

converts adenosine triphosphate (ATP) to cAMP, which goes on to interact with downstream target proteins. In addition to the differential expression of respective adenylyl cylase isoforms in different tissues, each isoform is modulated by a different combination of second messengers. Primary forms of adenylyl cylase found in CNS tissue include adenylyl cylase 1 and 8, which are both activated by forskolin and Ca^{+2}, and inhibited by $G_{i\alpha}$ (Cooper *et al.* 2003).

Cyclic AMP operates on a number of downstream target molecules including cAMP-dependent protein kinase (PKA) (Kim *et al.* 2005). When two molecules of cAMP bind to the two regulatory subunits of tetrameric PKA, two catalytic subunits are liberated for phosphorylation of downstream proteins. Cyclic AMP can also modulate cyclic nucleotide-gated Ca^{+2} channels (CNG). As cAMP levels increase, cAMP binds to the cytoplasmic surface of CNGs which causes channel opening and a resulting influx of Ca^{+2}. Influx of Ca^{+2} modulates a variety of intracellular targets including PKC and CaMK.

A variety of inhibitors and mimics, including cholera toxin, sodium fluoride, and other enterotoxins have been used (Seamon *et al.* 1981) to manipulate cAMP levels intracellularly. Unfortunately, most of these methods required disruption of either physiological cellular conditions or the use of multiple effector molecules in order to elicit a response. Analogs of cAMP have also been used to activate similar downstream targets. The diterpene forskolin from the roots of *Coleus forskohlii* has been reported to activate adenylyl cyclase in cardiac and brain tissue. Activation occurs in a rapid and reversible manner at a half maximal effective concentration of 5-10 μM by mimicking activated G_{α} under physiological conditions and is thus suitable for use in tissue culture

as well as *in situ* (Seamon *et al.* 1981). All isoforms except adenylyl cyclase 9 are stimulated by forskolin when it binds hydrophobically to the P-site of adenyly cyclase (Cooper *et al.* 2003).

My thesis work tested the hypothesis that mAB J1-31 recognizes a phospho-epitope that occurs on both GFAP and lamin B, and that this epitope may be important in the intracellular pathway leading to activation of astrocytes – reactive astrogliosis. This was tested by immunocytochemical methods and immuno-magnetic bead precipitation. To test the role of phosphorylation via adenylyl cyclase mediated pathways, forskolin was used to induce activation of F98 cells *in vitro,* and was observed to cause an increase in mAB J1-31 labeling intensity of nuclear and cytoplasmic structures; structures known from previous work to be labeled by anti-lamin B and anti-GFAP, respectively. However, under the same conditions there was no observable change in affinity for antibodies recognizing lamin B or GFAP

MATERIALS AND METHODS

Cell Culture and Experimental Treatments

All materials and reagents were supplied by Sigma St. Louis, MO, unless otherwise specified. F98 rat glioblastoma cells were obtained from the American Tissue Culture Collection (ATCC, #CRL-2397, Manassas, VA) and were cultured on coverslips or in flasks as described previously (Weigum *et al.,* 2003) for 24-72 hours prior to treatment and subsequent fixation. All treatment reagents were dissolved in 10 ml fresh culture medium at the concentrations indicated in Table 1. Cells were exposed to the reagents by replacing 10 ml of culture medium with 10 ml of the experimental treatment medium. Reagents listed in Table 1 include forskolin, an adenylyl cyclase activator; dbcAMP, a cAMP analog; H89, a selective inhibitor of PKA; R_p-cAMPS, a selective inhibitor of PKA regulatory subunits and verapamil, a Ca^{+2} channel inhibitor. Each experimental treatment lasted 10 minutes followed by a 45 minute forskolin or dbcAMP treatment. The forskolin or dbcAMP treatments were applied by replacing the culture medium that contained the inhibitor with media containing agents that increase [cAMP].

Table 1. Treatment reagents listed with respective dilutions used and solvent suspended in which each is dissolved. Final concentrations were based on previous studies using these inhibitors (Seamon *et al.* 1981; Safavi-Abbasi *et al.* 2001; Chijiwa *et al.* 1989; Gjertsen *et al.* 1995; Agrawal *et al.* 2000 respectively).

Agent	Final Concentration	Solvent	Source
forskolin	4μM	DMSO	LC Laboratories, Woburn, MA
dbcAMP	10mM	H_2O	Sigma, St. Louis, MO
H89	48nM	DMSO	CalBiochem, San Diego, CA
R_p-cAMPS	200μM	H_2O	Sigma, St. Louis, MO
verapamil	2μM	H_2O	CalBiochem, San Diego, CA

At the end of the treatment period, coverslips bearing cells were removed, washed in phosphate buffered saline (pH 7.4, 137mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mMKH_2PO_4)(PBS) in order to remove all residual medium remaining on the culture surface, then fixed by immersion in ice cold methanol (-25°C) for 2 minutes. After fixation, cells on coverslips were allowed to air dry and were stored at -20°C, then rehydrated in PBS at room temperature prior to immunostaining.

Immunocytochemistry

Coverslips bearing cells were treated with 20% non-fat powdered milk in PBS for 2 hours at room temperature to block non-specific binding. The coverslips were then washed three times in PBS containing 0.2% Tween-20 (PBST) over a period of 30 minutes. Non-specific binding of antibodies was prevented by treating all cover slips with 20% nonfat powdered milk prepared in PBS. Incubation at room temperature for 2 hours was followed by three ten-minute washes in PBS/PBST. Cover slips were then incubated in primary antibody overnight at 4°C.

Table 2. Primary antibodies used in immunocytochemical and western analysis

Primary antibody	Dilution used	Source
J1-31 hybridoma	No dilution of culture supernatant	ATCC, Manassas, VA
mAB anti-GFAP	1:2000	Chemicon, Temecula, CA
mAB anti-lamin B2	1:200	Chemicon, Temecula, CA
Goat-anti-mouse IgG conjugated to AlexaFluor488	1:200	Molecular Probes, Eugene, OR
Nucleic Acid binding probe; TO-PRO-3 iodine	1:1000	Molecular Probes, Eugene, OR

Coverslips were incubated overnight with agitation at 4°C in primary antibody diluted in PBS (see Table 2), washed three times in PBS, then incubated in secondary antibody at room temperature for 2 hours. Appropriate secondary antibodies were diluted as recommended by their suppliers (Table 2). During the final 30 minutes of secondary incubation, the DNA intercalating agent TO-PRO-3 (Molecular Probes, Eugene, OR) was added as a counter stain for nuclei. Immuno-staining negative controls were performed by replacing the primary antibody with conditioned F98 culture medium. After incubation in secondary antibody, the coverslips were again washed three times in PBS, dipped in water, then mounted using 90% glycerol in PBS.

Image Acquisition

Images were acquired using an Olympus IX-70 (Melville, NY) fitted with a Bio-Rad MRC 1024 confocal scan-head via the Keller port. All images collected for qualitative and quantitative analysis were done so at an optical magnification of 600x. Gain and dynamic range settings were calibrated on experimentally treated cells, and then

kept unchanged for recording of experimental control and immuno-stained images thus allowing quantitative comparison to be made. Final production of images for publishing was done on an Apple Macintosh G5 (Cupertino,CA) running Adobe Photoshop CS (San Jose, CA).

Quantification of Staining Intensity

Confocal projections were imported into NIH Image 1.6 (National Institutes of Health, Bethesda, MD) software where a 9x9 grid was superimposed over each image (Fig.1) resulting in 81 sample boxes per image. A random number generator developed and offered by the Distributed Systems Group at Trinity University (Dublin, Ireland) was used to acquire coordinates for 10 sample boxes on each image. Mean pixel intensity was measured for the green channel only of cells included in the sampling box by highlighting the selected area. A histogram of the selected area indicated a distribution of pixel intensity and the mean intensity of the selected area. Mean pixel intensities (PI) were exported to S-Plus 6.1 (Insightful, http://www.insightful.com/) for single factor ANOVA followed by a post hoc Tukey test to determine statistical significance between treatments. $P \leq 0.05$ was considered to indicate significance. The Tukey test was used to determine the significant differences between group means in an analysis of variance.

Cell Lysates and Immunoprecipitation Using Magnetic Beads

Cell monolayers were scraped from culture flasks using a rubber policeman and collected after a brief centrifugation. Once cells were washed in PBS they were added to 5 volumes of lysis buffer (PBS containing 0.1% Triton X-100, 1mM EDTA, 1mg/ml PMSF) and incubated on ice for 30 minutes. The lysates were run through a 20 gauge needle to shear chromosomal DNA. Aliquots of lysate were combined (4 parts to 1 part)

with 5X sample buffer (60 mM Tris-HCl, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromphenol blue and 1 mM EGTA) and frozen for later analysis by denaturing electrophoresis and western analysis as previously described (Weigum *et al.*, 2003, see Table 2 for antibodies used). Other aliquots were used in immuno-isolation experiments.

Isolation of J1-31 Ag Using Magnetic Beads

Immunomagnetic beads coupled to mAB J1-31 were used to isolate the J1-31 antigen(s) from cell lysates, cellulose coated iron oxide beads (Cortex Biochem., San Leandro, CA) were acquired in de-ionized water in 20% ethanol at a density of $3.2x10^{10}$ particles/ml. Greater than 75% of the particles are 2-4μm in diameter composed of cellulose/iron oxide (Fe_3O_4), 50:50. Activation of the beads for attachment of mAB J1-31 utilized cyanogen bromide (protocol MagaCell™ 501) as described below. Beads were brought to room temperature with rolling for approximately 30 minutes. Beads were attracted to and adhered to a magnet through the glassware, allowing the "supernatant" to be removed. The beads were then resuspended in 20 ml water.

After 3 washes in water, beads were suspended and washed in 0.05M phosphate buffer (pH 11.5) two times. Then freshly prepared cyanogen bromide (1g in 15ml of 0.05M phosphate buffer, pH11.5) was added slowly over 15 minutes while the pH was controlled using 2M NaOH to maintain at 11.5. Beads were then washed three times in cold deionized water followed by two washes in 0.1M bicarbonate buffer at pH 8.6. J1-31 hybridoma culture supernatant was concentrated using Millipore-Centriplus (Amicon, Watertown, MA) centrifugally at 4°C to 25% of the original volume. The beads were then combined with concentrated J1-31 hybridoma supernatant for 48 hours with

continuous agitation at 4°C. After incubation with mAB J1-31 a magnet was used to collect the beads. The beads were then washed in bicarbonate buffer. Next the beads were washed in bicarbonate buffer containing ethanolamine (3 ml per liter) then incubated in the same solution for 30 minutes at room temperature. Beads were then washed in 0.1M sodium acetate buffer pH 4.0 and incubated in the same solution for 30 minutes at room temperature. The beads were finally washed four times in working buffer (1X PBS, 1mM EDTA, 1mg/ml PMSF) containing 0.1% sodium azide to prevent contamination.

The mAB J1-31 coated beads were then combined with F98 total cell lysates and incubated with continuous agitation for 12 hours at 4°C. The beads were then collected with a magnet and washed four times in PBS. To free the antigen from the J1-31 mAB, the beads were bathed in 0.1M glycine (pH 2.5) for 10 minutes. The beads, now presumably freed of J1-31 antigen(s) were again collected by a magnet, and the resulting supernatant collected and neutralized by the addition of an equal amount of 1xPBS containing protease inhibitors (1mM EDTA, 1mg/ml PMSF) pH 7.4. Samples of bead elution were prepared for SDS-PAGE and western analysis as described above for lysate and stored at -20°C.

RESULTS

J1-31 labeling intensity is increased consequent to activation of adenylyl cyclase.

Treatment of F98 cells after 3 days in culture with forskolin for 45 minutes induced an increase in mAB J1-31 labeling primarily associated with the nucleus as compared to cells not exposed to forskolin. As seen in Fig. 2 panels A and B, this increase in labeling intensity appeared to occurs in the form of nuclear rings and "dots". Quantification of the pixel intensity increase revealed a significant difference ($p<0.001$) between non-treated cells (PI = 32 ± 0.79) and forskolin treated cells (PI = 69 ± 1.73) (Fig. 7) with the average value of pixel intensity greater than doubled. A similar, forskolin-dependent increase in labeling intensity was not observed when the same cells were immunostained with antibodies specific for lamin B and GFAP (Fig. 6); in that case, pixel intensity remained unchanged.

The forskolin-dependent increase in J1-31 is not mediated through PKA.

Treatment of F98 cells at 3 days with H89, followed by forskolin, did not alter the degree to which mAB J1-31 labeled nuclear antigens (Fig. 3B,C). Further, quantification revealed there to be no significant difference between cells treated with forskolin, having an average pixel intensity (PI) of 69 ± 1.73, and H89/forskolin-treated cells with an average PI of 65 ± 3.48 (Fig. 7). Treatment of F98 cells with H89 in the absence of forskolin produced similar labeling patterns and intensity as observed in cells not treated with forskolin (PI=31 ± 0.19) (Fig. 3 panel D). Additional studies were performed using the regulatory subunit inhibitor R_p-cAMPS to inhibit PKA activity prior to forskolin

treatment (Fig. 4). Quantification of mean pixel intensity revealed similar results as those found when H89 was used to inhibit PKA (Fig. 7). PI values for R_p-cAMPS followed by forskolin showed no significant difference in mean pixel intensity (PI=68 ± 0.19; Fig. 7) compared to those seen when only forskolin was used (PI=69 ± 1.73). Finally when only R_p-cAMPS was used pixel intensity was near the same levels seen when no treatment was used (PI=32 ± 0.19)(Fig. 8).

The forskolin-dependent increase in J1-31 labeling is blocked by verapamil.

Treatment of F98 cells with verapamil prevented the forskolin-dependent increase in labeling intensity (Fig. 5B, C; Fig. 7). The difference in intensity of staining between forskolin-treated cells (PI = 69 ± 1.73) and verapamil/forskolin (PI = 31.92 ± 0.19) treated cells was statistically significant ($p<0.001$). Treatment of F98 cells with verapamil alone resulted in a similar pattern of labeling with similar intensity as cells that were not treated (Fig. 5D).

Immunoprecipitation with magnetic beads

Proteins eluted from J1-31 coated magnetic beads included lamin B and GFAP, as identified by immunostaining of dot blots (Fig. 8). SDS-PAGE consistently revealed three strong bands of molecular mass of approximately 100,000, 70,000, 50,000 and 30,000. Western analysis of these gels provided unexpected results with both mAB J1-31 and anti-GFAP labeling a M_r 100,000 protein. Anti-GFAP also revealed the expected M_r 50,000 (see Figure 8), and no bands were consistently labeled by anti-lamin B antibodies.

Figure 1

Confocal image representing only data collected in the green channel imported to ImageJ for superimposition of measuring grid.

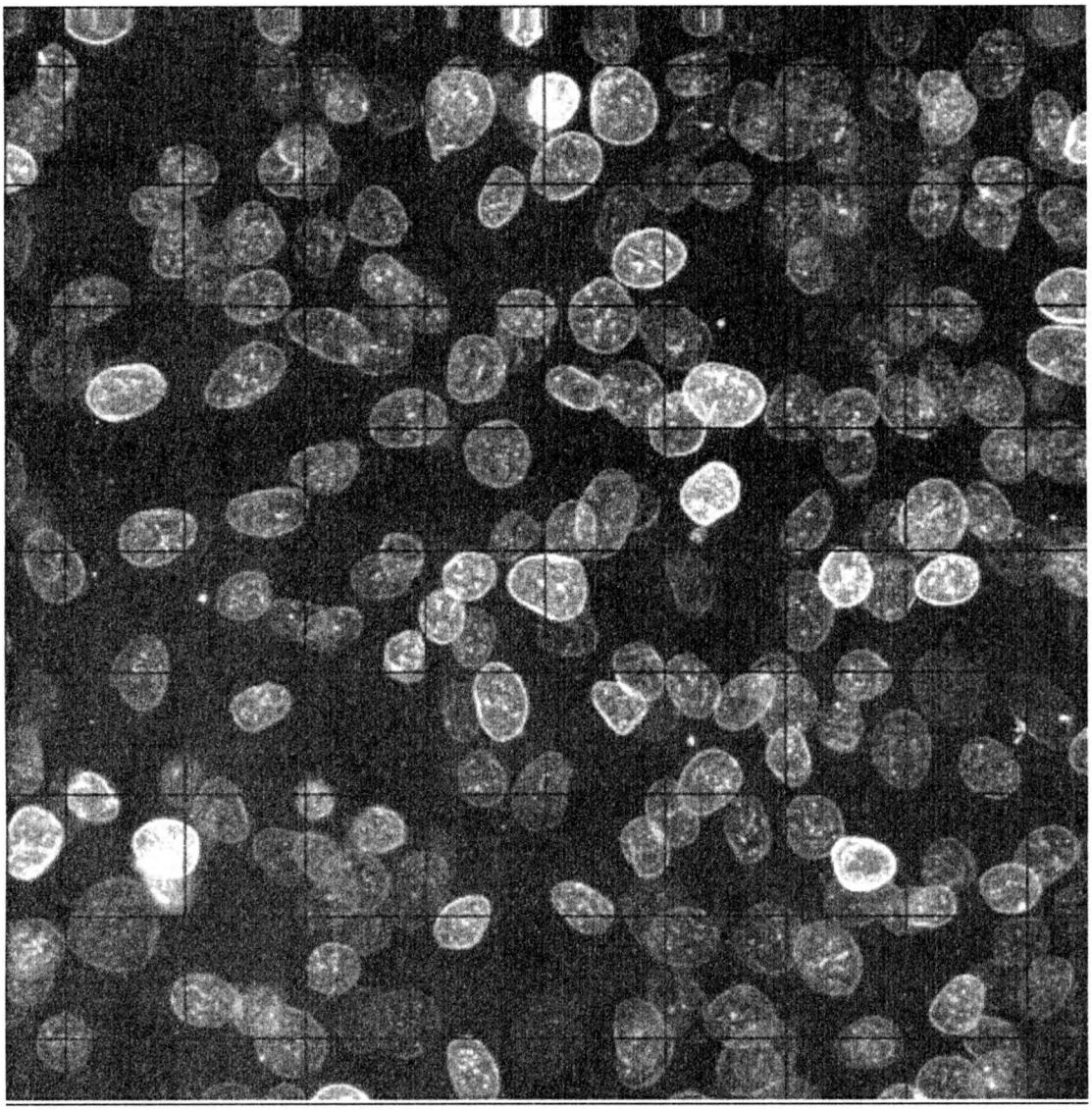


Figure 2

Confocal image prepared from a digital projection of F98 cells. Cells were cultured for 3 days followed by a replacement of medium with treatment reagents mixed in fresh medium. Treatment for 45 minutes was carried out under normal culture conditions with subsequent fixation in methanol and processing for immunocytochemical analysis as described in the text. Primary antibody in non-control samples (A-C) was mAB J1-31 with a primary control (D) of conditioned culture media and all panels labeled with a secondary conjugated to AlexFluor488.

A. No treatment.

B. Forskolin treatment for 45 minutes.

C. DMSO vehicle control for forskolin.

D. Primary antibody control labeled with conditioned culture media

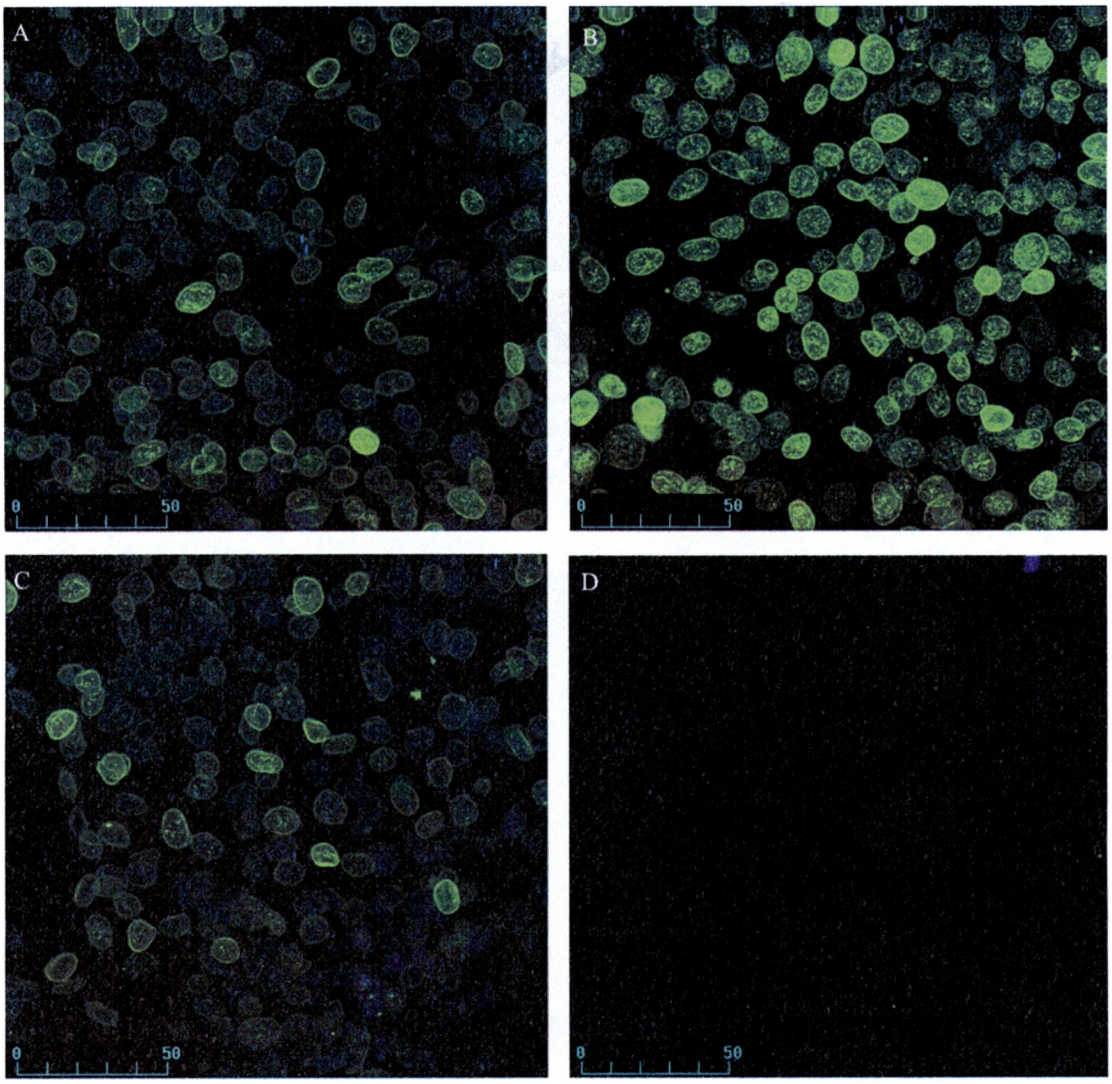

A
0
50
B
0
50
C
0
50
D
0
50

Figure 3

Confocal image prepared from a digital projection of F98 cells. Cells were cultured for 3 days followed by a replacement of medium with treatment reagents mixed in fresh medium. Treatment for 45 minutes was carried out under normal culture conditions with subsequent fixation in methanol and processing for immunocytochemical analysis, as described in the text. All panels probed with mAB J1-31 and labeled with a secondary conjugated to Alexafluor 488.

A. No treatment

B. forskolin treatment

C. H89 followed by forskolin treatment

D. H89 treatment

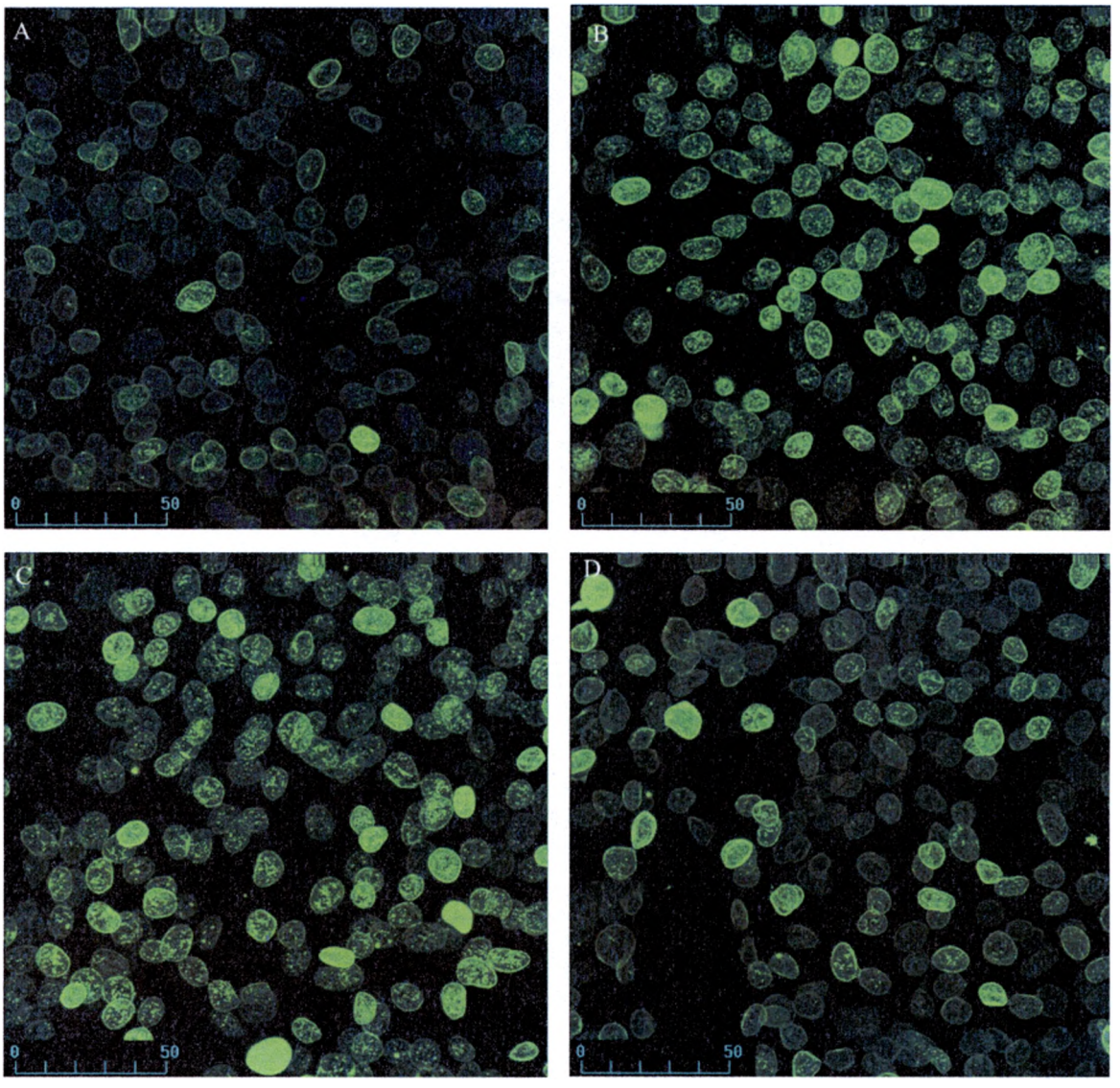

A
0
50
B
0
50
C
0
50
D
0
50

Figure 4

Confocal image prepared from a digital projection of F98 cells. Cells were cultured for 3 days followed by a replacement of medium with treatment reagents mixed in fresh medium. Treatment for 45 minutes was carried out under normal culture conditions with subsequent fixation in methanol and processing for immunocytochemical analysis, as described in the text. All panels probed with mAB J1-31 and labeled with a secondary conjugated to Alexafluor 488.

A. No treatment

B. forskolin treatment

C. Rp-cAMPS followed by forskolin treatment

D. Rp-cAMPS treatment

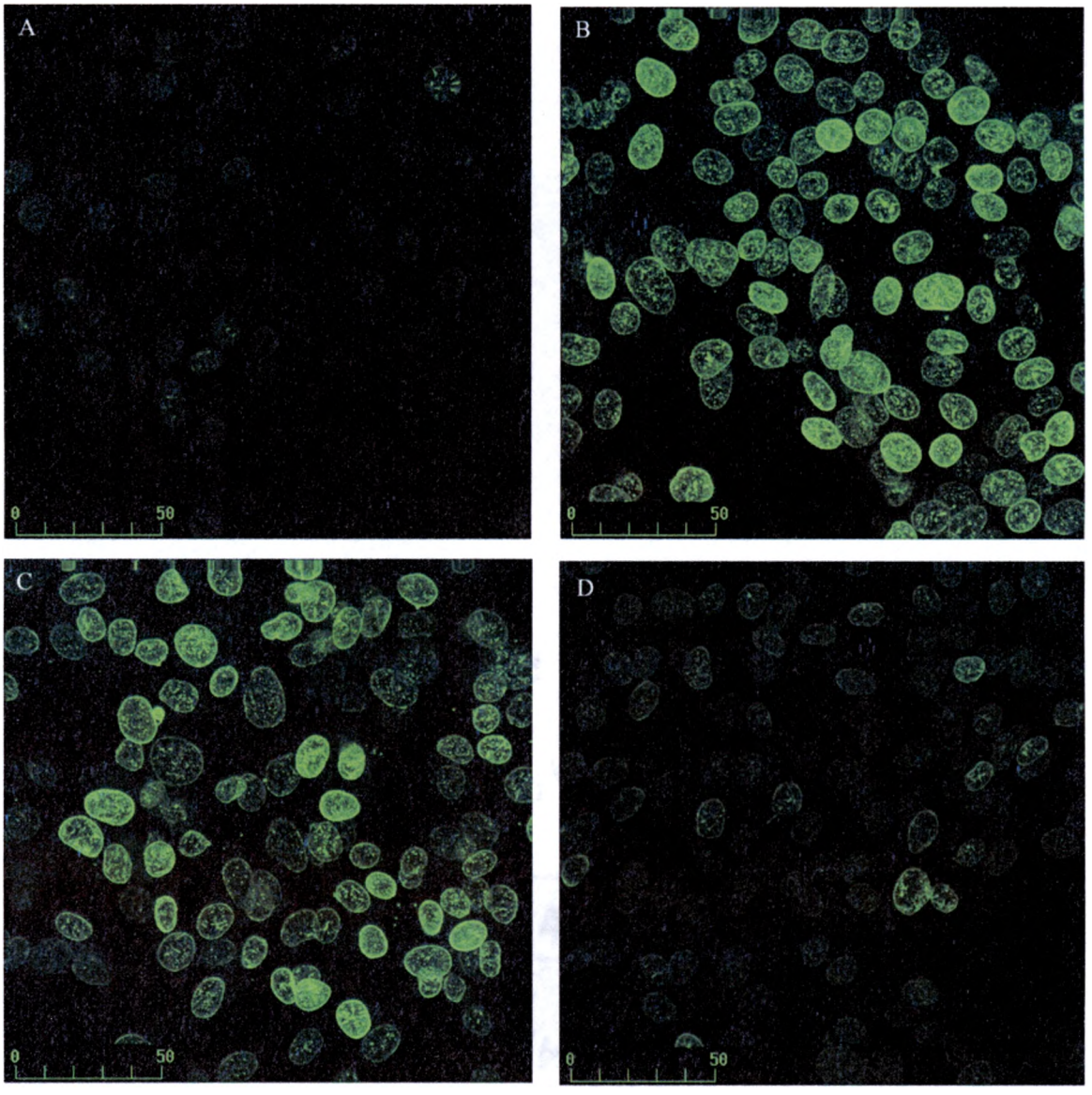

A
0
50
B
0
50
C
0
50
D
0
50

Figure 5

Confocal image prepared from a digital projection of F98 cells. Cells were cultured for 3 days followed by a replacement of medium with treatment reagents mixed in fresh medium. Treatment for 45 minutes was carried out under normal culture conditions with subsequent fixation in methanol and processing for immunocytochemical analysis, as described in the text. All panels probed with mAB J1-31 and labeled with a secondary conjugated to Alexafluor 488.

A. No treatment

B. forskolin treatment

C. verapamil followed by forskolin

D. verapamil alone

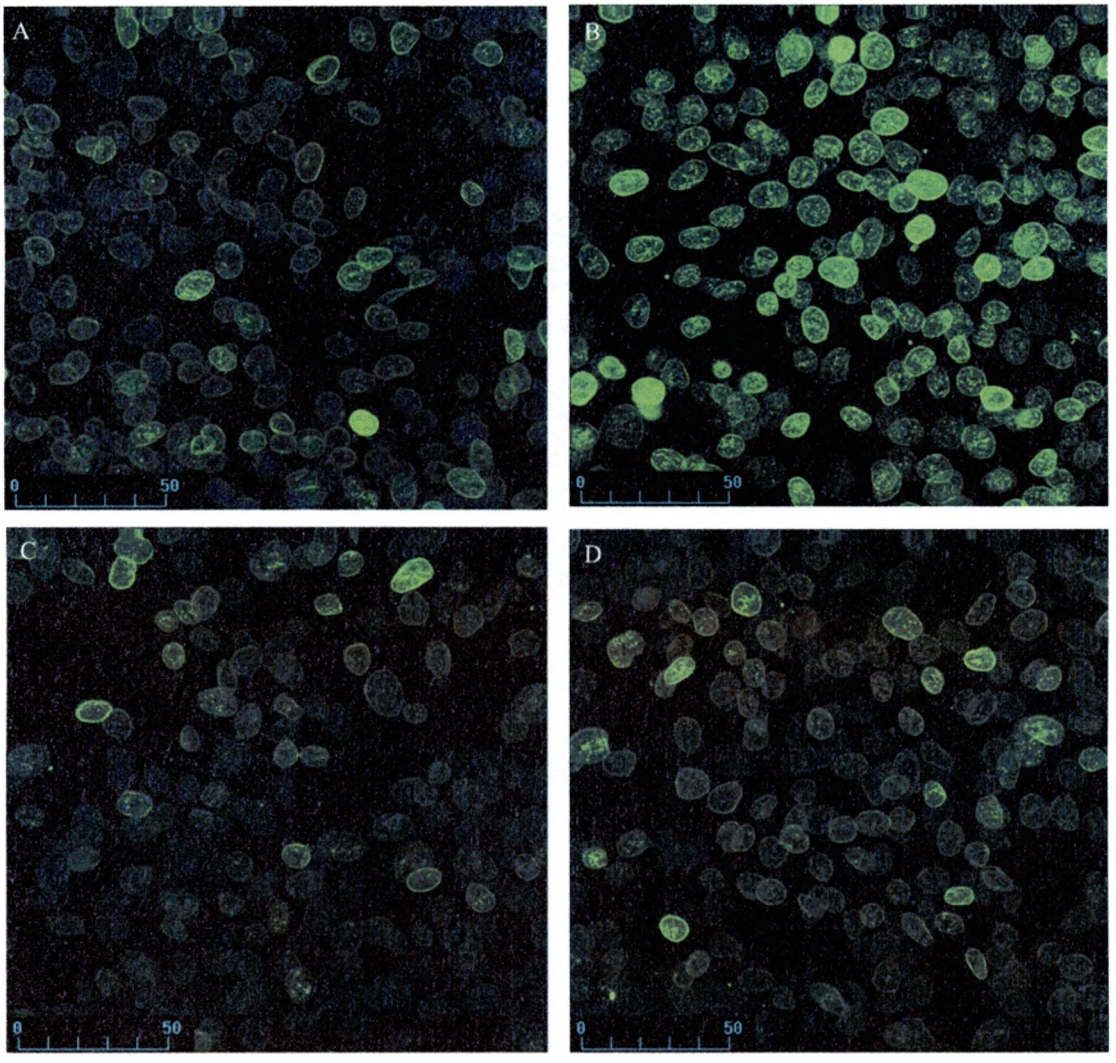

A
0
50
B
0
50
C
0
50
D
0
50

Figure 6

Confocal image prepared from a digital projection of F98 cells. Cells were cultured for 3 days followed by a replacement of media with treatment reagents mixed in fresh media. Treatment for 45 minutes was carried out under normal culture conditions with subsequent fixation in methanol and processing for immunocytochemical analysis, as described in the text. All panels in the right column are of cells treated with forskolin while the left column shows cells not exposed to forskolin.

A, B. probed with mAB J1-31

C, D. probed with mAB anti-Lamin B (Chemicon)

E, F. probed with mAB anti-GFAP (Chemicon)

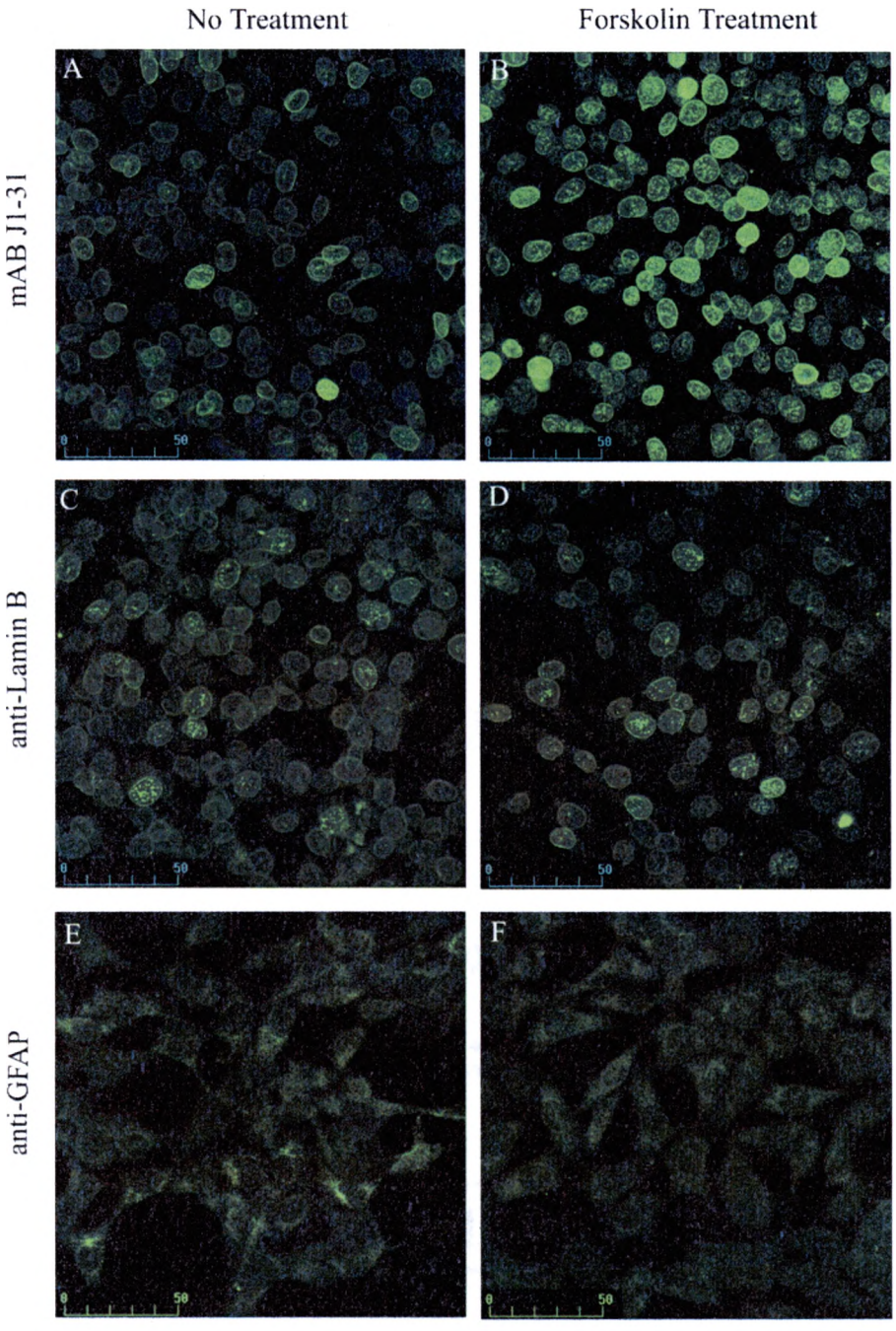

No Treatment
Forskolin Treatment
mAB J1-31
anti-Lamin B
anti-GFAP
A
B
C
D
E
F
0
50

Figure 7

Mean pixel intensity listed as a function of treatment (n=3). All optical settings were optimized for forskolin treated mAB J1-31 labeling then maintained the same for all. Error bars represent ±1 SEM.

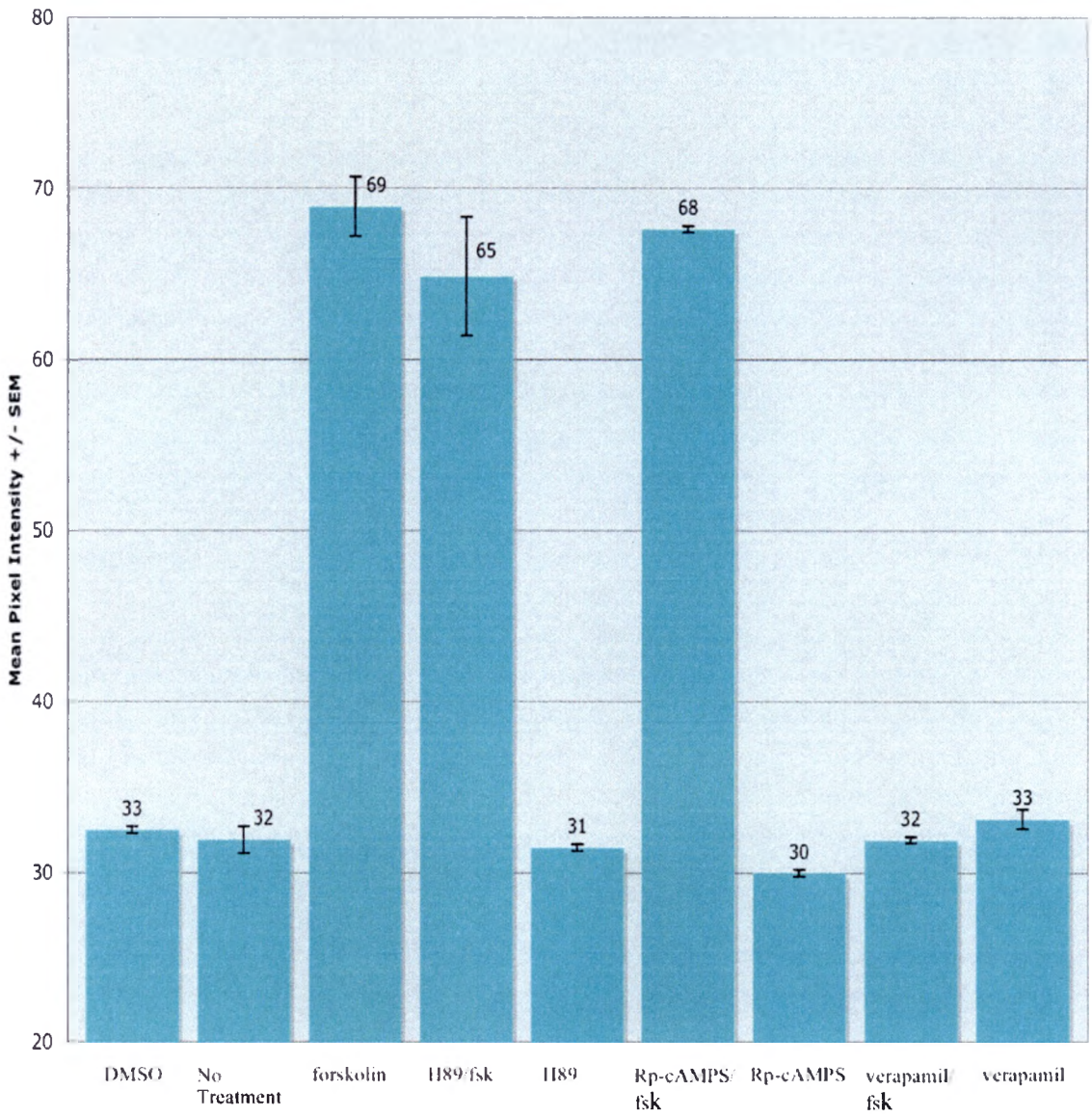
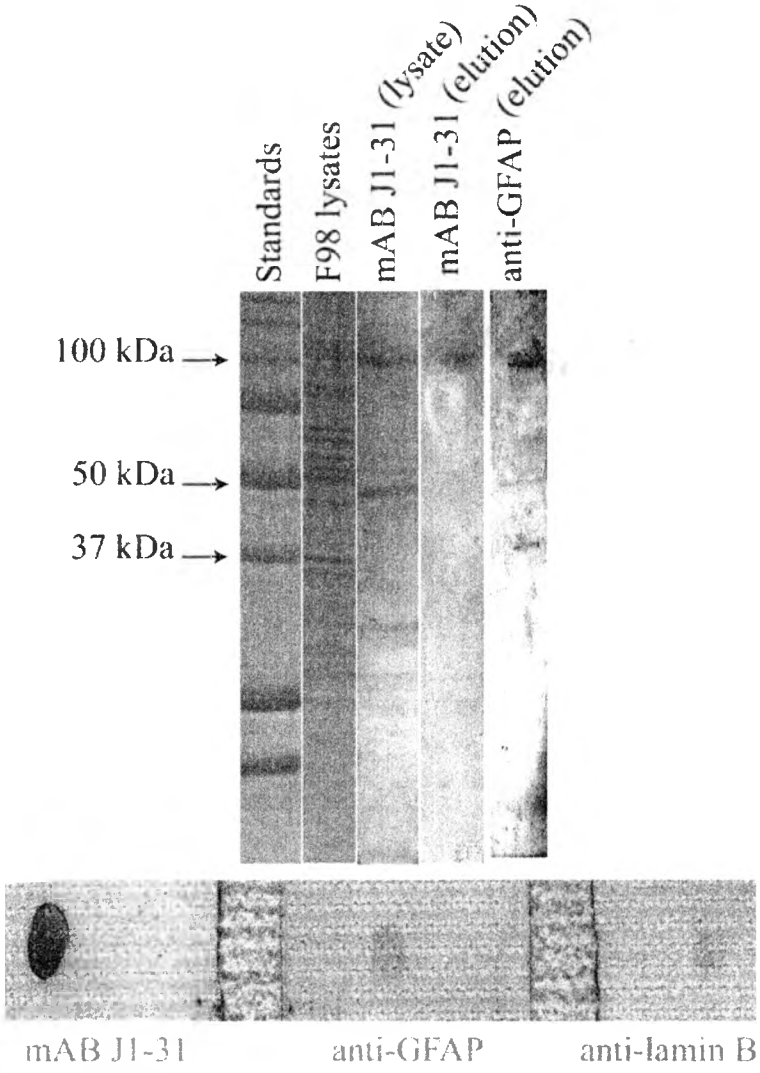

Mean Pixel Intensity +/- SEM
80
70
60
50
40
30
20
33
32
69
65
31
68
30
32
33
DMSO
No Treatment
forskolin
H89/fsk
H89
Rp-cAMPS/ fsk
Rp-cAMPS
verapamil/ fsk
verapamil

Figure 8

SDS-PAGE and immunoblot analysis of F98 glioma cell lysates. MW standards (1); Coomassie stained for total protein (2); immunoblot of total lysates with mAB J1-31 (3); immunoblot of bead elution with mAB J1-31 (4); immunoblot of bead elution with anti-GFAP (5). Below are "dot" immunoblots of bead elution with mAB J1-31 (a); anti-GFAP (b); and anti-lamin B (c).


Standards
F98 lysates
mAB J1-31 (lysate)
mAB J1-31 (elution)
anti-GFAP (elution)
100 kDa
50 kDa
37 kDa
mAB J1-31
anti-GFAP
anti-lamin B

DISCUSSION

mAB J1-31 labeling increases in F98 cells treated with forskolin

Monoclonal antibody J1-31 has been postulated to bind specifically to a phosphorylated epitope on both GFAP and lamin B (García *et al.* 2003); the results described here are consistent with this epitope modification. As mentioned previously, GFAP and lamin B are both intermediate filaments and phosphorylation is known to regulate dynamic reorganization during cell division (Herrmann *et al.* 2000). In concert with this, each IF mentioned has consensus sequences for phosphorylation by PKA, PKC and CaMK, all of which have been shown to phosphorylate serine residues located in the amino head region (Matsuzawa *et al.* 1998). Of the putative phosphorylation amino acid sequences in lamin and GFAP identified by García *et al.* (2003), a single serine (Ser-68) residue was found that was located in a PKA consensus sequence. This finding raised the possibility that activation of adenylyl cyclase and subsequent activation of PKA would enhance labeling of lamin and GFAP by mAB J1-31 if that serine residue were located in the J1-31 epitope. To test this possibility, forskolin was used to activate adenylyl cyclase thereby increasing cAMP levels and presumably PKA activity. Monoclonal antibody J1-31 labeling was found to increase significantly in F98 glioblastoma cells after treatment with forskolin for 45 minutes. Forskolin-induced labeling changes were primarily associated with the nucleus (Fig.2). Nuclear labeling has also been observed by Singh *et al.* (1994) in astrocytes that have been activated. The colocalization between mAB J1-31

and anti-lamin B labeling on discrete nuclear structures reported by García *et al.* (2003) suggests that in the nucleus mAB J1-31 binds to an epitope on lamin B.

As described above, forskolin activates adenylyl cyclase and elevates cAMP concentrations. In order to test the role PKA played in the forskolin-induced response, I selectively inhibited PKA by using H89 and R_p-cAMPS in independent studies. Curiously, however, inhibition of PKA did not appear to significantly block the forskolin-induced increase in J1-31 labeling seen previously (Fig. 3 and 4). H89 has been described as a cell-permeable, potent inhibitor of PKA catalytic subunits with a K_i=48nM. R_p-cAMPS is used as a irreversible PKA regulatory subunit inhibitor with a K_i=200μM. Given this result, it seems unlikely that the J1-31 antigen is phosphorylated by PKA dependent pathway but is phosphorylated in a cAMP-dependent manner.

Ca^{+2} influx results in phosphorylation of the J1-31 epitope

In F98 cells, the J1-31 labeling intensity changes do not appear a result of PKA activation by cAMP; therefore, the ability of cAMP to elicit a Ca^{+2} influx fits nicely with additional second messenger pathways that lead to protein phosphorylation. Specifically, if Ca^{+2} were to enter the cell through cAMP-activated CNG channels, Ca^{+2} -modulated protein kinases could in turn be activated. As mentioned above, CNG channels are expressed in CNS tissue although there does not appear to be any record of their expression in glioblastoma specifically. Such would however help to explain some of the observations described in the present study. Additional components of this model include the presence of Ca^{+2} dependent protein kinase consensus sequences found on both intermediate filaments that mAB J1-31 recognizes. To test the involvement of such kinases, the Ca^{+2} channel inhibitor, verapamil, was used prior to treatment with forskolin.

In the presence of verapamil, a dramatic inhibition of the forskolin-induced J1-31 labeling changes occurred (Fig. 5). Verapamil is best known as in inhibitor of L-type Ca^{+2} channels in cardiac and smooth muscle, and these channels are also found in some nerve tissues (Agrawal *et al.* 2000). These results suggest that the forskolin-induced J1-31 labeling increase occurs in a Ca^{+2} dependent manner. Because Ca^{+2} activates a number of protein kinases, and there are consensus sequences for such kinases located in the amino head region of both GFAP and lamin B (Ser-13, Ser-34) (Matsuzawa *et al.* 1998), it seems likely that the cAMP-dependent influx of Ca^{+2} may be involved in the increase in J1-31 antigenicity following forskolin treatment. Although these findings do not indicate which Ca^{+2} -dependent kinase is responsible, they may provide insight into the signaling pathways of reactive astrogliosis.

Immunomagnetic isolation of the J1-31 antigen

The identity of the J1-31 antigen was further investigated using immuno-magnetic bead techniques. The source for J1-31 antigen was provided by F98 cell lysates that had been in culture for two days; the point at which J1-31 has been reported to be at highest levels in these cell cultures (Weigum 2002). More commonly used immunoaffinity column techniques have been unsuccessful in the past because the J1-31 antigens are largely insoluble. This characteristic was thought to prevent the antigen from entering an affinity column and thus prompted the development of a novel approach; immunomagnetic bead isolation. By effectively taking the affinity column to the antigen, the technique described above was used in hopes of isolating the J1-31 antigens from rapidly growing F98 cells lysates. Elutants from the bead isolation were separated and probed using SDS-PAGE and immunobloting respectively. A band (M_r 100,000) present

in the bead elution that was positively probed by antibodies against both J1-31 and GFAP was thought to be the dimeric form of GFAP. J1-31 mAB labeled a band (M_r 50,000) in total cell lysates, however it failed to do the same in the well containing bead elutants. Antibodies against GFAP did however label a band at the molecular weight of monomeric GFAP or M_r 50,000. Curiously anti-GFAP antibodies also labeled a band with a molecular mass of 65,000 that could be the product of unintended co-immunoprecipitation. The results described, above although partially consistent with antigen identity described by García *et al.* (2003), present additional question regarding the novel bands labeled by anti-GFAP in bead elutant. More specifically, the results presented here raise the possibility that the J1-31 antigen may be as Malhotra *et al.* (1984) first thought; an IFAP associated with GFAP. Future studies should focus on dissecting these results to test the anomalies described above.

Future studies

More specific inhibitors of Ca^{+2} dependent kinases should be used to further dissect the pathway that leads to J1-31 epitope phosphorylation. A more specific understanding of the kinase is involved may provide leads about the specific J1-31 epitope. Specific epitope characterization would provide us with a tool to investigate reactive astrogliosis. Additional studies should also focus on isolation of the J1-31 antigen by means of immunomagnetic bead techniques for the purpose of sequence determination. Attempts to resolve the unexpected bands should focus on additional wash steps with mild tonic buffers in order to interrupt any non-covalent interactions the J1-31 antigen may experience with other proteins. Considering the harsh pH buffers that were necessary to elute that J1-31 antigens there is the possibility that mild tonic buffers

would not alter the antigens of interest, but could remove proteins not recognized by mAB J1-31. Additional bands were labeled at lower molecular weight (~30kDa) by mAB J1-31 in total lysates that corresponded to what García *et al.* 2003 concluded might be degradation products of GFAP. Unfortunately anti-GFAP antibodies do not label these bands. This result does not preclude them from being degradation products that are simply not recognized by the anti-GFAP antibody. In order to promote greater yield of isolated protein, it may be advantageous to treat a confluent monolayer of F98 cells *in vitro* with forskolin prior to lysis. According to the data presented above, this should increase the amount of antigen present and in turn may help to correct the inconsistent isolation of what is thought to be a second antigen of mAB J1-31; lamin B.

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VITA

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