Huntingtin Interacting Protein 1 Is a Novel Brain Tumor Marker that Associates with Epidermal Growth Factor Receptor

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Abstract

Huntingtin interacting protein 1 (HIP1) is a multidomain oncoprotein whose expression correlates with increased epidermal growth factor receptor (EGFR) levels in certain tumors. For example, HIP1-transformed fibroblasts and HIP1-positive breast cancers have elevated EGFR protein levels. The combined association of HIP1 with huntingtin, the protein that is mutated in Huntington's disease, and the known overexpression of EGFR in glial brain tumors prompted us to explore HIP1 expression in a group of patients with different types of brain cancer. We report here that HIP1 is overexpressed with high frequency in brain cancers and that this overexpression correlates with EGFR and platelet-derived growth factor β receptor expression. Furthermore, serum samples from patients with brain cancer contained anti-HIP1 antibodies more frequently than age-matched brain cancer–free controls. Finally, we report that HIP1 physically associates with EGFR and that this association is independent of the lipid, clathrin, and actin interacting domains of HIP1. These findings suggest that HIP1 may up-regulate or maintain EGFR overexpression in primary brain tumors by directly interacting with the receptor. This novel HIP1-EGFR interaction may work with or independent of HIP1 modulation of EGFR degradation via clathrin-mediated membrane trafficking pathways. Further investigation of HIP1 function in brain cancer biology and validation of its use as a prognostic or predictive brain tumor marker are now warranted. [Cancer Res 2007;67(8):3609–15]

Introduction

Gliomas are the most common primary tumor of the central nervous system (1) and one of the most common molecular defects in these tumors is overexpression/mutation of the epidermal growth factor receptor (EGFR; refs. 2–5). In fact, because of the high frequency of EGFR abnormalities in glioblastoma multiforme, EGFR small-molecule inhibitors (gefitinib/Iressa and erlotinib/Tarceva) are currently undergoing clinical trials in humans (6–9). A clear understanding of the novel pathways that specifically modulate EGFR signaling in brain tumors will be critical when evaluating or devising better brain tumor therapies.

Huntingtin interacting protein 1 (HIP1) and its only known mammalian relative HIP1-related (HIP1r) are clathrin- and inositol lipid–binding proteins that may be involved in neurodegeneration based on the finding that HIP1 interacts with huntingtin, the protein mutated in Huntington's disease (10, 11). HIP1 was first associated with cancer when it was identified as part of the oncogenic HIP1/platelet-derived growth factor β receptor (PDGFβR) fusion protein that resulted from a t(5;7) chromosomal translocation in a patient with chronic myelomonocytic leukemia (12). Subsequent studies have strengthened the role of HIP1 in tumorigenesis by showing HIP1 overexpression in multiple human epithelial tumors including prostate, colon, and breast cancers (13, 14) and that anti-HIP1 antibodies predict the presence of prostate cancer (15). In addition, HIP1 overexpression transforms fibroblasts, and this transformation is associated with altered receptor trafficking and elevated EGFR levels (14). HIP1 is the first clathrin-binding protein directly implicated in human cancer biology, and the dysregulation of growth factor receptor endocytosis provides a novel pathway to pursue for potential cancer therapy targeting (16).

Although HIP1 is thought to function in clathrin-mediated membrane trafficking and its overexpression alters the degradation of growth factor receptors (17) such as EGFR, the precise role(s) of HIP1 in endocytic, actin, lipid, protein degradation, and other undiscovered pathways remain to be defined. We do know that HIP1 family members associate with proteins that are required for endocytosis (e.g., clathrin and AP2; refs. 18–23) and that HIP1 knockdown in HeLa cells inhibits the uptake of transferrin (24). In contrast, the deletion of mouse HIP1 and HIP1r *in vivo* does not completely disrupt clathrin-mediated endocytosis despite the fact that the HIP1/HIP1r–deficient mice display severe degenerative defects and premature death in early adulthood.[3] This surprising *in vivo* data indicates either that HIP1 has additional roles in the cell or that other proteins may partially compensate for HIP1/HIP1r function. Indeed, HIP1 has been shown to interact with the androgen receptor (25) as well as with the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (26). HIP1 and HIP1r also prolong the half-life of both PDGFβR and EGFR on ligand stimulation (17). These diverse effects on distinct types of receptors suggest that HIP1 may affect pathways that are distinct from clathrin-mediated trafficking.

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

S.V. Bradley participated in the design, execution, and interpretation of all of the experiments. E.C. Holland provided sera from brain tumor patients and interpreted the results from the serum experiments. G.Y. Liu obtained data that documented endogenous EGFR association with HIP1. D. Thomas generated and stained the brain tissue microarrays. T.S. Hyun scored the brain tumor arrays for HIP1, PDGFβR, and EGFR expression. T.S. Ross wrote the manuscript with S.V. Bradley and participated in the design and interpretation of all described experiments.

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[3] S.V. Bradley, T.S. Hyun, K.I. Oravecz-Wilson, et al. Degenerative phenotypes caused by the combined deficiency of murine HIP1 and HIP1r are rescued by human HIP1, submitted for publication.

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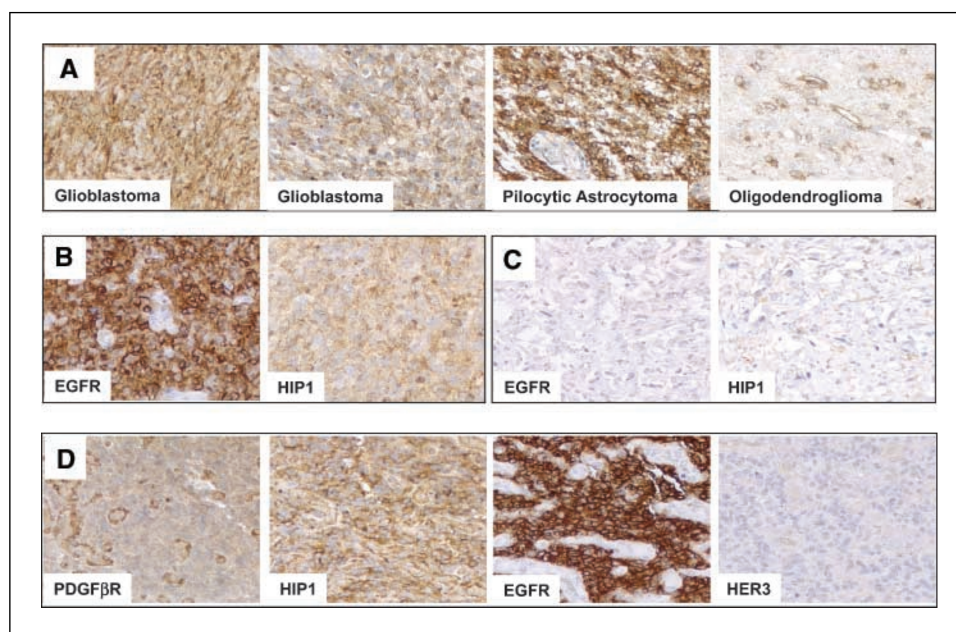

Figure 1. HIP1 expression in brain tumor tissue. Tissue microarrays constructed from normal and neoplastic brain tissues were stained for HIP1 using the human monoclonal antibody HIP1/4B10. Multiple spots from each tumor were scored as follows: 0 (no staining), 1+ (low staining), 2+ (intermediate staining), and 3+ (high staining) by at least two independent observers. An overall positive score was assigned when the average score between reads was ≥2. *A,* glioblastoma multiforme (*Glioblastoma*) with all tumor cells 3+ for HIP1 staining; glioblastoma multiforme with 50% of the tumor cells 3+ for HIP1 staining; pilocytic astrocytoma 3+ for HIP1 staining; and oligodendroglioma with 1+ low staining for HIP1 except for the endothelial cells of blood vessels. *B,* EGFR-positive and HIP1 3+ high-grade glioblastoma multiforme. *C,* EGFR-negative, HIP1-negative glioblastoma multiforme. *D,* PDGFβR-positive, HIP1-positive, EGFR-positive, and HER3-negative glioblastoma multiforme.

The interaction of HIP1 with huntingtin (10, 11), the protein mutated in Huntington's disease, together with its interaction with the AMPA receptor (26), suggests that HIP1 may play a role in neurobiology. Furthermore, HIP1 expression is enriched in the periventricular germinal zones of the developing mouse brain, as well as in neurospheres, both of which are composed of neural progenitor cells (27). This expression in normal progenitors, together with the fact that HIP1 was previously shown to be expressed in brain tumors from a small group of brain cancer patients (13), supports the idea that brain tumor cells may use mechanisms that normal progenitors use for survival and proliferation. In addition, because HIP1 overexpression is associated with EGFR overexpression and EGFR and PDGFβR are overexpressed in brain cancers (28, 29), it was logical to characterize HIP1 protein levels in brain tumor tissue. We examined the expression of HIP1 in neoplastic brain tissues from a large cohort of patients (n = 78), as well as in normal brain tissues from control individuals (n = 75; warm autopsies). We also examined the expression of PDGFβR and EGFR in neoplastic brain tissues from a group of glioblastoma patients (n = 28) and compared it with levels of HIP1 expression. In addition, we show for the first time that HIP1 and HIP1r physically interact with EGFR and discovered that these interactions do not depend on the binding of HIP1 family members to lipid, clathrin, or actin.

Materials and Methods

Coimmunoprecipitation of EGFR with the HIP1 family. Full-length and mutant EGFR, HIP1, and HIP1r cDNA constructs in pcDNA3 have previously been described (13, 17). A 10-cm dish of 70% confluent 293T cells was transfected with 20 μg of total DNA. Thirteen hours after transfection, the cells were lysed using an all-purpose lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 5 mmol/L EGTA, 10% glycerol, Complete EDTA-free protease inhibitor tablets (Roche), 30 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, and 100 μmol/L sodium orthovandate]. One milligram of protein was incubated with preimmune serum, polyclonal anti-HIP1 specific serum, UM323 (8/7/2000 bleed), or anti-HIP1r specific serum, UM374 (2/18/2002 bleed) overnight at 4°C. One hundred microliters of a 50:50 slurry of protein G-sepharose beads and lysis buffer were then incubated with the lysate-antibody mixture for 30 min, with rotation, at room temperature. The protein G pellets were washed four times with 1 mL of lysis buffer. The entire pellet was dissolved in 20-μL SDS sample buffer, boiled for 5 min, separated on 7% SDS-PAGE, and transferred to nitrocellulose. Antibodies used for Western blot analysis were the HIP1/4B10 antibody (monoclonal, human anti-HIP1 immunoglobulin G, 400 ng/mL) and an anti-EGFR antibody (sheep polyclonal antibody, Upstate Biotechnology, Charlottesville, VA; 1:100).

Patient sera samples. Sera from "normal" age-matched controls and patients with brain, skin (melanoma), ovarian, and colon cancers were obtained from the following institutions: Memorial Sloan Kettering Cancer Center (brain), The University of Michigan (controls, melanoma, colon), and the Mayo Clinic (ovarian). The overall ages and gender distribution of the individuals from whom sera were derived are shown in Table 2.

Brain tissue samples. Formalin-fixed, paraffin-embedded tissue blocks of high-grade brain tumors (WHO grades 3 and 4) were obtained from the files of the Department of Pathology, University of Michigan Medical Center (Ann Arbor, MI). Institutional Review Board approval was obtained and the diagnosis was confirmed by morphology. The diagnosis of high-grade glial tumors (anaplastic astrocytoma or oligodendroglioma and glioblastoma multiforme) was defined according to the recently published WHO criteria (2005). After pathologic review, a "high-grade brain tumor" tissue microarray was constructed from glioblastoma multiforme patients using the methods of Nocito et al. (30). Other brain tumor tissues, including oligodendroglioma, pilocytic astrocytoma, ependyoma, gliosis, glioma, medulloblastoma, and peripheral nerve sheath tumors, were screened using a previously generated brain cancer array (referred to as TMA132).

Normal brain samples were derived from men that were diagnosed with prostate cancer and underwent warm autopsy as part of the University of Michigan prostate cancer Specialized Program of Research Excellence program (n = 44) from the Research Genetics normal human array (n = 21), from the TMA132 brain cancer array (n = 7), or from the high-grade brain tumor array (n = 3).

Immunohistochemical staining for HIP1. Immunohistochemical staining was done on DAKO Autostainer (DAKO, Carpinteria, CA) using DAKO LSAB+ and diaminobenzidine as the chromogen. Deparaffinized sections of formalin-fixed tissue at 5-μm thickness were stained for HIP1 (mouse monoclonal antibody, 4B10; 1:10,000, ascites), EGFR, PDGFβR, and HER2/neu levels (DAKO) after microwave citric acid epitope retrieval. Appropriate negative (no primary antibody) and positive controls (prostate carcinoma) were stained in parallel with each set of tumors studied.

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Table 1. HIP1 expression in normal and neoplastic tissue samples

Tissue sample category	Positive	Negative	Frequency	PLR*
Normal brain tissue (n = 75)	21	54	0.28	
All brain tumors† (n = 78 total, 54 glial)	49	29	0.63 †	2.2
Glioma† (n = 7)	7	0	1.00 †	3.6
Oligodendroglioma‡ (n = 9)	7	2	0.78 ‡	2.8
GBM† (n = 38)	27	11	0.71 †	2.5
EGFR-positive GBM (n = 14)	9	3	0.79	2.8
PDGFβR-positive GBM (n = 6)	6	0	1.00	3.6
EGFR- or PDGFβR-positive GBM§ (n = 17)	14	3	0.82§	2.9
EGFR- and PDGFβR-negative GBM (n = 11)	5	6	0.45	1.6

Abbreviation: GBM, glioblastoma multiforme.
* PLR (positive likelihood ratio) = sensitivity / (1 − specificity).
† $P \leq 0.001$, compared with HIP1 staining in normal brain tissue.
‡ $P \leq 0.01$, compared with HIP1 staining in normal brain tissue.
§ $P \leq 0.05$, compared with EGFR- and PDGFβR-negative glioblastoma multiforme.

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Results

HIP1 is overexpressed in primary brain tumors. Tissue microarrays with 75 normal cortical brain tissue samples and 78 brain cancer tissue samples were stained for HIP1 and spots were scored as positive or negative (score <2) for HIP1 expression. For example, Fig. 1*A* displays a glioblastoma multiforme with 3+ positive HIP1 stain; a glioblastoma multiforme with a 2+ positive HIP1 stain; a pilocytic astrocytoma with a 3+ positive HIP1 stain; and an oligodendroglioma with a 1+ negative stain limited to the tumor vasculature. Overall, the frequency of HIP1 expression was significantly higher in primary brain tumor tissue than in normal cortical brain tissue (Table 1; 63% versus 28%; $P < 0.001$, Pearson χ^2). Common glial tumors (low- to high-grade gliomas, oligodendrogliomas, and glioblastoma multiformes) tended to express HIP1 more frequently than less common brain tumor types such as ependymoma, medulloblastoma, pilocytic astrocytoma, and peripheral nerve sheath tumors (Supplementary Table S1). Of note, the staining pattern for the high-grade brain tumors differed from normal tissue in that it was not concentrated in the tumor blood vessel endothelium but rather stained the actual tumor cells. This staining was quite different from previous staining patterns in normal brain tissue, indicating that the high level of HIP1 in the nonneoplastic brain was mainly due to its high level in the central nervous system blood vessel endothelium (13).

Increased frequency of anti-HIP1 antibodies in sera from brain cancer patients. Anti-HIP1 antibodies were present at a higher frequency in prostate cancer patients compared with age-matched male controls, presumably as a result of HIP1 overexpression in patient prostate cancer tissues (15). Because glial brain tumors express higher levels of HIP1 than normal brain tissue, sera from a group of 40 patients with glioblastomas (n = 30) or oligodendrogliomas (n = 10) were analyzed for the presence of anti-HIP1 antibodies (Table 2; Fig. 2). Ninety-three percent of the serum samples from this cohort of glioma patients were positive for anti-HIP1 antibodies compared with 38% of normal individuals ($P < 0.001$). Twenty-seven of 30 (90%) of the glioblastoma multiforme serum samples were positive for HIP1 antibodies ($P < 0.001$, significant difference compared with controls; Table 2), and all 10 (100%) of the oligodendroglioma serum samples were positive for HIP1 antibodies ($P < 0.001$; Table 2). Additionally, glioblastoma multiforme and oligodendroglioma sera displayed similar actual antibody titers (Fig. 2). In comparison, the frequencies of HIP1

Table 2. Frequency of a positive anti-HIP1 antibody blood test in cancer patients

Sera category	Positive	Negative	Frequency	PLR*	Age (±SD), y	Male (%)
Normal controls	23	37	0.38		55 ± 14	44
Brain cancer†	37	3	0.93 †	2.4	51 ± 13	43
Glioblastoma†	27	3	0.90 †	2.3	54 ± 12	44
Oligodendroglioma†	10	0	1.0 †	2.6	42 ± 9	40
Melanoma	32	43	0.43	1.1	54 ± 15	56
Ovarian cancer	7	18	0.28	0.7	59 ± 13	0
Colon cancer	9	15	0.38	1.0	68 ± 10	58

* PLR (positive likelihood ratio) = sensitivity / (1 − specificity).
† $P \leq 0.001$, compared with normal control sera.

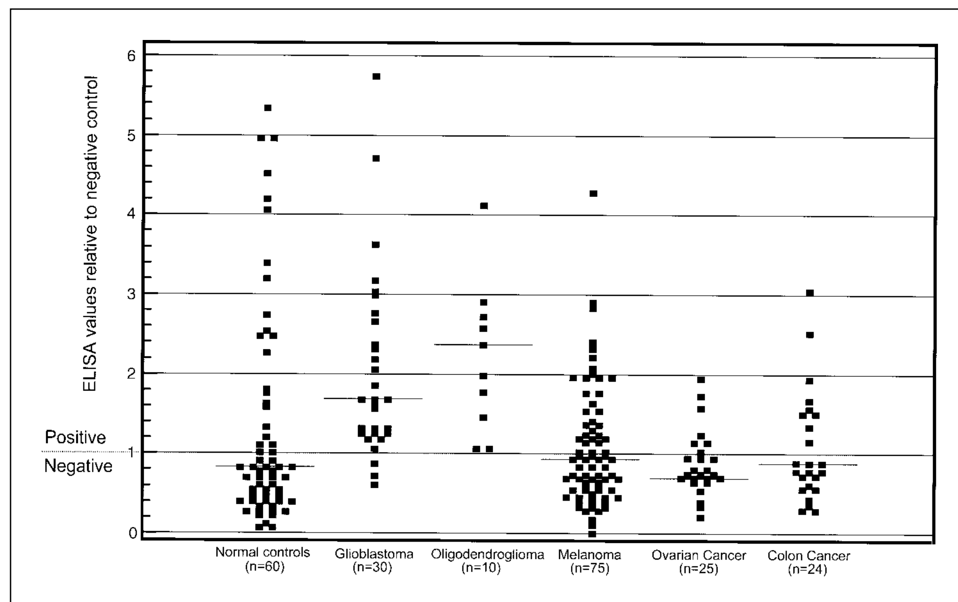

Figure 2. Increased frequency of anti-HIP1 antibodies in sera from human brain cancer patients. Significantly more patients with brain cancer were positive for anti-HIP1 antibodies in their sera, compared with the sera of normal healthy control patients ($P < 0.001$), as measured by ELISA (15). An ELISA value >1, compared with the negative control, was considered positive. Sixty age-matched control sera samples were compared with sera from 30 glioblastoma multiforme and 10 oligodendroglioma patients. Sera from patients with melanoma, ovarian cancer, or colon cancer were not significantly more frequently positive than sera from control individuals without a cancer diagnosis. *Horizontal line,* median value for each group.

antibody positive tests were similar in sera from patients with melanoma, ovarian cancer, colon cancer, and control individuals (Table 2; Fig. 2). Other than in the ovarian cancer group, males and females were equally distributed in the different cancer groups and the presence of HIP1 antibodies did not differ between genders. These data suggest that increased anti-HIP1 antibodies is specific to only a subset of cancers with HIP1 overexpression, such as brain (this study) and prostate cancer (15) patients.

HIP1 overexpression in brain tumors correlates with growth factor receptor expression in brain cancer. EGFR, PDGFβR, and HER3 are established brain tumor markers (28, 29). Taken together with the fact that HIP1 increases levels of both EGFR and PDGFβR in cultured cells (17), these observations suggest that HIP1 expression may correlate with growth factor receptor overexpression in the brain tumors. To test this, glioblastomas were stained for HIP1, EGFR, PDGFβR, and HER3 expression (Table 1). HIP1 staining frequently correlated with EGFR staining (Fig. 1*B–D*). Overall, 14 (79%), 6 (21%), and 3 (11%) glioblastomas from this high-grade brain tumor microarray expressed EGFR, PDGFβR, and/or HER3, respectively (Fig. 1*D*). HIP1 overexpression was also observed in 11 of the 14 (79%) EGFR positive glioblastomas (Table 1, *row 6*). This was significantly different from normal tissue in which only 28% expressed HIP1 (Table 1, *row 1*). All (100%) of the PDGFβR positive glioblastomas expressed HIP1 (Table 1, *row 7*). In contrast, only one of three HER3-positive glioblastomas showed concomitant HIP1 expression (data not shown). The frequency of HIP1 expression was significantly higher (82%) in tumors that were either EGFR or PDGFβR positive (Table 1, *row 8*) than in EGFR- and PDGFβR-negative tumors (45%; Table 1, *row 8* versus *row 9*; $P < 0.05$). These data indicated that EGFR and PDGFβR expression in glioblastomas correlates with HIP1 overexpression. The concomitant overexpression of HIP1 and growth factor receptors in primary neoplastic tissue samples could be the result of altered receptor trafficking or degradation mediated by HIP1. Another possibility is that HIP1 interacts directly with receptors and that a posttranslational interaction increases the overall levels, activation, sensitivity, or localization of the growth factor receptors. Therefore, we tested whether HIP1 directly interacts with growth factor receptors, such as EGFR.

HIP1 associates with EGFR. Because HIP1 overexpression in glioblastoma correlated with EGFR overexpression and because HIP1 previously was shown to bind to clathrin and AP2, which are fundamental components of growth factor receptor endocytosis, we tested if there was a physical association of HIP1 with EGFR. We also evaluated whether this association depended on binding to lipids, clathrin, or actin. First, the association of HIP1 with endogenous EGFR in the liver of a human HIP1 transgenic mouse[3] was tested (Fig. 3*A*). A small reproducible fraction of the total endogenous EGFR indeed was coimmunoprecipitated with HIP1 (*lane 2*). Although this indicated that EGFR is found in a complex with HIP1, it is possible that this interaction is indirect, mediated via the known interaction of HIP1 with clathrin (20–23).

HIP1 family association with EGFR is independent of lipid-, clathrin-, and actin-binding domains. To determine whether the physical association of HIP1 with EGFR required the clathrin-, lipid-, or actin-binding activity of HIP1, human 293T cells, which express low endogenous levels of endogenous EGFR and HIP1/HIP1r, were cotransfected with various HIP1 or HIP1r wild-type or mutant cDNAs. These mutants were differentially capable of lipid, clathrin, or actin interactions (13, 17) together with the full-length EGFR cDNA. Transfected cells were lysed and extracts were immunoprecipitated with polyclonal antibodies against either human HIP1 or human HIP1r, and then blotted for EGFR. Because the levels of transiently transfected proteins are orders of magnitude higher than any endogenous HIP1, HIP1r, or EGFR, we reasoned that this would serve as a pseudo-*in vitro* assay for association of various mutants with EGFR. As expected from the *in vivo* data in Fig. 3*A*, both full-length HIP1 (Fig. 3*B, lane 3*) and full-length HIP1r (Fig. 3*D, lanes 4* and *5*) reproducibly associated with EGFR protein.

Using this *in vitro* assay, several HIP1 and HIP1r mutants then were tested for their ability to associate with EGFR. For example, as we expected, the ΔANTH lipid-binding domain deletion mutants of HIP1 and HIP1r still associated with EGFR [Fig. 3*B* (*lane 4*) and *D* (*lanes 7* and *8*)]. Less expected was the fact that the HIP1 ΔLD and HIP1r "Δ153-632" deletion mutants that do not bind clathrin via the classic LMD (13) or coiled-coil domains

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(17), respectively, retained the ability to interact with EGFR [Fig. 3*C* (*lane 9*) and *D* (*lane 6*)]. Finally, the HIP1 and HIP1r talin homology domain deletion mutants, ΔTH, also retained the capacity to bind EGFR [Fig. 3*C* (*lane 10*) and *D* (*lanes 7* and *8*)]. These data indicated that lipid, clathrin, and actin binding are not required for members of the HIP1 family to associate with EGFR. These data also indicated that the region of HIP1 required for interaction with EGFR lies between amino acids 381 and 814 of human HIP1 (Fig. 4*A*), and that the region of HIP1r required for interaction with EGFR lies between amino acids 633 and 822 of human HIP1r (Fig. 4*B*). This region of HIP1r does not include the coiled-coil or leucine zipper and, although very homologous (70%) with HIP1, does not encode known consensus sequences. Interestingly, previous data indicated that amino acids 690 to 752 of HIP1 were necessary for the transforming activity of the leukemogenic HIP1/PDGFβR fusion protein (Fig. 4*A*, *horizontal striped zone*) and that these amino acids do not include the HIP1 coiled-coil or leucine zipper (31). Thus, the interaction of this HIP1 sequence with growth factor receptors may contribute to the mechanisms of transformation by both overexpressed HIP1 as well as the HIP1/PDGFβR fusion protein. Finally, the ability of HIP1 and HIP1r to associate with EGFR independent of their AP2-, clathrin-, and actin-binding domains suggests that HIP1 and HIP1r directly interact with EGFR. Thus, activities of HIP1, in addition to its role in endocytosis, may contribute to tumorigenesis.

Discussion

Many different molecular changes have been identified and used in human and mouse glial brain tumors, including EGFR overexpression, *Tp53* mutation, *INK4a/b* gene deletion, and *PTEN* mutation (32). Interestingly, although EGFR overexpression has not been clearly prognostic in high-grade brain tumor–bearing patients, it has been predictive in some studies. For example, EGFR-positive anaplastic astrocytoma patients more frequently display a clinical course similar to that of glioblastoma multiforme patients (33). Therefore, EGFR overexpression may be a surrogate for other more direct changes. Additional markers of prognosis, prediction, and dissection of glial cancer biology are sorely needed.

Several candidate brain tumor genes can modulate EGFR and should be investigated as both targets and markers. HIP1 is included in this group for the following reasons. HIP1 is overexpressed in a variety of solid tumors (13); HIP1 overexpression transforms fibroblasts and this transformation is associated with EGFR up-regulation (14); HIP1 posttranslationally stabilizes EGFR (17); HIP1 binds to endocytic factors and thus could define a novel

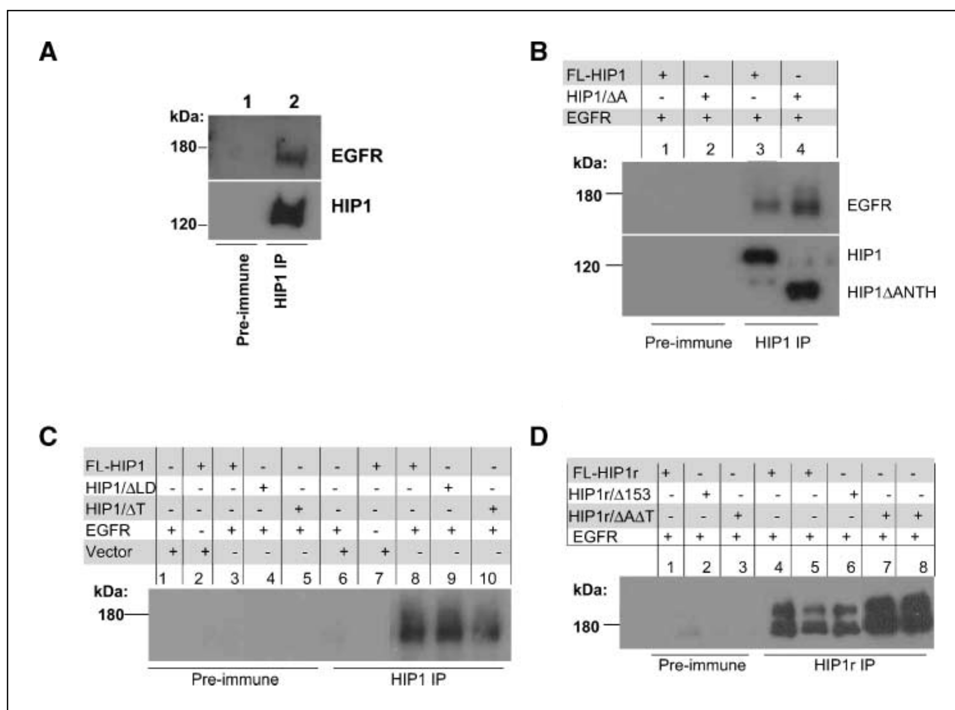

Figure 3. Association of HIP1 family members with EGFR independent of their lipid, clathrin, AP2, or actin interacting domains. *A,* endogenous EGFR associates with HIP1. Liver extracts from HIP1 transgenic mice were analyzed for an endogenous association between HIP1 and EGFR because the levels of EGFR in murine liver were higher than in other tissues, and human HIP1 was expressed at high enough levels to detect the endogenous interaction. Liver extract (4.9 mg) derived from a transgenic mouse that expressed human HIP1 in the liver was immunoprecipitated with either preimmune sera (*lane 1*) or UM323, a polyclonal antibody specific to the COOH-terminal end of HIP1 (*lane 2*), separated by 6% SDS-PAGE and blotted for HIP1 (monoclonal HIP1/4B10) and EGFR (sheep polyclonal, Upstate Biotechnology). *B,* association of HIP1 and the lipid-binding deletion mutant of HIP1 (HIP1/ΔANTH) with EGFR *in vitro*. Ten-centimeter dishes of 70% confluent 293T cells were transfected with 20 μg of total empty vector, EGFR, and HIP1 DNA as indicated above the blot. Thirteen hours after transfection, the cells were lysed and 1 mg of total protein was precipitated with the polyclonal anti-HIP1 antibody, UM323, or a preimmune bleed from the same rabbit as a negative control. Immunoprecipitates then were blotted for EGFR and HIP1. Lanes 1 and 3 were transfected with HIP1 and EGFR. Lanes 2 and 4 were transfected with HIP1/ΔANTH and EGFR. *C,* HIP1 associates with EGFR independent of clathrin or actin binding. Immunoprecipitation of transfected 293T cells, as described in (*B*), indicated that association of HIP1 with EGFR was dependent on transfected HIP1 (*lane 6*) but independent of clathrin/AP2 (ΔLD, *lane 9*) and actin (ΔTH, *lane 10*) interacting domains, as well as the lipid interacting ANTH domain. *D,* HIP1r associates with EGFR independent of lipid, clathrin, or actin binding. Immunoprecipitation of HIP1r-transfected 293T cells, as in (*B*), indicated that association of HIP1r with EGFR was independent of the HIP1r clathrin (Δ153, *lane 6*), lipid, or actin binding (ΔAΔT, *lanes 7* and *8*) domains.

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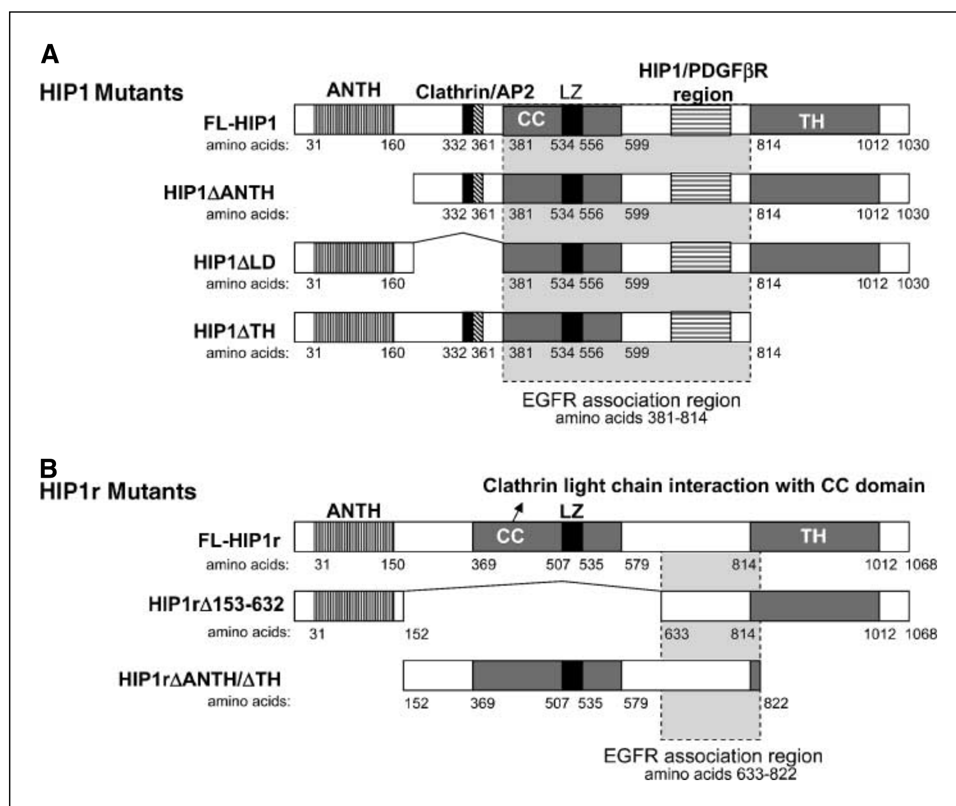

Figure 4. EGFR interacts with the HIP1 family via overlapping regions. *A,* region for EGFR association with HIP1 spans HIP1 amino acids 381 to 814. Overlapping with this EGFR association region is a region (*horizontal striped zone*) that was previously shown to be necessary for HIP1/PDGFβR hematopoietic cell transformation (31). *B,* region for EGFR association with HIP1r spans amino acids 633 to 822. *ANTH,* AP180 NH_2-terminal homology; *CC,* coiled-coil; *LZ,* leucine zipper; *TH,* talin homology.

cellular pathway to target in growth factor overexpressing tumors (20–23); and, because HIP1 interacts with huntingtin, the protein mutated in Huntington's disease (10, 11), abnormal HIP1 expression might alter the biology of brain cells leading to transformation. Interestingly, alteration of the chromosomal 7 region that contains the human *HIP1* locus at 7q11.2 and of the 7p21 region that contains the *EGFR* locus was recently implicated in a glioblastoma multiforme derived from an unusually young man (22 years old; ref. 34). Finally, trisomic chromosome 7 has been described as a frequent abnormality in glioblastoma multiforme tissue (35).

Here we report the expression of HIP1 in brain cancer tissue and identify for the first time its physical interaction with EGFR. We described how HIP1 is overexpressed in a variety glial tumors and how this overexpression is reflected by an increased frequency of anti-HIP1 antibodies in the sera of patients with glioblastoma multiforme and oligodendroglioma. We also showed that HIP1 overexpression correlates with EGFR and PDGFβR overexpression in glioblastomas. These data suggest that the detection of HIP1 in brain tumor tissue and anti-HIP1 antibodies in the blood could aid in the surveillance for different types of brain tumors. Further analysis of these tissue and blood tests in even larger cohorts of brain cancer patients and controls will allow for validation of the correlations of the test results with clinical outcomes.

We are particularly interested in determining whether HIP1 overexpression can be used to identify subsets of patients who could benefit from novel therapeutic strategies that inhibit endocytosis or other pathways modulated by overexpression of the HIP1 family. It also will be important to determine, using larger groups of patients or mouse models of brain cancer, whether HIP1 is involved in the transition of lower-grade astrocytomas to high-grade glial tumors that include the most deadly glioblastoma multiformes.

Because HIP1 expression correlated with elevated growth factor receptor expression, such as EGFR in brain tumors, we asked whether HIP1 could directly associate with EGFR. Indeed, a specific association of HIP1 with EGFR was detected. Because HIP1 was previously shown to interact with lipids, clathrin, and actin, we predicted that one of these interactions (most likely clathrin) may indirectly mediate the observed interaction between HIP1 and EGFR. Remarkably, we found that both HIP1 and HIP1r directly associate with EGFR independent of lipid, clathrin, AP2, and actin binding sites. This observation is consistent with our recent *in vivo* studies of double-deficient HIP1/HIP1r mutant mice. In these mice, we found that endocytosis and EGFR degradation are not necessarily disrupted despite the profound effects of HIP1 loss-of-function on normal physiology.[3]

The ability of HIP1 family members to associate with EGFR suggests that the HIP1 family may directly extend the half-life and expression level of EGFR independent of their role in receptor-mediated endocytosis and degradation by trafficking to the lysosome. We previously showed that the lipid-binding domain of HIP1 is necessary to stabilize EGFR activity, indicating that the physical interaction and stabilization activities may be distinct, rather than linked, HIP1 activities. *In vitro* analysis using smaller deletion mutants of HIP1 and HIP1r, better definition of the region of EGFR that associates with HIP1 and HIP1r sequences, and interactions with other growth factor receptors (e.g., PDGFβR) should be pursued.

In summary, this is the first description of HIP1 expression in a large group of human brain tumors and the first demonstration of the direct physical association of the HIP1 family with EGFR. Future studies will include prospective analysis of tissue and blood from

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larger patient cohorts to identify clinical correlations. In addition, more detailed *in vivo* and *in vitro* studies of the contributing role(s) of HIP1 in brain tumorigenesis will be pursued. Such studies will determine if HIP1 overexpression is sufficient and/or necessary for glial tumor formation using genetically modified mice; if there are genetic alterations (mutation or amplification) of the human HIP1 locus; and if anti-HIP1 antibodies predict clinical outcomes in humans. Finally, the data herein support a search for molecules that target HIP1 therapeutically.

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