sZIP, an Alternative Splice Variant of ZIP, Antagonizes Transcription Repression and Growth Inhibition by ZIP*

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Recently, we reported a novel transcriptional repressor, ZIP (for zinc finger and G-patch domain-containing), which recruits the Mi-2/NuRD (nucleosome remodeling and deacetylase) complex and represses the expression of epidermal growth factor receptor (EGFR). In doing so, ZIP inhibits cell proliferation and suppresses breast carcinogenesis. Here, we report the cloning and the characterization of an alternatively spliced isoform of ZIP, sZIP. sZIP is an N-terminal truncated form of ZIP, lacking the zinc finger but retaining part of the G-patch domain and C-terminal coiled-coil domain of ZIP. We showed that sZIP could interact with the NuRD complex but lost its DNA-binding capacity. We demonstrated that sZIP antagonizes the transcription repression by ZIP by competing for the binding of the NuRD complex and that sZIP alleviates the growth inhibitory effect of ZIP on hepatocarcinoma cells through attenuating the transcriptional repression of EGFR. Our data provide a finely tuned mechanism for EGFR regulation and add another player for transcription repression.

Alternative splicing is the process by which a single primary transcript yields different mature RNA molecules, leading to the production of multiple protein isoforms with diverse and even antagonistic functions. In humans, up to 70% of genes are alternatively spliced (1, 2), which greatly increases the diversity of proteins that can be encoded by the surprisingly low number of human genes. The resulting changes in amino acid sequence could potentially lead to the alteration of the binding properties of proteins, their intracellular localization, their enzymatic activity, or their stability and posttranslational modifications (3, 4), which may, in turn, render a gain, subtle modulation, or even a complete loss of function of a particular protein. Therefore, alternative splicing represents a key strategy in eukaryotes to generate a complex set of proteome in dealing with diverse environments and cellular micromilieu.

In our previous work (5), we identified and functionally characterized a novel gene-specific transcription repressor and a potential tumor suppressor, ZIP. ZIP is a modular protein with several important functional domains: a CCCH zinc finger, a Tudor domain, a G-patch, and a coiled-coil domain. Our experiments demonstrated that ZIP is capable of binding to specific DNA sequences and recruits the NuRD complex via the interaction of its C-terminal coiled-coil domain with the Mi-2 subunit of the NuRD complex. We further showed that ZIP acts to repress epidermal growth factor receptor (EGFR) transcription and to inhibit the proliferation of breast cancer cells in vitro and to suppress breast carcinogenesis in vivo (5).

Here, we describe the cloning and characterization of an alternative splice variant of ZIP, named sZIP for short ZIP. We showed that sZIP could interact with the NuRD complex but not with DNA. We demonstrated that sZIP antagonizes the transcription repression activity of ZIP, attenuates the transcriptional repression of EGFR by ZIP, and alleviates the growth inhibition of hepatocarcinoma cells by ZIP.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Antibodies used were as follows: αβ-actin from Sigma; αMTA2 from Upstate Biotechnology; αMi-2 from Santa Cruz Biotechnology; and αEGFR from SAB (Signalway Antibody Co., Ltd.). The antibodies against sZIP/ZIP were generated by immunizing rabbits with a chemically synthesized C-terminal epitope (EQRKADTHKKMTEF) of sZIP/ZIP protein. GST, FLAG-tagged constructs, GFP, and GAL4 fusion constructs were generated by standard molecular techniques.

5’ RNA Ligase-mediated Rapid Amplification of cDNA Ends—Total RNA preparations from HepG2, 293T, and MCF-7 were used to determine the transcription start site and 3’ end of ZIP mRNA. Rapid amplification of 5’ and 3’ cDNA ends was carried out using a FirstChoice RNA ligase-mediated rapid amplification of cDNA ends kit (Ambion) according to the manufacturer’s instructions. The gene-specific primers used in 5’ nested PCR and 3’ nested PCR were sZIP-5’-Outer (5’-GCTGACGC-CACGCTAT-3’), sZIP-5’-Inner (5’-CCCCGTGGCCACTCTT-3’), sZIP-3’-Outer (5’-TGGTGAGGAGTCTGGAGC-3’), and sZIP-3’-Inner (5’-GAAATGTGGAGTCGGAGAC-3’). The nested PCR-generated fragments were then cloned and sequenced.

Cell Culture and Reporter Assay—The HepG2, 293T, and HeLa cell lines were obtained from the American Type Culture Collection (Manassas, VA). They were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transfections were carried out using Lip-
fectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Luciferase activity was measured using a Dual-Luciferase kit (Promega, Madison, WI) (6–8). Each experiment was performed in triplicate and repeated at least three times.

Fluorescence Confocal Microscopy—HeLa cells were transfected with appropriate plasmids using Lipofectamine 2000. Twenty-four h after transfection, cells were washed with PBS, fixed in 4% paraformaldehyde, and permeabilized with 1% Triton X-100. Cells were washed four times, and a final concentration of 0.1 µg/ml 4,6-diamidino-2-phenylindole dihydrochloride (Sigma) was included in the final wash to stain nuclei. Images were visualized with an Olympus inverted microscope equipped with a charge-coupled camera. The resulting images were deconvolved with Deltavision software. For immunostaining, 24 h after transfections, cells were washed with PBS, fixed with 4% paraformaldehyde, and incubated with appropriate primary antibodies followed by addition of rhodamine-conjugated secondary antibodies (9).

Cyclic Amplification and Selection of Target (CASTing) Assay—A library of single-stranded oligonucleotides containing the sequences 5’-GACTCGAGACTCTCTAGATGCGCA-(N)20GCATG-3’ was generated. To produce double-stranded oligonucleotides, 400 pmol of the library was incubated in 100 µl of polymerase reaction buffer containing 1,200 pmol of reverse primer (5’-ATCGAAGCTTCACTGACATAGGC-3’), 200 µM of each deoxynucleoside triphosphate, and 10 units of Ex Taq polymerase (Takara Bio.) and amplified with following procedure: 5 min at 95 °C, 20 min at 65 °C, and 20 min at 72 °C. The double-stranded oligonucleotides were purified using the QIAquick nucleotide removal kit (Qiagen) and incubated with GST-fused ZIP or sZIP proteins bound to glutathione beads in a binding buffer containing 25 mM HEPES-KOH, pH 7.5, 100 mM KCl, 5 mM dithiothreitol, 5% glycerol, 1 mM MgCl2, 100 µM ZnCl2, 0.1% Nonidet P-40, 100 µg/ml poly(dI-dC) and 1 mg/ml bovine serum albumin. After a 30-min rotating incubation at room temperature, the beads were washed for eight times with cold binding buffer without poly(dI-dC) and then boiled for 5 min in sterilized H2O. The eluted oligonucleotides were used for PCR amplification. The amplified products were subsequently used for a second round of selection. After nine rounds of amplification, PCR products were cloned into pGEM-Teasy vector, transformed into DH5α competent host cells, and sequenced.

Immunoprecipitation and Western Blotting—HepG2 or HeLa cells were transfected with sZIP or ZIP expression plas-
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mid. Forty-eight h after transfection, cellular lysates were prepared by incubating the cells in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40) for 20 min at 4 °C. This was followed by centrifugation at 14,000 × g for 15 min at 4 °C. For immunoprecipitation, 500 µg of protein was incubated with specific antibodies (1–2 µg) for 12 h at 4 °C with a constant rotation; 50 µl of 50% protein A or G agarose beads was then added, and the incubation was continued for an additional 2 h. Beads were then washed five times using the lysis buffer. Between washes, the beads were collected by centrifugation in 2× SDS-PAGE loading buffer and boiling for 5 min. The resultant materials from immunoprecipitation or cell lysates were resolved using 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. For Western blotting analysis, membranes were incubated with appropriate antibodies for 1 h at room temperature or overnight at 4 °C followed by incubation with a secondary antibody. Immunoreactive bands were visualized using Western blotting Luminol reagent (Santa Cruz Biotechnology) (10–13).

ChIP/qChIP—ChIP/qChIP experiments were performed according to the procedure described previously (14–17). The following primer pairs were used for EGFR gene sequence: 5’-AAAAACCCACCGTTCAG-3’ (forward) and 5’-GCAAGTCC-ACCCCATC-3’ (reverse).

Colony Formation Assay—HepG2 cells were stably transfected with ZIP, sZIP, or ZIP+sZIP. The cells were maintained in culture media for 14 days supplemented with 0.4 mg/ml G418 and then stained with crystal violet.

Cell Flow Cytometry—HepG2 cells stably transfected with ZIP, sZIP, or both were synchronized by serum starvation for 72 h, and released with 10% fetal bovine serum for an appropriate period of time; cells were then trypsinized, washed with PBS, and fixed in 70% ethanol at 4 °C overnight. After being washed with PBS, cells were incubated with RNase A (Sigma) in PBS for 30 min at 37 °C and then stained with 50 mg/ml propidium iodide. Cell cycle data were collected with FACS Calibur (Becton Dickinson) and analyzed with ModFit LT 3.0 (Verity Software House Inc., Topsham, ME).

RESULTS

Cloning and Characterization of sZIP—In our previous work (5), we cloned a novel transcriptional repressor gene, ZIP (for zinc finger and G-patch domain-containing of its protein product), from a mammary cDNA library. The cDNA of ZIP is 1,882 bp in length (GenBank™ accession number BC032612) and contains an open reading frame encoding for a protein of 511 amino acids. The corresponding gene consists of seven exons and six introns (Fig. 1A). ZIP harbors a CCCH or C3H1 type of zinc finger, a Tudor domain, a G-patch domain, a coiled-coil domain, and a nuclear localization signal (Fig. 1A).

Molecular profiling the tissue distribution pattern of ZIP by Northern blotting using a probe spanning bases 948–1514 of the human ZIP cDNA revealed that, in addition to the transcript encoding for ZIP protein, a short form transcript was also detected, primarily in the liver and kidneys (5). We then performed 5’ RACE and 3’ RACE to determine the 5’ and 3’ ends of sZIP, and cloned and sequenced the full-length cDNA of sZIP. The results indicated that the mRNA of sZIP is 868 bp in length and compared with ZIP transcript, sZIP lacks exons 2–4 and part of exon 5, corresponding to nucleotides 121–1089 and amino acid residues 1–323 of ZIP (Fig. 1A). As a result, sZIP contains an open reading frame encoding for a protein of 188 amino acids, including part of G-patch domain, the nuclear localization signal, and the C-terminal coiled-coil domain (Fig. 1A). The predicted molecular mass of sZIP protein is ~20.7 kDa, with a theoretical isoelectric point of 10.69, much higher than that of ZIP (pI of 5.49).

To investigate the expression of endogenous sZIP protein, cellular extracts from various cell lines were prepared, and Western blotting was performed with polyclonal antibodies against sZIP, which we generated in rabbits with a C-terminal epitope (EQRKADTHKKMTEF) of sZIP protein but that also

FIGURE 2. sZIP antagonizes the intrinsic transcription repressive activity of ZIP. A, sZIP represses transcription when fused to the heterogeneous DNA-binding domain. 293T cells were transfected with indicated constructs together with GAL4-SV40 luciferase reporter (GAL4-SV40-Luc). Twenty-four h after transfection, cells were harvested, and luciferase activity was measured and normalized to Renilla activity. Each bar represents the mean ± S.D. for triplicate experiments. B, the transcription repression activity of ZIP is alleviated by sZIP. 293T cells were plated in 24-well plates 1 day before transfection. For transfections, 200 ng of GAL4-SV40-Luc reporter plasmid, 100 ng of GAL4DBD, or 100 ng of GAL4DBD-ZIP constructs plus increasing amounts of FLAG-sZIP (0, 10, or 100 ng) constructs were used. Total plasmid DNAs were made up to 400 ng with pcDNA3.1 empty vectors in each transfection. Cells were collected 24 h after transfection. Luciferase activity was measured and normalized to Renilla activity. Each bar represents the mean ± S.D. for triplicate experiments. C, the protein expression of GAL4-ZIP, GAL4-sZIP, and FLAG-sZIP was confirmed by Western blotting with anti-GAL4 or anti-FLAG.

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recognize ZIP because the amino acid sequence of sZIP is completely the same as that of C-terminal portion of ZIP. The results indicated that endogenous sZIP is a protein of ~20 kDa (Fig. 1B, left panel), confirming the predicted molecular weight. In addition, the protein expression patterns of ZIP and sZIP are similar in HeLa, 293T, and HepG2 cells, although the overall expression levels of sZIP are lower than that of ZIP (Fig. 1B, left panel). Moreover, the specificity of the antibodies was validated by knocking down the expression of sZIP using its specific siRNAs, which resulted in decreased protein levels of both sZIP and ZIP (Fig. 1B, right panel).

ZIP protein is primarily localized in the nucleus (5). To gain insight into the biological function of the sZIP protein, we also examined its subcellular localization. Fluorescent imaging of green fluorescent protein (GFP)-fused sZIP (GFP-sZIP) and immunostaining of FLAG-sZIP (FLAG-sZIP) in HeLa cells indicated that sZIP is also localized in the nucleus (Fig. 1C), suggesting that sZIP may function, similar to ZIP, primarily in the nucleus.

**sZIP Antagonizes the Intrinsic Transcription Repressive Activity of ZIP**—ZIP is a nuclear protein and functions as a transcription repressor (5). sZIP is an alternatively spliced form of ZIP and is also localized in the nucleus. To understand the cellular function of sZIP, we first investigated the role of sZIP, if any, in transcription regulation. For this purpose, 293T cells were co-transfected with GAL4-ZIP or/and GAL4-sZIP and GAL4-SV40-driven luciferase construct (GAL4-SV40-Luc). Forty eight h after transfection, cellular extracts were prepared, and the reporter activity was measured. The results indicated that transfection of either GAL4-ZIP or GAL4-sZIP resulted in a repression of the reporter activity (Fig. 2A), although the repressive activity of GAL4-sZIP was weaker compared with that of GAL4-ZIP. However, co-transfection of FLAG-sZIP with GAL4-ZIP led to a relieved repression of the reporter activity by ZIP (Fig. 2B), suggesting that sZIP acts to antagonize the transcription repression activity of ZIP. The protein expression of GAL4-ZIP, GAL4-sZIP, and FLAG-sZIP was examined by Western blotting with anti-GAL4 and anti-FLAG antibodies (Fig. 2C).

**Molecular Basis for the Antagonistic Activity of sZIP**—In its transcription repression function, ZIP has been shown to recognize a specific DNA sequence through its CCCH zinc finger domain and recruit the NuRD complex through its coiled-coil domain (5). Structurally, sZIP lacks the zinc finger but retains the coiled-coil domain. To further support the antagonistic effect of sZIP on the transcription repression activity of ZIP and
to understand the molecular basis of this antagonism, we next performed cyclic amplification and selection of target (CASTing) assays to investigate whether sZIP indeed lost the DNA-binding capacity. To this end, glutathione S-transferase-fused ZIP (GST-ZIP) or GST-sZIP that was immobilized on glutathione-Sepharose 4B beads was used to screen a double-stranded random oligonucleotide library. As shown in Fig. 3A, although, compared with the DNA-binding pattern of ZIP, weaker binding to DNA sequences by GST-sZIP was detected at the first two rounds of binding and amplification reaction, no DNA-binding by GST-sZIP was detected in subsequent rounds of the CASTing assays, suggesting that sZIP is not a DNA-binding protein. Consistent with this result, although FLAG-ZIP was detected on the EGFR promoter with ChIP assays, FLAG-sZIP was not (Fig. 3B). Further study by quantitative ChIP (qChIP) indicated that sZIP overexpression resulted in a significant reduction of the binding of ZIP and Mi-2 to the EGFR promoter region (Fig. 3C).

As mentioned above, ZIP recruits the NuRD complex through the interaction of its coiled-coil domain with the Mi-2 subunit of the NuRD complex. Because sZIP protein retains the coiled-coil domain, we tested whether sZIP could also interact with the NuRD complex. For this purpose, HeLa cells were transfected with FLAG-sZIP. Cellular extracts were prepared, and co-immunoprecipitation assays were performed with anti-FLAG followed by immunoblotting with antibodies against the components of the NuRD complex. The results indicated that all components of the NuRD complex, including HDAC1, HDAC2, MTA2, RbAp46/48, and Mi-2, could be indeed immunoprecipitated with FLAG-sZIP (Fig. 3D).

In light of the observation that sZIP antagonized the transcription repression activity of ZIP and the results that showed both ZIP and sZIP are capable of physically associating with the NuRD complex, it is conceivable that the antagonistic effect of sZIP on the transcription repression activity of ZIP is through the competition between ZIP and sZIP for the binding of the NuRD complex. In support of this proposition, overexpression of sZIP in HeLa cells resulted in a dramatic decrease in the amount of Mi-2 and MTA2 that was immunoprecipitated with ZIP (Fig. 3E).

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**FIGURE 4.** The biological significance of antagonizing ZIP by sZIP. A, sZIP alleviates the repression of EGFR expression by ZIP. FLAG-sZIP or/and FLAG-ZIP was transfected into HepG2 cells. The expression of EGFR in these cells was measured by Western blotting with antibodies against EGFR. The expression of FLAG-ZIP and FLAG-sZIP was confirmed by Western blotting with anti-FLAG antibodies. B, colony formation assay. HepG2 cells stably expressing ZIP or/and sZIP were maintained in culture media containing 0.4 mg/ml G418 for 14 days before staining with crystal violet and being counted for colony numbers. Each bar represents the mean ± S.D. for triplicate experiments. C, sZIP alleviates the inhibition of cell cycle progression by ZIP. HepG2 cells were transfected with vector, ZIP, sZIP, or ZIP+sZIP. Twenty four h after transfection, the cells were switched to conditioned medium with 0.5% fetal bovine serum for another 72 h. The cells were then cultured in medium containing 10% fetal bovine serum for 12 h and were collected for cell cycle analysis by flow cytometry.

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expression, and overexpression of sZIP alone was accompanied by increases in EGFR expression. However, co-transfection of ZIP and sZIP resulted in a relieved repression of the EGFR expression by ZIP (Fig. 4A). The overexpression of sZIP or/and ZIP in these experiments was confirmed by Western blotting (Fig. 4A). Collectively, these results support the argument that sZIP acts to antagonize the transcription repression of EGFR expression by ZIP.

The Biological Effect of the Transcription Regulation of EGFR by sZIP—To determine whether the antagonistic effect of sZIP on the transcription repression of EGFR by sZIP could extend to a physiologically relevant response, we next examined the effect of sZIP on cell proliferation and colony formation. For these experiments, we created HepG2 cell clones that stably express ZIP, sZIP, or ZIP plus sZIP, and performed colony formation assays. The results indicated that overexpression of ZIP was associated with a decreased colony number and sZIP overexpression was associated with an increased colony number. In comparison with overexpression of ZIP, co-expression of ZIP and sZIP led to an increased colony number (Fig. 4B). Moreover, flow cytometry analysis revealed that cell cycle progression of HepG2 cells was significantly delayed when ZIP was overexpressed; there was a significant accumulation of cells in G0/G1 phase under this condition. Conversely, overexpression of sZIP was associated with a decreased colony number and sZIP overexpression was accompanied by an increased colony number. Collectively, these experiments support a role for sZIP in alleviating the growth inhibitory effect of ZIP.

DISCUSSION

The EGFR family proteins, also known as ErbB receptors, are receptor protein tyrosine kinases and are among the most intensively studied groups of membrane-spanning cell surface receptors (18–21). All ErbB receptors/ligands are intimately involved in the regulation of cell growth, differentiation, and survival, and their dysregulation contributes to a wide range of epithelial cancers (22–25). This dysregulation has also been associated with a poor prognosis and resistance to the conventional therapies (26, 27). These discoveries have led to the strategic development of several kinds of EGFR inhibitors, five of which have gained the United States Food and Drug Administration approval for the treatment of patients with non-small-cell lung cancer (gefitinib and erlotinib), metastatic colorectal cancer (cetuximab and panitumumab), head and neck (cetuximab), pancreatic cancer (erlotinib), and breast (lapatinib) cancer (26, 28).

Despite the extensive molecular and functional characterization of EGFR and numerous research efforts in pursuing anti-EGFR cancer therapy, little is known about the mechanism underlying the regulation/deregulation of EGFR expression. Recently, we reported a novel transcriptional repressor, ZIP, which represses the expression of EGFR by recruiting the NuRD complex (5). In the present study, we report an alternative splice variant of ZIP, sZIP, which is an N-terminal truncated isoform of ZIP. Luciferase reporter assays indicated that sZIP antagonized transcriptional repression activity of ZIP. Further studies demonstrated that sZIP could interact with the NuRD complex but not with DNA. We showed that sZIP acts to attenuate the transcription repression of EGFR by ZIP and to alleviate the growth inhibitory effect of ZIP.

Keeping an appropriate amount of EGFR is critical for the normal physiology of the cells (29). The transcription repression of EGFR expression by ZIP and the antagonistic function of sZIP may represent a finely tuned mechanism for EGFR regulation. Further studies are needed to investigate the molecular scope and the significance of this mechanism in physiological conditions. In this regard, it is intriguing to note that the mRNA of sZIP was highly expressed in the liver and kidneys. Whether or not the antagonistic function of sZIP exhibits a tissue specific pattern and what role(s) of the tissue-specific activity of sZIP might contribute to the normal development and the homeostasis of the liver and kidneys will be interesting issues to investigate. On the other hand, abundant studies indicate a positive correlation of increased amounts or/and activity of EGFR with shortened survival of cancer patients, poor response to chemotherapy, and even failure of endocrine therapy (29, 30). Thus, it will be worthwhile to examine the genetic and epigenetic changes, if any, of ZIP/sZIP, and to explore the contribution of these changes to the cancer development.

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