Expression of the src homology 3 (SH3) domain-containing expressed in tumorigenic astrocytes (SETA) gene is associated with the tumorigenic state in astrocytes. SETA encodes a variety of adapter proteins containing either one or two SH3 domains, as suggested by the sequence heterogeneity of isolated cDNAs. Using both SH3 domains in a yeast two-hybrid screen of a glioblastoma cell cDNA library, we isolated the rat homolog of the ALG-2-interacting protein 1 or ALG-2-interacting protein X (AIP1/Alix). In vitro confrontation experiments showed that the SH3-N domain of SETA interacted with the proline-rich C terminus of AIP1. In co-immunoprecipitation experiments, SETA and AIP1 interacted and could form a complex with apoptosis-linked gene 2 protein. Endogenous SETA and AIP1 proteins showed similar patterns of staining in primary rat astrocytes. Misexpression of a variety of SETA protein isoforms in these astrocytes revealed that they localized to the actin cytoskeleton. Furthermore, SETA proteins containing the SH3-N domain were able to sensitize astrocytes to apoptosis induced by UV irradiation. Expression of the isolated SH3-N domain had the greatest effect in these experiments, indicating that interference in the interaction between endogenous SETA and AIP1 sensitizes astrocytes to apoptosis in response to DNA damage.

The src homology 3 (SH3)3 domain-containing expressed in tumorigenic astrocytes (SETA) gene was isolated from differentiating glial cells and implicated in primary brain tumors on the basis of its expression in malignant astrocytes in culture and gliomas in the adult brain in vivo (1). While SETA mRNA is expressed in the developing rodent brain at high levels, it is barely detectable in the adult rat or mouse cortex or in normal human brain. However, approximately half of all experimentally induced rat gliomas as well as human astrocytomas of grade II, III, and IV express the gene, and it is also found in oligodendrogliomas and oligoastrocytomas (1). Furthermore, expression of SETA in a culture model of astrocytoma progression based on astrocytes from p53 knockout mice (2, 3) was closely associated with the ability of these cells to form tumors when reintroduced into animals (1). In this model system, SETA shared expression patterns with established glioma-associated genes including epidermal growth factor receptor, platelet-derived growth factor receptors α and β, vascular endothelial cell growth factor, and protein kinase C-δ (3). These expression data suggest that the re-expression of the SETA protein in astrocytes in the mature central nervous system contributes to their malignant transformation and progression, prompting an investigation of its mode of action at the molecular level.

The SETA gene encodes proteins that contain SH3 domains, which are involved in high affinity, specific protein-protein interactions. While these domains are found in proteins with enzymatic function, such as Src kinase, they are also common in adapter molecules whose function is to promote the interaction of members of signal transduction pathways. SETA appears to belong in this group and to be a member of a new subfamily of adapter molecules that includes the CD2AP and CMS proteins (4, 5). In addition to SH3 domains, these proteins appear to have coiled-coil motifs at their C termini and two PXXP motifs, which are themselves the cognates of SH3 domains. Therefore, they appear to have at least three modalities of binding to other proteins, suggesting involvement in a complex series of protein-protein interactions. While CD2AP has been shown to interact with the CD2 molecule in T-cells (4), CMS has been shown to interact with actin, p130cas, the p85 subunit of phosphatidylinositol 3-kinase, src family kinases, and Grb2 (5), suggesting that this family of proteins may play a role in cell architecture and mitogenic signaling.

As a first step toward understanding the function of SETA, we have taken the direct approach of isolating a binding partner with a yeast two-hybrid library screen. The protein we isolated is the rat homolog of the mouse gene, apoptosis-linked gene 2 (ALG-2)-interacting protein 1 or ALG-2-interacting protein X (AIP1/Alix), which was recently isolated by two groups performing yeast two-hybrid screens with the ALG-2 (6, 7). In vitro confrontation and co-immunoprecipitation from transiently transfected cells demonstrated that the SH3-N domain of SETA interacts with the proline-rich C terminus of AIP1 and that SETA, AIP1, and ALG-2 can form a complex.

ALG-2 was originally isolated in a “death trap” screen in T-cells, and an antisense ALG-2 cDNA promoted survival after apoptosis had been induced by a variety of stimuli (8). Conversely, when ALG-2 was overexpressed in fibroblasts it sensitized them to cell death. Therefore, it has been suggested that the 22-kDa calcium-binding protein ALG-2 is a necessary component of the apoptotic machinery (8). AIP1 is a 105-kDa pro-
tein with a proline-rich C terminus that has 10 PXXP sequence motifs of the kind that bind to SH3 domains (7). While the mechanism of action of AIP1 alone or in conjunction with ALG-2 is not understood, it is clear that perturbing the levels of these proteins in a variety of cells alters their response to apoptotic stimuli without affecting the background level of apoptosis (7, 8). In this paper, we show that these genes are expressed in astrocytes and glialoma cells, which also express endogenous SETA proteins. Furthermore, introducing SETA proteins capable of binding to AIP1 sensitized astrocytes to UV light-induced cell death, while control cells or those expressing a part of the SETA protein that does not bind AIP1 had no effect. These data suggest that SETA, AIP1, and ALG-2 represent a new set of proteins with a role in modulating apoptosis in astrocytes and with a potential role in the formation of gliomas.

EXPERIMENTAL PROCEDURES

 Yeast Two-hybrid Screen—The SETA SH3-NC bait construct insert was generated by polymerase chain reaction from a cDNA template using proofreading DeepVent DNA polymerase (New England Biolabs), and the absence of mutations was confirmed by sequencing. It included amino acids 24–258 (ERQRR...LPSDF; see Fig. 2A) according to the numbering of the longer SETA isoform described in Ref. 1 and GenBank™ AF131867. This insert was introduced into the pBD-GAL4 phagemid vector (Stratagene) and was used to screen a CG4 rat glial progenitor cell library (1) previously constructed in HybriZAP and rescued into the pAD-GAL4 plasmid form (Stratagene). Two rounds of yeast colony screening for the ability to grow in the absence of histidine and for a positive β-galactosidase reaction were performed. Isolated plasmids were sequenced, and the sequences were compared with gene data bases.

 In Vitro Confrontation—SETA SH3 cDNAs (see legend to Fig. 2A for details) were cloned in frame into pGEX-KG, and glutathione S-transferase (GST) fusion proteins were generated and purified on glutathione-Sepharose 4B, washed in several changes of buffer, and separated on an SDS-polyacrylamide gel, which was pre-selected using glutathione-Sepharose 4B, washed in several changes of 5 mM Tris, pH 7, 100 mM NaCl, 1 mM EGTA, 0.96 mM CaCl2, and 0.1% Nonidet P-40, which contains 5 mM free calcium. Complexes were collected using glutathione-Sepharose 4B, washed in several changes of buffer, and separated on an SDS-polyacrylamide gel, which was prepared for fluorography and exposed to film.

 Co-immunoprecipitation following Transient Expression in 293 Cells—SETA cDNAs (see legend to Fig. 2A for details) were cloned in frame into pCDNA4-Xpress/His (Ser-719 to the C terminus, in 6-cm tissue culture dishes with 5 ml of medium were exposed to 5 μM free calcium. Complexes were col-

RESULTS

SETHER Interacts with the Apoptosis-linked Genes AIP1 and ALG-2—The SETA gene encodes an adapter molecule with SH3 domains, suggesting that it functions by binding with high affinity and specificity to other proteins. Therefore, a yeast two-hybrid cDNA library screen was performed to isolate potential binding partners of SETA. A bait construct encoding the two SH3 domains of SETA and the intervening sequence (for details, see “Experimental Procedures”), fused in frame to the DNA binding domain of the GAL4, was used to screen a CG4 glial progenitor cell cDNA library. SETA was originally isolated from these cells (1), making it likely that physiologically relevant binding partners would be represented. Analysis of only 250,000 co-transformants led to the isolation of eight positive clones after two rounds of screening. Sequence analysis revealed that they represented two overlapping cDNAs for the rat homolog of the mouse AIP1/Alix gene (Fig. 1), which had been previously isolated as a binding partner of ALG-2 (6, 7). Interestingly, the partial rat cDNA clone isolated by us (Fig. 1) was only one amino acid shorter than the mouse cDNA isolated by yeast two-hybrid screening with ALG-2 (7), suggesting that the same region of AIP1 interacts with both ALG-2 and SETA.

Examination of the predicted protein sequence of the partial rat AIP1 cDNA revealed extensive sequence homology to the mouse AIP1 molecule (Fig. 1). Both predicted proteins encoded a proline-rich region downstream of the N-terminal SH3 domain, which comprised 30% of the residues are proline, as compared with an overall frequency of 8% proline in the long form of AIP1 (6, 7). Ten PXXP motifs, such as are found at the core of SH3 binding peptides (11), can be found in this sequence, suggesting that it represents the region that interacts with SETA. Interestingly, although the rodent AIP1 proteins are highly homol-
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The SETA SH3-NC encoding GST fusion protein (Fig. 2C), which contains the same region of SETA as was used in the bait construct employed in the yeast two-hybrid screen, interacted with radiolabeled AIP1 made by coupled in vitro transcription and translation (Fig. 2B) and then collected on glutathione-Sepharose. The SETA SH3-NC encoding GST fusion protein (Fig. 2A), which contains the same region of SETA as was used in the bait construct employed in the yeast two-hybrid screen, interacted with AIP1 in vitro (Fig. 2B). Furthermore, the isolated N-terminal SETA SH3 domain, fused to GST, was able to bind to mouse AIP1 in these experiments (Fig. 2B). However, the C-terminal SH3 domain did not bind to AIP1 (Fig. 2A), which was not due to a general lack of function of this fusion protein, since it has been shown to bind to another novel SETA binding protein recently isolated in our laboratory (data not shown). As expected from previous studies, the ALG-2 protein was able to bind full-length AIP1 in this assay (6, 7). In contrast to the interaction between ALG-2 and AIP1, the SETA SH3-N protein was able to bind to AIP1 in the absence of calcium, as would be expected from an SH3 domain-mediated interaction. Last, none of the GST fusion proteins interacted with radiolabeled β-galactosidase protein (Fig. 2B).

The use of isolated SETA SH3 domains in these experiments provides the most precise definition of which regions of this protein are capable of interacting with AIP1, as functionality depends on correct folding of the SH3 domain, requiring the presence of their entire sequence. However, we were able to further define the region of AIP1 that bound to SETA by determining that neither the SH3-NC nor SH3-N proteins were able to interact with a truncated AIP1 protein, terminating at the internal EcoRI site at position 636 (Fig. 1), which lacks the proline-rich region of AIP1 (data not shown). Together, these data suggest that the interaction between SETA and AIP1 can occur outside of yeast and is mediated by SETA’s N-terminal SH3 domain and AIP1’s proline-rich C terminus.

To test whether SETA and AIP1 also interact in cells, epitope-tagged proteins were transiently introduced into 293 cells, which can be transfected at high efficiency, and cell lysates were analyzed by immunoprecipitation and Western blotting (Fig. 2C). A FLAG-tagged AIP1 cDNA was co-transfected with a C-terminally V5-tagged SETA SH3-NC expression construct, an N-terminally Xpress-tagged SETA NCcc construct or control lacZ constructs tagged with either epitope tag. Although in our hands the V5 epitope tag performed better in all experiments than the Xpress tag, the latter was chosen to tag SETA NCcc N-terminally in order to minimize the possibility of interfering with the structural integrity of the predicted C-terminal coiled-coil. Precipitates from the resultant lysates were collected with anti-V5, anti-Xpress, or anti-FLAG antibodies or with bacterially made ALG-2-GST. The resultant precipitates were then analyzed in Western blots probed with anti-V5, anti-Xpress, anti-SETA, or anti-FLAG antibodies.

Antibodies against epitope tags were used in these experiments to specifically study transfected isoforms of SETA. In order to establish that immunoprecipitation with these antibodies recovered the transfected SETA proteins, lysates of 293 cells transfected with SETA SH3-NC and SETA NCcc were precipitated with anti-V5 or anti-Xpress antibodies, respectively, and immunoblotted with polyclonal anti-SETA antibodies. As shown in Fig. 2C, lanes 1 and 2, bands of the expected size were obtained, demonstrating that these reagents were specifically recovering SETA proteins.

To test whether SETA and AIP1 could bind to ALG-2 in one complex, we precipitated lysates with bacterially made GST-ALG-2 protein. In line with previous studies, GST-ALG-2 could precipitate AIP1-FLAG from cell lysates (Fig. 2C, lane 3), which was not present in lysates of cells transfected with the lacZ control construct (Fig. 2C, lane 4). Bands common to both lanes 3 and 4 are anti-FLAG antibody cross-reacting proteins. To further support the suggestion that SETA and AIP1 interact in cells, lysates were immunoprecipitated with anti-FLAG antibodies recognizing AIP1. SETA SH3-NC or SETA NCcc was detected in these lysates with anti-V5 or anti-Xpress antibody, respectively (Fig. 2C, lanes 5 and 6). Therefore, complexes of AIP1 and SETA proteins could be recovered by immunoprecipitation with antibodies directed at either protein.

To test whether SETA and AIP1 could bind to ALG-2 in one complex, we precipitated lysates with bacterially made GST-ALG-2 protein. In line with previous studies, GST-ALG-2 could precipitate AIP1-FLAG from cell lysates (Fig. 2C, lane 7) (7). Furthermore, these lysates also contained SETA SH3-NC or SETA NCcc protein (Fig. 2C, lanes 8 and 9). Taken together, these data suggest that SETA and AIP1 can interact in cells. Furthermore, the detection of SETA proteins in precipitates made with GST-ALG-2, which does not interact directly with SETA, suggests that these two proteins can interact with AIP1 simultaneously.

Wild-type Rat and p53−/− Mouse Astrocytes and Glioma Cells Express AIP1 and ALG-2—The observation that SETA, AIP1, and ALG-2 can interact raises the question of whether they are co-expressed in cells. SETA was originally identified as a gene associated with malignancy in astrocytes and is expressed in p53−/− astrocytes derived from p53 knockout mice capable of forming tumors as well as glioma-derived cell lines.
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FIG. 2. SETA interacts with AIP1 and ALG-2 in vitro and in cells. A, schematic describing the SETA constructs used in this study. SETA NCce is identical to the sequence reported in GenBank accession no. AF131867, while SETA SH3-C represents sub-regions of the molecule as indicated. Also shown are the epitope tags used to identify recombinant SETA proteins; SETA NCce was tagged N-terminally with the Xpress tag, and SETA SH3 constructs were tagged C-terminally with V5. B, in vitro confrontation between SETA or ALG-2 GST fusion proteins and radiolabeled AIP1 or β-galactosidase (lacZ) proteins produced by in vitro coupled transcription and translation (TNT). SETA SH3-NC and -N bind to AIP1, as does the positive control ALG-2 protein, while SETA SH3-C does not. The interaction between SETA SH3-N and AIP1 occurs in the absence of calcium. Neither SETA nor ALG-2 interact with β-galactosidase (lacZ). The TNT proteins alone are run on the right as a reference, C, co-immunoprecipitation of SETA and AIP1 proteins from transiently transfected cells. FLAG-tagged AIP1 and V5-tagged SETA SH3-NC, Xpress-tagged SETA NCce, or Xpress-tagged lacZ were co-transfected into 293 cells. Immunoprecipitates were generated using anti-V5 (lane 1), anti-Xpress (lanes 2–4), or anti-FLAG (lanes 5 and 6) antibodies or bacterially expressed GST ALG-2 fusion protein (lanes 7 and 8) and analyzed by Western blotting using anti-SETA (lanes 1 and 2), anti-FLAG (lanes 3, 4, and 7), anti-V5 (lanes 5 and 8), and anti-Xpress (lanes 6 and 9), or antibodies, as indicated. Molecular masses in kDa are shown at the right; aa, amino acids.

Therefore, we analyzed these cells, as well as normal rat astrocytes expressing various SETA protein isoforms (see below), for AIP1 and ALG-2 mRNA expression. As shown in Fig. 3, all of the cells tested expressed AIP1 and ALG-2 mRNA. Two AIP1 mRNA species were observed, with one just below the 28 S at about 7 kb, as described previously (7). ALG-2 mRNA ran as a single band below the 18 S at approximately 1 kb (8). AIP1 and ALG-2 expression did not correlate with the ability of p53−/− astrocytes to form tumors, since similar levels of expression were found in cells grown in DMEM plus 10% fetal calf serum or in DMEM plus 20 ng/ml epidermal growth factor, which are capable of forming tumors, and DMEM plus 10% basic fibroblast growth factor, which are not (3). Rat astrocytes with a normal p53 genotype also expressed both AIP1 and ALG-2 mRNA, regardless of whether they had been engineered to express SETA constructs or vector alone (see below). Last, both genes were analyzed using a rat primary cell line, GV2C8, and two human glioma cell lines, LNZ308 and A172, all known to express SETA mRNA (1). These data demonstrate that SETA, AIP1, and ALG-2 are co-expressed in a variety of cells relevant to the study of glial cell transformation and so have the opportunity to interact.

SETA Modulates Apoptosis in Astrocytes in Response to UV Irradiation—To directly examine the question of whether the association of SETA with the known regulators of apoptosis ALG-2 and AIP1 allows it to modulate this process, primary rat astrocyte cell lines that expressed various SETA protein forms (shown in Fig. 2A) were established. Initial experiments in astrocytes demonstrated that the the plasmid expression vectors used in 293 cells (Fig. 2C) did not achieve high enough efficiencies of transient transfection or maintain stable expression of SETA or control proteins in this cell type. Therefore, the epitope-tagged SETA cDNAs were engineered into a retroviral expression construct immediately downstream of the packaging signal and upstream of an internal ribosome entry site and the gene for zeocin resistance (for details, see “Experimental Procedures”). Infection of primary rat astrocytes with retroviruses generated from these constructs and selection with zeocin for 10 days resulted in populations of cells that showed greater than 98% expression of SETA proteins, which was stable over time, as determined by immunohistochemistry (data not shown). All further experiments were performed with these selected cell populations, thereby eliminating issues relating to clonal variation. Populations of astrocytes engineered with SETA SH3-NC in the antisense orientation or with vector alone were also isolated as controls. All of these populations expressed endogenous AIP1 and ALG-2 mRNA (Fig. 3).

Western blotting of zeocin-selected rat astrocyte populations with a polyclonal anti-SETA antibody revealed expression of endogenous SETA that increased with time in culture (Fig. 4). Astrocytes cultured for fewer than five passages after the com-
FIG. 3. Northern analysis of AIP1 and ALG-2 expression. Varying quantities of poly(A)⁺ mRNA were subjected to Northern blotting and hybridized with random primed probes derived from the cDNAs for AIP1 or ALG-2 as indicated. Two bands were seen in AIP1-probed Northern blots with one just below 28 S at about 4 kbp and one above the 28 S at approximately 7 kb (7). ALG-2 mRNA ran as a single band below 18 S at approximately 1 kbp.

FIG. 4. SETA Western blot of rat astrocytes. Cell extracts derived from rat astrocytes infected with 1726/zeo retroviruses encoding no additional gene (lanes 1 and 8), SETA SH3-N (lanes 2 and 9), SETA SH3-C (lanes 3 and 10), SETA SH3-NC (lanes 4 and 11), SETA NCcc (lanes 5, 7, and 12), or SETA SH3-NC in the antisense orientation (lanes 6 and 13) or extracts derived from p53⁻/⁻ mouse astrocytes were subjected to Western blotting with anti-SETA or V5 antibodies as indicated. Gels run in parallel and stained with Coomassie Blue are shown below to demonstrate similar loading of protein in all lanes. Endogenous SETA appears as a series of bands as described under "Results," while the introduced SETA SH3-N and -C proteins are revealed by V5 antibody (lanes 2 and 3), the introduced SETA SH3-NC is shown in lanes 4 and 11, and SETA NCcc is shown in lanes 5, 7, and 12.

Completion of selection showed variable, low levels of endogenous SETA, with bands near 60, 95, and 150 kDa (Fig. 4, lanes 1–6). At higher than 10 passages, the level of endogenous SETA was increased, as demonstrated by the inclusion of cell extract of high passage SETA NCcc-expressing astrocytes on the same Western blot as the low passage cells (Fig. 4, lane 7). This higher level of SETA expression was found in all cell lines at higher passages (Fig. 4, lanes 8–14). In high passage astrocytes, SETA protein bands in addition to those seen in low passage cells were detected, including two more bands near 50 kDa, a band at 35 kDa, and some lower molecular mass bands.

In addition to endogenous SETA, these Western blots revealed the presence of the introduced SETA SH3-NC and NCcc proteins and showed that their expression was stable over time. SETA SH3-NC appears as a strong band near 42 kDa (Fig. 4, lanes 4 and 11), while NCcc is detected just underneath the endogenous band at 95 kDa (lanes 5 and 12). Neither SETA SH3-N nor -C proteins were recognized by polyclonal anti-SETA antibodies, but they could be detected by monoclonal anti-V5 antibody via their V5 epitope tag both at low passage (Fig. 4, lanes 2 and 3, inset) and at high passage (data not shown). Epitope tag antibodies V5 for SETA SH3-NC and Xpress for SETA NCcc were also used to confirm the identity of these proteins on separate blots (data not shown).

The pattern of bands observed in normal rat astrocytes was different from that seen in p53⁻/⁻ mouse astrocytes reported previously (1) and shown here for comparison (Fig. 4, lane 14). While both cell types showed the triple of SETA proteins near 50 kDa, primary rat astrocytes also expressed a much larger form of SETA near 180 kDa and smaller forms at 35 kDa and below. In addition, the band near 95 kDa in rat astrocytes appeared to migrate faster in p53⁻/⁻ mouse astrocytes.

To determine the effect of SETA protein misexpression on apoptosis, populations of astrocytes expressing various SETA proteins were exposed to 5 mJ/cm² of ultraviolet irradiation and analyzed by TUNEL and annexin V labeling 24 h later (Fig. 5). In all experiments, the percentage of TUNEL-positive or annexin V-positive cells was higher in astrocyte cultures expressing SETA SH3-N, -C, or NCcc than in those expressing vector alone, SETA SH3-C, or SETA antisense NC, which showed similar and lower levels of apoptotic cells. Control cultures that received no irradiation showed barely detectable levels of apoptosis, regardless of which retroviral vector they contained (not shown). Together, these experiments demonstrated that the overexpression of SETA isoforms encoding the SH3-N domain increased the rate of apoptosis in astrocyte cell cultures in response to UV irradiation.

SETA Proteins Associate with the Actin Cytoskeleton—Normal astrocytes harboring the control retroviral vector or expressing various introduced SETA isoforms were prepared for immunohistochemistry using either polyclonal anti-SETA or anti-AIP1 antibodies or monoclonal antibodies directed at the epitope tags encoded by these exogenous proteins (Fig. 6). Cells transduced with the vector alone showed no reactivity with the antibodies directed at the epitope tags (e.g., the V5 antibody; Fig. 6A). However, when stained with anti-SETA antibodies, they did show endogenous SETA expression (Fig. 6B) as expected from the Western blot results (Fig. 4). Similarly, vector-transduced cells also reacted with anti-AIP1 polyclonal antibodies (Fig. 6C). The pattern of cell staining obtained with anti-SETA and anti-AIP antibodies was similar, being cyto-
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Fig. 5. Expression of SETA proteins encoding the SH3-N domain sensitizes cells to apoptosis in response to UV irradiation. Astrocytes expressing various SETA constructs were challenged with 5 mJ/cm² UV irradiation and prepared for analysis by annexin V or TUNEL 24 h later. The percentage of annexin V- or TUNEL-positive cells is shown. Cells expressing SETA constructs that make a protein containing the SH3-N domain have significantly higher rates of apoptosis than control cultures, with the most dramatic increase seen in cultures expressing SETA SH3-N. Data shown are mean ± S.E. from four or more independent experiments, with three coverslips per experiment and over 200 cells counted per coverslip.

Fig. 6. SETA proteins localize to the actin cytoskeleton. Astrocytes harboring the control vector (A–C) or expressing SETA NCcc (D), SH3-NC (E–K), SH3-N (F), or SH3-C (G) were analyzed by immunohistostaining. Cells were stained with anti-V5 antibody (Fig. 6, A–C), or Xpress antibody (Fig. 6, D–G). Cells in A were counterstained with propidium iodide to reveal the nuclei. Cells expressing SETA SH3-NC and stained with anti-V5 detected by secondary fluorescein isothiocyanate-conjugated antibodies (I and K) were counterstained with phalloidin-tetramethylrhodamine isothiocyanate conjugate secondary antibody (J). The arrows in H–K show areas that were stained in both colors. Cells stained with secondary antibodies alone showed no signal (not shown).

In order to examine the localization of introduced SETA proteins, cells expressing SETA NCcc were stained with Xpress antibody (Fig. 6D), while those expressing SETA SH3-NC (Fig. 6, E and H–K), SH3-N (Fig. 6F), or SH3-C (Fig. 6G) were stained with V5 antibody (Fig. 6, E–G, I, and K). In all cases, SETA proteins were found in the cytoplasm of cells, and the smaller SH3-NC, -N, and -C proteins could also be found in the nucleus in some cells (Fig. 6, D–G). However, SETA proteins also appeared to associate with cytoskeletal elements, since a proportion of the signal had a filamentous appearance. In order to investigate which component of the cytoskeleton SETA proteins associated with, cells were counterstained with phalloidin or anti-paxillin. This analysis revealed that SETA NCcc, SH3-NC, -N, and -C all localized to the actin cytoskeleton, including regions that were paxillin-positive as shown in Fig. 6 for cells expressing SETA SH3-NC. The arrows in Fig. 6, H–K, reveal areas that are both phalloidin-positive (Fig. 6H) and SETA-positive (Fig. 6I) or both paxillin-positive (Fig. 6J) and SETA-positive (Fig. 6K). Since cells expressing SETA SH3-C (Fig. 6G) also exhibited this pattern, this suggests that it is unlikely to be mediated exclusively by interaction with proteins recognized by the SH3-N domain, such as AIP1.

DISCUSSION

Expression of SETA in cultured mouse p53−/− astrocytes and in astrocytes in the adult brain is associated with the tumorigenic state (1), suggesting that it may contribute to the process of glioma formation. The gene encodes SH3 domain-containing adapter molecules that are likely to exert their function by binding with high affinity and specificity to other proteins. A yeast two-hybrid screen of a rat CG4 glial progenitor cell cDNA library with the two SH3 domains of SETA NCc as bait resulted in our isolation of the rat homolog of the AIP1/Alix gene, which has been described as a binding partner of ALG-2 (8). In vitro confrontation experiments and co-immunoprecipitation from transiently transfected 293 cells confirmed that SETA and AIP1 bind and showed that the SH3-N domain of SETA and the proline-rich region of AIP1 are responsible for this interaction. The ability to recover SETA-AIP1 complexes with ALG-2 suggests that SETA and ALG-2 may be able to bind to AIP1 simultaneously. Furthermore, SETA, AIP1, and ALG-2 are all co-expressed in normal and transformed astrocytes, as well as rat and human glioma cell lines, making their possible interaction plausible in cells relevant to understanding glioma.

In view of the established role that AIP1 and ALG-2 play in apoptosis (7, 8, 13), a direct analysis of the role of SETA in apoptosis in astrocytes was performed. Clonally complex pop-
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volutions of normal rat astrocyes expressing SETA NCcc as well as partial SETA protein constructs were isolated using a bicistronic, zeocin-resistance-encoding retrovirus. Analysis of the localization of introduced SETA proteins in astrocytes revealed that they appeared to localize to the actin cytoskeleton. The base-line levels of apoptosis in astrocytes expressing SETA proteins were not changed when compared with the vector controls and were less than 1% in all cultures tested. However, when cells were exposed to UV irradiation, it was found that exogenous SETA proteins that included the SH3-N domain increased the number of apoptotic cells. Astrocyte cultures expressing SETA NCcc, SH3-NC, or SH3-N all had elevated levels of apoptosis, as measured by annexin V staining and TUNEL, when compared with cells expressing vector alone, SETA SH3-C, or antisense SETA SH3-NC. The isolated SETA SH3-N protein had the greatest effect in these experiments.

It appears from these data that SETA proteins capable of interacting with the AIP1-ALG-2 complex modulate apoptosis in response to UV irradiation in astrocytes, while not affecting their background rate of apoptosis. Similar findings of a contingent effect on apoptosis have been made for ALG-2 and AIP1. Reducing levels of ALG-2 protein by antisense in T-cells protected them from apoptosis induced by a variety of stimuli including T-cell receptor stimulation, dexamethasone, and staurosporine (8). Conversely, overexpressing ALG-2 in fibroblasts sensitized them to cell death in response to phorbol ester and calcium ionophore without increasing rates of apoptosis in unstimulated cells (8). In the case of AIP1, overexpression of a truncated form, TH28, protected HeLa and COS cells from serum starvation, etoposide, and staurosporine (7). Therefore, it seems likely that SETA, AIP1, and ALG-2 are involved in a complex that modulates apoptotic signals rather than generating them de novo.

The information available at present on the mechanism by which AIP1 and ALG-2 affect apoptosis allows for the simple hypothesis that the ALG-2-AIP1 complex is a component of a proapoptotic signaling pathway. The possibility that ALG-2 is the limiting component is suggested by the observation that reducing levels protects cells, while overexpression of ALG-2 sensitizes them (8). As proposed by Vito et al. (7), this model allows the further hypothesis that TH28, the truncated form of AIP1, is a dominant negative form of AIP1 that protects cells by interfering with the function of the ALG-2-AIP1 complex. The observation that the protective effect of overexpressing TH28, which binds ALG-2, can be overcome by co-expressing additional ALG-2 (7) could then be interpreted as indicating that under these circumstances enough ALG-2-AIP1 complexes are formed to allow apoptosis to occur. The data presented here could fit into this model by suggesting that binding of AIP1 by the introduced SETA proteins containing the SH3-N domain promotes the activity of the AIP1-ALG-2 complex.

The proapoptotic effect of introduced SETA NCc, SH3-NC, and SH3-N raises the question as to whether they interfere with the function of the endogenous SETA protein(s), synergize with it, or have novel and different functions. While it is difficult to make a strong case for any of these possibilities in the absence of a detailed understanding of how SETA functions, the observation that the isolated SH3 domain, SETA SH3-N, had the greatest effect in these experiments favors the first possibility. It is difficult to see how this small protein with only one binding group could act in the same way as larger SETA proteins with several binding modalities. According to this model, the SETA isoforms used here interfere with the anti-apoptotic influence of endogenous SETA proteins. Although it is easy to see how partial SETA proteins such as the SH3-NC and -N isoforms could act as a dominant negative protein, this is less obvious for SETA NCc. However, comparison of SETA cDNA sequences with those of its closest relatives, CD2AP (4) and CMS (5), both of which have three SH3 domains, suggests that an additional SH3 domain more N-terminal to SH3-N remains to be isolated. Furthermore, the multiple isoforms of endogenous SETA evident in the complex pattern revealed by Western blot remain to be fully characterized. The original cloning of SETA suggested that there are at least two alternative N termini, encoding both the SH3-N and -C domains or only the SH3-C domain (1). In addition, the possibility of two alternate C termini are suggested by cDNAs, with one encoding a coiled-coil (1). Analysis of additional cDNA clones and genomic clones for SETA is currently under way. A further consideration is that, just as overexpression of TH28 only affected apoptosis in response to some stimuli but not others (7), SETA's effect may be different in response to different apoptotic stimuli. Experiments using different stimuli are being performed.

The observation that SETA proteins are associated with the cytoskeleton suggests that this may be a common characteristic of the subfamily of SH3 domain-containing adapter molecules that also includes CD2AP and CMS. CD2AP associates with CD2 in T-cells and is involved in its clustering and cytoskeletal polarization by linking it to the cytoskeleton (4). Similarly, the CMS protein is associated with the cytoskeleton, and its misexpression causes an alteration in actin fiber arrangement (5). Interestingly, CMS encodes putative actin binding sequences in its C terminus, which are not conserved in SETA. However, since SETA proteins, including the isolated SETA SH3-N and -C domains, were able to localize to actin in rat astrocytes, this suggests that SETA and CMS may both bind to similar elements of the cytoskeleton but in different ways. Furthermore, the observation that CMS, CD2AP, and SETA all share two PXXP motifs, which in CMS have been shown to bind to src, p85 subunit of phosphatidylinositol 3-kinase, and Grb2 suggests that members of this group may be downstream of a variety of signaling pathways. These observations combined with the data presented here suggest that the new family of adapter molecules that includes SETA, CD2AP, and CMS may act at a point of integration of mitogenic signals, cytoskeletal architecture, and apoptosis (14).

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