

IQGAP1 Protein Specifies Amplifying Cancer Cells in Glioblastoma Multiforme

Laurent Balenci,¹ Ian D. Clarke,² Peter B. Dirks,² Nicole Assard,¹ François Ducray,³ Anne Jouvet,³ Marie-Françoise Belin,³ Jérôme Honnorat,³ and Jacques Baudier¹

¹Institut National de la Santé et de la Recherche Médicale EMI 0104, Département de Réponse et Dynamique Cellulaires, CEA Grenoble, Grenoble, France; ²The Arthur and Sonia Labatt Brain Tumor Research Center, The Hospital for Sick Children, Toronto, Ontario, Canada; and ³Institut National de la Santé et de la Recherche Médicale Unité 433, Institut Fédératif des Neurosciences de Lyon, Faculté Laennec, Lyon, France

Abstract

The accurate identification and thorough characterization of tumorigenic cells in glioblastomas are essential to enhance our understanding of their malignant behavior and for the design of strategies that target this important cell population. We report here that, in rat brain, the scaffolding protein IQGAP1 is a marker of brain nestin⁺ amplifying neural progenitor cells. In a rat model of glioma, IQGAP1 also characterizes a subpopulation of nestin⁺ amplifying tumor cells in glioblastoma-like tumors but not in tumors with oligodendroglioma features. We next confirmed that IQGAP1 represents a new marker that may help to discriminate human glioblastoma from oligodendrogliomas. In human glioblastoma exclusively, IQGAP1 specifies a subpopulation of amplifying nestin⁺ cancer cells. Neoplastic IQGAP1⁺ cells from glioblastoma can be expanded in culture and possess all the characteristics of cancer stem-like progenitors. The similarities between amplifying neural progenitors and glioblastoma amplifying cancer cells may have significant implications for understanding the biology of glioblastoma. (Cancer Res 2006; 66(18): 9074-82)

Introduction

Gliomas are the most common primary malignant brain tumors and are classified into two major groups: oligodendrogliomas and astrocytomas, including glioblastomas. It is implicit in such definitions that these neoplasms originate from either oligodendrocytes or astrocytes. There is, however, evidence that these brain tumors can also result from the transformation of undifferentiated glial progenitor cells or cells with stem cell characteristics (1–3). The first indication that some brain tumors can arise from transformation of neural stem cells or neural progenitors comes from mouse models, in which combined expression of Ras and Akt oncogenes under the control of the nestin promoter induced glioblastoma formation (4). In adult rodent brain, nestin is considered as a marker for both neural stem cell and neural progenitors (5, 6). The identification of transformed neural stem cell-like cells in cultures derived from human medulloblastomas and glioblastoma tumors

has brought further support to the hypothesis that these tumors contain cancer stem cells that may participate in brain tumorigenesis (7–11). More recently, a population of brain tumor-initiating cells, with characteristics of stem cells (nestin⁺/CD133⁺), has been purified from human gliomas (8). In the more aggressive glioblastomas, this population represents up to 30% of the total tumor cell population (8). The neural stem cell concept for the origin of gliomas sheds light on their heterogeneity but also raises questions as to understand the high malignancy of some of these tumors, such as glioblastoma (3). A model has been proposed where cancer stem cells generate transformed progenitor cells that divide rapidly but are incapable of complete differentiation *in vivo* (3). The accurate identification and thorough characterization of the tumor amplifying progenitor cells is now essential to enhance our understanding of malignant gliomas. To reach this goal, it will be necessary to identify more definitive markers of these cells. Moreover, if these markers have recognized specific functions, they might help to develop new therapeutic strategies for more effective cancer treatments.

We first report here that the IQGAP1 protein is a reliable marker of nestin⁺ amplifying neural progenitors in rat brain. Mammalian IQGAP1 is considered to be a scaffolding protein at the crossroads of several signaling pathways involved in the control of cell adhesion (12, 13), polarization (14, 15), and directional migration (15, 16). A recent study has identified IQGAP1 as a key component of neuronal motility signal transduction (17). Several studies have also implicated IQGAP1 in epithelial carcinogenesis and metastasis (18). Consistent with its possible implication in tumorigenesis, IQGAP1 is highly abundant in rat and human glioma cell lines (19). To investigate possible regulation of IQGAP1 during neurocarcinogenesis, we analyzed IQGAP1 expression in a rat model of ethylnitrosourea (ENU)-induced glioma (20). We show that IQGAP1 specifies a subpopulation of amplifying tumor cells in glioblastoma-like tumors but not in tumors with oligodendroglioma features. We next confirmed in human glioma the specific expression of IQGAP1 in glioblastoma amplifying cancer cells. Our results may have considerable implications for further understanding the biology of these invasive tumors and the development of specific and more effective therapies.

Materials and Methods

Antibodies. The following primary antibodies were used: IQGAP1 (H-109; rabbit polyclonal IgG, Santa Cruz Biotechnology, Inc., Tebu-bio, France), nestin (mouse monoclonal IgG, clone Rat-401, Developmental Studies Hybridoma Bank, Iowa City, IA), specific human nestin (mouse monoclonal, Chemicon International, Inc., Euromedex, France), NG2 (mouse monoclonal, Upstate Laboratories, Upstate, Euromedex, France), Olig-2 (rabbit polyclonal, generous gift from Dr. H. Chneiweiss, INSERM U114, Collège de France, Paris, France), GFAP (mouse monoclonal IgG, Chemicon; chicken polyclonal,

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

J. Honnorat and J. Baudier contributed equally to this work.

Requests for reprints: Jacques Baudier, Institut National de la Santé et de la Recherche Médicale EMI 0104/TS-DRDC, CEA Grenoble, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France. Phone: 33-4-38-78-43-28; Fax: 33-4-38-78-50-58; E-mail: jbaudier@cea.fr.

©2006 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-06-0761

Abcam, France; rabbit polyclonal IgG, DAKO Cytomation, S.A, France), PECAM (CD31; mouse monoclonal, BD PharMingen, BD Biosciences, France), specific human PECAM (CD31; mouse monoclonal, DAKO), Ki-67 (mouse monoclonal IgG, Novocastra Laboratories, Ltd., AbCys, S.A, France), specific human Ki-67 (rabbit polyclonal, Zymed Laboratories, Inc., Clinisciences, France), β III-tubulin (Tuj-1; rabbit polyclonal, Eurogentec, France), β -tubulin (mouse monoclonal, a gift from Dr. D. Job, INSERM U366, Laboratoire du Cytosquelette, Grenoble, France), and CD133/1 and CD133/2 (mouse monoclonal, Miltenyi Biotec, France). Secondary anti-mouse and anti-rat antibodies conjugated to cyanin 3 or cyanin 5 were from Jackson ImmunoResearch Laboratories (Interchim, France). Secondary anti-mouse and anti-rat antibodies conjugated to Alexa Fluor 488 were from Molecular Probes, Inc., (Invitrogen, France). Secondary anti-chicken polyclonal IgY (ab6569) was from Abcam.

Transplacental administration of ENU. All procedures on animals were approved by Institut National de la Santé et de la Recherche Médicale in compliance with European and French law. OFA Sprague-Dawley strain adult rats were provided by Charles River Laboratories (France). Pregnant rats were injected i.v. via tail vein with 60 mg/kg ENU (Sigma-Aldrich, France) at gestational day 19 (20).

Human tumors. Tumor samples were obtained during surgery from patients of the Neurological Hospital of Lyon (Lyon, France). Informed consent was obtained for all patients before the surgery as approved by the Research Ethics Board at the Hospices Civils de Lyon (Lyon, France). All patients had surgery for the first time and never received chemotherapy or radiotherapy. Samples were collected immediately after surgical resection, snap frozen in liquid nitrogen, and stored at -196°C in liquid nitrogen (NeuroBioTec Banques, Biological Resources Center of Hospices Civils de Lyon). A sample of glioblastoma 280, which was used for sphere culture, was progressively frozen in RPMI 1640/20% FCS/10% DMSO and then stored at -196°C in liquid nitrogen before to be used in culture. Hemalin phloxine saffron (HPS)-stained slide of all frozen samples was reviewed by a neuropathologist (A.J.) and graded with the WHO grading classification to confirm the diagnosis and exclude samples without typical aspects of the tumor. All samples containing significant regions of normal brain (>10%) and/or excessively large amounts of necrotic material were excluded. Clinical presentation of all patients was reviewed by physicians (F.D. and J.H.) to select only patients with typical clinical and radiological presentation of glioblastomas, low-grade oligodendrogliomas, or anaplastic oligodendrogliomas. Using all these clinical and histologic criteria, nine glioblastomas (samples no. 280, 410, 431, 492, 581, 532, 596, 658, and 733) and nine oligodendrogliomas (grade 2 WHO oligodendroglioma samples no. 344, 409, 512, 559, and 592; grade 3 WHO oligodendroglioma samples no. 450, 552, 701, and 756) were selected.

Immunohistochemistry. Three- to 8-month-old animals were deeply anesthetized and killed by transcardial perfusion of saline solution (150 mmol/L NaCl) followed by 4% paraformaldehyde. After 24 hours in 4% paraformaldehyde, brains were cryopreserved and 20- μm cryostat (Leica CM 3000 Camera, AG, Germany) sections were cut. Cryopreserved human biopsies were cut into 20- μm cryostat sections and postfixed with 4% paraformaldehyde. Cryosections were permeabilized in TBS containing 0.2% Triton X-100 and blocked in 5% normal goat serum-TBS (NGS-TBS). After incubation with primary antibodies in NGS-TBS overnight at 4°C , sections were washed in TBS and stained with the appropriate secondary antibodies. Sections were counterstained with nuclear marker Hoechst 33258 (1 $\mu\text{g}/\text{mL}$). Images were obtained with a Carl Zeiss, AG, Germany (Axiovert 200 M) microscope and with Leica (TCS SP2) confocal microscope.

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100. After incubation with primary antibodies in NGS-TBS overnight at 4°C , cells were washed in TBS and stained with the appropriate secondary antibodies. For CD133 immunostaining, neurospheres were fixed and partially permeabilized with 4% paraformaldehyde.

Western blotting. Cultured neurospheres and postsurgical human glioma samples were lysed in SDS sample buffer. Proteins were run on 6% SDS-PAGE gel and transferred to a nitrocellulose membrane. Immunoblotting was done with the different antibodies described above and revealed with adequate secondary antibodies coupled to peroxidase.

Blots were revealed by chemiluminescence according to the manufacturer's instructions (enhanced chemiluminescence, Amersham Biosciences, GE Healthcare, France). All Western blot analyses were done in duplicates.

Rat neurosphere culture. Three-month-old rat was killed by decapitation. Brains were removed and placed in PBS and the ventricular walls were dissected, transferred in dissociation medium containing trypsin (5,000 units; Sigma), 0.67 mg/mL hyaluronidase (2,000 units/mg; Sigma), and 0.2 mg/mL kynurenic acid (Sigma), and kept 30 minutes in incubator (37°C , 5% CO_2). Tissues were washed in DMEM with 20% fetal bovine serum (FBS) to inactivate the enzyme activity and then carefully triturated with a Pasteur glass pipette. After homogenization, cells were centrifuged and resuspended in chemically defined medium: proliferation neurosphere medium (DMEM/F12/B27 complement/0.1% bovine serum albumin) supplemented with 20 ng/mL epidermal growth factor (EGF) and 20 ng/mL basic fibroblast growth factor (bFGF; ref. 21).

Primary human tumor sphere culture. Human tumor sample corresponding to glioblastoma 280 was intensively washed in growth factor serum-free neural stem cell medium (9) acutely dissociated in trypsin/EDTA solution. After filtration, primary tumor cells were cultivated in noncoated bacterial dishes in DMEM/F12 medium containing B27 complement and 20 ng/mL of both EGF and bFGF and leukemia inhibitory factor (1,000 units; Chemicon). For differentiation experiments, neurospheres were plated onto polylysine-coated (Sigma) glass coverslips in a defined medium containing DMEM/F12/B27 supplemented with either 3% FBS or in 48-hour starved conditioned medium of EaHY endothelial cell line.

Intracranial cell transplantation into nonobese diabetic-severe combined immunodeficient mice. Purified CD133⁺ cells from a human glioblastoma were injected stereotactically into immunodepressive non-obese diabetic-severe combined immunodeficient (NOD-SCID) mouse frontal cortex as described previously (8). Two months after cell injection, mouse brains were embedded in paraffin. Paraffin-embedded, 5- μm formalin-fixed tissue sections were mounted on microscope slides. Tissue sections were then treated as described previously (8).

Results

IQGAP1 is expressed by amplifying neural progenitors in the adult rat brain. Affinity-purified antibodies against an NH_2 -terminal epitope that is conserved in rodent and human protein were used to probe IQGAP1 protein in adult rat brain. The specificity of these antibodies was first shown by Western blot and indirect immunofluorescence analysis using mouse brain derived from wild-type and *iqgap1*^{-/-} animals (data provided to reviewers for examination). In adult rat brain extracts, IQGAP1 antibodies recognize a single protein band with the expected molecular weight of IQGAP1 (M_r 180 kDa; Fig. 1A, lane 1). By immunohistochemistry analyses on rat brain sections, we determined IQGAP1 immunoreactivity in endothelial cells, in the epithelial ependymal cells lining the lateral ventricles, and in neural progenitor cells of the germinal anterior subventricular zone (aSVZ) and of the rostral migratory stream (RMS; Fig. 1B and C). In the aSVZ, IQGAP1 is associated with clusters of neural progenitor cells coimmunostained with nestin marker (Fig. 1B, a and b). These IQGAP1⁺ cells were stained by a mitotic nuclear marker antigen Ki-67 (Fig. 1B, c and d), suggesting that they correspond to amplifying neural progenitors. In the RMS, IQGAP1 persists in proliferating neural progenitors, which are closely associated with blood vessels (Fig. 1C, a-c).

Amplifying neural progenitors have been isolated from adult rat SVZ and grown as neurospheres as previously described for mice neural progenitors (21). Western blot (Fig. 1A, lane 2) and indirect immunofluorescence (Fig. 1D) on neurospheres confirmed the coexpression of IQGAP1 and nestin in neural progenitors. The glial marker GFAP is not detected in neurospheres (Fig. 1A, lane 2),

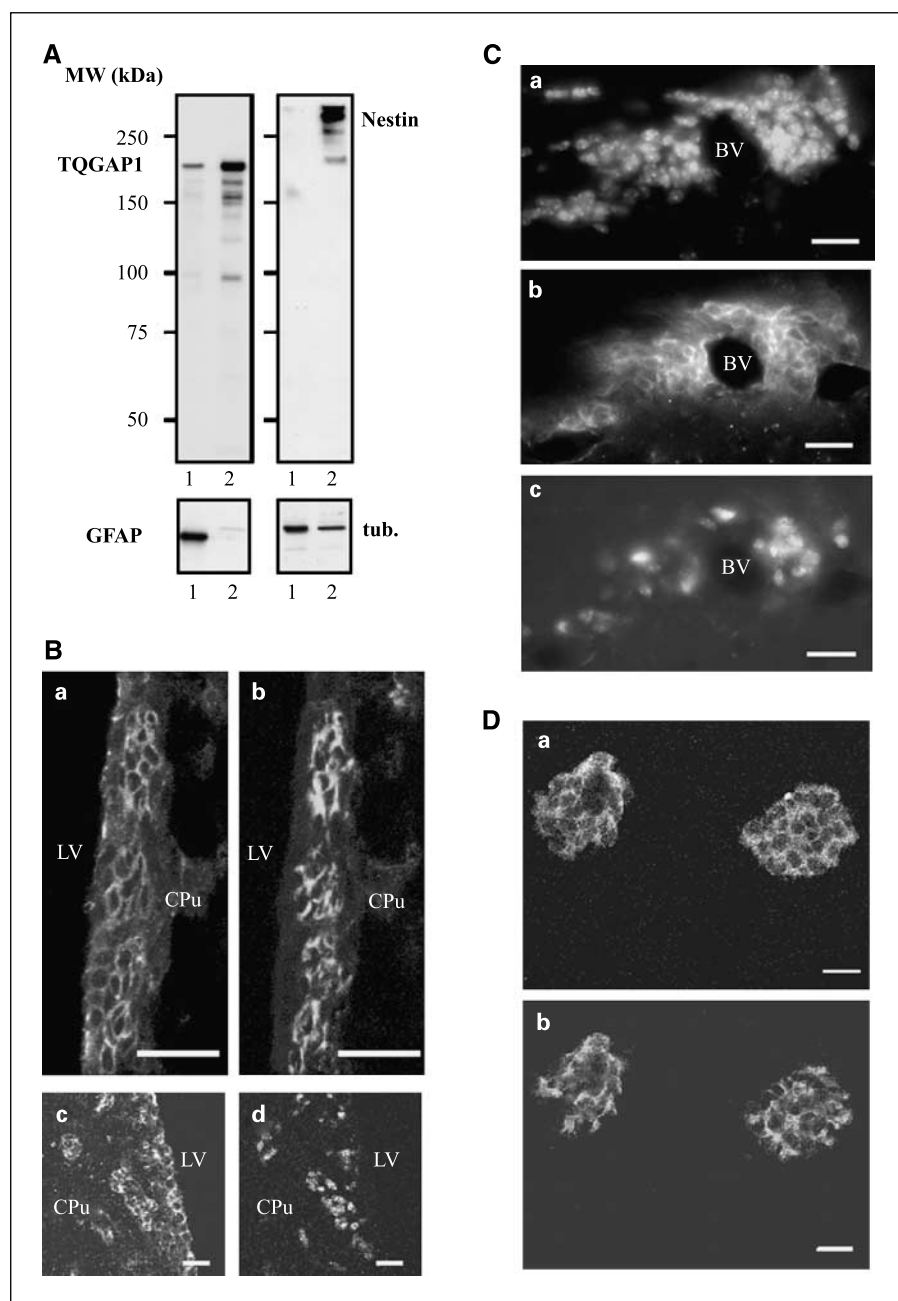


Figure 1. IQGAP1 is expressed in amplifying neural progenitor cells in adult rat brain. **A**, Western blot analysis of total adult rat brain extract (*lane 1*) and of neurospheres derived from adult rat SVZ (*lane 2*) with anti-IQGAP1, anti-nestin, anti-GFAP, or anti- β -tubulin (*tub.*). **B**, confocal microscope analysis of coronal rat brain section showing the aSVZ double immunostained with IQGAP1 antibodies (*a* and *c*) and either nestin (*b*) or Ki-67 (*d*) antibody. LV, lateral ventricle; CPu, caudate nucleus. Bar, 20 μ m. **C**, optical microscope analysis of sagittal section of rat brain RMS triple immunostained with Hoechst for DNA (*a*), IQGAP1 antibodies (*b*), and Ki-67 antibody (*c*). BV, blood vessel. Bar, 20 μ m. **D**, neurospheres derived from adult rat SVZ grown in the presence of EGF and FGF were double labeled with IQGAP1 (*a*) and nestin (*b*) antibodies. Bar, 20 μ m.

whereas the protein is highly abundant in total brain extracts (Fig. 1A, *lane 1*).

IQGAP1 specifies a population of nestin⁺ tumor cells in a rat model of glioblastoma-like tumor. In differentiated cells of the adult rat brain, IQGAP1 immunoreactivity is below the detection limit. However, IQGAP1 protein is highly abundant in rat and human cell lines derived from glioma (19). We therefore investigated possible regulation of IQGAP1 expression during neurocarcinogenesis.

In the rat, a single transplacental injection ENU into pregnant female rat at E19 induces the development of brain tumors in virtually 100% of offspring after several months (20). Many animals developed more than one intraparenchymal tumors. Three months after injection, 25 intraparenchymal tumors in 10 rats were

examined for their histologic and immunophenotypic characteristics. Immunohistochemical characterizations included expression of the astrocyte marker (GFAP), the oligodendrocyte progenitor markers (Olig-2/NG2), and the neural progenitor markers (nestin and IQGAP1). Two types of tumors have been distinguished based on these criteria. Representative results are shown in Figs. 2 and 3.

Nineteen primitive hyperplastic lesions examined have histologic characteristics that resemble human oligodendroglioma. Figure 2 shows a representative image of such hyperplastic lesion located in the angle of the lateral ventricle between the corpus callosum and the caudate nucleus. Histologically, these tumors show moderate cellularity with relatively monomorphous round nuclei and an arborized capillary network. As reported for human oligodendrogliomas (22), the majority of tumor cells have a uniform

phenotype characterized by intense immunoreactivities for the oligodendrocyte precursor markers, including the nuclear transcription factor Olig-2 and the cell surface NG2 proteoglycan (Fig. 2, *A* and *B*) and the platelet-derived growth factor- α receptor (data not shown). Within these tumors, IQGAP1 immunoreactivity is exclusively confined to the capillary network (Fig. 2, *c*). A costaining with the endothelial marker PECAM confirmed the restriction of IQGAP1 in oligodendroglia-like endothelial cells (Supplementary Fig. S1). These tumors also incorporate reactive astrocytes identified based on their morphology and immunoreactivity for GFAP marker (Fig. 2, *d*). Tumor cells, as well as reactive astrocytes, are not stained with nestin antibodies, and only very few cells express the proliferation-associated Ki-67 antigen (data not shown). Low mitotic index is also a feature of human oligodendrogloma.

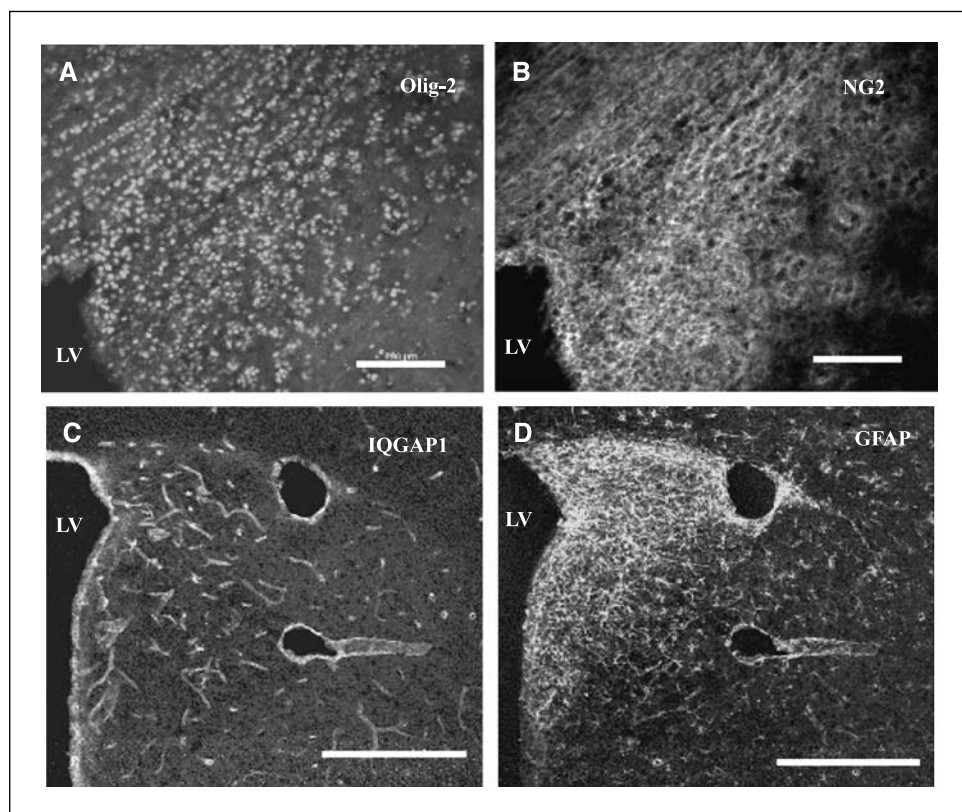
The second type of parenchymal tumor is histologically more heterogeneous and reveals an apparent complexity of phenotypes that closely resembled human glioblastoma. These tumors are characterized by clusters of IQGAP1⁺ cells surrounded by GFAP⁺ cells (Fig. 3, *a* and *b*). The IQGAP1⁺ cells are coimmunostained with nestin (Fig. 3, *c-e*). The tumor GFAP⁺ cells have mixed phenotypes depending on their localization within the tumor bulk. The GFAP⁺ astrocyte population, which unsheathed clusters of IQGAP1⁺/nestin⁺ cells, can be colabeled with nestin. In contrast, GFAP⁺ astrocytes, which accumulate to the periphery of the tumors, are nestin⁻ (data not shown). These nestin⁻ astrocytes are likely associated with reactive gliosis that accompanies tumor development. The cellular heterogeneity of the tumors is also illustrated by the presence of many tumor cells with strong NG2 immunoreactivity (data not shown). However, in contrast to oligodendrogloma-like tumors, the NG2⁺ tumor cells are totally devoid of

nuclear Olig-2 immunoreactivity (data not shown). We noted a large number of proliferating Ki-67⁺ cells in these tumors in agreement with a malignant grade. Most of the Ki-67⁺ cells form clusters unsheathed within astrocyte processes (Fig. 3, *f-h*). Double immunostaining with Ki-67 and IQGAP1 antibodies shows that most of the Ki-67⁺ cells are IQGAP1⁺ (Fig. 3, *i-l*), indicating that IQGAP1⁺ cells are part of the proliferating component of the tumor. Hoechst staining does not reveal fragmented nuclei, confirming that Ki-67⁺ cells are not undergoing apoptosis (Fig. 3, *i*).

IQGAP1 expression identifies tumor amplifying cells in human glioblastoma. Having identified IQGAP1 as a specific marker of amplifying tumor cells in a rat model of glioblastoma, we next investigated whether IQGAP1 might also help to discriminate human glioblastoma from oligodendrogloma. We first compared the expression of IQGAP1 and nestin in nine human glioblastoma and nine human oligodendrogloma (grades 2 and 3) by Western blot (Fig. 4A). Results show that nestin is highly expressed in tumors classified as glioblastoma and almost undetectable in oligodendrogloma with low or anaplastic features. Western blot analysis also reveals a correlation between nestin expression and IQGAP1 content in all glioblastoma extracts. Significant nestin expression was observed only in one oligodendrogloma (patient 756). Interestingly, this tumor had characteristics of glioblastoma in some regions. In contrast, the astrocyte marker GFAP does not discriminate glioblastoma from oligodendrogloma.

Triple indirect immunofluorescence analysis with IQGAP1, GFAP, and the endothelial marker PECAM (Fig. 4B) reveals that, within low-grade oligodendrogloma (Fig. 4B, *a-d*) and in anaplastic oligodendrogloma characterized by higher vascular development (Fig. 4B, *e-h*), IQGAP1 immunoreactivity is exclusively associated with endothelial cells. This contrasts with glioblastoma, where

Figure 2. Immunohistochemical characterization of rat oligodendrogloma-like tumors. Representative oligodendrogloma-like tumor in the angle of the lateral ventricle between the corpus callosum and the caudate nucleus from a 90-day-old rat exposed to ENU and double immunostained with either Olig-2 (*A*) or NG2 (*B*) antibodies or with IQGAP1 (*C*) and GFAP (*D*) antibodies. Bar, 200 μ m.



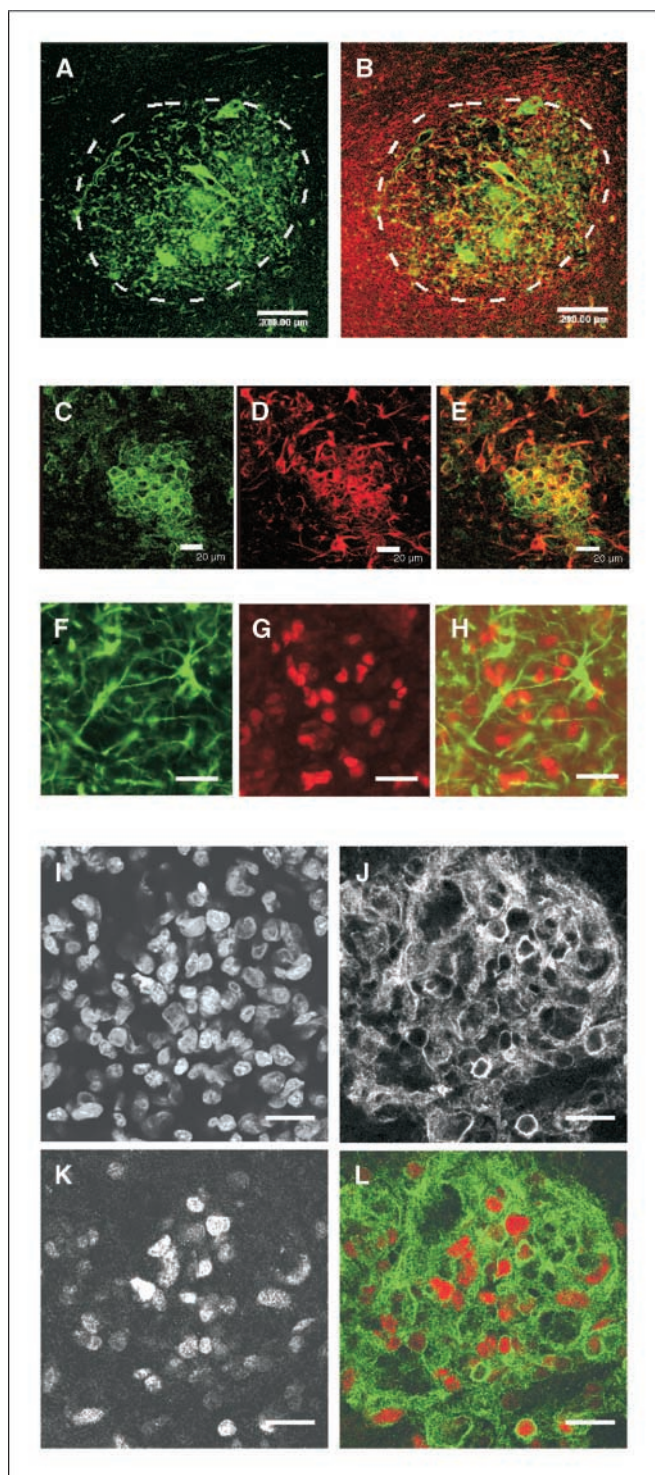


Figure 3. Immunohistochemical characterization of rat glioblastoma-like tumors. Representative glioblastoma-like tumor in the cortex above the corpus callosum from a 90-day-old rat. *A* and *B*, low-power view section immunostained with IQGAP1 (green, *A* and *B*) and GFAP (red, *B*) antibodies shows clusters of IQGAP1⁺ cells surrounded by GFAP⁺ astrocytes. Dashed line, tumor is outlined. Bar, 200 μ m. *C* to *E*, double immunostaining with IQGAP1 and nestin antibodies shows that clusters of IQGAP1⁺ cells (green, *C* and *E*) express nestin (red, *D* and *E*). Bar, 20 μ m. *F* to *H*, double immunostaining with anti-GFAP (green, *f* and *h*) and anti-Ki-67 (red, *G* and *H*) antibodies shows that tumor cells labeled with Ki-67 are not GFAP⁺. Bar, 50 μ m. *I* to *L*, cluster of IQGAP1⁺ cells stained with Hoechst for DNA (*I*) and double immunostained with anti-IQGAP1 (*J* and *L*) and anti-Ki-67 (*K* and *L*) antibodies shows that tumor cells labeled with Ki-67 correspond to IQGAP1⁺ cells. Bar, 50 μ m.

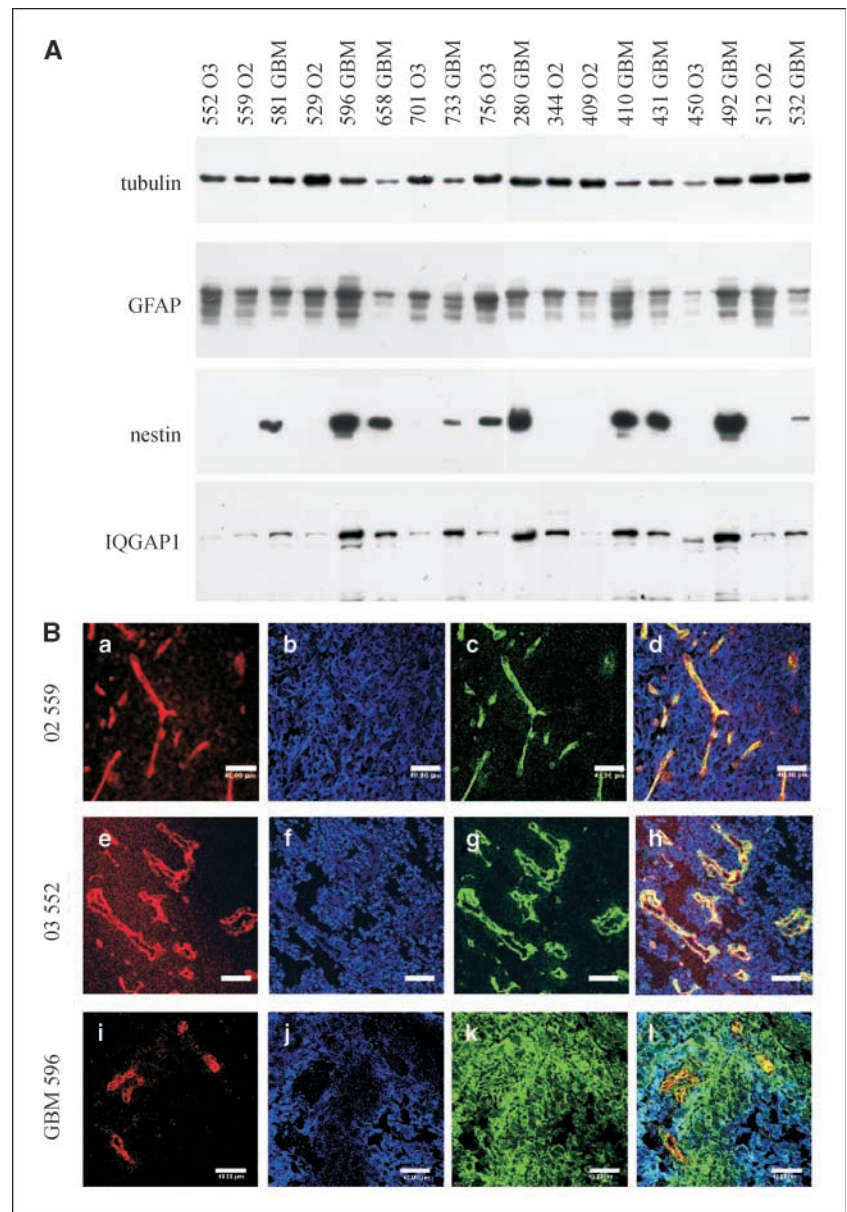
IQGAP1 immunoreactivity is present both in endothelial cells and also in a cell population that is not stained with GFAP (Fig. 4*B, i-l*). In all glioblastoma, the IQGAP1⁺/GFAP⁻ cells are preferentially located around blood vessels.

Based on these observations, we did a systematic characterization of the IQGAP1⁺/GFAP⁻ cells in human glioblastoma biopsies. Representative results obtained with glioblastoma sample 280 are shown.

HPS coloration on tumor sections shows histologic features of a human glioblastoma (Supplementary Fig. S2a). A GFAP staining done on the same tumor sample shows that certain tumor cell subtypes are not positive for this astrocyte marker showing the cell heterogeneity in human glioblastoma (Supplementary Fig. S2b). We next used indirect immunofluorescence to refine the immunologic phenotype of the different tumor cell populations (Fig. 5, *A-I*). Triple immunostaining with GFAP, nestin, and IQGAP1 reveals clusters of cells coexpressing IQGAP1 and nestin surrounded by GFAP⁺ cells (Fig. 5, *A-C*). The GFAP⁺ tumor cells, which surround the IQGAP1⁺/nestin⁺ clusters, remain heavily stained with nestin (Fig. 5, *A* and *B*). At higher magnification, the IQGAP1⁺/nestin⁺ clusters show a clear colocalization of IQGAP1 and nestin (Fig. 5, *D-F*). Triple immunostaining with IQGAP1, GFAP, and the endothelial marker PECAM confirms that the IQGAP⁺ cells accumulate around blood vessels (Fig. 5, *G-I*). On serial sections of this human glioblastoma sample, we also analyzed the proliferation status of the different tumor cell populations (Fig. 5, *J-R*). Low magnification observation of the tumor double immunostained with GFAP and Ki-67 clearly shows that the majority of proliferating cells (Ki-67⁺) are located within clusters surrounded by GFAP⁺ cells (Fig. 5, *J-L*). Triple immunostaining with GFAP, nestin, and Ki-67 constantly revealed that the clusters of Ki-67⁺ cells correspond to nestin⁺/GFAP⁻ cells (Fig. 5, *M-O*). It has not been possible to do colabeling of Ki-67 with IQGAP1 due to the rabbit origin of the two antibodies. Nevertheless, taking into account that clusters of nestin⁺/GFAP⁻ cells are IQGAP1⁺ (Fig. 5, *D-F*) and that clusters of nestin⁺/GFAP⁻ cells are Ki-67⁺ (Fig. 5, *M-O*), we can logically conclude that Ki-67⁺/nestin⁺/GFAP⁻ cells correspond to IQGAP1⁺ tumor cells. There are some nestin⁺/GFAP⁺ cells present within the tumor mass that are also Ki-67⁺ (Fig. 5, *P-R*). These cells may correspond to tumor cells that have acquired more differentiated phenotype but still retain the capacity to proliferate.

IQGAP1⁺/nestin⁺ cells derived from glioblastoma have *in vitro* and *in vivo* characteristics of amplifying cancer progenitor cells. To confirm that the IQGAP1⁺/nestin⁺/GFAP⁻ cells present in human glioblastoma represent the tumor amplifying cells, we produced neurospheres from postsurgery specimens of glioblastoma 280 as described in Materials and Methods. Western blot analysis revealed that IQGAP1 is highly expressed in the total tumor extract (Fig. 6*A, lane 1*) and in the corresponding cancerous neurospheres (Fig. 6*A, lane 2*). These cancerous neurospheres do not express GFAP, whereas the protein is present in the original total tumor extract (Fig. 6*A, lanes 1* and 2). Two recognized marker of tumorigenic glioblastoma stem-like progenitors were then chosen to characterize the expanded spheres: nestin and CD133 (8, 11). Double indirect immunofluorescence reveals that the IQGAP1⁺ cells within neurospheres express both nestin and CD133 (Fig. 6*B* and *C*). The intensity of staining between IQGAP1 and nestin may vary among cells. These differences might either reflect subtle heterogeneity of neurosphere cells or are due to a difference in antigen accessibility. The

Figure 4. Immunohistochemical characterization of human glioma. **A**, samples of human brain tumors, representing grade 2 or 3 oligodendroglioma (O2 and O3) and glioblastoma (GBM), were analyzed by Western blot for β -tubulin, GFAP, nestin, and IQGAP1 content. **B**, triple immunofluorescence analysis of low-grade oligodendroglioma (patient 559; *a-d*), high-grade oligodendroglioma (patient 552; *e-h*), and glioblastoma (patient 596; *i-l*) stained with PECAM antibodies for endothelial cells (*a*, *e*, and *i*), GFAP (*b*, *f*, and *j*), and IQGAP1 (*c*, *g*, and *k*). Bar, 40 μ m.



neurospheres can be dissociated in a single cell and they are able to reform a secondary neurospheres for a long time, confirming their self-renewal potency. These cells have been expanded for >30 passages with an average doubling time estimated to 3 days. Moreover, the cell immunophenotypes remained constant throughout passages.

A property of tumorigenic glioblastoma stem-like progenitors grown as neurospheres is their multipotency (9, 11). To examine the *in vitro* multipotency of the 280 glioblastoma-derived neurospheres, cells were cultured in different culture conditions. Neurospheres were firstly plated onto polylysine-coated coverslips in a low serum-containing medium. Results show that cells spread out from the spheres and differentiate down astrocyte lineage, which characterizes the original tumor phenotype. Most of the cells express GFAP, and many of them coexpress nestin and still proliferate as revealed by Ki-67 staining (Supplementary Fig. S3A). Only rare cells express the neuronal marker Tuj-1 (data not shown). To analyze the neurogenic potential of the glioblastoma amplifying

cancer cells, we plated neurospheres onto polylysine-coated slides in conditioned medium of endothelial cells. Enhancement of neuron production from multipotent neural progenitors by factors secreted by endothelial cells has been previously reported (23). In this culture condition, after 4 days, most of the glioblastoma-derived cells express the neuronal marker Tuj-1 (Supplementary Fig. S3B). As observed by others (9), a fraction of cells were colabeled with both neuronal and glial markers. This abnormal type of cells likely reflects aberrant differentiation program in tumor neural progenitors.

CD133⁺ cells can be purified from human glioblastoma, and as few as 100 CD133⁺ cells can produce a tumor when injected into the brain of NOD-SCID mouse (8). Analysis of IQGAP1 immunoreactivity in the xenograft at an early stage of the neoplastic process reveals that most of the amplifying tumor cells are heavily immunostained with IQGAP1 antibodies (Fig. 6D, *a* and *b*). Double immunofluorescence staining reveals that the IQGAP1⁺ xenograft tumor cells do also express CD133 immunoreactivity (Fig. 6D,

c and d), confirming the specific expression of IQGAP1 and CD133 in glioblastoma amplifying cancer progenitor cells *in vivo*.

Discussion

Detailed investigation of IQGAP1 expression in adult rat brain by indirect immunofluorescence has revealed a restricted pattern of accumulation of the protein in brain endothelial cells, in the epithelial ependymal cells lining the ventricles, and in amplifying

neural progenitor cells abundant in the germinative SVZ and RMS. We have confirmed the expression of IQGAP1 protein in amplifying neural progenitors isolated from adult rat SVZ and grown as neurospheres *in vitro*. In the rat brain and in neurosphere cells, IQGAP1⁺ cells also express the neural progenitor marker nestin. The IQGAP1⁺/nestin⁺ neural progenitors associate as dense clusters and are generally concentrated around blood vessels. This location places neural progenitor cells in close proximity to the endothelial cells, facilitating interaction and communication between these two cell types. These cellular interactions are important to control neural progenitor self-renewal and differentiation (23–25). In a rat model of glioblastoma-like tumors, IQGAP1 is also expressed by a subpopulation of nestin⁺/GFAP⁻ cells. The mitotic cells present in the tumors express IQGAP1, indicating that the IQGAP1⁺/nestin⁺ cells are part of the proliferating component of the tumor. Our results corroborate previous study showing that, in ENU-induced rat glioma, nestin⁺ cells frequently incorporated bromodeoxyuridine (BrdUrd), whereas other tumor cells expressing GFAP were only rarely colabeled with BrdUrd (20). Rat cell lines can be derived from ENU-induced glioma. One example is the C6 cell line used as a cellular model of brain glioma. A side population of tumorigenic cancer stem-like cells persists in the C6 glioma cell line (26). We have purified C6 side population cells by flow cytometry (26) and shown that they express both IQGAP1 and nestin (data not shown). These observations suggest that the IQGAP1⁺/nestin⁺ cells may be important in the early stages of brain tumorigenesis. Several possibilities exist for the source of such cells. One possibility is that a precursor cell or a differentiated cell in the parenchyma undergoes transformation, after which it expresses nestin and IQGAP1. Alternatively, a nestin⁺/IQGAP1⁺ neural progenitor in the SVZ and the RMS could migrate to this area after transformation. Very recent studies have shown that amplifying neural progenitors capable of proliferation and multipotential differentiation can indeed migrate to the brain parenchyma to produce new neurons and glia (27).

In contrast to rat glioblastoma, IQGAP1⁺/nestin⁺ tumor cells are absent in rat tumors with oligodendroglial phenotypes. Because oligodendroglioma-like tumors express oligodendrocyte progenitor markers (Olig-2/NG2), it is likely that that these tumors are related to oligodendrocyte progenitors as already suggested for human oligodendrogliomas (22). Oligodendrogliomas might arise from a stem cell that acquires oligodendrocyte progenitor phenotypes determined by environment (3) or might directly derive from multipotent oligodendroglial progenitors (28). The exact origin of

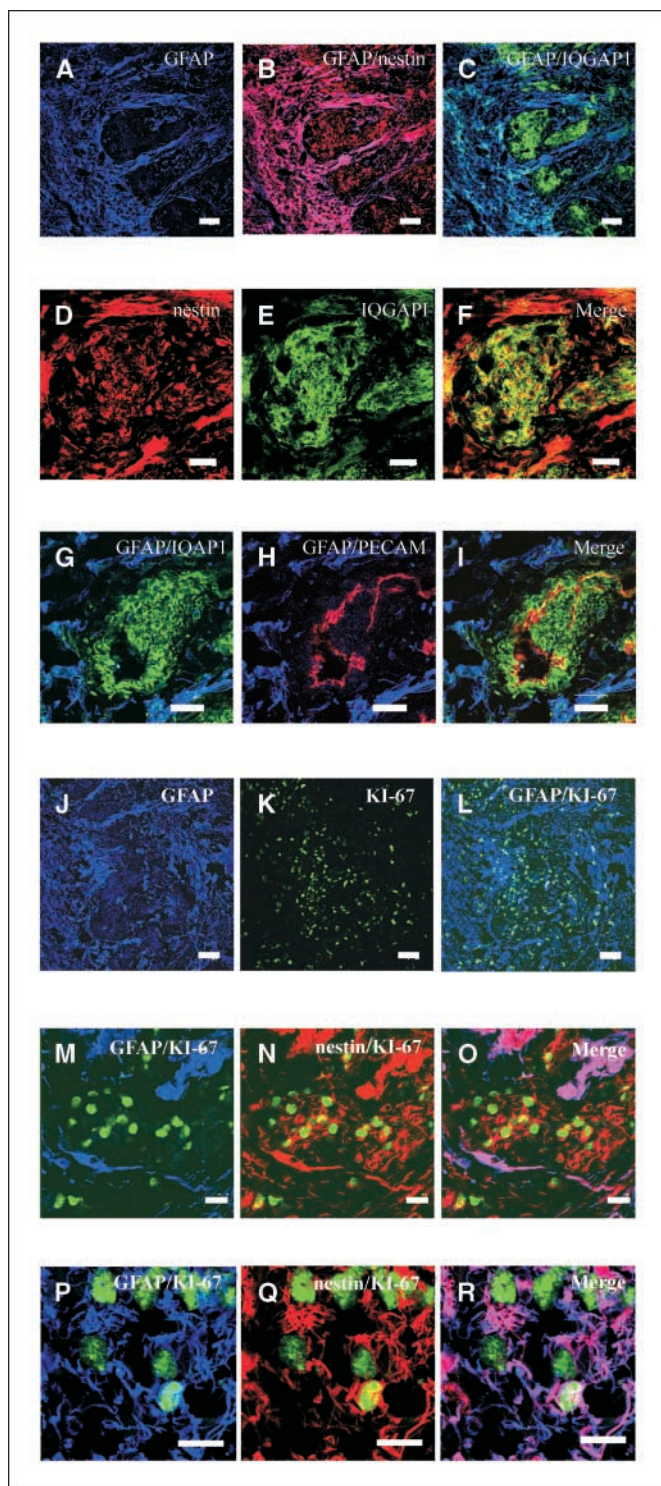


Figure 5. IQGAP1 expression specifies tumor amplifying cells in human glioblastoma. Human glioblastoma (patient 280) sections were analyzed by indirect immunofluorescence and confocal microscopy. A to C, triple indirect immunofluorescence analysis with anti-GFAP (blue), anti-nestin (red), and anti-IQGAP1 (green) antibodies shows that nestin⁺/IQGAP1⁺ cells form clusters surrounded by GFAP⁺/nestin⁺ cells. Bar, 80 μ m. D to F, higher magnification of the nestin⁺/IQGAP1⁺ clusters shows overlap between the two markers. Bar, 40 μ m. G to I, triple immunostaining with anti-GFAP (blue), anti-IQGAP1 (green), and anti-PECAM (red) antibodies shows that IQGAP1⁺ cells form perivascular niches surrounded by GFAP⁺ cells. Bar, 40 μ m. J to L, double immunostaining with anti-GFAP and anti-Ki-67 antibodies shows that cells with the highest mitotic index are located within clusters surrounded by GFAP⁺ cells. Bar, 80 μ m. M to O, higher magnification of a cluster of nestin⁺/GFAP⁻ cells triple immunostained with anti-GFAP (blue), anti-nestin (red), and anti-Ki-67 (green) antibodies shows that nestin⁺/GFAP⁻ cells actively proliferate. Bar, 20 μ m. P to R, triple immunostaining with anti-GFAP (blue), anti-nestin (red), and anti-Ki-67 (green) antibodies shows that some cells that form the tumor mass and that express both nestin and GFAP are also able to proliferate. Bar, 20 μ m.

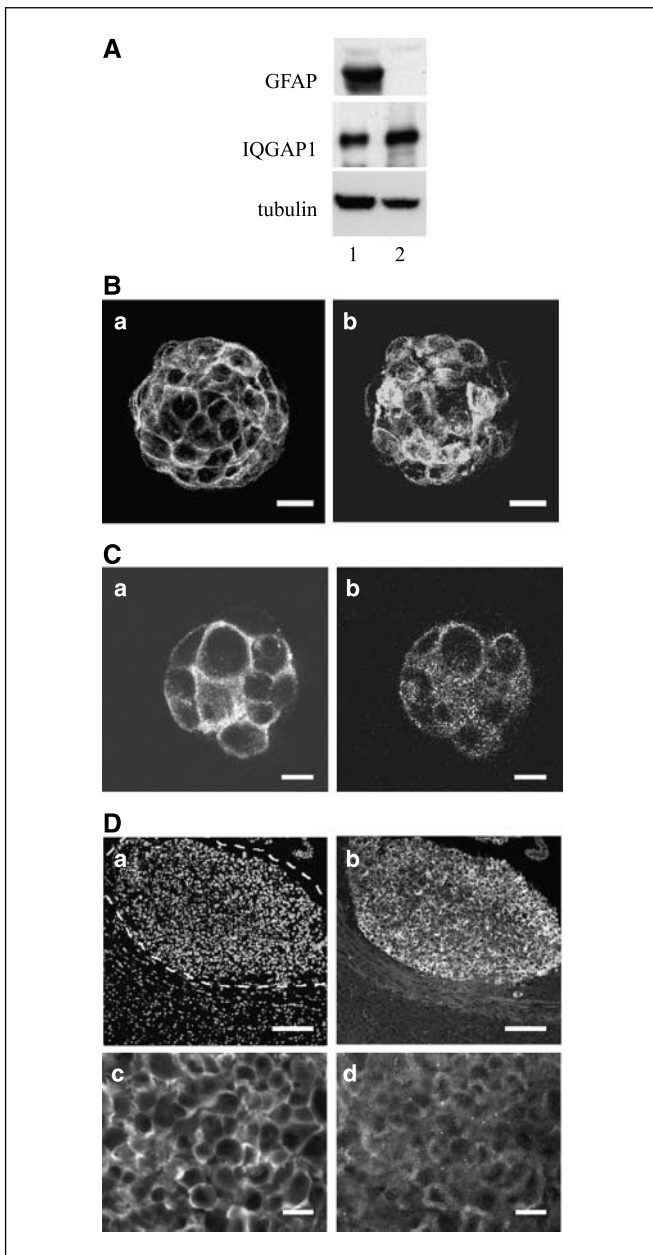


Figure 6. IQGAP1⁺ cells derived from glioblastoma have *in vitro* and *in vivo* characteristics of amplifying cancer cells. **A**, sample of human glioblastoma (280; lane 1) and corresponding neurospheres (lane 2) was analyzed by Western blot for GFAP, IQGAP1, and β -tubulin content. **B**, 280 glioblastoma-derived neurospheres grown in the presence of EGF and FGF were double labeled with IQGAP1 (a) and nestin (b) antibodies. Bar, 20 μ m. **C**, 280 glioblastoma-derived neurospheres grown in the presence of EGF and FGF were double labeled with IQGAP1 (a) and CD133 (b) antibodies. Bar, 20 μ m. **D**, immunohistochemical characterization of human glioblastoma xenograft. a and b, section of mouse xenografted tumor derived from human CD133⁺ glioblastoma tumor cells was stained for DNA with Hoechst (a) and immunostained with IQGAP1 antibodies (b). Dashed line, tumor is outlined. Bar, 200 μ m. c and d, xenograft was double labeled with IQGAP1 (c) and CD133 (d) antibodies. Bar, 20 μ m.

oligodendrogliomas remains to be further studied. To validate our results with rat brain tumor model, we next studied IQGAP1 expression in human oligodendroglioma and glioblastoma. We confirmed that IQGAP1 is a reliable marker that may help to distinguish oligodendroglioma from glioblastoma. Although in

both tumors IQGAP1 is expressed by endothelial cells, only in glioblastoma it specifies a population of amplifying tumor cells. Remarkably, IQGAP1 is absent from the astrocytic contingency of the tumors, which often induces bias in brain tumor classification. IQGAP1 may thus represent a protein marker that may have significant implication in the classification of human brain tumors. The human glioblastoma IQGAP1⁺ cells can be expanded *in vitro* as neurospheres and exhibit features of multipotent glioblastoma tumorigenic neural precursors (Fig. 6; Supplementary Fig. S3). *In vivo*, the amplifying IQGAP1⁺ cancer cells are surrounded by tumor cells that have down-regulated IQGAP1 expression and express the differentiation-associated marker antigen GFAP. Nonetheless, GFAP expression does not correlate with full differentiation, as the cells still express the stem/progenitor cell marker nestin. Although, these GFAP⁺/nestin⁺ cells are still able to proliferate, *in vivo* and *in vitro*, they have lost their tumorigenicity (8). All together, these findings suggest that the amplifying IQGAP1⁺ cancer cells are closer to a multipotent progenitor cells and represent the most aggressive cancer cell population in glioblastoma. The clusters of IQGAP1⁺ tumor cells most likely correspond to the glioblastoma amplifying cancer cells that represent up to 30% of the glioblastoma tumor cell population (8) and maybe the cell population, which has to be target for more effective cancer therapies of glioblastoma. The close association between IQGAP1⁺ amplifying cancer cells with blood vessels might contribute to maintain cancer cells undifferentiated with high tumorigenic potential. Several studies have already pointed out that endothelial cells are important in the regulation of neural progenitor proliferation and differentiation (23–25). The close association between amplifying cancer cells and endothelial cells also raises the possibility that the tumorigenic cells may use blood vessels to migrate and disseminate throughout the brain parenchyma. Indeed, in both rat and human glioblastomas, the perivascular clusters of amplifying cancer cells are not concentrated within a single tumor area but are found dispersed within the tumor mass. The migratory potential of tumorigenic cells could explain the local recurrence of gliomas after surgical resection or radiotherapy that targets the tumor mass but does not necessarily include the zones where cancer cells might have migrated. The IQGAP1 signaling pathway might play an essential role in the control of these migration and invasion processes. IQGAP1 is a key component of cell motility signal transduction (16–18). In neural precursors, in response to intracellular Ca²⁺ elevation, IQGAP1 forms a complex with Rho family GTPases, CLIP-170 and Lis1, and enhances neuronal motility (17). In endothelial cells, IQGAP1 interacts with the vascular endothelial growth factor (VEGF) receptor 2 (VEGF-R2) to promote endothelial cell migration (16). VEGF-R2 is also expressed by neural progenitors and mediates the chemotactic activities of VEGF on neural progenitors (29). We have found that neurospheres derived from wild-type mice respond to the VEGF-mediated chemokinesis but not those derived from *iqgap1* null mice, suggesting that IQGAP1 signaling pathway might play an essential role in the control of neural progenitor migration.⁴ Considering the similarities between IQGAP1⁺ amplifying neural progenitors and IQGAP1⁺ glioblastoma amplifying cancer cells, the proposed function of IQGAP1 in the regulation of neural progenitor migration can be extended to the amplifying tumor cells in human glioblastoma. A better understanding of the

⁴ L. Balenci, A. Bernards, and J. Baudier, unpublished data.

IQGAP1 signaling in amplifying tumor cells may open new avenue to develop pharmacologic approach aiming to neutralize these cells, which represent the most aggressive cell type in glioblastoma.

Acknowledgments

Received 2/27/2006; revised 7/1/2006; accepted 7/25/2006.

Grant support: Association pour la Recherche sur le Cancer grant 4725 and Région Rhône-Alpes Emergence grant 8HC04H00 (J. Baudier) and Institut National du Cancer grant PL114 (J. Baudier and J. Honnorat).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. H. Chneiweiss for generous gift of Olig-2 antibodies, Prof. J. LaMarre (Guelph University, Guelph, Ontario, Canada) for critical reading of the article, Dr. D. Grunwald for assistance in confocal microscopy analysis, and Dr. J.C. Deloulme for advices in neurosphere cultures.

References

- Oliver TG, Wechsler-Reya RJ. Getting at the root and stem of brain tumors. *Neuron* 2004;42:885–8.
- Clarke MF. Neurobiology: at the root of brain cancer. *Nature* 2004;432:281–2.
- Sanai N, Alvarez-Buylla A, Berger MS. Neural stem cells and the origin of gliomas. *N Engl J Med* 2005;353:811–22.
- Holland EC, Celestino J, Dai C, Schaefer L, Sawaya RE, Fuller GN. Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. *Nat Genet* 2000;25:55–7.
- Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. *Cell* 1990;60:585–95.
- Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J Neurosci* 1997;17:5046–61.
- Hemmati HD, Nakano I, Lazareff JA, et al. Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci U S A* 2003;100:15178–83.
- Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature* 2004;432:396–401.
- Galli R, Binda E, Orfanelli U, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res* 2004;64:7011–21.
- Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003;63:5821–8.
- Yuan X, Curtin J, Xiong Y, et al. Isolation of cancer stem cells from adult glioblastoma multiforme. *Oncogene* 2004;23:9392–400.
- Katata T, Irie K, Fukuhara A, et al. Involvement of nectin in the localization of IQGAP1 at the cell-cell adhesion sites through the actin cytoskeleton in Madin-Darby canine kidney cells. *Oncogene* 2003;22:2097–109.
- Lui WY, Mruk DD, Cheng CY. Interactions among IQGAP1, Cdc42, and the cadherin/catenin protein complex regulate Sertoli-germ cell adherens junction dynamics in the testis. *J Cell Physiol* 2005;202:49–66.
- Fukata M, Watanabe T, Noritake J, et al. Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170. *Cell* 2002;109:873–85.
- Watanabe T, Wang S, Noritake J, et al. Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration. *Dev Cell* 2004;7:871–83.
- Yamaoka-Tojo M, Ushio-Fukai M, Hilenski L, et al. IQGAP1, a novel vascular endothelial growth factor receptor binding protein, is involved in reactive oxygen species-dependent endothelial migration and proliferation. *Circ Res* 2004;95:276–83.
- Kholmanskikh SS, Koeller HB, Wynshaw-Boris A, Gomez T, Letourneau PC, Ross ME. Calcium-dependent interaction of Lis1 with IQGAP1 and Cdc42 promotes neuronal motility. *Nat Neurosci* 2006;9:50–7.
- Briggs MW, Sacks DB. IQGAP proteins are integral components of cytoskeletal regulation. *EMBO Rep* 2003;4:571–4.
- Mbele GO, Deloulme JC, Gentil BJ, et al. The zinc- and calcium-binding S100B interacts and co-localizes with IQGAP1 during dynamic rearrangement of cell membranes. *J Biol Chem* 2002;277:49998–50007.
- Jang T, Litofsky NS, Smith TW, Ross AH, Recht LD. Aberrant nestin expression during ethylnitrosourea-(ENU)-induced neurocarcinogenesis. *Neurobiol Dis* 2004;15:544–52.
- Doetsch F, Petreanu L, Caille I, Garcia-Verdugo JM, Alvarez-Buylla A. EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. *Neuron* 2002;36:1021–34.
- Shoshan Y, Nishiyama A, Chang A, et al. Expression of oligodendrocyte progenitor cell antigens by gliomas: implications for the histogenesis of brain tumors. *Proc Natl Acad Sci U S A* 1999;96:10361–6.
- Shen Q, Goderie SK, Jin L, et al. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science* 2004;304:1338–40.
- Wurmser AE, Palmer TD, Gage FH. Neuroscience. Cellular interactions in the stem cell niche. *Science* 2004;304:1253–5.
- Palmer TD, Willhoite AR, Gage FH. Vascular niche for adult hippocampal neurogenesis. *J Comp Neurol* 2000;425:479–94.
- Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. *Proc Natl Acad Sci U S A* 2004;101:781–6.
- Bull ND, Bartlett PF. The adult mouse hippocampal progenitor is neurogenic but not a stem cell. *J Neurosci* 2005;25:10815–21.
- Aguirre A, Gallo V. Postnatal neurogenesis and gliogenesis in the olfactory bulb from NG2-expressing progenitors of the subventricular zone. *J Neurosci* 2004;24:10530–41.
- Zhang H, Vutskits L, Pepper MS, Kiss JZ. VEGF is a chemoattractant for FGF-2-stimulated neural progenitors. *J Cell Biol* 2003;163:1375–84.