Analysis of the Calcium-modulated Proteins, S100 and Calmodulin, and Their Target Proteins During C6 Glioma Cell Differentiation

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Abstract. We have analyzed the levels, subcellular distribution, and target proteins of two calcium-modulated proteins, S100 and calmodulin, in differentiated and undifferentiated rat C6 glioma cells. Undifferentiated and differentiated C6 cells express primarily the S100β polypeptide, and the S100β levels are fourfold higher in differentiated compared to undifferentiated cells. Double fluorescent labeling studies of undifferentiated cells demonstrated that S100β staining localized to a small region of the perinuclear cytoplasm and colocalized with the microtubule organizing center and Golgi apparatus. Analysis of differentiated C6 cells demonstrated that S100β distribution and S100β-binding protein profile changed significantly upon differentiation. In addition, the brain-specific isozyme of one S100-binding protein, fructose-1,6-bisphosphate aldolase C, can be detected in differentiated but not undifferentiated C6 cells. While changes in the subcellular distribution of calmodulin were not observed during differentiation, calmodulin levels and calmodulin-binding protein profiles did change. Altogether these data suggest that S100β and calmodulin regulate different processes in glial cells and that the regulation of the expression, subcellular distribution, and target proteins of S100β and calmodulin during differentiation is a complex process which involves multiple mechanisms.

THE interaction of calcium with calcium binding proteins is one mechanism by which calcium is thought to regulate cellular processes. One class of calcium binding proteins, the calcium-modulated proteins, appear to act as effector proteins and transduce calcium signals into a cellular response by modulating the activity of other proteins; i.e., target proteins. Two examples of calcium-modulated proteins are calmodulin and the S100 family of proteins. Calmodulin is an ubiquitous, eukaryotic protein that activates several enzymes and appears to regulate many cellular processes (for review see Klee and Vanaman, 1982). The S100 fraction is composed primarily of two similar proteins, S100α and S100β, which share ~50% identity in amino acid sequence (Isobe and Okuyama, 1978; 1981). Available data suggest that like calmodulin, S100 proteins may also be multifunctional effector proteins (Baudier and Cole, 1988; Hagiwara et al., 1988; Zimmer and Van Eldik, 1986). S100 proteins and calmodulin are particularly abundant in brain tissue, and the levels of both proteins change during nervous system development (Zuckerman et al., 1970; Stewart and Urban, 1972; Haglid et al., 1977). These data suggest that S100 and calmodulin play an important role in the function and differentiation of neural cells in vivo.

Rat C6 glioma cells (Benda et al., 1968) possess antigens and enzymes characteristic of both astrocytes and oligodendrocytes, and retain patterns of hormonal responsiveness that are similar to those found in normal glial cells in brain and in primary cultures (deVellis et al., 1977; McCarthy and deVellis, 1980). In addition, morphological differentiation of C6 cells occurs under conditions that elevate intracellular cAMP (Willingham, 1976). During differentiation, C6 cells develop long processes and their morphology resembles that of mature astrocytes. Under these conditions specific biochemical changes occur, including induction of astrocytic markers such as glial fibrillary acidic protein (Raju et al., 1980), 2′:3′-cyclic nucleotide 3′ phosphohydrolase S100 proteins (McMorris, 1977; Tabuchi et al., 1981), and the C isozyme of fructose-1,6-bisphosphate aldolase (Kumanishi et al., 1975). It is interesting to note that, in addition to being an astrocytic marker (Sato et al., 1972; Kumanishi et al., 1985), the C isozyme of aldolase has been identified as an intracellular target protein of S100 (Zimmer and Van Eldik, 1986). Since C6 cells express both S100 (Labourdette and Marks, 1975; Zimmer and Van Eldik, 1988) and calmodulin (Brostrom and Wolff, 1974; Zimmer and Van Eldik, 1988), this cell line is an excellent system for studying the mechanisms by which multiple calcium-modulated proteins function during astrocyte differentiation.

Previous studies in our laboratory (Zimmer and Van Eldik, 1988) focused on the regulation of S100 and calmodulin during growth of C6 cells in vitro. We found that S100β is the predominant S100 protein in rat C6 cells and that it may

be associated with a specific intracellular structure(s): the Golgi apparatus and/or microtubule organizing center (MTOC).[1] Our studies also suggested that changes in S100β and S100β-binding proteins may be involved in regulating S100-mediated processes in C6 cells and that the levels of calmodulin and S100β may be differentially regulated in these cells. Other laboratories have examined S100 during differentiation of C6 glioma cells. In these studies, low serum or serum-free media and/or the addition of dibutyryl cAMP or agents which increase the intracellular level of cAMP have been used to induce differentiation of these cells. However, there are discrepancies in the literature regarding the extent of morphological differentiation of C6 cells under various culture conditions (Fan and Uzman, 1977; Steinbach and Schubert, 1975). Some investigators have reported that the levels of the S100 proteins do not change during differentiation (Donta, 1973), while others have reported that S100 protein levels increase during differentiation (Labourdette and Mandel, 1980; Higashida et al., 1985). These previous studies have not used S100α- and S100β-specific antibodies to determine if the changes in S100 levels represent changes in the level of one or both of the S100 proteins. In addition, other mechanisms of regulation such as alterations in the subcellular distribution of the S100 proteins and/or alterations in the complement of S100 target proteins in C6 cells have not been examined. To date, none of the studies on differentiation of rat C6 cells has analyzed the regulation of calmodulin during this process.

To better understand the function of multiple calcium-modulated proteins in glial cells, we have compared the level, subcellular distribution and target proteins of S100 and calmodulin in undifferentiated and differentiated C6 glioma cells. In our studies, we have also examined partially differentiated cells in an attempt to clarify the conflicting reports in the literature regarding the extent of morphological differentiation and S100 levels in these cells. In addition, the relationship of S100β with specific intracellular structures, in particular the Golgi apparatus and MTOC, has been examined. Altogether our data suggest that regulation of the expression, subcellular distribution, and target proteins of S100β and calmodulin during differentiation is a complex process which involves multiple mechanisms.

Materials and Methods

Cell Culture and Drug Treatment of Cells

The rat C6 glioma cell line was obtained from the American Type Culture Collection (Rockville, MD) and grown in F10 media (Gibco Laboratories, Grand Island, NY) supplemented with 15% (vol/vol) horse serum (HyClone Laboratories, Sterile Systems, Inc., Logan, UT), 2.5% (vol/vol) FCS (HyClone Laboratories, Sterile Systems Inc.), 2 μg/ml fungizone (E. R. Squibb & Sons, Princeton, NJ), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco Laboratories). For adaptation to growth in serum-free media, C6 cells were grown in serum-containing media for 2 d after trypsinization, the culture media was then changed to PC-1 serum-free media (Ventrex Laboratories, Inc., Portland, ME) containing 5% (vol/vol) horse serum and antibiotics for 2 d, then PC-1 media containing 1% (vol/vol) horse serum and antibiotics for 2 d, and finally PC-1 media containing only antibiotics. Stock cultures were maintained in T-flasks of 25 cm^2 surface area (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) and grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Stock cultures were fed every 2 d and subcultured every 7 d by trypsinization. For differentiation and drug treatment experiments, cells were prepared as described below. In all studies cells were allowed to grow for at least 48 h and fed at least once before treatment.

To depolymerize microtubules, cells were treated with 1 μg/ml *N*-deacyl-*N*-methyl-colchicine (demecolcine) (Sigma Chemical Co., St. Louis, MO) for 1-4 h at 37°C. Demecolcine was stored at −20°C as a 1 mg/ml stock solution in water.

Differentiation of C6 Cells

To induce morphological differentiation, cells were fed with fresh media containing N^6-2′-O-dibutyryl cAMP (dcAMP) (Sigma Chemical Co.) at a final concentration of 1 mM. The cells were incubated in dcAMP for 48 h before cell fractionation or immunofluorescence microscopy studies. At the end of dcAMP treatment the cells were ~80% confluent. Control cells grown in media only (either serum-containing or serum-free) were also harvested at similar cell densities.

Purification of Proteins

S100α, S100β, and calmodulin were purified from bovine brain as previously described (Hertzberg and Van Eldik, 1987; Lukas and Watterson, 1988). Rat brain aldolase was purified by using the procedure of Penhoet et al. (1969). Rabbit skeletal muscle aldolase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Aldolase C was purified from rat brain as previously described (Zimmer and Van Eldik, 1986).

Antibody Preparation and Characterization

Affinity-purified sheep antitubulin antibody was obtained from Southern Biotechnology Associates (Birmingham, AL). Rabbit antisera against bovine calmodulin (Van Eldik and Watterson, 1981), a synthetic peptide of vertebrate calmodulin (Van Eldik et al., 1983), and bovine brain S100α and S100β (Zimmer and Van Eldik, 1987) were prepared as previously described. Immunoglobulin fractions were prepared by ammonium sulfate fractionation (0-50%) and DEAE-cellulose chromatography (Fahey and Terry, 1978).

Radioimmunoassay

Competition radioimmunoassays for calmodulin (Van Eldik and Watterson, 1981) and S100α and S100β (Zimmer and Van Eldik, 1987) were done as previously described.

Fluorescence Microscopy

Trypsinized cells were plated in 60-mm plastic petri dishes (Falcon Labware, Becton, Dickinson & Co.) containing 11 × 22-mm glass coverslips. At the appropriate cell density, the cells were treated with dcAMP or colchicine as described above. After rinsing in 0.137 M NaCl, 0.003 M KCl, 0.004 M Na_2HPO_4, 0.015 M KH_2PO_4 buffer, pH 7.5 (PBS), the coverslips were fixed in 2.5% (vol/vol) paraformaldehyde (Polysciences, Inc., Warrington, PA) in PBS for 20 min at room temperature, incubated in cold acetone at −20°C for 5 min, and rinsed briefly in PBS. In addition to acetone, digitonin (Sigma Chemical Co.) was also used as a permeabilizing agent. For these experiments, the coverslips were rinsed briefly in PBS and incubated in various concentrations of digitonin in PBS for 2 min at room temperature. The digitonin-permeabilized coverslips were then rinsed in PBS, fixed in paraformaldehyde as described above, and rinsed briefly in PBS. After permeabilization, the coverslips were incubated in 50 μl of rabbit anti-S100β IgG diluted 1:2 or undiluted affinity-purified sheep antitubulin antibody (Southern Biotechnology Associates) in PBS for 60 min at 37°C. After rinsing briefly in PBS followed by two 15-min washes in PBS, the coverslips were incubated in 50 μl of FITC-conjugated goat anti-rabbit or rabbit anti-goat secondary antibody (ICN ImmunoBiologicals, Lisle, IL) at a 1:32 dilution in PBS. After two 15-min washes in PBS, the coverslips were mounted in glycerol-PBS (9:1), pH 8.0, on glass slides. In some experiments, the coverslips were incubated in TRITC-conjugated wheat germ agglutinin (WGA) (E. Y. Laboratories, Inc., San Mateo, CA) diluted 1:20 in PBS for 20 min at room temperature, rinsed with PBS, and then mounted. Cells were examined with a Leitz Diavert microscope equipped for epifluorescence with appropriate rhodamine and fluorescein filters and

1. *Abbreviations used in this paper*: dcAMP, N^6-2′-O-dibutyryl cAMP; MTOC, microtubule organizing center; WGA, wheat germ agglutinin.

phase contrast optics. Photographs were taken on Kodak Tri-X film (ASA 1600) with a 50× oil (NA 1.0) planapochromat objective.

Preparation of Cell Pellet Fractions

Extracts of cell pellets were prepared as previously described (Zimmer and Van Eldik, 1987). Briefly, after homogenization the particulate fraction was removed by centrifugation at 15,000 *g* for 30 min. The homogenate supernatant was fractionated by ammonium sulfate precipitation into four fractions: a 0–45% pellet, a 45–60% pellet, a 60–80% pH 4 pellet, and a 60–80% pH 4 supernatant. All samples were dialyzed against 1 mM ammonium bicarbonate, lyophilized, resuspended in water, and stored at −80°C. Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as a standard.

Gel Overlay Analysis

Electrophoresis on 12.5% (wt/vol) acrylamide-(SDS) minigels and overlay analysis with iodinated proteins were done as previously described (Zimmer and Van Eldik, 1987).

Gel Electrophoresis and Detection of Aldolase Hybrids

Aldolase hybrids were separated by gel electrophoresis and detected by an activity stain as previously described (Zimmer and Van Eldik, 1986).

Results

Relationship of S100β and Cellular Organelles

Our previous immunolocalization studies (Zimmer and Van Eldik, 1988) demonstrated that S100β is localized to a small region of the perinuclear cytoplasm in C6 cells; we postulated that this region of the cytoplasm might contain the Golgi apparatus and/or MTOC. To test directly the hypothesis that S100β is associated with the Golgi apparatus in C6 cells, a double-labeling experiment was performed in which TRITC-WGA was used to localize the Golgi apparatus (Virtanen et al., 1980) and an anti-S100β antibody and a FITC-conjugated secondary antibody were used to localize S100β. The results of these experiments are shown in Fig. 1. When 0.2% digitonin was used to permeabilize the cells, S100β (Fig. 1 *a*) and TRITC-WGA (Fig. 1 *b*) staining colocalized to the same region of the perinuclear cytoplasm. However, the TRITC-WGA staining pattern was vesicular while the S100β staining pattern was diffuse. The Golgi apparatus also colocalized with the MTOC as revealed by double-labeling experiments in which antitubulin antibodies were used to localize the MTOC (Fig. 1 *e*) and TRITC-WGA was used to localize the Golgi apparatus (Fig. 1 *f*). Similar results were obtained when cold acetone rather than digitonin was used to permeabilize the cells (Fig. 2, *a* and *c*). These double-labeling experiments demonstrate that the perinuclear cytoplasm of C6 cells, which contains S100β immunoreactivity, also contains the Golgi apparatus and MTOC.

Previous studies have suggested that S100β might be actively secreted from C6 cells (Van Eldik and Zimmer, 1987). Since many secreted proteins are located on the inside of the Golgi membranes, these studies raise the possibility that S100β is located on the inside of the Golgi membranes. To investigate further the relationship of S100β and the Golgi apparatus, concentrations of digitonin, which would selectively permeabilize the plasma membrane and not the Golgi membranes, were used to permeabilize the cell before labeling (Fiskum et al., 1980). In these experiments antibodies to tubulin (which is located on the outside of the Golgi membranes) and TRITC-WGA (which binds to glycosylated proteins found inside the Golgi membranes) were used to monitor the permeability of the plasma membrane and Golgi membranes, respectively. When the digitonin concentration was lowered to 0.01%, a low level of TRITC-WGA labeling was observed throughout the cytoplasm in a punctate pattern (Fig. 1, *d* and *h*) but no intense perinuclear labeling was seen. These results indicate that in cells permeabilized with 0.01% digitonin the Golgi membranes are not permeable to WGA and suggest that they would not be permeable to other macromolecules such as antibodies. Incubation of the cells in antitubulin antibody (Fig. 1 *g*) produced a labeling pattern which was identical to that seen in cells permeabilized with 0.2% digitonin (Fig. 1 *e*) or acetone, confirming that the plasma membrane of cells treated with 0.01% digitonin was permeable to antibody molecules. In cells treated with 0.01% digitonin and incubated in S100β antibodies (Fig. 1 *c*), the staining observed was very similar to that seen in cells that were permeabilized with 0.2% digitonin (Fig. 1 *a*); i.e., intense staining in a small region of the perinuclear cytoplasm with a low level diffuse cytoplasmic staining. It should be noted that these experiments are not quantitative and a small reduction in S100β staining intensity in cells in which the Golgi membranes are not permeable would not be detected. Thus, our studies cannot rule out the possibility that a small portion of the intracellular S100β immunoreactivity is located inside the Golgi membranes. Nonetheless, the fact that S100β immunoreactivity is present in C6 cells in which the Golgi membranes are not permeable to antibody molecules demonstrates that most of the S100β immunoreactivity is located outside of the Golgi membranes in the cytoplasm of the cell.

The colocalization of S100β with the MTOC and previous reports which have shown that S100β colocalizes with the mitotic spindle (Zimmer and Van Eldik, 1988) and affects in vitro microtubule assembly (Donato, 1985; Baudier and Cole, 1988) suggest that one function of S100β may be the regulation of cell motility and/or cell architecture via interaction with microtubules and/or microtubule-associated proteins. To examine more closely the association of S100β with the MTOC, we examined the subcellular distribution of S100β in C6 cells in which the integrity of the microtubule network had been disrupted. In C6 cells treated with 1 or 5 μg/ml colchicine, no antitubulin staining was observed (Fig. 2 *f*) suggesting that colchicine treatment disrupted the cytoplasmic microtubules. When colchicine-treated cells were incubated in TRITC-WGA (Fig. 2 *d*), a punctate staining pattern was observed throughout the cytoplasm, rather than a small region of the perinuclear cytoplasm, suggesting that the Golgi apparatus had been disrupted. The dependency of the perinuclear arrangement of the Golgi apparatus on an intact microtubule complex has been observed in many cell types (Rogalski and Singer, 1984). When colchicine-treated C6 cells were incubated in S100β antibodies a very low level of diffuse staining throughout the cytoplasm was observed (Fig. 2 *b*) and this staining did not colocalize with the TRITC-WGA–labeled vesicles. Thus, significant levels of S100β immunoreactivity do not redistribute with the Golgi vesicles in colchicine-treated C6 cells. These data are consistent with the selective permeabilization studies described above and suggest that significant levels of S100β are not found in the inside of the Golgi apparatus or intimately associated with the outside surface of the Golgi membranes.

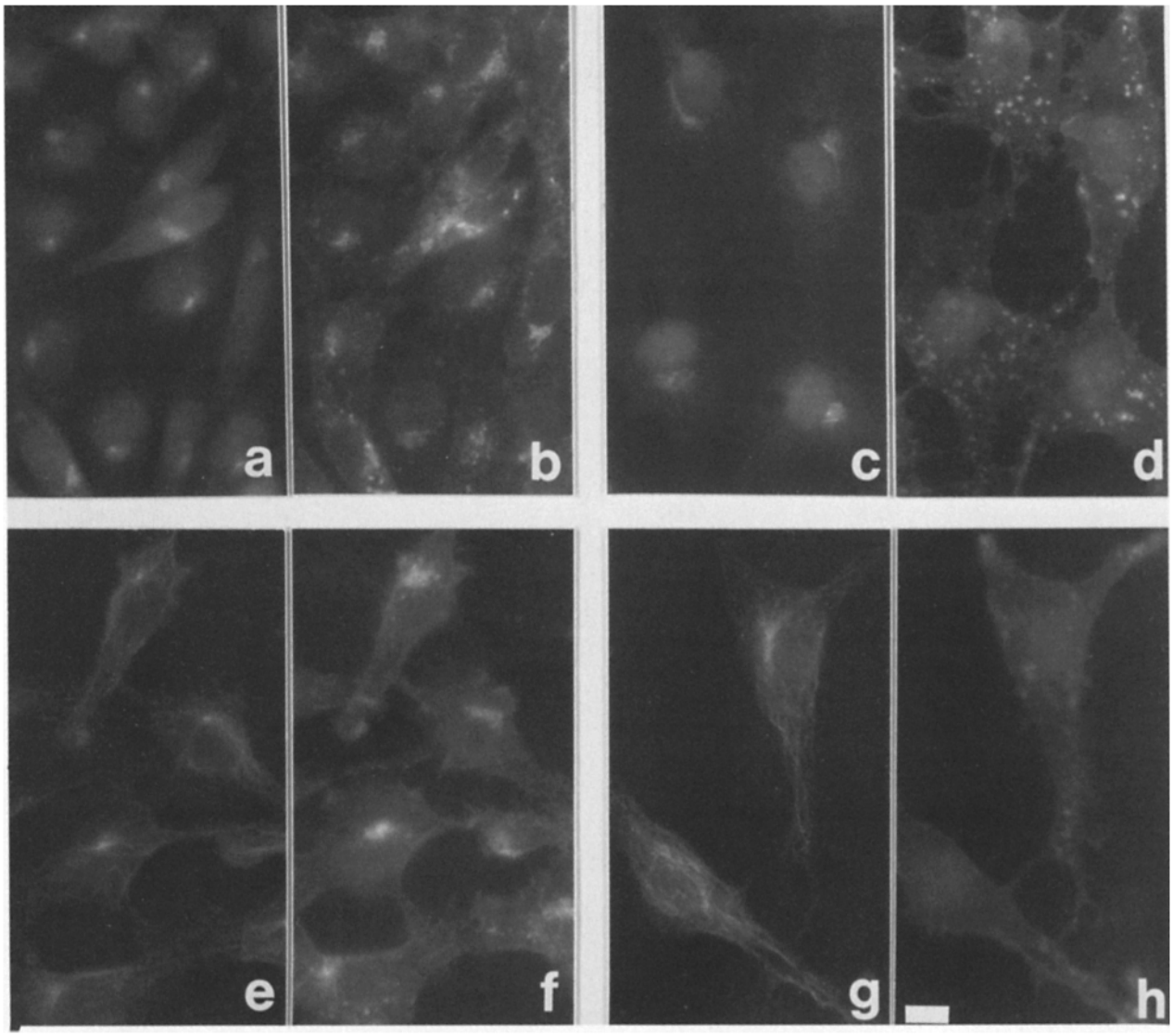

Figure 1. Subcellular localization of S100β and tubulin in digitonin-permeabilized rat C6 cells. Fluorescent micrographs of C6 cells which were permeabilized with 0.2 (*a*, *b*, *e*, and *f*) or 0.01% digitonin (*c*, *d*, *g*, and *h*) are shown. The permeabilized cells were labeled with an anti-S100β IgG fraction, followed by a FITC-conjugated goat anti-rabbit secondary antibody and TRITC-WGA (*a–d*) or with an antitubulin antibody followed by an FITC-conjugated rabbit anti-sheep secondary antibody and TRITC-WGA (*e–h*). Cells in *a*, *c*, *e*, and *g* were viewed by optics selective for fluorescein to visualize the distribution of S100β (*a* and *c*) or microtubules (*e* and *g*). The same fields were viewed with optics selective for rhodamine to visualize the distribution of WGA binding sites (*b*, *d*, *f*, and *h*). Bar, 100 μm.

These results along with the observation that S100β distribution in the perinuclear cytoplasm is dependent on an intact cytoskeleton suggest that S100β is associated with the MTOC.

Morphological Differentiation of C6 Cells

In previous studies (Fan and Uzman, 1977; Steinbach and Schubert, 1975) the degree of morphological differentiation of C6 cells has varied from laboratory to laboratory and may be a result of the different culture conditions (i.e., presence or absence of serum) used in the different laboratories. To determine whether serum affects morphological differentiation of C6 cells, we examined the effect of dcAMP on the morphology of C6 cells grown in serum-containing and serum-free media. Since previous studies in our laboratory (Zimmer and Van Eldik, 1988) have demonstrated that the levels of S100 and calmodulin vary with cell density, all of the cells used in the present study were harvested at ~80% confluency. In serum-containing media, C6 cells had a bipolar appearance (Fig. 3 *a*). C6 cells grown in serum-containing media supplemented with dcAMP (Fig. 3 *b*) and C6 cells adapted to growth in serum-free media (Fig. 3 *c*) had similar morphologies, appearing larger and more elongated than cells grown in serum-containing media. We refer to these cells as being partially differentiated although there is no direct evidence that these cells represent a true intermediate in the differentiation process.

When C6 cells that had been adapted to growth in serum-free media were treated with dcAMP (Fig. 3 *d*), the cell bodies became rounded and elaborated numerous, long pro-

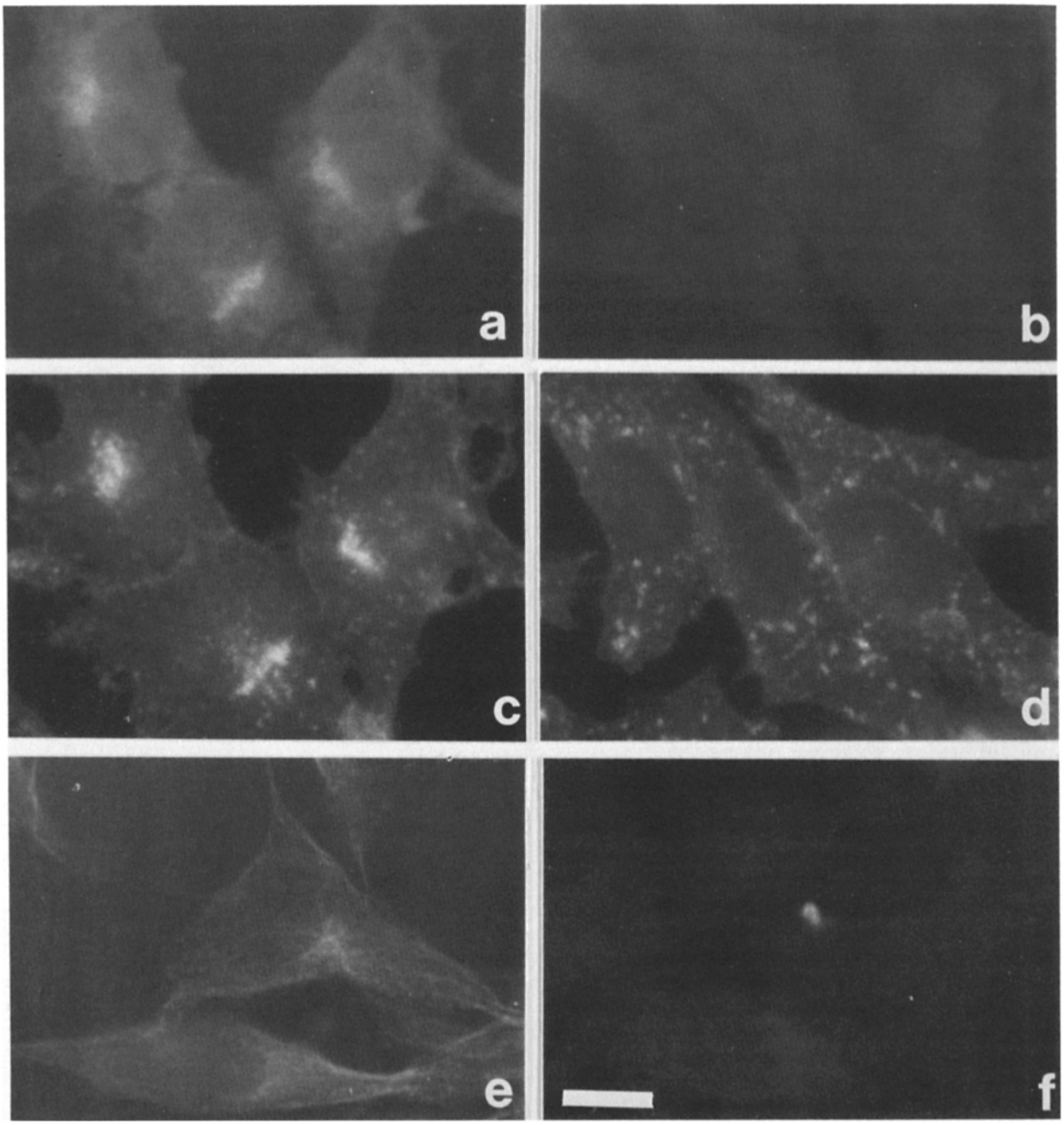

Figure 2. Subcellular localization of S100β in colchicine-treated rat C6 cells. Fluorescent micrographs of C6 cells that were incubated for 4 h in media containing 1 μg/ml colchicine (*b*, *d*, and *f*) or in media containing an equivalent volume of water (*a*, *c*, and *e*). Cells were permeabilized with cold acetone and labeled with an S100β IgG fraction, followed by an FITC-conjugated secondary antibody and TRITC-WGA (*a–d*), or with an antitubulin antibody (*e* and *f*) followed by an FITC-conjugated secondary antibody. Cells in *a*, *b*, *e*, and *f* were viewed by optics selective for fluorescein to visualize S100β (*a* and *b*) or tubulin (*e* and *f*) distribution. The fields shown in *a* and *b* were also viewed with optics selective for rhodamine to visualize the distribution of WGA binding sites (*c* and *d*). Bar, 10 μm.

cesses. The morphology of these cells resembled that of mature astrocytes. It should be noted that this cell population was heterogeneous with some cells being bipolar and others multipolar. In control experiments in which sodium butyrate was added to the media instead of dcAMP, no morphological changes were observed, suggesting that increased intracellular cAMP levels, and not butyrate, were responsible for the morphological changes observed (data not shown). Other cAMP analogs, including 8-bromo-cAMP, 8-(4-chlorophenylthio)-cAMP, N^6-benzoyl-cAMP, and N^6-monobutyryl-cAMP, also induced morphological changes identical to those seen with dcAMP, suggesting that increased intracellular cAMP levels were responsible for the morphological changes observed. Altogether our data demonstrate that dcAMP causes a morphological differentiation of C6 cells and that the effects of dcAMP on C6 cell morphology vary depending on the serum composition of the media.

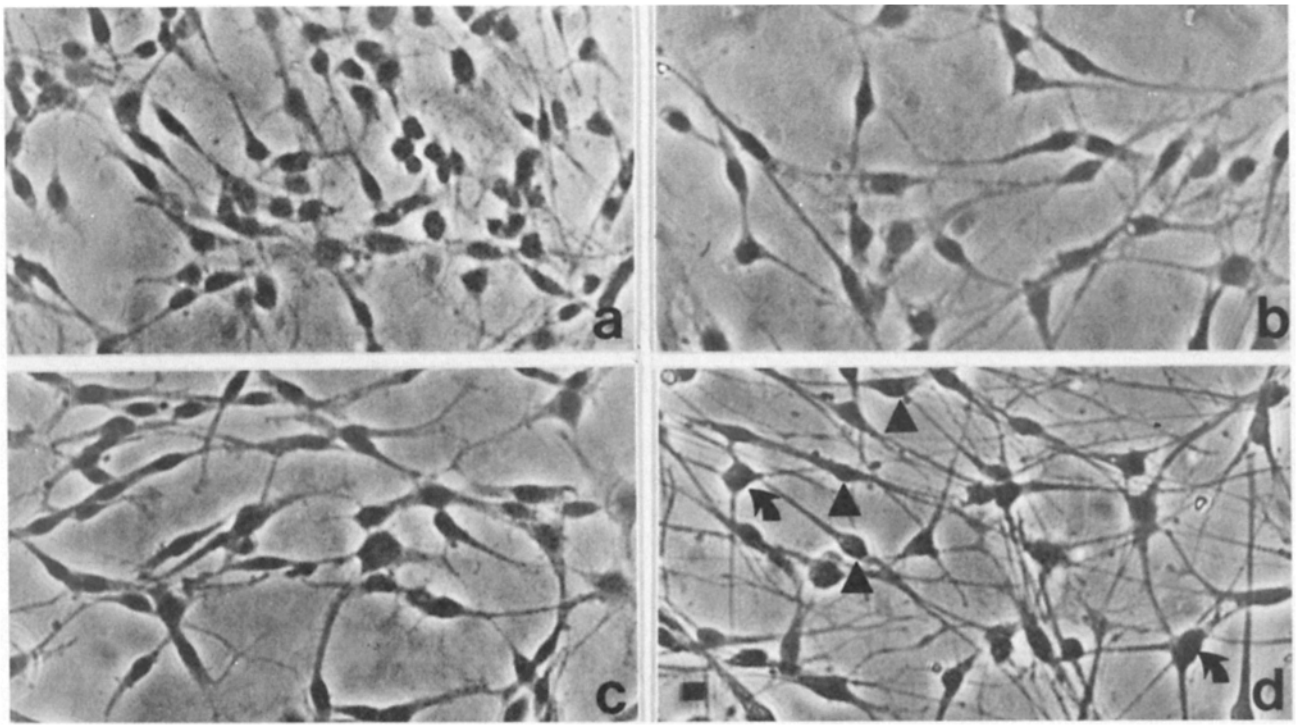

Figure 3. Morphological features of rat C6 cells grown under various culture conditions. Phase contrast micrographs of C6 cells grown in (*a*) serum-containing media, (*b*) serum-containing media supplemented with 1 mM dcAMP, (*c*) serum-free media, and (*d*) serum-free media supplemented with 1 mM dcAMP. Treatment of C6 cells grown in serum-free media with 1 mM dcAMP produced a heterogeneous population of cells which contained both bipolar (*arrowheads*) and multipolar (*arrows*) cells. Bar, 1 μm.

Intracellular Levels of S100α, S100β, and Calmodulin

The levels of S100α, S100β, and calmodulin in differentiated, partially differentiated, and undifferentiated C6 cells were determined by radioimmunoassay using rabbit polyclonal antisera. The S100α and S100β antisera used in these studies exhibited no reactivity with purified calmodulin and exhibited <5% cross-reactivity with S100β or S100α, respectively (Zimmer and Van Eldik, 1987). Similarly, the calmodulin antisera exhibited no reactivity with purified S100α or S100β (Van Eldik et al., 1983). The competition curves obtained for the various C6 fractions were parallel to those obtained with the purified S100 or calmodulin standards. In addition, the fractionation properties of S100 and calmodulin were identical in differentiated and undifferentiated cells.

As shown in Fig. 4, differentiated C6 cells contained fourfold more S100β than undifferentiated C6 cells (75.6 ± 8 fg/cell vs. 12.9 ± 2.5 fg/cell). Differentiated and undifferentiated C6 cells contained predominantly the S100β polypeptide. In fact the S100α levels were so low that it was not possible to determine if the S100α immunoreactivity detected was due to cross-reactivity of the S100β antibody or a very small amount of S100α present in the cells. The calmodulin levels were approximately threefold higher in differentiated (223.6 ± 10.9 fg/cell) compared with undifferentiated C6 cells (72.5 ± 5.5 fg/cell). In partially differentiated C6 cells—i.e., cells grown in serum-containing media supplemented with dcAMP or in serum-free media—S100β (28.8 ± 4.7 fg/cell) and calmodulin (145.8 ± 10.5) levels were approximately half the levels seen in fully differentiated cells. When cells were treated with sodium butyrate, instead of dcAMP, the S100β and calmodulin levels in C6 cells did not change (data not shown), in agreement with previous studies (Hirschfeld and Bressler, 1987). Thus, the levels of both S100β and calmodulin increase during morphological differentiation of C6 cells and appear to correlate with the degree of morphological differentiation.

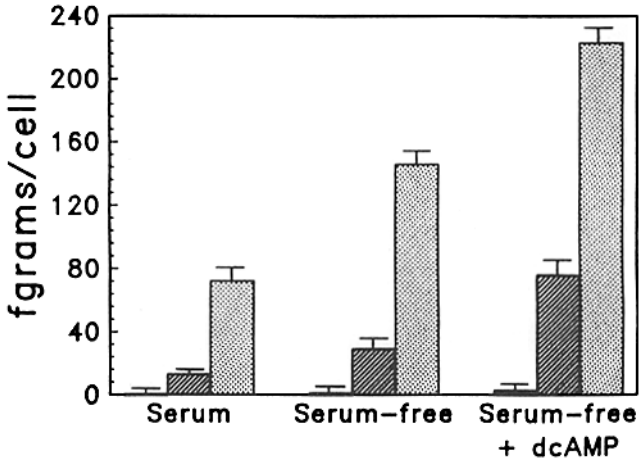

Figure 4. Immunoreactive levels of intracellular S100α, S100β, and calmodulin in rat C6 cells grown under various culture conditions. The levels of S100α (*solid bars*), S100β (*striped bars*), and calmodulin (*stippled bars*) were determined by radioimmunoassay. The mean fg of S100α, S100β, and calmodulin per cell ± the SEM was then calculated. The total number of determinations using three different preparations for each condition was 3.

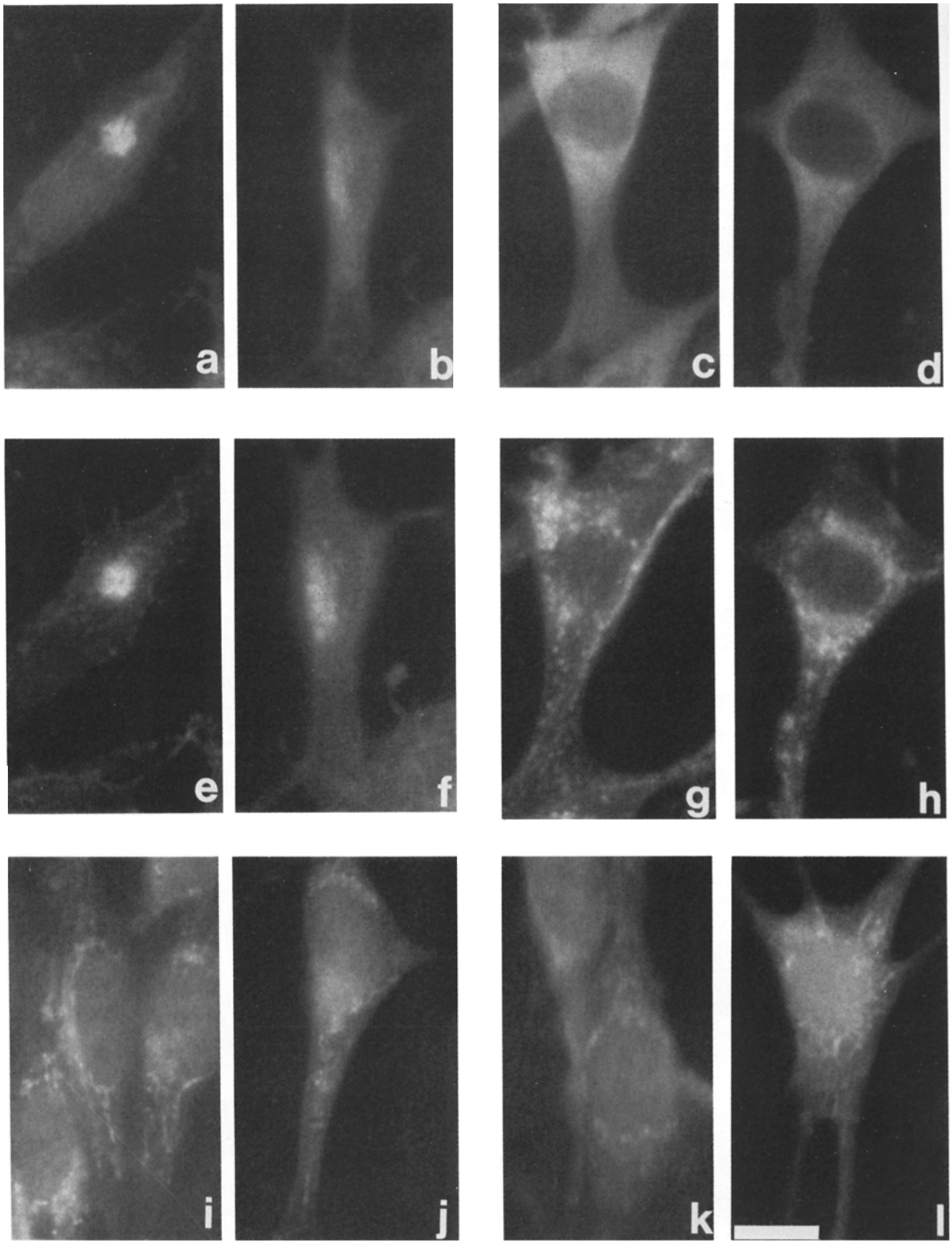

Figure 5. Subcellular distribution of S100β, TRITC-WGA, and calmodulin in rat C6 cells grown under various conditions. Fluorescent micrographs of C6 cells grown in serum-containing media (*a*, *e*, and *i*), serum-containing media supplemented with 1 mM dcAMP (*b*, *f*, and *j*), serum-free media (*c*, *g*, and *k*), and serum-free media supplemented with 1 mM dcAMP (*d*, *h*, and *l*) are shown. Cells were permeabilized with cold acetone and incubated in an antiS100β IgG fraction (*a–d*) or an anticalmodulin IgG fraction (*i–l*) followed by an FITC-conjugated goat anti-rabbit secondary antibody and TRITC-conjugated WGA. Cells in *a–d* and *i–l* were viewed with optics selective for fluorescein to visualize the distribution of S100β (*a–d*) or calmodulin (*i–l*). The same fields shown in *a–d* were viewed with optics selective for rhodamine to visualize the distribution of WGA binding sites (*e–h*). Bar, 1 μm.

Subcellular Distribution of S100β and Calmodulin in Differentiated C6 Cells

To determine whether the increased levels of S100β and calmodulin in differentiated C6 cells resulted in a redistribution of these proteins in the cytoplasm, the subcellular distributions of S100β and calmodulin in differentiated and undifferentiated C6 cells were compared using indirect immunofluorescence microscopy. In differentiated C6 cells, S100β staining throughout the cytoplasm was observed (Fig. 5 *d*) and the staining in the perinuclear cytoplasm appeared more intense than the staining in the nonperinuclear cytoplasm. This staining pattern was significantly different from the staining pattern seen in undifferentiated cells; i.e., intense staining in a small region of the perinuclear cytoplasm (Fig. 5 *a*). These data suggest that during C6 cell differentiation, S100β redistributes from a small region of the perinuclear cytoplasm into the entire cytoplasm with somewhat higher concentrations being found throughout the perinuclear cytoplasm. In contrast, the calmodulin staining pattern did not change during differentiation of C6 cells. In differentiated (Fig. 5 *l*), partially differentiated (Fig. 5, *j* and *k*), and undifferentiated (Fig. 5 *i*) cells, calmodulin exhibited a punctate staining throughout the cytoplasm.

While populations of partially differentiated C6 cells (those grown in serum-containing media supplemented with dcAMP or in serum-free media) had indistinguishable morphologies, these two populations of cells could be distinguished on the basis of S100β subcellular distribution. In C6 cells grown in serum-containing media supplemented with dcAMP (Fig. 5 *b*), the S100β staining pattern was similar to that seen in undifferentiated C6 cells; i.e., staining in a small region of the perinuclear cytoplasm. In contrast, cells grown in serum-free media (Fig. 5 *c*) exhibited an S100β staining pattern that was similar to that seen in differentiated cells; i.e., a diffuse staining pattern throughout the perinuclear cytoplasm and a less intense diffuse staining throughout the nonperinuclear cytoplasm. The redistribution of S100β during differentiation is accompanied by changes in the subcellular distribution of the Golgi apparatus as determined by TRITC-WGA staining. In differentiated C6 cells (Fig. 5 *h*) TRITC-WGA labeling is located in a vesicular pattern throughout the perinuclear cytoplasm rather than the small region of the perinuclear cytoplasm seen in undifferentiated cells (Fig. 5 *e*). The two populations of partially differentiated cells could also be distinguished on the basis of TRITC-WGA staining patterns. C6 cells grown in serum-containing media supplemented with dcAMP (Fig. 5 *f*) exhibited a TRITC-WGA staining pattern, which was very similar to that seen in undifferentiated cells (Fig. 5 *e*), while those grown in serum-free media (Fig. 5 *g*) had a TRITC-WGA staining pattern, which was identical to that seen in differentiated cells (Fig. 5 *h*). Thus, changes in the subcellular distribution of the Golgi membranes occur during in vitro differentiation of C6 cells but do not correlate with the degree of morphological differentiation.

S100 and Calmodulin Target Proteins in C6 Cells

Calcium-modulated proteins such as calmodulin and S100 can interact with and modulate the activity of other proteins. Thus, calcium-dependent cellular processes may be regulated by changes in these target proteins instead of or in addition to changes in the levels and/or subcellular distribution of the calcium-modulated proteins. Therefore, a gel overlay procedure was used to examine S100β- and calmodulin-binding proteins in differentiated and undifferentiated C6 cells (Fig. 6). This is a qualitative procedure that has been used in previous studies of S100- and calmodulin-binding proteins (Burgess et al., 1984; Zimmer and Van Eldik, 1987) and allows rapid screening and detection of binding proteins in crude extracts.

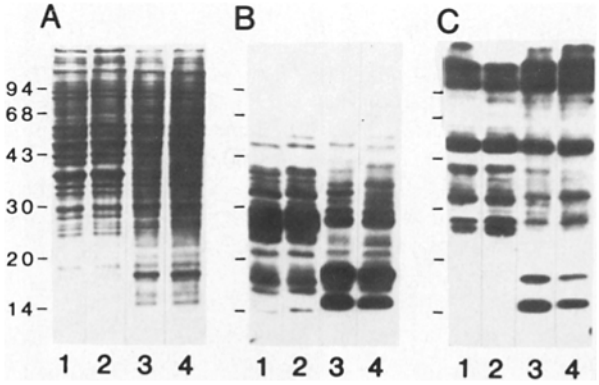

Figure 6. S100- and calmodulin-binding proteins in C6 cells grown under various culture conditions. The supernatant fractions of C6 cells grown in serum-containing media (lane *1*), serum-containing media supplemented with 1 mM dcAMP (lane *2*), serum-free media (lane *3*), or serum-free media supplemented with 1 mM dcAMP (lane *4*) were subjected to electrophoresis in 12.5% (wt/vol) acrylamide-SDS gels, and ^{125}I-S100β- and ^{125}I-calmodulin-binding proteins were detected by using the gel overlay technique. (*A*) Coomassie Blue–stained gel profile. Corresponding autoradiograms of gels incubated in (*B*) ^{125}I-S100β, or (*C*) ^{125}I-calmodulin in the presence of 0.1 mM calcium. Molecular weight standards are shown ($\times 10^{-3}$) on the left.

Comparison of the S100β-binding protein profile of undifferentiated (Fig. 6 *B*, lane *1*) and differentiated C6 cells (Fig. 6 *B*, lane *4*) demonstrated a reduction in the intensity of binding proteins in the 20,000–43,000 mol wt range and an increase in the intensity of binding proteins in the 14,000–20,000 mol wt range in differentiated cells. The calmodulin-binding proteins in the 43,000–150,000 mol wt range did not appear to change in intensity during differentiation (compare Fig. 6 *C*, lanes *1* and *4*). However, calmodulin-binding proteins in the 25,000–40,000 mol wt range decreased in intensity while those in the 14,000–17,000 mol wt range increased in intensity in differentiated cells. Overall, the S100β- and calmodulin-binding protein profiles were distinct, suggesting that S100β and calmodulin have different intracellular targets in C6 cells. However, in both differentiated and undifferentiated cells some S100- and calmodulin-binding proteins have similar apparent molecular weights, suggesting that S100β and calmodulin may have some common target proteins in C6 cells. Partially differentiated C6 cells (those grown in serum-containing media supplemented with dcAMP [Fig. 6, lanes *2*] or in serum-free media [Fig. 6, lanes *3*]) had different S100β- (Fig. 6 *B*, lanes *2* and *3*) and calmodulin-binding protein profiles (Fig. 6 *C*, lanes *2* and *3*) even though these cells have similar morphologies. Thus, major changes in S100β- and calmodulin-binding protein profiles occur during differentiation of C6 cells but do not correlate with the degree of morphological differentiation.

Aldolase Hybrids in C6 Cells

While the gel overlay technique is useful for screening heterogeneous protein fractions for S100-binding activity, it does not allow one to detect changes in the isozymic composition of a binding protein. The effect of S100 on one S100-binding protein, fructose-1,6-bisphosphate aldolase, varies with each isozyme: the C, or brain-specific, isozyme shows calcium-dependent stimulation by S100 while the A isozyme shows calcium-independent stimulation by S100 (Zimmer and Van Eldik, 1986). In addition, previous studies have suggested that the isozymic composition of aldolase in glial cells may be developmentally regulated (Sato et al., 1972; Kumanishi et al., 1985). To determine whether the aldolase isozymes changed during C6 cell differentiation, we used a nondenaturing gel system to examine the aldolase isozyme composition of C6 cells at various stages of differentiation. Native aldolase is a tetramer, and in cells and tissues such as muscle, which contain only the A isozyme, a single aldolase hybrid (A_4) is seen on nondenaturing gels (Fig. 7, lane *1*). In contrast, in tissues such as brain, which contain both the A and C aldolase isozymes, five aldolase hybrids (A_4, A_3C_1, A_2C_2, A_1C_3, and C_4) are visible on nondenaturing gels (Fig. 7, lane *2*). Undifferentiated C6 cells contain only the A isozyme as evidenced by the presence of only the A_4 aldolase hybrid (Fig. 7, lane *3*). However, differentiated C6 cells contain both the A and C isozymes as evidenced by the presence of aldolase hybrids which contain C subunits (predominantly the A_2C_2, A_1C_3, and C_4 hybrids) (Fig. 7, lane *6*). Examination of the aldolase isozyme composition in partially differentiated cells revealed that, while these cells had indistinguishable morphologies (cells grown in serum-containing media supplemented with dcAMP (Fig, 7, lane *4*) or in serum-free media (Fig. 7, lane *5*), they had distinct aldolase isozyme patterns. Cells grown in serum-containing media containing dcAMP contained only the A aldolase isozyme as evidenced by the presence of only the A_4 aldolase hybrid on nondenaturing gels. In contrast, cells grown in serum-free media contained both the A and C aldolase isozymes as evidenced by the presence of aldolase hybrids on nondenaturing gels that contain the C isozyme (predominantly the A_3C_1, A_2C_2, and A_1C_3 hybrids). Thus, our data suggest that there is an increase in the level of the C aldolase isozyme in differentiated C6 cells, and this increase does not correlate with the degree of morphological differentiation.

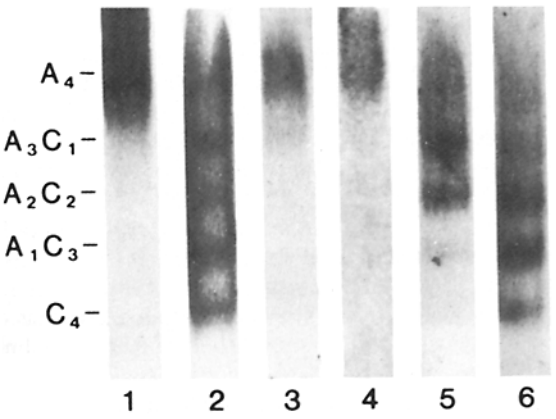

Figure 7. Aldolase hybrids in C6 cells grown under various culture conditions. An aldolase-enriched fraction (45–60% ammonium sulfate fraction) from C6 cells grown in serum-containing media (lane *3*), serum-containing media supplemented with 1 mM dcAMP (lane *4*), serum-free media (lane *5*), and serum-free media supplemented with 1 mM dcAMP (lane *6*) were subjected to electrophoresis on 5% nondenaturing polyacrylamide gels. Commercially prepared muscle aldolase and purified rat brain aldolase are shown in lanes *1* and *2*, respectively. Aldolase hybrids were visualized by using an activity stain.

Discussion

We have examined the level, subcellular distribution, and target proteins of the calcium-modulated proteins S100β and calmodulin in undifferentiated, partially differentiated, and differentiated glial cells, and these results are summarized in Table I. The levels, subcellular distribution, and binding-protein profiles for S100β were distinct in differentiated and undifferentiated cells, suggesting that S100β mediates new cellular processes in differentiated cells. While the levels and binding protein profiles for calmodulin were also distinct in differentiated and undifferentiated cells, the subcellular distribution of calmodulin was not altered. When partially differentiated populations of C6 cells were examined, we found that some of these parameters correlated with the degree of morphological differentiation while others did not (see Table I). Thus, the in vitro differentiation of rat glioma cells is a complex event in which multiple mechanisms appear to regulate the function of calmodulin and S100β.

Our results regarding S100 levels in C6 cells at various stages of differentiation agree with previous studies using in vitro and in vivo systems to analyze glial cell function. Other investigators have observed a 2–2.5-fold increase in S100 levels in C6 cells grown in serum-containing media supplemented with dcAMP (Labourdette and Mandel, 1980; Higashida et al., 1985). Increases in S100 levels in C6 cells grown in serum-free media have also been reported. However, we observed a 200% increase compared with the 50% increase seen by others (Fan and Uzman, 1977). This difference may reflect different compositions of the serum-free media. There are no previous reports regarding S100 levels in C6 cells grown in serum-free media supplemented with dcAMP. While these previous studies did not determine which S100 polypeptide(s) was responsible for the increased S100 levels, our study demonstrates that increased levels of S100 of rat glioma cells are due to increased levels of the S100β polypeptide. This is in agreement with in vivo studies that have shown that only the S100β polypeptide is expressed in rat brain (Baudier et al., 1985; Kuwano et al., 1986). Our results are also consistent with developmental studies of rat that have demonstrated increased S100 levels in brain during development that correlate with differentiation of glial cells (Cicero et al., 1972; Ghandour et al., 1981; Kuwano et al., 1987).

The different binding protein profiles and subcellular distributions of S100β and calmodulin suggest that these proteins modulate the activity of different intracellular proteins and thus have distinct functions in glial cells. However, there are some areas of the cell cytoplasm that contain both S100β and calmodulin immunoreactivity and some S100β- and calmodulin-binding proteins that have similar molecular weights on SDS gels suggesting that S100 and calmodulin may share certain intracellular targets. An apparent increase

Table I. Properties of C6 Cells under Various Culture Conditions

Culture conditions	Morphology	Protein levels*		Distribution		Target proteins		Aldolase
		S100β	Calmodulin	S100β	Calmodulin	S100β‡	Calmodulin‡	
Serum	Bipolar	1	1	Intense staining small region perinuclear cytoplasm	Punctate staining throughout cytoplasm	20,000–43,000 14,000–20,000	43,000–150,000 25,000–40,000 14,000–17,000	A
Serum + dcAMP	Larger, elongated	2	1.5	Intense staining small region perinuclear cytoplasm	Punctate staining throughout cytoplasm	20,000–43,000 14,000–20,000	43,000–150,000 25,000–40,000 14,000–17,000	A
Serum free	Larger, elongated	2	1.5	Diffuse staining throughout cytoplasm	Punctate staining throughout cytoplasm	20,000–43,000↓ 14,000–20,000↑	43,000–150,000 25,000–40,000↓ 14,000–17,000↑	A and C
Serum free + dcAMP	Astrocyte-like	4	3	Diffuse staining throughout cytoplasm	Punctate staining throughout cytoplasm	20,000–43,000↓ 14,000–20,000↑	43,000–150,000 25,000–40,000↓ 14,000–17,000↑	A and C

* Normalized to values seen with serum.
‡ Increases in intensity are indicated by ↑ and decreases by ↓.

in the C isozymic form of aldolase, an S100-binding protein, occurs during glial cell differentiation. These results are consistent with in vivo studies which have demonstrated that aldolase C is found in glial cells in vivo (Thompson et al., 1982) and with in vitro studies which suggest that the level of aldolase C correlates with the degree of glioma cell differentiation (Kumanshi et al., 1975).

Our study also provides new information regarding the association of S100β with specific intracellular structures. Using double fluorescent labeling techniques, we have been able to demonstrate that S100β immunoreactivity colocalizes with the Golgi apparatus and MTOC in undifferentiated C6 cells and that this localization is dependent on an intact microtubule complex. Selective permeabilization techniques suggest that significant levels of S100β are present on the cytoplasmic surface and not on the inside of the Golgi apparatus. Altogether these data suggest that S100β is closely associated with the MTOC in undifferentiated C6 cells. The significant change in the subcellular distribution of S100β in differentiated cells to include the entire cytoplasm suggests that S100β mediates different processes in differentiated glial cells.

The association of S100β with the MTOC suggests that one function of S100 in glial cells may be the regulation of cell architecture and/or motility via interaction of S100 with microtubules or microtubule-associated proteins. Previous immunolocalization studies on mitotic C6 cells have demonstrated that S100β colocalizes with the mitotic spindle (Zimmer and Van Eldik, 1988), suggesting that S100 may have a similar function in mitotic cells. Studies from other laboratories (Donato, 1986, 1988) have demonstrated that S100 can affect microtubule assembly in vitro supporting the hypothesis that S100 may regulate cell shape and/or motility. The molecular mechanisms of S100 regulation of microtubule assembly in vivo are unknown. However, studies by Baudier et al. (1987, 1988) have demonstrated that S100 interacts in vitro, in a calcium-dependent manner, with τ proteins. These previous studies suggest that S100 may act via a Ca^{+2}-S100-τ complex, analogous to that proposed for calmodulin (Lee and Wolff, 1984). More information regarding the exact nature of the association of S100 with the MTOC and spindle apparatus, and S100 target proteins associated with the MTOC and spindle will be necessary before the role of S100 in regulating cell architecture and motility can be ascertained.

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