Identification of the Putative Brain Tumor Antigen BF7/GE2 as the (De)Toxifying Enzyme Microsomal Epoxide Hydrolase

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ABSTRACT

Malignant gliomas are the main cause of death from primary brain tumors. Despite surgery, radiation, and chemotherapy, patients have a median survival of less than a few years; therefore, it is imperative to investigate new ways of treatment. The development of new therapeutic strategies for brain tumors is dependent on a better understanding of the differences between normal and tumoral brain cells. Our group had described previously a M, 48,000 antigen defined by reactivity with two monoclonal antibodies (GE2 and BF7) obtained by immunization of mice with human glioblastoma cells.

Here, we describe the identification of the GE2/BF7 antigen as microsomal epoxide hydrolase (mEH), a drug-metabolizing enzyme that is involved both in toxification and detoxification of carcinogens. We initially used immunooaffinity purification using GE2 and BF7 and analyzed the purified proteins by microsequencing. Edman degradation identified 15 amino acids of the NH2-terminal sequence that were 100% identical to mEH. To further confirm the identity of the BF7/GE2 antigen as mEH, we showed that the protein immunopurified with GE2 and BF7 was recognized by an anti-mEH antibody and that in vitro and in vivo synthesized human mEH is recognized by BF7 and GE2 antibodies. Furthermore, anti-mEH antibody recognizes an antigen expressed both in gliomas and reactive astrocytes, as do BF7 and GE2. Finally, we demonstrate that in contrast to what has been reported in rat embryo fibroblasts, p53 does not regulate mEH mRNA expression in glioma cells.

INTRODUCTION

Malignant gliomas represent 65% of primary brain tumors. They remain refractory to conventional treatments such as surgery, radiation, and chemotherapy. Patients suffering from their most malignant representative, glioblastoma, have a median survival of <1 year, despite the best available treatments. Therefore, a deeper understanding of neuro-oncogenesis as well as new therapeutic avenues are imperative.

The development of new therapeutic strategies is dependent on a better understanding of the differences between normal and tumoral brain cells. A putative brain tumor-associated antigen was identified previously by reactivity with two monoclonal antibodies, GE2 and BF7. These antibodies were obtained after screening 345 hybridomas resulting from a fusion between spleen cells of a mouse immunized with human glioblastoma cell line LN-18 and mouse P3 × 63Ag8 myeloma cells (1). Initial testing in an indirect antibody binding RIA showed that GE2 and BF7 reacted preferentially with glioma cell lines (1, 2).

Additional studies demonstrated that these two Mabs3 recognize different epitopes of a common antigen with an estimated size of M, 48,000 (3, 4). Immunostaining showed expression of this antigen in the cytoplasm of cells from glioblastomas, in other tumors of neuroectodermal origin, and in normal liver hepatocytes (2, 5). In contrast, astrocytes from normal brain were stained only weakly in the white matter (5–7). Thus, the expression of the antigen recognized by GE2 and BF7 appeared associated with brain tumors and has the potential to improve our current understanding of brain tumor biology.

In this report, we describe the isolation of the GE2/BF7 antigen by an immunoaffinity purification approach. It was identified as mEH, a drug-metabolizing enzyme that is involved both in toxification and detoxification of carcinogens (8–12).

MATERIALS AND METHODS

Cell Culture and Transfections. Human glioblastoma cell lines (LN-71, LN-729, LN-Z308, LN382T, and 2024) and their culture conditions were described previously (13–15). Doxycycline (Sigma Chemical Co.) was added to a final concentration of 2 µg/ml. Cells growing on coverslips at 60–80% confluency were transiently transfected with the Geneporter reagent (Gene Therapy Systems, Inc.) using 10 µg of DNA with 50 µl of Geneporter reagent for 10-cm culture dishes, according to the manufacturer’s instructions.

Immunoprecipitation and Immunoaffinity Purification. Cell extracts from LN-71 glioblastoma cells were prepared as follows. Cells at 70–90% confluency were washed twice with ice-cold PBS and then scraped off the plates in RIPA buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 1% NP-40, and 1% sodium deoxycholate] supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 3 µg/ml leupeptin, 3 µg/ml pepstatin A, and 3 µg/ml aprotinin). Cell suspensions were subjected to three cycles of freeze-thawing, followed by filtering through a 0.22 µm Millex filter (Millipore). The protein concentrations of the extracts were determined by BCA protein assay (Pierce, Rockford, IL). Extracts were preadsorbed with protein G-Sepharose to diminish elution of proteins binding nonspecifically to the beads. Immunoprecipitation was performed as described previously (16).

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3 The abbreviations used are: Mab, monoclonal antibody; mEH, microsomal epoxide hydrolase; sEH, soluble EH, GFAP, glial fibrillary acidic protein; DAPI, 4',6-diamidino-2-phenylindole; PVDF, polyvinylidene difluoride; LCCP, Leman coiled-coil protein; pAb, polyclonal antibody; GST, glutathione S-transferase; RIA, radioimmunoassay.

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Immunostaining. Tissue sections and cell lines were fixed and stained as described previously using the Vectastain ABC Elite kit (Vector Laboratories, Inc.) and amino-ethyl-carbazole as chromogen (13, 18). Isotope-matched mouse immunoglobulins were used as negative controls. For the double immunofluorescence experiments, the anti Myc-tag 9E10 Mab was used at 1:25 dilution (in PBS, 1% BSA) and the rabbit antirat mEH and BF7 Mab at 1:10. Coverslips were incubated simultaneously with both primary antibodies for 90 min at room temperature, followed by three washing steps with PBS. Mabs were detected with a FITC-conjugated donkey antimoos IgG (Jackson Immunoresearch Lab, Inc.) and the polyclonal antibody was detected with Cy-conjugated donkey antirabbit IgG (Jackson Immunoresearch Lab, Inc.) at dilutions of 1:200 and 1:800, respectively. After incubation for 45 min with a mix of the two secondary antibodies, coverslips were washed three times with PBS and then mounted in antifade medium (DAKO). In the last washing step, DAPI (Boehringer Mannheim, Indianapolis, IN) was added at a final concentration of 1 μg/ml for 3 min to stain the nuclei. Pictures were photographed as slides and subsequently scanned and processed using the Adobe Photoshop software.

SDS-PAGE and Western Blots. Proteins were fractionated on 10% SDS polyacrylamide gels. Silver staining of proteins was performed according to manufacturer’s instructions (Pharmacia Biotech AB). For Western blots, proteins were electrophoresed on Hybond-C membranes (Amersham, Aylesbury, United Kingdom). The primary antibody was used a rabbit polyclonal antibody against the purified rat mEH (19). The secondary antibody was used a goat antirabbit immunoglobulin linked to peroxidase (Nordic). Blots were developed with the BM Chemiluminescence Blotting Substrate POD kit (Boehringer Mannheim).

Protein Sequencing. Purified BF7 antigen was subjected to electrophoresis on a 10% SDS-PAGE and subsequently transferred onto PVDF membrane (Millipore). Protein was revealed by Coomassie blue staining [25% methanol, 7.5% acetic acid, and 0.25% brilliant blue R-250 (Sigma)] for 3 min and subsequent destaining (25% methanol, 7.5% acetic acid) until bands became visible. The M, 49,000 band of the antigen was cut out and sequenced by Edman degradation using Hewlett Packard Model G1005A-4 columns.

Plasmids. To generate the expression construct CMV-MycT/mEH, we replaced the Rab3A DNA sequence of the plasmid Rab3A-myc with the complete coding part of human mEH cDNA. The plasmid Rab3A-myc has a pcDNA3.1 backbone and includes the Myc-tag between the HindIII and BamHI restriction sites in front of the cDNA of Rab3A that is cloned between BamHI and EcoRI restriction sites. Primers hu/mEH1 (5'-TTGGATCCATGTTGGCTAAGAATCCTCCTA-3') and hu/mEH3 (5'-TATGGGGCTTCATTGCGCCGCTCCTACCG-3') were used to amplify by PCR the complete coding sequence of mEH using plasmid pBluescript SK/mEH as template. Restriction sites for BamHI and Apol (underlined) in primers were used for cloning into the expression vector. The control construct CMV-MycT/LCCP was generated in a similar way by replacing the Rab3A cDNA sequence with a PCR fragment harboring the complete cDNA sequence of the gene encoding the LCCP (GenBank AF175966). Primer BamHI/ATG (5'-TTGGATCCATTGACCACTGGAGGCGCTGCTGAGGGAG-3'), which contains a BamHI restriction site, was used in combination with T3 (5'-ATTTACACCCCTCTACTA-3') on pBluescript KS/LCCP as template. The PCR fragment obtained was restricted with BamHI and NotI to replace the Rab3A cDNA. Integrity of the PCR-derived cDNA inserts was verified by sequencing.

In Vitro Transcription and Translation. The construct CMV-MycT/mEH was used as template for the in vitro transcription reaction of the mEH mRNA. The reaction was performed according to the protocol of the mCAP RNA Capping Kit (Stratagene, La Jolla, CA). RNA integrity was verified by analysis of an aliquot on a 1% agarose gel. In vitro translation was performed in a 40-μl reaction with [35S]methionine using a rabbit reticulocyte lysate based kit as described (in vitro Express Translation kit; Stratagene). Ten μl of the reaction were used per immunoprecipitation assay, whereas 5 μl served as control for protein generation.

Northern Blots. Total RNA was extracted with the TRIzol reagent as described by the manufacturer’s protocol (Life Technologies, Inc.). Northern blot analysis was performed as described previously (20). After the transfer and RNA cross-linking, the membrane was soaked in 5% acetic acid for 15 min at room temperature. 18S and 28S RNAs were stained with a methylene blue solution [0.04% methylene blue in 0.5 m sodium acetate (pH 5.2)] for 5 min, scanned, and subsequently destained in water. Probes were generated using a random primed DNA labeling kit (Boehringer Mannheim). The following probes were used: (a) mEH, a 1.4-kb PCR fragment of human mEH cDNA sequence [primers hu/mEH1 and hu/mEH3 (see above) were applied on the template pBluescript SK/mEH to amplify the fragment]; (b) p21, a 2.1-kb NotI cDNA fragment that was isolated from the plasmid pCEP-WAF1 (21); and (c) p53, a 1.8-kb BamHI cDNA fragment that was isolated from the plasmid p53-53N3 (22).

RESULTS
GE2 and BF7 Recognize an Antigen Expressed in Tumoral and Reactive Astrocytes. The reactivity of GE2 and BF7 antibodies, originally derived from a mouse immunized with glioblastoma cell line LN-18, against various human brain tumors has been described in the 1980s (5, 6). These studies showed that most glioma cell lines (see Fig. 1A for an example) and gliomas react with these antibodies. These initial studies suggested that two types of positive stainings can be distinguished in the tumors: (a) moderate cytoplasmic staining of a fraction of glioma cells (Fig. 1, C and D); and (b) strong staining in the cytoplasm and cellular processes of cells with the phenotypic appearance of reactive fibrillary astrocytes (Fig. 1, B and E). The latter staining was also prominent in peritumoral areas (Fig. 1B), during brain inflammation, and in areas associated with epileptic seizures (not shown).

Imunooaffinity Purification of GE2/BF7 Antigen Shows Identity to Human mEH. To identify the GE2/BF7 antigen, we used an immunoaffinity purification approach. We chose glioma cell line LN-71 as an antigen source because it is strongly stained by both Mabs (Fig. 1A). To evaluate the relative amount of antigen present in total cellular extracts of LN-71, we performed immunoprecipitation experiments. Proteins precipitated with the Mabs GE2 and BF7 were separated on SDS-PAGE and detected by silver staining (Fig. 2A). No reducing agent was added to prevent interference of cleaved immunoglobulins with the precipitated antigen that appears as multiple bands under these conditions. These preliminary experiments allowed us to estimate that the GE2/BF7 antigen represents ~0.1% of total protein in LN-71 extracts. This percentage was suitable to proceed with immunoaffinity purification. To perform up-scaled immunoprecipitation experiments, we established a reusable system of chemically cross-linked BF7 Mab to protein G-Sepharose. Purified antigen was subjected to SDS-PAGE, blotted onto a PVDF membrane, and detected by Coomassie staining as a protein with an estimated size of M, 48,000–49,000 (Fig. 2B). The antigen was cut out of the membrane and subjected to Edman degradation. This analysis revealed the following NH2-terminal sequence: MWLEILLTSLVGLFA1. A BLAST search revealed that this sequence has 100% identity to human microsomal epoxide hydrolase, an enzyme of M, 49,000 involved in both activation and detoxification of carcinogens (12).

GE2/BF7 Antigen Is Recognized by a Polyclonal Antibody Directed at Rat mEH. To examine whether an anti-mEH antibody might recognize the antigen defined by BF7 and GE2, we performed a Western blot analysis using a pAb directed against purified rat mEH. The pAb recognized the affinity-purified human GE2/BF7 antigen (Fig. 3A). The size of the human antigen was very similar to the size of purified rat mEH used as a positive control. GE2 and BF7 were described previously as Mabs that recognize different epitopes of a common glioma antigen, as demonstrated by a reciprocal binding inhibition test (1). To confirm this result, we performed Western blot analysis on proteins immunoprecipitated from LN-71 cellular extracts with GE2, BF7, and two unrelated Mabs. In contrast to fibronectin and GFAP-directed Mabs, GE2 and BF7 precipitated a protein of identical size.
that is also recognized by a pAb directed at rat mEH (Fig. 3B). This experiment also suggests that BF7 has a higher affinity toward the LN-71 glioma cell line-derived antigen as compared with GE2. These experiments demonstrate that proteins immunoprecipitated with GE2 and BF7 are recognized by a pAb directed at rat mEH.

**In Vitro and in Vivo Synthesized Human mEH Is Recognized by GE2 and BF7 Antibodies.** To conversely prove that mEH is recognized by GE2 and BF7 antibodies, we synthesized human mEH in *vitro* using a rabbit reticulocyte lysate (see “Materials and Methods”). [35S]Methionine-labeled mEH was immunoprecipitated with GE2 and BF7 and subsequently analyzed by SDS-PAGE. GE2 but not BF7 was able to efficiently precipitate the major Mr 49,000 product of the *in vitro* translation reaction (Fig. 4). We have shown before that BF7 was more efficient in precipitating cell line-derived antigen (Fig. 3B). Therefore, this experiment suggests that in contrast to GE2, BF7 might recognize an epitope requiring posttranslational modification or
that in vitro mEH is not correctly folded, resulting in the loss of the BF7 epitope.

To verify whether BF7 recognizes human mEH when expressed in a mammalian cell, we generated an expression construct where the cDNA of mEH is cloned in-frame behind a Myc-tag (CMV-MycT/mEH). Human glioma cell line LN-229 was selected for this experiment because it expresses very little endogenous mEH, as judged by immunostaining using GE2 and BF7 (not shown). Cells were transiently transfected with the mEH expression construct and analyzed by immunostaining. DAPI staining of nuclei was applied to visualize all cells (Fig. 5, A, D, and G). The Myc-tag-directed Mab 9E10 was used to detect transfected cells that express the tagged human mEH enzyme (Fig. 5B). All Myc-tag-positive cells stained with the pAb directed against rat mEH, proving proper expression of the fusion protein (Fig. 5C). We next examined whether transfected cells expressing human mEH were recognized by BF7 and found that all cells positive for mEH expression were stained by BF7 as well (Fig. 5, E and F). To exclude that mEH staining was attributable to transfection-mediated changes in gene expression, a control construct harboring an unrelated cDNA (LCCP) behind the Myc-tag (CMV-MycT/LCCP) was transiently transfected into LN-229 cells. Transfected cells stained by 9E10 were negative for mEH staining (Fig. 5, H and I), providing evidence for specificity of the observed result in Fig. 5C. These results further demonstrate that the antigen recognized by BF7 in vivo is indeed human mEH.

mEH mRNA Expression Is Not Regulated by p53 in Glioma Cells. mEH has been described as a p53-inducible gene in rat embryo fibroblasts (23). Inactivation of p53 is a frequent event in gliomas and has been considered as a major predictor for response to chemotherapy in some cancers (24). Therefore, we wanted to investigate whether mEH is regulated by p53 in glioma cell lines. We used two p53-inducible systems. One is based on an endogenous temperaturesensitive p53 protein present in cell line LN382T (15). In these cells, p53 activity switches from inactive to active by changing the temperature from 37°C to 34°C. In the second system (2024), a doxycycline-inducible (25) exogenous p53 gene has been introduced in the p53-null cell line LN-Z308 (14). p53 expression is induced in these cells by conditional treatment with doxycycline.\(^4\) Induction of wild-type p53 activity by shift to the permissive temperature of 34°C for LN382T cells or treatment with doxycycline in the case of 2024 cells did not lead to up-regulation of mEH mRNA expression by Northern blotting (Fig. 6). Doxycycline had a slightly inhibitory effect on mEH mRNA expression in 2024 as well as in the parental cell line LN-Z308. To confirm conditional expression of functional p53 protein, the same Northern blot was rehybridized with a probe for the p53 target gene p21\(^{CDKN1}\). These results demonstrate that, unlike in rat embryo fibroblasts, mEH expression is not regulated by p53 in glioma cell lines. This conclusion is also supported by our finding that mEH mRNA levels in 10 glioma cell lines analyzed did not correlate with genomic TP53 gene status (not shown).

**DISCUSSION**

This study identifies the putative glioma-associated antigen GE2/BF7 (1, 2, 4, 7) as mEH, a (de)toxicifying enzyme, using an immunofinity purification approach. Epidemiological studies indicate that at the origin of most human tumors, there is exposure to environmental mutagens and carcinogens (26). Humans are exposed to a vast number of natural epoxides, such as mycotoxins that are frequent contaminants of food (8, 27). Many xenobiotics such as the mycotoxin

\(^4\) M. Albertoni et al., manuscript in preparation.
aflatoxin B₁ and benzo(a)pyrene, found in tobacco smoke, are metabolized by cytochrome P-450-dependent monooxygenases to genotoxic epoxides. To protect themselves against these harmful compounds, cells need an efficient detoxification system.

mEH catalyzes the hydrolyses of reactive epoxides, some of which are known mutagens or carcinogens, to their less harmful and more easily secretable dihydrodiol derivatives. In this way, mEH acts in concert with other phase I and II detoxifying enzymes, such as cytochrome P-450 and glutathione transferase (GST) isoforms (10–12). Besides its role in detoxification of xenobiotics, mEH has on the other hand also been implicated in the formation of even more reactive mutagenic secondary products (10). mEH has been identified as the preneoplastic antigen induced by hepatocarcinogens such as 2-acetylaminofluorene, nitrosamines, aflatoxin B₁, and others in rat liver hyperplastic nodules (Ref. 28 and references therein). The physiological importance of this interesting finding in liver tumorigenesis remains to be resolved.

Our finding that mEH is the putative brain tumor-associated antigen defined previously by monoclonal antibodies BF7 and GE2 (1, 2, 4, 7) opens some questions regarding the role of this enzyme in glioma genesis and development. Polymorphisms in the mEH gene have been associated with different types of cancer (29–31) and have been correlated with altered enzymatic activity attributable to modified protein stability (32). Tyrosine and arginine at position 113 and 139, respectively, are considered to provide high enzymatic activity, whereas histidine at both positions will lead to low activity. High-activity alleles have been associated with an increased risk for developing lung and ovarian cancer, presumably because of enhanced formation of carcinogenic diol-epoxide derivatives (29, 30). On the other hand, low-activity alleles with consequent inefficient detoxification have been associated with susceptibility to hepatocellular carcinoma in areas of aflatoxin B₁ exposure (31) and to susceptibility to emphysema (33). Although some of the genetic alterations associated with glioma development have been identified (34), early oncogenesis of these tumors is not well understood. In animal models, carcinogens such as ethylnitrosourea or methylcholanthrene can induce gliomas (35). In humans, contact with vinyl chloride has been associated with increased incidence of glioblastoma (36). The ultimate carcinogenic metabolite of vinyl chloride is its epoxide, which is a substrate for mEH (37). Therefore, it would be interesting to determine allelic frequencies of polymorphisms in the mEH gene of brain tumors to establish whether a correlation exists between mEH activity and glioma genesis.

Madden et al. (23) have identified mEH as a p53-inducible gene in rat embryo fibroblasts. Furthermore, they were able to demonstrate a strong growth-inhibitory effect of rat mEH on T98G glioma cells in a colony formation assay. Regarding p53 as guardian of the genome, it would make perfect sense to induce a protein whose function is to protect cells from genetic aberrations provoked by reactive epoxides. However, we found that mEH mRNA levels did not correlate with TP53 gene status in glioma cell lines (data not shown). Furthermore, mEH mRNA levels were not regulated by p53 in glioma cell lines with inducible TP53 genes.

The identification of mEH as the putative glioma-associated antigen GE2/BF7 raises the interesting issue of its role in chemoresistance of gliomas. In hepatocytes, the adaptation to growth under toxic carcinogen exposure has been paralleled with an acquired clinical drug resistance (38). Modulation of the detoxification system in such cells includes decreased levels of cytochrome P-450 and increased expression of mEH and GST (28). Such changes could lead to less...
Finally, the finding of high and specific expression of mEH in some pathological tissues could have an impact on new therapeutic strategies. A compound that is nontoxic per se but activatable by mEH could be administered to patients. One such compound could be leukotxin, which has to be activated by mEH or sEH to become cytotoxic (43). Such a system would be superior to the prodrug-activating approaches currently being evaluated in the clinic (thymidine kinase/ganciclovir or cytochrome P-450/cyclophosphamide, for example), where the enzyme has to be first administered by gene therapy (Ref. 44 and references therein). In this case, mEH enzymatic activity is already present in the pathological tissue. Co-administration of a high affinity inhibitor specific for sEH (45) would avoid prodrug activation in other tissues. To prevent adverse side effects of systemic administration of the prodrug in organs with high mEH expression such as liver, lung, and pancreas (30), the local implantation of a biodegradable polymer device could be envisaged to reach high local drug concentrations (46). The application of such a strategy to the brain for the treatment of tumors with elevated mEH expression or epileptic foci will first require careful characterization of endogenous expression of mEH/sEH in all brain areas. Also, it will be important to establish whether elevated mEH activity in reactive astrocytes associated with epileptic foci is intrinsic to the disease or is secondary to the treatment of the patient with medication such as phenytoin or carbamezepine.

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