Selective distribution of protein kinase A regulatory subunit RII α in rodent gliomas

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Differential diagnosis of brain tumor types is mainly based on cell morphology and could benefit from additional markers. The cAMP second-messenger system is involved in regulating cell proliferation and differentiation and is conceivably modulated during cancer transformation. The cAMP second-messenger system mainly activates protein kinases, which are in part docked to cytoskeleton, membranes, or organelles by anchoring proteins, forming protein aggregates that are detergent insoluble and not freely diffusible and that are characteristic for each cell type. The intracellular distribution of the detergent-insoluble regulatory subunits (R) of the cAMP-dependent protein kinase has been examined in mouse and rat glioma cells both in vitro and in vivo by immunohistochemistry. In normal rodent brains, the RII α regulatory subunit is detergent insoluble only in ependymal cells, while in the rest of the brain it is present in soluble form. Immunohistochemistry shows that in both mouse and rat glioma cell lines, RII α is mainly detergent insoluble. RII α is localized close to the nucleus, associated with smooth vesicles in the trans-Golgi network area. Both paclitaxel and vinblastine cause a redistribution of RII α within the cell. Under conditions that increased intracellular cAMP, apoptosis of glioma cells was observed, and it was accompanied by RII α redistribution. Also in vivo, detergent-insoluble RII α can be observed in mouse and rat gliomas, where it delineates the border between normal brain tissue and glioma.

Therefore, intracellular distribution of detergent-insoluble RII α can assist in detecting tumor cells within the brain, thus making the histologic diagnosis of brain tumors more accurate, and may represent an additional target for therapy. *Neuro-Oncology* 10, 958–967, 2008 (Posted to *Neuro-Oncology* [serial online], Doc. D07-00266, August 14, 2008. URL <http://neuro-oncology.dukejournals.org>; DOI: 10.1215/15228517-2008-054)

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Differential diagnosis of brain tumors requires careful histological examination. Up until now, immunohistochemical markers and molecular biology techniques have been used, but the variable and often elusive expression of different markers by the most malignant tumors has hampered the use of a standardized diagnostic method.

Prognosis depends on malignancy grade and is very poor for higher grade gliomas;¹ therefore, additional markers that improve the detection of malignant cells could lead to more accurate identification of these cells and easier tumor classification.

The major focus of research in oncogenesis is on alterations of oncogenes or oncosuppressors. The subsequent dysregulation of different signal transduction pathways may be involved in the pathogenesis of many tumors or in regulating tumor growth.

The cAMP second-messenger pathway is present in all eukaryotic cells, where it controls a large variety of vital cellular functions, ranging from ion channel permeability to gene expression. Observations suggest that the cAMP system is involved in regulating differentiation and proliferation of many normal and transformed cell

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types.^{2,3} cAMP analogues are known to inhibit proliferation and to induce differentiation of neuroblastoma and glioma cells in culture.⁴⁻⁶ It is noteworthy that in some cases cAMP has opposite effects on different cell types; for example, in PC12 cells and Sertoli cells, it stimulates cell growth,^{7,8} but in NIH3T3 cells and adipocytes, it inhibits growth.^{9,10}

cAMP acts mainly through cAMP-dependent protein kinases (PKA), consisting of two regulatory subunits which, upon binding two cAMP molecules, release two catalytic subunits. The catalytic subunits phosphorylate many target proteins, thus modifying their biological activity. Four different isoforms of regulatory subunits have been characterized (RI α , RI β , RII α , and RII β), each one forming homodimers that bind the catalytic subunits.

Apparently, the four regulatory isoforms have similar biochemical characteristics, and, in the absence of cAMP, their only function seems to be the inhibition of catalytic subunits. In contrast, it is known that PKA regulatory isoforms are differentially expressed in many tissues, with RI β and RII β being restricted mainly to the nervous system and some endocrine glands.¹¹ Moreover, regulatory isoforms may be either soluble or selectively bound to subcellular structures (e.g., cytoskeleton, Golgi apparatus) through a large family of isoform-specific anchoring proteins, A-kinase anchor proteins (AKAPs).¹² In this way, a different distribution of PKA in subcellular microdomains can be obtained, depending on the AKAPs and regulatory subunits expressed in a cell.

In cancer cells, the expression of cAMP-dependent protein kinases has been investigated, with different and apparently partially conflicting results.

RI α loss of heterozygosity is associated with endocrine tumors in the Carney complex: the decrease in RI α makes more PKA catalytic subunits available, enhancing PKA phosphorylating activity, ultimately leading to tumorigenesis. This finding suggests RI α as a candidate tumor-suppressor gene.^{13,14}

In contrast, in other human cancers, an increase in RI α correlates with more aggressive behavior and worse prognosis, whereas decreased expression of RI α correlates with decreased tumor cell growth.¹⁵⁻¹⁷ In prostate carcinoma cells, increase in RII β expression leads to inhibition of tumor growth, whereas increase in RI α has opposite effects,¹⁵ suggesting different involvement of the PKA regulatory subunits in different cancers. In addition, the development of Ewing sarcoma requires the involvement of cAMP response element binding (CREB) proteins, pointing to a cAMP-mediated pathway.¹⁸

In the most aggressive glioma, glioblastoma multiforme, protein kinase C is involved in tumor growth.¹⁹ Activation of the cAMP pathway, especially through activation of the RII regulatory subunits, causes differentiation and apoptosis of glioma cells.²⁰ AKAP1 was recently found to be upregulated in human glioblastoma specimens, as was phosphodiesterase 1A, a cAMP-degradating enzyme,²¹ and extracellular PKA catalytic subunit has been used as a cancer marker.²²

Our laboratory described a differential distribution in rodent brain of the four PKA regulatory subunits

bound to cytoskeleton and membranes and organelles. Docking at particular cell sites, this detergent-insoluble PKA fraction provides a large amount of kinases readily available in the proximity of target proteins, thus allowing more efficient tuning of intracellular signaling. Different brain-cell types can be characterized by PKA intracellular distribution. Detergent-insoluble RII β is present in most neural and glial cells,²³ whereas insoluble RI α is neuronal and restricted to some brain nuclei.²⁴ Insoluble RI β is present only in some neuron types, mainly in olfactory-bulb mitral cells and cerebellar Purkinje cells,²⁵ and insoluble RII α is localized only on the ependymal cells lining the ventricles.²³ Each subunit appears at specific times during development,²³ suggesting that a different balance between regulatory isoform expression and intracellular localization can be responsible for different properties of the same cell during the normal developmental process. Therefore, it is possible that a different picture emerges when cells have been transformed, acquiring a cancer cell phenotype.

In this article, the distribution of the detergent-insoluble PKA regulatory isoforms in glioma cells is reported, along with the modulation of this distribution by different agents. Two models were investigated: GL261 mouse glioblastoma cells and rat F98 glioma cells.²⁶⁻²⁸ The cells were analyzed *in vitro* and *in vivo* after inoculation in the brain of syngenic hosts.

Materials and Methods

Reagents

Cell culture reagents were from Gibco (Milan, Italy). All other chemicals were from Sigma (Milan, Italy), unless otherwise stated.

Cell Culture and Pharmacological Treatments

Mouse GL261 glioblastoma cells, rat F98 glioma, and human Gli36 glioblastoma cells were grown and maintained in monolayer in Dulbecco modified Eagle's medium (DMEM)/Ham's F12 (for GL261 cells) or DMEM (for F98) with streptomycin/penicillin (100 U/ml), 10% fetal bovine serum, at 37°C, 5% CO₂. Primary glial cell cultures were obtained from telencephalon of 3-day-old mice and maintained in DMEM supplemented with antibiotics and 20% fetal bovine serum.

For pharmacological treatments, GL261 cells were plated on six-well plates. After 48 h, one of the following substances was added to the culture, maintained in DMEM/F12: 500 μ M glutamate (as an apoptosis-inducing drug); two clinically used antineoplastic agents, 100 nM paclitaxel, 200 nM vinblastine; a series of chemicals affecting the intracellular cAMP pathway, 10 μ M forskolin, 500 μ M isobutylmethylxanthine (IBMX), 100 μ M teophyllin, 2 mM caffeine, 500 μ M 8-bromo-cAMP, 500 μ M 6-dibutyl-cAMP, and 12 μ M H89.

Cells were incubated for 24 h (72 h for paclitaxel treatment), and they were then scraped and cytocentrifuged 10 min at 6,000 rpm on microscope slides, fixed

with methanol, and stained with Weigert's hematoxylin. A total of 600 cells was counted from each slide, and the percentage of apoptotic cells was calculated. Apoptosis was determined by morphological criteria as a reduction in cell volume and condensation of chromatin or cell-membrane blebbing. At least six control and six treated slides were counted for each treatment. Monovariate analysis of variance was used to test the difference in the percentages of apoptotic cells between control and treated cells.

In Vivo Studies

In vivo studies were approved by the Italian Ministry of Health and conducted according to the European laws on animal experiments and welfare. A total of 8×10^4 GL261 cells was resuspended in 2 μ l DMEM/F12 and injected over 5 min in the caudate nucleus of the syngenic C57/Bl6J mice (1 mm anterior and 1.3 mm lateral to the bregma, 2.5 mm below the pial surface), anesthetized with ketamine (75 mg/kg) and xilazine (20 mg/kg). A total of 8×10^6 F98 cells was resuspended in 8 μ l DMEM and injected 1 mm anterior and 1.5 mm lateral to the bregma, 3.7 mm below the pial surface, over 15 min in Fischer rats anesthetized as above. Animals were monitored daily for the appearance of neurological signs and for weight loss. At the appearance of neurological signs, the animals were sacrificed with overdose anesthesia (three times the surgical dose). Brains were immediately excised and frozen in liquid nitrogen.

Western Blots

Cells were scraped and resuspended in 40 volumes of phosphate-buffered saline (PBS; 10 mM phosphate, 150 mM NaCl, 1 mM EDTA, Triton X-100 2%, pH 7.6) with protease inhibitor cocktail (Roche, Milan, Italy) and forced 30 times through a 27-gauge needle. They were then centrifuged 15 min at 6,000 rpm. The supernatant was collected and the pellet was washed again with the same volume of PBS and centrifuged as above; the second supernatant was discarded and the pellet resuspended in the same volume of PBS. Pellets were run on a 12% polyacrilamide gel, then blotted on nitrocellulose membrane, blocked 60 min with 2% bovine serum albumin, incubated overnight with primary antibodies, RII α or RI α (both made in rabbit; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:5,000), then incubated for 4 h with horseradish peroxidase-conjugated antirabbit secondary antibody (Sigma; 1:10,000) and revealed through chemiluminescence (Amersham Advanced ECL, Milan, Italy).

Immunocytochemistry and Immunohistochemistry

Cells were grown on cover slips, washed in PBS, and frozen. Cells were thawed and air dried, incubated for 5 min in 2% Triton X-100 in PBS at 18°C and fixed in 5% formalin supplemented with 1% Triton X-100 for 1 min at 37°C.

Brains were cut on a cryostat. The 24- μ m slices were

air dried and postfixed using one of the following protocols: (a) 1 h in 5% formalin at 18°C, followed by 30 min in 2% Triton X-100 in PBS or (b) incubation in Triton X-100 2% in PBS at 18°C for 30 min, followed by fixation in 5% formalin and Triton X-100 2% at 37°C for 1 min. Primary antibodies were incubated overnight. The following antibodies were used: RII α (rabbit, Chemicon, Temecula, CA, USA), 1:80 on brain slices, 1:100 on cells; RII β (rabbit; Chemicon), 1:80 on brain slices, 1:100 on cells; RII α (rabbit; Santa Cruz Biotechnology), 1:200 on brain slices, 1:500 on cells; RI α (rabbit; Santa Cruz Biotechnology), 1:200 on slices, 1:500 on cells; vimentin, 1:80 on slices, 1:250 in cells (mouse; Sigma); glial fibrillary acidic protein (GFAP), 1:200 on slices, 1:500 on cells (mouse; Sigma); neurofilament 200, 1:200 on slices, 1:500 on cells (mouse; Sigma); neurofilament 68, 1:200 on slices, 1:500 on cells (mouse; Sigma); golgin 97 (mouse; Molecular Probes, Eugene, OR, USA), 1:40 on slices, 1:80 on cells. Secondary antibodies were incubated 30 min at 37°C and were used 1:200 on tissue, 1:500 on cells: anti-rabbit IgG Alexafluor 594 (Molecular Probes), antirabbit IgG fluorescein-conjugate (Sigma), antimouse IgG fluorescein-conjugate (Sigma), antimouse IgG Alexafluor 568 (Molecular Probes). Cell nuclei were counterstained with bisbenzimidazole (Sigma).

Positive and negative controls were always performed in each session and included control slides for positive immunolabeling; that is, mouse brain sections were incubated with each of the above antibodies, for which the pattern of labeling has already been described for the different areas;²³⁻²⁵ as negative controls, the cells and tumors were reacted omitting the primary antibody, and for controlling background staining, the cells and tumors were incubated with normal rabbit serum and normal mouse serum (data not shown).

For colocalization experiments, after immunohistochemistry, cells or tissue were incubated in the presence of 8-thioacetamidofluorescein-cAMP, which allows the visualization of RI α as reported elsewhere.²³⁻²⁵

After immunohistochemistry, sections were counterstained with hematoxylin and eosin to confirm tumor presence, localization, and extension. A Leica epifluorescence microscope (20 $\times$, 40 $\times$, 100 $\times$ objectives) was used for immunofluorescence and histology. Images were captured with the resident software at 782 $\times$ 582 pixels with a color digital camera, using the same parameters within each experiment.

Micrographs of differential interference contrast or fluorescence images were taken on golgin and RII α doubly-labeled cells, with an Olympus Camedia 5050 digital camera mounted on an Olympus BX51 microscope, a 60 $\times$ oil-immersion objective (1.25 numerical aperture). The following filter sets were selected for fluorescence microscopy: 480-nm excitation filter, 500-nm dichroic mirror, and 515-nm bandpass filter for green fluorochromes, or 540-nm excitation filter, 580 nm dichroic mirror, and 620 nm barrier filter for red fluorochromes. Contrast was enhanced by maximum 10% when necessary, with Corel Photo Paint; final figures were prepared and lettered using Corel Draw 12 (Corel Corporation, Ottawa, Canada).

Electron Microscopy

For immunoelectron microscopy, GL261 cells were fixed in 4% paraformaldehyde and embedded in LR White resin (Electron Microscopy Sciences, Hatfield, PA, USA) as previously described.²⁹ Ultrathin sections were processed for immunocytochemistry by using a rabbit anti-RII α antibody (Santa Cruz Biotechnology; dilution 1:20) revealed by a secondary 12-nm gold-conjugated antibody (Jackson ImmunoResearch, West Grove, PA, USA).²⁹ Double labeling was performed after golgin 97 (1:20) and RII α (1:20) antibody incubation with 6-nm and 12-nm gold-conjugated antibodies (Jackson ImmunoResearch). As controls, some grids were treated with the incubation mixture without the primary antibody. The specimens were observed in a Philips Morgagni transmission electron microscope equipped with a Megaview III camera for digital image acquisition.

Results

Antibody Specificity and PKA Regulatory Subunit Identification

Different antibodies were tested in cells and tissues. The anti-RII α antibody from Santa Cruz Biotechnology provided very bright histochemical images in glioma cells, which were almost identical to the fainter images provided by anti-RII α from Chemicon. In Western blots of normal brain extracts, the Santa Cruz anti-RII α antibody recognized both RII α and β isoforms. Labeling was not affected by preincubation with 1 μ M RI α . In Western blots of cell and normal brain extracts, anti-RII α and RII β antibodies (Chemicon) recognized only one single band of a slightly different molecular weight. Although concurrent incubation with RI α had no effect, excess RII α abolished RII α labeling without affecting RII β labeling. Similar results were obtained from immunohistochemistry of the brain sections.

Biochemical fractionation of GL261 glioma cells and primary glial culture in supernatant and in a detergent-insoluble pellet showed that both RI and RII regulatory subunits were present in the pellet (Fig. 1A).

Fig. 1. RII α distribution in mouse and rat glioma. (A) Western blot of mouse primary glial cell and GL261 cells. Lanes 1–4 are probed against RI, lanes 5–8 against RII. Both proteins are present in Triton-insoluble pellets. M, molecular weight markers: 1 and 5, soluble fraction from glial cell primary culture; 2 and 6, insoluble fraction from glial cell primary culture; 3 and 7, GL261 soluble fraction; 4 and 8, GL261 insoluble fraction. (B) Percentage of apoptosis (+ SEM) in GL261 cells treated with various agents. All treated cells present a statistically significant increase in apoptosis (analysis of variance, $p < 0.05$), compared to control cells. (C) In a GL261 cell, RII α immunostaining (green) appears as a cluster of small dots on one side of the cell nucleus (blue). (D) RII α (green) localization in GL261 cells is not coincident with the intermediate filament vimentin (red). Scale bar, 10 μ m for C and D. IBMX, isobutylmethylxanthine; 8Br-cAMP, 8-bromo-cAMP; DB-cAMP, 6-dibutyl-cAMP.

Induction of Apoptosis

GL261 cells were treated with drugs that either increase the concentration of cAMP or inhibit the catalytic activity of the cAMP-dependent kinases to reveal the possible effect of cAMP modification on cell survival. A statistically significant increase in apoptosis was observed, similar to that elicited by two well-known drugs in clinical use, paclitaxel and vinblastine, as already reported for other cancer cell lines (Fig. 1B).

RII α Intracellular Localization

In both GL261 and F98 cells, only RII α insoluble aggregates were detected by immunocytochemistry under different fixation protocols, using the different anti-RII α antibodies. Under the same conditions, RI α aggregates were not detected, even though RI α was present in Western blots, presumably because of a more distributed localization that hindered immunohistochemical detection above background.

RII α immunolabeling (green; Fig. 1C) was present close to the nucleus (blue), in a restricted region on one side, and apparently was organized as a cluster of many small dots. All the cells were labeled, although with different intensities. A small percentage of cells appeared elongated, with large processes, indicating a possible differentiation of the cell: in these cells, RII α labeling

appeared fainter, although structured in the same way inside the cell.

RII α appeared to be clearly separated from vimentin-labeled filaments (Fig. 1D), suggesting that RII α is not associated with this intermediate filament protein. When cells were labeled with anti-golgin 97, a trans-Golgi network marker, RII α was associated in the same area, although it did not exactly overlap (Fig. 2A). This localization was confirmed by immunoelectron microscopy, which showed that RII α was associated with smooth vesicles (Fig. 2B), which were also labeled with golgin (Fig. 2C).

GFAP (green), a marker for mature astroglia in the normal brain, had a filamentous appearance in glioma cells (Fig. 3A). Usually, cells that were strongly labeled with GFAP, and hence that were more differentiated, showed a fainter RII α labeling, and vice versa. Under some conditions, changes in the pattern of RII α labeling were detected. Treatment with forskolin, an activator of adenylate cyclase, results in an increase in cAMP, triggering differentiation,²⁰ so that the cells acquire an elongated appearance, with many well-developed processes. After forskolin treatment, in many cells RII α aggregates were not exclusively confined close to the nucleus (Fig. 3B: RII α , red and GFAP, green).

In cells treated with H89, an inhibitor of the PKA catalytic activity, GFAP labeling was present only in a few cells. RII α labeling was more dispersed than in control

Fig. 2. RII α and golgin distribution in rat glioma cells. (A) Detail of an F98 cell doubly immunolabeled for RII α and golgin: differential interference contrast image of cells (far left), RII α (green) and golgin (red) staining, and the superimposed image (far right). The two signals (yellow) are present in the same subcellular compartment. Scale bar, 10 μ m. (B) Transmission electron micrograph of a GL261 cell, immunolabeled with anti-RII α antibody. The gold grains are mostly distributed at the periphery of Golgi vesicles (v), whereas the nucleus (N) and mitochondria (m) are devoid of signal. Scale bar, 0.5 μ m. (C) Transmission electron micrograph of a smooth vesicle in a GL261 cell, doubly immunolabeled for RII (larger gold grains, black arrow) and golgin (smaller grains, open arrow). The vesicle appears to be labeled by both antibodies.

Fig. 3. RII α distribution in GL261 cells. (A) In untreated cells, RII α (red) appears localized on one side of the cell and does not overlap with the astroglia-specific intermediate filament glial fibrillary acidic protein (GFAP; green). (B) GL261 cells, after treatment with the diterpene activator of adenylate cyclase, forskolin, which stimulates cAMP production. RII α (red) is apparently more loosely distributed around the nuclei. GFAP (green) strongly stains cellular processes. (C) GL261 cells treated with H89, an inhibitor of cAMP-dependent protein kinase catalytic activity. RII α (red) appears scattered around the nuclei, in particular in cells which lack GFAP expression. If present, the GFAP (green) filaments are not clearly defined. (D) GL261 cells after paclitaxel treatment. RII α (red) is hardly detectable around cell nuclei, and vimentin (green) lacks the filamentous appearance in the few cells that are surviving. Scale bar, 10 μ m for all panels.

cells but could be observed around the nucleus (Fig. 3C). After treatment with paclitaxel, a microtubule stabilizer, a few cells survived. In these cells, RII α labeling (Fig. 3D, red) was uniformly distributed around the nucleus, whereas vimentin labeling (green) was weaker than in controls, and filaments could no longer be recognized.

Vinblastine, which inhibits functional microtubule assembly, induced a dramatic redistribution of RII α , which appeared scattered through the cell cytoplasm (Fig. 4A). Double labeling (Fig. 4B) showed that although RII α redistributed, vimentin did not seem to be affected by vinblastine treatment.

In Vivo Immunohistochemistry

Hematoxylin and eosin staining of a mouse brain after GL261 tumor growth shows the boundary between glioma and brain parenchyma (Fig. 4C). Similarly, RII α labeling (Fig. 4D, red) also highlighted the border between the brain and the tumor; after fixation at room temperature and subsequent permeabilization, RII α appeared restricted to the tumor area. Cells were densely packed and small in size; therefore, in contrast to *in vitro* observations, RII α labeling appeared more restricted to a small labeled area in a perinuclear posi-

tion, present only in the cells within the tumor. At higher magnification (Fig. 5A), mouse tumor labeling consisted of densely packed dots, similar to *in vitro* labeling. Similar results were observed in a rat glioma cell line (F98) and in human glioblastoma cells (Gli36), confirming that RII α peculiar distribution can be a characteristic of glioma cell types.

In F98 cell culture, RII α (Fig. 5B, red) appeared as a cluster of dots restricted to one side of the nucleus (blue). RII α labeling of the glioma in the rat brain resembled that in the mouse, being located on one side of the cell nucleus (Fig. 5C, red). As in the mouse, at lower magnification (Fig. 5D), RII α distribution appeared restricted to the tumor mass, thus defining the boundary between the glioma and the brain parenchyma. RII α showed a similar perinuclear distribution in the human glioblastoma cell line Gli36 (Fig. 5E).

Labeling Conditions

Glioma labeling, as well as its differentiation from the normal brain tissue, was dependent on the fixation procedure and on the primary antibody. In sections fixed in formalin at room temperature and subsequently permeabilized with Triton, bright labeling of the glioma could

Fig. 4. RII α distribution in GL261 cells and mouse glioma. (A) GL261 cells treated with vinblastine. RII α (red) appears scattered all around the nucleus. (B) GL261 cells treated with vinblastine. RII α (red) appears scattered all around the nucleus, whereas vimentin staining (green) appears not affected by the treatment. (C) Hematoxylin-eosin staining of a mouse brain hosting a glioma (left). The border of the tumor (arrow) can be easily identified. (D) RII α (red) also highlights the tumor border (arrow). The transition from tumor to normal parenchyma is easier to visualize than in hematoxylin and eosin-stained sections (C). The cells inside the tumor mass are labeled, as well as some isolated RII α -labeled cells that appear at the border, surrounded by unlabeled tissue. Their detection is easier than in hematoxylin and eosin-stained sections (see C). Scale bar, 10 μ m for A and B; 50 μ m for C and D.

be observed with Santa Cruz Biotechnology antibodies; the present data show that this labeling was related to the RII α isoform. Under this condition, no labeling could be observed in the adjacent normal brain tissue, as previously described.²³

In contrast, when sections were first permeabilized with Triton and subsequently fixed with formalin, the same antibody showed a bright labeling, both in glioma and in normal tissue. At high magnification, the pattern of labeling was quite different in the brain and tumor areas. Only when permeabilization preceded fixation was labeling with anti-RII α or RII β (Chemicon) antibodies possible, and microscopy showed that glioma labeling was related only to RII α , whereas RII β was present in the brain but not in glioma. These data suggest that RII β detection in the brain requires unmasking before fixation. Brain labeling was related to RII β , with the exception of ependymal cells, which are the only cell type that shows insoluble RII α in the normal brain, as already reported.²³ These data suggest that RII α and RII β have different biochemical properties and can be detected under different conditions, RII α in the tumor cells being easily accessible to antibodies and revealed under milder conditions.

Colocalization experiments indicated that, under the

same conditions, RI α detergent-insoluble aggregates were never detected in glioma cells in culture and in the tumor (data not shown); they were instead observed in neurons of some regions of the normal mouse brain, as previously described.²⁴

Discussion

The role of the cAMP pathway in cell differentiation and oncogenesis is still unclear. Strict regulation of the RI/RII ratio seems to be crucial for a normal cell cycle. Some human tumors have a predominance of RI over RII, the extent of RI increase being related to malignancy. Induction of differentiation correlates with restoration of a normal RI/RII ratio.¹⁶

Among brain tumors, glioblastoma is the most diffuse and the one with the poorest prognosis.¹ Up until now, only a few molecular markers, such as intermediate-filament-associated proteins, laminin-8, nestin, epidermal growth factor receptor, and tenascin,³⁰⁻³⁴ have been used to differentiate glioma cells from adjacent parenchyma, but none of them is unique to glioma cells, making both therapeutic molecular targeting and immunohistochemical diagnosis problematic. Prompted by these considerations, we screened mouse and rat glioma cell

Fig. 5. RII α distribution in mouse and rat glioma. (A) Glioma obtained from a mouse after GL261 inoculation in vivo. RII α (green) is distributed in clusters of small dots localized near nuclei (blue). Their appearance resembles those detected in cultured glioma cells. (B) Distribution of RII α (red) in F98 cells resembles those seen in GL261 cells, consisting of a cluster of dots located on one side of each nucleus (blue). (C) Glioma obtained from a rat after F98 inoculation in vivo. RII α (red) is distributed in clusters of small dots localized near nuclei (blue). (D) Glioma obtained from a rat after inoculation of F98 cells. As in the mouse, RII α (red) highlights the tumor border (arrow). (E) RII α immunolabeling (red) of the human glioblastoma cells Gli36: the labeling is similar to that in the mouse and rat glioma cell lines. The nucleus is stained blue. Scale bar, 10 μ m for A, B, C, and E; 25 μ m for D.

lines for the presence and intracellular localization of the four PKA regulatory subunits, an issue apparently never addressed before. Although earlier reports pointed to the presence of only RII in glioma cells,^{35,36} the present experiments show detergent-insoluble RI isoforms in Western blot, but only RII α hot spots can be detected by immunohistochemistry. This result can be explained by more distributed RI localization, which does not reach a local intensity higher than the immunofluorescence background.

The RII α isoform presents an unusual intracellular organization in glioma cells from mice, rats, and humans: instead of being soluble, it is mainly compart-

mentalized in detergent-insoluble form, conceivably localized in the trans-Golgi network area, as shown by golgin-97 colocalization.³⁷

The functional meaning of this supramolecular organization is not clear: in principle, aggregates of insoluble regulatory subunits may either concentrate catalytic subunits close to target proteins or act as traps for free catalytic subunits, thus creating phosphorylation-free microdomains, or they may bind free cAMP molecules, thus decreasing their intracellular diffusion. Also unclear is the reason that the insoluble aggregates in glioma cells consist of RII α , while in the rest of the brain they consist mainly of the RII β isoform.

In breast cancer and in prostate carcinoma cell lines, paclitaxel, forskolin, and vincristine activate PKA, which hyperphosphorylates Bcl2, leading to apoptosis.³⁸ A similar mechanism may underlie the cAMP-induced cell death observed in the present experiments in glioma cell lines. In glioma cells, paclitaxel also induces a reorganization of vimentin intermediate filaments, an effect apparent in our paclitaxel-treated cells as well.³⁹

Aggregates of detergent-insoluble RII α are not specific for glioma cells; immunolabeling of ependymal cells was previously described in the normal brain, although with a different localization, at the base of the cilia, and with morphology suggesting a different structure.²³ RII α insoluble aggregates are not detectable in the rest of the brain. Moreover, the structural basis of RII α aggregates in gliomas is, presumably, different from those of RII β in the brain. It is noteworthy that a different sensitivity to detergent treatment is evident: RII β aggregates in the brain can be visualized only if permeabilization is made before fixation, whereas RII α in tumor cells do not need such treatment. In cell culture, RII α aggregates seem more typical of undifferentiated glioma cells, as suggested by GFAP and vimentin colocalization.

In contrast to normal brain parenchyma, in which RI α and RII β insoluble aggregates are detected, mouse and rat glioma cells present a striking cluster of insoluble RII α . This is consistent with a report on human neuroblastoma SH-SY5Y cells that showed an increase in RII α after cAMP activation.⁴⁰ Moreover, in a different

glioma cell line, an increase in cAMP induced differentiation and apoptosis,²⁰ suggesting a mechanism common to different glioma models. In humans, some proteins, including AKAPs and phosphodiesterases, are modulated in glioblastoma specimens,²¹ suggesting involvement of the cAMP signaling system in glioblastoma.

The presence of RII α in gliomas is possibly correlated with genetic abnormalities on chromosomes 7 and 17 detected in glioblastoma specimens.^{41,42} We emphasize the novel result that these abnormalities span the PKA-, RI α -, RI β -, and RII β -coding genes, suggesting possible upregulation of RII α to compensate for other PKA regulatory isoform deficits.

In conclusion, the present data show that in glioma cells, RII α is distributed at particular sites, unlike its distribution in normal brain tissue. Moreover, glioma cells are sensitive to cAMP modifications. The presence of a detectable biochemical marker in glioma cells could possibly assist in diagnosis and points to an additional pathway that can be targeted for therapy.

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