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Identification of a novel gene product, *RIG*, that is down-regulated in human glioblastoma

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Genetic deletions to chromosome 10 have been extensively documented for human glioblastomas (GBMs). To identify gene products that may be involved in malignant progression, a subtractive hybridization was performed between GBM cells and hybrid cells suppressed for tumorigenicity following microcell transfer of chromosome 10. One novel cDNA isolated from this subtraction showed consistent upregulation (~4 to 10-fold) that correlated with the nontumorigenic phenotype of the hybrid cells. Subsequent analysis resulted in the identification of a full length cDNA (2,569 bp) termed RIG (*r*egulated *i*n *g*lioma). *RIG* expression was either not detected or detected only at low levels in cultured glioma cells and primary glioblastoma specimens compared to normal brain cells. The 2.6 kb *RIG* mRNA was expressed predominantly in normal brain with lower levels in heart and lung. Sequence analysis showed no significant homology to known gene products. Genomic alterations of *RIG* were present in ~25% of glioma cell lines examined. Also, *RIG* mapped to chromosome 11p15.1, a region that is known to be altered in malignant astrocytomas. The differential expression pattern, tissue distribution and chromosomal location of *RIG* suggests it serves as a molecular marker for or may play a role in the malignant progression of GBMs.

Keywords: glioblastoma; chromosome 11; differential expression

Introduction

Astrocytic tumors are the most common form of primary brain tumors and their histological classification ranges from low grade astrocytomas to glioblastoma multiforme (GBM), the end-stage and most malignant form of the disease. A number of chromosomal abnormalities are observed during the formation of GBM including amplifications of chromosome 7, specifically the EGF-R gene, and deletions of chromosomes 9p, 10p, 10q, 11p, 13, 17p and 22 (Wong *et al*., 1987; James *et al*., 1988; Bigner *et al*., 1990; Fults *et al*., 1992; Rasheed *et al*., 1992, 1995; Ransom *et al*., 1992; Steck *et al*., 1995). Although candidate genes corresponding to some of these genetic losses have been identified (Fults *et al*., 1989; James *et al*., 1989; Kamb *et al*., 1994; Henson *et al*., 1994), the specific genes mapping to the remaining loci remain elusive. For instance, loss of heterozygosity on chromosome 11 has been shown to occur in 30–40% of astrocytic tumors, yet no gene has been identified as the target of this loss (Fults *et al*., 1992; Sonoda *et al*., 1995). By far the most frequent cytogenetic and molecular abnormality observed in GBM is the deletion of large regions of chromosome 10, however no specific region critical to the development of GBM has been identified on this chromosome (Bigner *et al*., 1988; James *et al*., 1988; Ransom *et al*., 1992; Karlbom *et al*., 1993). The efforts to define a critical interval have been hindered by the fact that the loss of large regions or an entire copy of the chromosome occurs in 70–95% of these tumors. Consequently a minimally deleted region has been difficult to delineate (Rasheed *et al*., 1992, 1995; Ransom *et al*., 1992).

Subtractive hybridization is one attractive approach used to define genes that are differentially expressed between normal and tumor cells, because it does not require knowledge of the genomic location of target genes. This technique has been used previously to study differential expression in many tumor systems and to identify candidate genes involved in the suppression of tumorigenesis (Dowdy *et al*., 1991; Lee *et al*., 1991; Hutchins *et al*., 1991; Schweinfest *et al*., 1993; Murphy *et al*., 1993). To address the functional significance of the loss of chromosome 10 in GBM, suppressed hybrid cells were previously created by transferring a chromosome 10 into recipient GBM cells (Pershouse *et al*., 1993; Ligon *et al*., submitted). The resulting hybrid cells and their tumorigenic counterparts were then used to perform a cDNA/mRNA subtractive hybridization. A number of cDNAs were identified through this subtraction, including several whose expression suggested that the presence of an intact, functioning chromosome 10 may have influenced the differentiation status of glioma cells (Ligon *et al*., submitted). In this paper we describe the isolation, characterization and mapping of a novel subtracted cDNA (clone 17) whose expression was coincident with the nontumorigenic phenotype.

Results

Identification of a novel cDNA

We used the clone 17 cDNA isolated by subtractive hybridization to probe a fetal brain cDNA library and identify 32 clones (Ligon *et al*., submitted). One cDNA in particular was selected because it closely approximated the message size (2.6 kb) previously detected by

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Northern analysis with clone 17, and because this longer cDNA (*RIG*, *r*egulated *i*n *g*lioma) was found to contain clone 17 in its entirety. Subsequently, Northern analysis using *RIG* demonstrated a differential expression pattern identical to that of clone 17 in suppressed hybrid cells and their tumorigenic counterparts (Figure 1a). Specifically, *RIG* was expressed four to 10-fold more in nontumorigenic hybrid cells than in GBM cells. A panel of human adult tissues was studied for *RIG* expression and highest mRNA levels were observed in brain, with lower levels detectable in heart and lung (Figure 1b). Placenta, skeletal muscle, kidney, pancreas and liver showed no expression even upon extended exposure of the Northern blot. In addition to *RIG*, five other clones (1.7–2.8 kb) were also identified by this screening and were found to be related by partial sequence homology with the original clone 17. However, *RIG* was the only cDNA that contained the entire clone 17 sequence, indicating that clone 17 likely represented a fragment of *RIG*, and not of any of the related clones. This homology, however, was not the sole reason for the similar expression patterns observed for clone 17 and RIG since identical results were obtained even when clone 17 was removed from *RIG* by enzymatic digestion prior to Northern analysis.

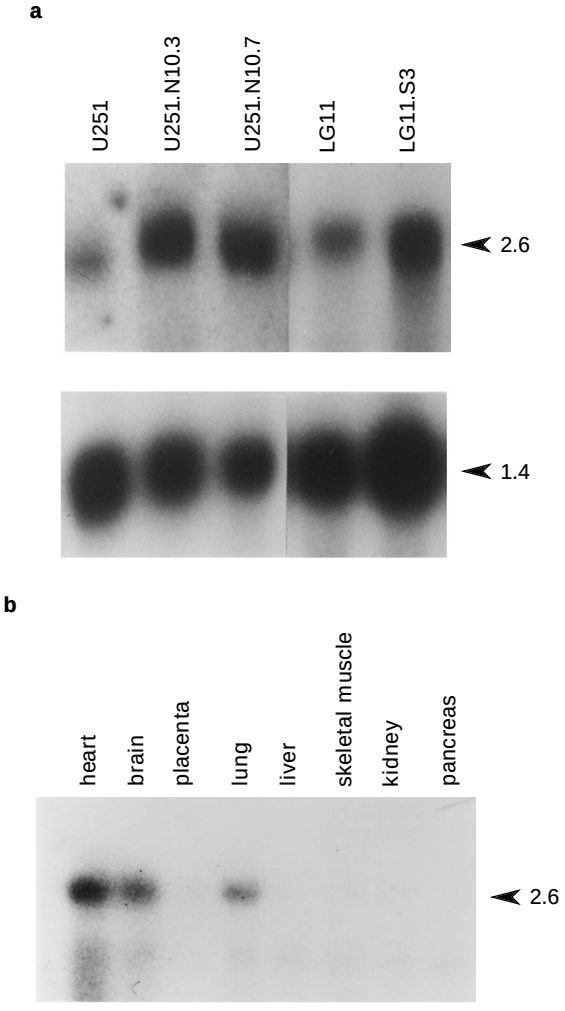

Figure 1 (**a**) Northern blot analysis of mRNA from cultured GBM cells and hybrid cells containing an inserted chromosome 10. *RIG* cDNA was radiolabeled and hybridized against mRNA isolated from cultured cells. Upper panel: (A) preferentially expressed 2.6 kb message is apparent in LG11.S3 cells relative to LG11, and in U251.N10.6 and U251.N10.7 cells relative to U251. Lower panel: Hybridization with GAPDH. Autoradiograms were subjected to densitometric analysis to calculate the ~ threefold decrease of mRNA in U251 cells and a fourfold decrease for LG11 cell compared to the hybrid cells. Relative molecular weight of the mRNAs is depicted on the right. (**b**) Tissue distribution of *RIG* expression is identical to that observed for clone 17. This blot shows increased loading of mRNA in the heart lane relative to brain and lung, yet both tissues showed less *RIG* expression than brain (0.4 × in heart and 0.3 × in lung, relative to brain levels). No message was detected in placenta, liver, skeletal muscle, kidney or pancreas. Signal intensities were quantitated based on control hybridization to β-actin (not shown)

Sequence analysis defined a 2569 bp *RIG* cDNA (Figure 2) and was confirmed by analysing three additional *RIG* cDNAs from independent nontumorigenic specimens. No nucleic acid homologies to any known genes were detected following searches of the relevant databases (EMBL, Genbank) using the BLAST program (Altschul *et al*., 1990). However, a 98% homology was detected between *RIG* and a 286 bp anonymous expressed sequence tag (EST IB666; Khan *et al*., 1992). More recently, this homology was extended to include several related ESTs (EST56233, 59369, 48217 and 59418), all previously isolated from fetal brain libraries.

Sequencing of the RIG cDNA did not identify either a poly(A)$^+$ tail or consensus sequence, so sense and antisense riboprobes were generated and hybridized against Northern blots to determine the correct cDNA orientation (data not shown). This analysis identified the coding strand and oriented the cDNA such that the IB666 sequence appeared in the 3′-untranslated region (Figure 2). Both 5′ and 3′ rapid amplification of cDNA (RACE) were performed to determine whether the *RIG* cDNA sequence was complete, but neither analysis identified any additional sequence. However, since the *RIG* cDNA already approximated the message size determined by Northern analysis, this suggested that the clone was essentially full length.

An examination of seventeen cultured glioma cell lines or primary glioma cell cultures identified five glioma cell lines that showed no *RIG* expression at all (D54, D77, U343, U87, U138). The majority of other cultured gliomas examined showed low levels of *RIG* message (Figure 3). This pattern of absent (or reduced) expression in the tumorigenic GBM cells was relative to message levels seen in cultured nonneoplastic fetal brain cells and human astrocytes (FB138, 13-week astrocytes) and was also similar to levels observed in chromosome 10-containing hybrid cells. However, three cell lines (PH-2, EFC-2 and U118) showed levels of *RIG* expression approximating that of the normal cells. It is worth noting that PH-2 cells were derived from an ependymoma in which alteration of chromosome 10 has not been detected and is not a common event. Similarly, U118 was derived from a mixed tumor with ependymomal and anaplastic astrocytoma components for which the status of chromosome 10 is unknown. The significance of increased *RIG* in EFC-2 cells is unclear, but may be related to genomic alteration at the *RIG* locus. Furthermore, no *RIG* mRNA could be detected in four neuroblastoma and three medulloblastoma-derived cell lines, suggesting that *RIG* expression may be restricted to cells of glial lineage.

To test for any biological significance for preferential expression of *RIG*, a series of brain tumor

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5'- GATGTNCAGACCACAGGAATACCTAATGCCTTTTTTCTCTTCCTGTCTTTGTCCCTCACACTACAGCAGGCCCCTCCCTTCCCTCTTCAACCTCATCCTC
101  CCTCCCCACAGGCCCAGAGAACCAGTTGGGCTTTGTTCTCCTGCAGGCTATGGTTCATCATGCAAATAGCTCCTGTGTCAGAAATGCTTTTTGGCTTCAA
201  CTAACACCAGCTTTATCAATAATAATATCGGTGGATAACAGAAAAGTTTACTTAAGGTGTCCAGAGATGGTGGAGAACAGGATTGGTTTCCTCCTCAATG
301                                                                                          M  Q  L
401  TCAAGGACTCAAAGACTCTTTCTGTGGTAGGGCCACATCCTAAACCCTGTATCCTGTGATTATTTACCTGACAGGCAAAAGAGATTTTGCAGATGCAATT
       R  L  R  T  L  T  W  E  D  C  D  Y  L  P  D  R  A  K  E  I  L  Q  M  Q  L  R  L  R  T  L  T  W  E
501  AAGGTTAAGGACCTTGACGTGGGAAGATTGTGATTATTTACCTGACAGGGCAAAAGAGATTTTGCAGATGCAATTAAGGTTAAGGACCTTGACGTGGGAA
       D  Y  S  G  L  S  R  W  A  Q  F  D  H  M  G  P  Q  K  W  R  T  F  P  T  C  R  K  P  E  S  W  H  L
601  GATTATTCTGGATTATCTAGGTGGGCGCAATTTGATCACATGGGTCCCCAGAAGTGGAGAACCTTTCCCACCTGTAGAAAGCCAGAGAGCTGGCACCTGA
     R  R  T  E  L  S  L  Q  D  L  K  M  K  G  P  M  S  Q  G  M  P  V  T  Y  R  G
     GAAGGACAGAACTGTCATTGCAGGATTTGAAGATGAAGGGGCCCATGAGCCAAGGAATGCCAGTGACCTATAGAGGCTAAAAAACAGCAAGGAAATGGAC

701  TCTCCCCAGAGCCTCCAGAGGAATGCAGCCCTGTTGATCACATGATCACCAGATGGCTGCCCCAGAGCCAAATGTCGCTTCCTGAGCACCATACTCAAAG
801  GCAGGGGAAGTGGATGGAGGGCAGGAGCTCCATTCTTGTTTGCCACTCTCCTTTTGTCAATTGGGAAAAAATTCCAGAAACTCTGGGAGCCCTCCCCTTA
901  CATTTCCTGGGTCATGGGGCCAGCCCTAGCTGCTGGAGGGACTGAGAACAGCTGTTGAGCAGTTTACCTGACGGCATCTGCCATGGCTTGGCAGGAACTC
1001 TGGCTTTGGGAGAGAGCAGCAGCAAGGTATTCAAGCACCACCTCCACCCAGCCCCTCCCACATTTCACTCAGGACTGAGTAAAGGAGACACTCAGATGCT
1101 ACTCAGATGCTGGCTTCAGCTAAGTATTTTGCAAAGCCTCTCGTGTCTTACAAGTTTGTGGCTATCATGACAAAATGGAGCAGCCTACTATATCTACATA
1201 TACAACTATGGGGGACCTAGTTTTATCTCATTTACCACAATGTTTTCAATCATTTTTTGGATGACATAATTTTTAGCCTCTTCTCTAAATGCTTCCTCAA
1301 GCTTTCCTTGCCTTCCAGCCACTGCAAATGACTTGCAGTTTCCCCTACATGNCACCTGACCCTTGTGCCTCCCTCCCTCTGCCCATGNCCAGAAAGCCCT
1401 TTNCTGTGCCCTCTGGCTTCCTGATAAACTCCTATCATCTTCAAGAGCCAGTTCCCATGCCAGCTCTCCCCAAGTGCTCCACTGAGGCTTCCGTAACACC
1501 TCTGTTCCCACATCGGGTTGACTGTCTTTGTTTTGTCATTGCTTGCTCTGGCTGTGTCTCCCTCATTAGACTGGGATGCCTTCAAGGTAGGGACCCTATC
1601 TGGGTCAGCTTGGCACCCCAAAGCGTACCACAGCACCTGATNCTGAGGAGGCTCTCAGTAGATATCTGTTGAGTAACCAGAATGTAGGGTGGTCCTGATG
1701 GTTTCTGACATTGAATAGAAAACAGCTCCCTATTTGATCTTAAAATAATCACTATAACCTGGACATACTGTACTAGATGCTGTTTTTGTCTGACTTCTAC
1801 TCTGTCAATCTCTTTGCACCTCCATTTGTTCATCTGTGAAATGAAGAAAATGCTCATGGAGTTCAGTGAAGATTAAATGAATGAATATAGGTAGACTGCC
1901 TAATCTGGCACTTGCCACGCAGCTGACTTCAATATAGTAGCTCTAATATTATGGTCCTTGAGGATCTTACTGTCTTATGGCCCAGAACTGCATTTGATTA
2001 AAGAAGGCTNNCCTAAAAAAAGAGTCATACATATTCCATTTGTCCTTTCAGAAGGCCGTGAAGCATTTACACTCTTTAAGACAAATTCCCATCCAAAAAT
2101 AGTTAAGATTTCTAAAATATTTTGATGCTGAAAGAGGTGTGCTTCAGTTGGGTGGCAAATTTGCTTCTATGGAAGATTTTTAATACAGGTTGTTTCTATT
2201 TTACTTTTTCTGGCTGAAAGGATTTTACATTTATTCAAAGTCAAAAGGGAAAAGAAATCCAAGAACTACAGAAGAGCAGTTGAAGTGATTTATGCTTGAT
2301 TTCTAAATGCAACTTATGTTTATACATAATTTAAAACTCAAAGAAAGCATGCTTATACAATCATGTGCAACTTTAAACTTTAAGAACTCTGGATGAATAC
2401 TGGTGGCAACAGTCCATGACACCTGAAAACATCATTTGTGGAGTGAGCGTAGAGTTCAGTGTTCGCAGTCGCATATTACAACCATGTTTCACACAGCCCT
2501 GCTCGGTTTGATTTTCTCCACGTGGTTGATAATTGTCTTCAGTTGCTGCTAAGTGATTTTGCAAATTTCG---3'
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Figure 2 The sequence of *RIG* cDNA. The bold underline indicates the predicted *RIG* open reading frame with the corresponding amino acid sequence. The functional initiation codon is indicated in bold face. The thin underline represents the region with homology to clone 17

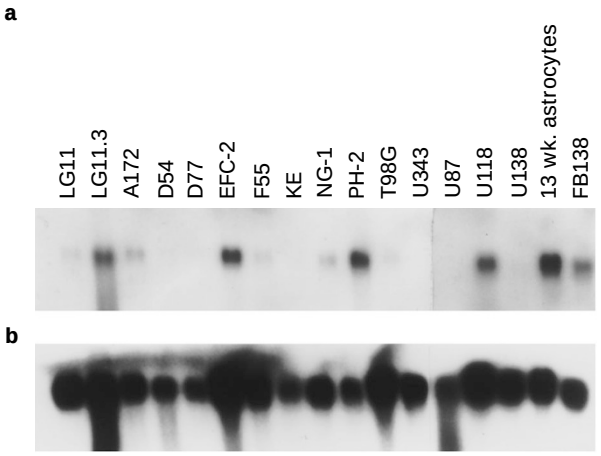

Figure 3 Northern blot analysis of *RIG* expression in cultured GBM cells. Upper panel: Note the increased expression of *RIG* in LG11.S3 cells compared with LG11 cells as well as significant levels of *RIG* message present in 13 week astrocytes (13 weeks) and fetal brain cells (FB138). No *RIG* expression, or decreased *RIG* expression, was detected in the majority of GBM samples examined. D77 and F55 are primary cell cultures of newly explanted human GBMs. Lower panel: Hybridization with GAPDH

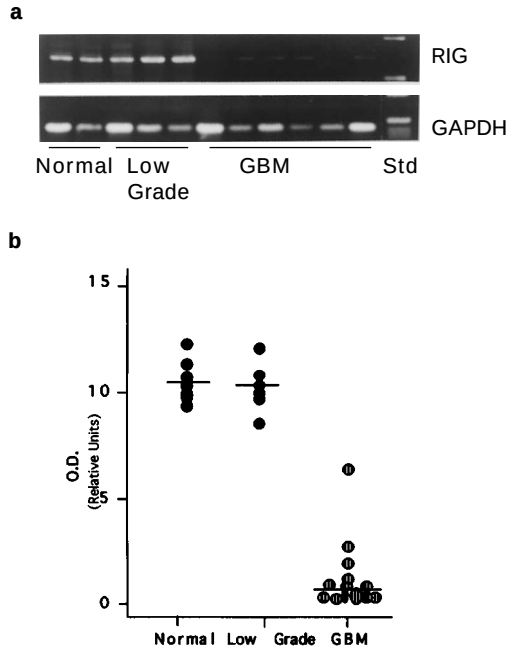

Figure 4 RT–PCR analysis of *RIG* expression of cDNA synthesized from frozen sections of normal brain, low grade astrocytomas, and glioblastomas. (**a**) The expression of *RIG* and GAPDH following RT–PCR. The mRNA was treated with DNAse prior to cDNA synthesis to remove contaminating genomic DNA. (**b**) The quantitation of PCR products were determined by densitometry and compared to GAPDH expression. The horizontal bar represents the mean of the determined ratio of *RIG*/GAPDH expression

specimens was examined by RT-PCR analysis (Figure 4). A significant decrease in *RIG* message was found in malignant GBM tumors compared to the levels observed in normal brain and in low-grade astrocytomas. Two GBM derived specimens did show increased expression of *RIG*, however, study of adjacent histological sections of these samples revealed the presence of normal cells, which are a likely source of increased *RIG* message. These results demonstrate that decreased expression of *RIG* is not restricted to cultured GBM cells but is also evidenced in tumor specimens.

Genomic structure of RIG

We examined the genomic structure of *RIG* in cultured glioma cell lines, normal astrocytic cells and tumor specimens. In the majority of samples examined, no genomic alterations of *RIG* were detectable. However,

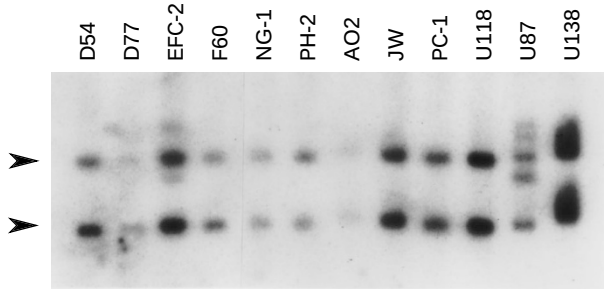

Figure 5 Southern blot analysis of DNA from cultured GBM cell lines probed with *RIG* cDNA. The 2.0 kb and 2.5 kb bands in the *Hind*III digests are depicted. Genomic alterations were observed in three GBM cultured cell lines: EFC-2, U87 and U138

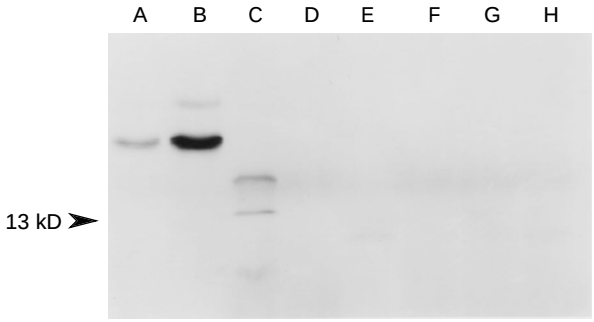

Figure 6 Coupled *in vitro* transcription and translation assay. *RIG* cDNA was cloned into the pGEMzf(−) and pBluescript (SK^-) vectors. Each construct was used in the TNT assay in the sense and antisense orientations. Only the sense orientations of each construct (lanes C and G) produced a 12 kD protein. The larger product visible in lane C is synthesized using an initiation codon located upstream of *RIG*, within the pGEM vector. This product is not synthesized when the pBluescript/*RIG* construct is transcribed and translated. Lanes: (**a**) positive control for T3 RNA polymerase; (**b**) positive control for T7 RNA polymerase; (**c**) *RIG*/pGEM, sense orientation; (**d**) *RIG*/pGEM, antisense orientation; (**e**) *RIG*/pGEM, negative control (no template); (**f**) *RIG*/pBluescript, antisense orientation; (**g**) *RIG*/pBluescript, sense orientation; (**h**) *RIG*/pBluescript, negative control (no template)

in three cultured GBM cell lines (U87, U138 and EFC-2) genomic alterations were detected following *Hind*III or *Eco*RI digests (Figure 5). The rearrangements identified in EFC-2 cells are notable because these cells consistently demonstrated increased expression of *RIG* which may be, in part, due to the gross changes in gene structure. In contrast, U87 cells express no *RIG* mRNA at all, while U138 cells express barely detectable quantities of *RIG*. Southern blot analyses were also used to determine that *RIG* is a relatively small gene, less than 5.1 kb in size. Furthermore, polymerase chain reaction (PCR) primers designed to amplify the predicted open reading frame amplified the same sized products whether cDNA or genomic DNA was used as a template, suggesting that *RIG* consists of a single coding exon (data not shown).

Predicted protein product of RIG

We identified a likely, though small (285 bp), open reading frame for RIG beginning at position 531 (see Figure 2). Evidence to support this codon as the correct site of initiation was obtained using coupled *in vitro* transcription and translation (TNT). This assay demonstrated that the *RIG* cDNA can direct the synthesis of an approximately 12 kD protein (Figure 6), in agreement with the predicted size (11.5 kD). In order to confirm this codon as the correct initiation site, we cloned the RIG cDNA into two independent vectors and then tested each construct in both the sense and antisense directions. The predicted protein (11.5 kD) could be identified in both the 'sense' TNT reactions but not with either of the 'antisense' reactions. As seen in Figure 6, a larger protein product (approximately 25 kD) was also identified when the TNT reaction was performed using the sense orientation of the *RIG*-pGEM construct. Since the size of this product could not be attributed to any reading frames within *RIG*, we examined the surrounding sequence and found an in-frame initiation codon within the multicloning site of the pGEM vector. Initiation of translation from this site would result in a 25 kD product. Indeed, this resulting fusion product was not detected in any of the TNT reactions that were performed using the *RIG*-Bluescript construct and can therefore be considered artifactual.

Amino acid sequence analysis of the predicted RIG protein using the BLASTp search program (including protein databases such as SWISS-PROT, Brookhaven Protein Data Bank, PIR and GenPept.) did not identify homology to any known proteins. The amino acid content was noted to be rich in arginine, threonine, and leucine residues. Two-dimensional electrophoresis of the TNT protein product confirmed the synthesis of a protein product with a relatively high pI.

Genomic mapping of RIG

A combination of somatic cell hybrid mapping and fluorescence *in situ* hybridization was used to map the genomic location of *RIG*. Hybridization of *RIG* to a somatic cell mapping panel of *Hind*III digested DNA showed two predominant bands of 2.0 and 2.5 kb only in DNA that contained human chromosome 11, thereby mapping *RIG* to this chromosome. Furthermore, a 3.2 kb band was observed in hamster DNA and a single 6.6 kb band in mouse DNA, indicating evolutionary conservation of the *RIG* gene amongst these rodents.

The *RIG* cDNA was used to screen a P1 library and one of the identified genomic clones was used as a probe for fluorescence *in situ* hybridization (FISH) of metaphase chromosomes. Positive signals were seen on the short arm of a pair of C group chromosomes with a fractional telomeric length (FTL) of 0.12 ± 0.02 (Figure 7a). FISH was repeated on G-banded chromosomes and identified the target as chromosome 11 and also allowed us to map the *RIG* gene to 11p15.1 (data not shown). We also performed FISH using metaphases prepared from U87 cells that observed only one signal on an apparently normal chromosome 11. The second signal was observed on a marker chromosome, illustrating a gross rearrangement of the 11p region. Although FISH results suggest that the *RIG* allele on this marker chromosome may not be affected, our southern analysis has demonstrated genomic rearrangements of *RIG* in these cells.

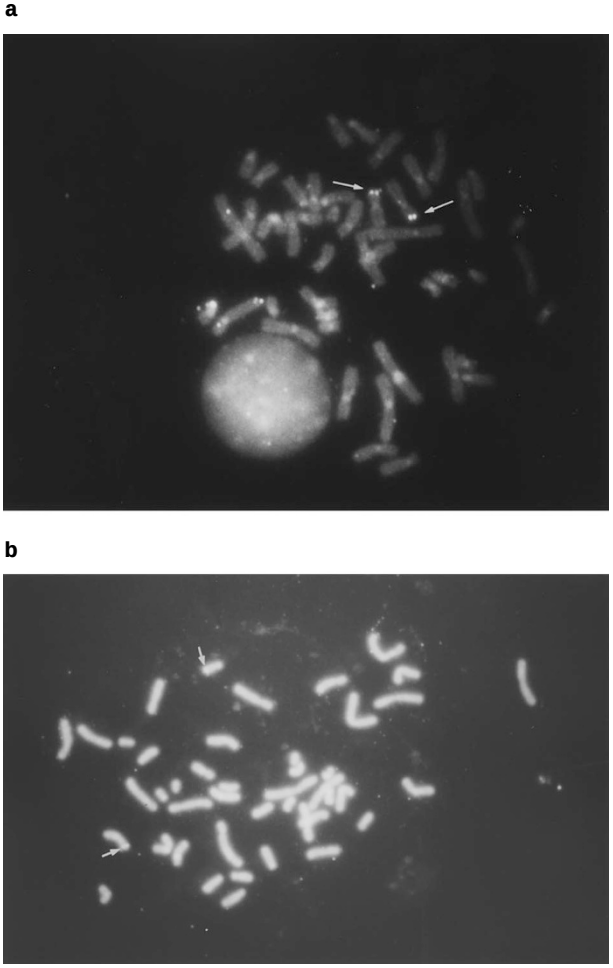

Figure 7 (**a**) Fluorescence *in situ* hybridization of *RIG*-genomic DNA with normal human lymphocyte metaphases. A P1 clone (#1088) containing the *RIG* gene was fluorescently labeled and hybridized against metaphase chromsomes. Two sets of positive signals indicate *RIG* maps to chromosome 11p. (**b**) Fluorescence *in situ* hybridization of the same P1 clone with metaphases isolated from U87 cells

Discussion

The role of tumor suppressor genes in oncogenesis has been well documented (Weinberg, 1991; Marshall, 1991; Stanbridge, 1992). Clearly, loss of specific genes or even entire chromosomes are only two methods by which the negative regulatory function of these genes may be compromised. Here we describe a novel cDNA (*RIG*), isolated through subtractive hybridization, which appears to be involved in, or act as a marker of, malignant GBM formation. *RIG* was initially identified as one of several genes which exhibited a differential expression between tumorigenic and suppressed GBM cells. This gene product was chosen for further analysis based on its expression predominantly in the brain and its large degree of preferential expression pattern.

Searches of relevant databases showed *RIG* to be a novel gene product whose only homology was to an anonymous EST previously cloned from an infant brain cDNA library (Khan *et al*., 1992). The abundance of this message in fetal brain was low, estimated to be 0.004% based on the frequency with which clones were isolated during the fetal brain library screening, although there is apparently increased expression in adult brain. A striking feature of *RIG* was its consistent preferential expression in normal human brain relative to gliomas. Significant levels of *RIG* message were observed in both cultured astrocytes and in normal adult brain. In contrast, decreased *RIG* expression was found in cultured GBM cells and primary GBM specimens compared to that of normal glia. Examination of a number of primary glioma samples ($n=17$) demonstrated significantly decreased levels of RIG mRNA, with five samples (30%) showing a complete lack of expression. Gliomas of histologic lower grade showed an expression level similar to normal brain suggesting that decreased expression of *RIG* may be correlated with the functional status of a glioma tumor suppressor gene(s) on chromosome 10. Deletions of chromosome 10 are predominantly associated with the malignant progression of GBM (James *et al*., 1988). Also supporting this possibility is the fact that *RIG* was originally isolated based on hybrid glioma cells containing a functional copy of chromosome 10 (Ligon *et al*., submitted). Furthermore, two cell lines derived from a low-grade astrocytoma and an ependymoma, both tumor types with no known alterations of chromosome 10, showed similarly high levels of *RIG* mRNA. Therefore, in this study, *RIG* expression is correlated with the presence of functional tumor suppressive genes on chromosome 10. Since the identity of the suppressive gene(s) on chromosome 10 is currently unknown, *RIG* may represent the first molecular marker to reflect the status of a glioma tumor suppressor gene.

The genomic location (11p15.1) and observed alterations to this locus raise some interesting possibilities for *RIG* involvement in oncogenesis. Loss of heterozygosity studies have previously implicated chromosome 11p15 in the genesis of multiple cancers, including Wilms tumor, small cell lung carcinoma, non-small cell lung carcinoma, bladder carcinoma, breast carcinoma and astrocytoma (Fults *et al*., 1992; Sonoda *et al*., 1995; Weston *et al*, 1989; Ludwig *et al*., 1991; Hao *et al*., 1993; Wingvist *et al*., 1993). Since most allelic deletion studies of astrocytomas have implicated the region 11p15.5 and not 11p15.1, it appears unlikely that *RIG* would be deleted in astrocytomas. However, allelic deletions around 11p15.1 were not investigated in most studies (Fults *et al*., 1992; Sonoda *et al*., 1995). We identified structural alterations of *RIG* in three cultured GBM cell lines. These rearrangements are notable because one GBM-derived cell line (EFC-2) was shown to express *RIG* at a level comparable to 'normal' cells, whereas the remaining two cell lines show very low or undetectable levels of *RIG* message. Together these data suggest a possible scenario in which *RIG* may act downstream of the 10q glioma tumor suppressor gene. Further studies are required to determine whether structural changes in *RIG* (or in associated cis elements) contribute to its preferential expression in low grade tumors, and to define the biological mechanism(s) through which *RIG* acts.

Transcription and translation studies confirmed that, at least *in vitro*, *RIG* can direct the synthesis of the predicted 11.5 kD protein product. The highly basic

nature of this protein allows for speculation regarding a possible interaction between a RIG protein and negatively charged molecules, perhaps including DNA. Alternatively, as no known protein motifs were detected in the amino acid sequence, the gene product of *RIG* may not be a protein, but rather an RNA molecule itself. Precedence for this can be found in studies of the H19 RNA, an RNA that has been suggested to act as a tumor suppressor *in vitro* and coincidentally also maps near *RIG* at 11p15.5 (Brannan *et al*., 1990; Jaber *et al*., 1994).

The identification of a set of cDNAs related to *RIG* by partial sequence homology is intriguing. Thus far, five independent clones have been identified sharing a partial homology with clone 17. The IB666 sequence (90–130 bp) appears to be confined to the 3′ untranslated region of the messages, and the possibility that this may represent a functional cis-element is presently under study. The further characterization of this novel family of genes may aid in our understanding of the oncogenesis of brain tumors, however, the expression of RIG should serve to reflect the status of a chromosome 10 tumor suppressor gene.

Materials and methods

Tissues and cells

The generation and culture of the somatic cell hybrids have been previously described (Pershouse *et al*., 1993; Ligon *et al*., submitted). The glioma cell cultures have been established in our laboratory either from glioma specimens or were obtained from ATCC (Yung *et al*., 1988). Glioma specimens were kindly provided and examined by Dr L Langford, Department of Pathology, UTMADCC.

DNA analysis

A human fetal brain expression library was obtained from Stratagene (La Jolla, CA) and screened with a radioactively labeled clone 17 cDNA. Labeling of the isolated insert was performed with [α^{32}P]dCTP using the random primed method (Feinberg and Vogelstein, 1984). Positive plaques were purified in three rounds of screening and the inserts were obtained by PCR following the procedures previously described (Ligon *et al*., submitted). The inserts were cloned into the pCRII vector using the TA cloning kit (InVitrogen, San Diego, CA). Southern blot analysis was performed using DNA isolated from cell cultures or tumor specimens according to standard laboratory procedures (Sambrook *et al*, 1989). The DNA was transferred to Hybond N^+ membranes (Amersham, Chicago, IL), u.v. cross-linked according to manufacturer's protocols and hybridized.

For sequencing, the cDNA insert was cloned into the *EcoRV* site of the pGEM5zf(−) vector (Promega, Madison, WI) before creating the deletion constructs. An array of inserts representing nested deletions spanning the *RIG* cDNA was then selected for sequencing using the T7 primer. A total of 15 overlapping inserts, each successively deleted by approximately 150–200 bp, was sequenced to obtain the full-length sequence of *RIG*. The sequence of the complementary strand was confirmed using both primer walking and automated sequencing.

Rapid amplification of cDNA ends (RACE) was performed in both directions (Frohman *et al*., 1988). For 3′ RACE, the following primers were synthesized: internal primers 5′-gggaaaagaaatccaagaact-3′ or 5′-gcatgcttatacaatcatgtg-3′ and oligo(dt) primer 5′-gactcgagtcgacatcgatttttttttttttttt-3′. The 5′ AmpliFINDER RACE kit (Clontech, Palo, Alto, CA) was used to perform 5′ RACE.

RNA analysis

Poly(A)$^+$ mRNA was isolated using Fast Track mRNA isolation kit (InVitrogen) from cells or from fresh frozen sections of human gliomas. mRNA (3 μg) was electrophoresed in formaldehyde gels, blotted onto Hybond N^+ membranes, and then hybridized (Sambrook *et al*., 1989). Alternatively, mRNA was treated with DNAse and used to synthesize cDNA with oligo dT primers and Superscript reverse transcriptase (GIBCO). Quantitative comparisons of RNAs were made by densitometric comparison to glyceraldehyde-3-phosphate dehydrogenase hybridization or (for PCR analysis) using primers specific for glyceraldehyde-3-phosphate.

An *in vitro* transcription kit (Promega) was used to synthesize riboprobes. Riboprobes were generated from the sense and antisense directions of the *RIG* cDNA to ascertain the orientation by northern analysis. Hybridization with the appropriate sense or antisense riboprobe was performed according to the manufacturer's instructions.

In vitro *transcription and translation*

The coupled TNT rabbit reticulocyte lysate system (Promega) was used to synthesize the *RIG* peptide product. *RIG* cDNA was first subcloned independently into two plasmid vectors, pBluescript SK^- (Stratagene) and pGEM5f(z) (Promega), and each construct was used to generate both sense and antisense transcripts from *RIG* cDNA. Translated reactions were then electrophoresed on a 5–15% gradient SDS-polyacrylamide gel, followed by fluorography and exposure to X-ray film.

Chromosomal localization

A commercially produced somatic cell hybrid mapping panel consisting of various mouse-human and hamster-human hybrids was obtained from BIOS (New Haven, CT). Southern blot analysis was performed with ^{32}P-labeled cDNA probes (*RIG*, clone 17). Fluorescence *in situ* hybridization was performed with a P1 clone obtained commercially by screening a P1 library with the *RIG* cDNA (BIOS P1 #1488). The P1 clone was biotinylated using nick-translations then hybridized with normal human lymphocyte metaphases. Slides were washed and detected with avidin-fluorescein isothiocyanate and amplified with anti-avidin as previously described (Steck *et al*., 1995; Pershouse *et al*., 1993).

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