Molecular Cloning and Characterization of the Human AKT1 Promoter Uncovers Its Up-regulation by the Src/Stat3 Pathway

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Sungman Park, Donghwa Kim, Satoshi Kaneko, Kristen M. Szewczyk, Santo V. Nicosia, Hua Yu, Richard Jove, and Jin Q. Cheng

From the Departments of Pathology and Interdisciplinary Oncology, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida College of Medicine, Tampa, Florida 33612

Akt1, also known as protein kinase B (PKB) α, is frequently activated in human cancers and has been implicated in many cell processes by phosphorylation of downstream molecules. However, transcriptional regulation of Akt1 has not been documented. Here, we report the isolation and characterization of the human AKT1 promoter and demonstrate transcriptional up-regulation of AKT1 by the Src/Stat3 pathway. Protein and mRNA levels of AKT1 are elevated in cells expressing constitutively active Stat3 as well as in v-Src-transformed cells. The AKT1 promoter contains five putative Stat3-binding motifs, the promoter failed to be activated in human cancers and has been implicated in many cell processes, such as cell proliferation, survival, and metabolism (8–11). Activation of Akt by growth factor depends on the integrity of the pleckstrin homology domain, which binds to phosphatidylinositol 3-kinase products PtdIns-3,4-P2 and PtdIns-3,4,5-P3, and on the phosphorylation of Thr308 (Thr309 in Akt2 and Thr305 in Akt3) in the activation loop and Ser473 (Ser474 in Akt2 and Ser472 in Akt3) in the C-terminal activation domain by PDK1 and ILK/DNA-PK (12–14). The activity of Akt is negatively regulated by PTEN, a tumor suppressor gene that is mutated in a number of human malignancies. PTEN encodes a dual-specificity protein and lipid phosphatase that reduces intracellular levels of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 in cells by converting them to PtdIns-4-P1 and PtdIns-4,5-P2, respectively, thereby inhibiting the phosphatidylinositol 3-kinase/Akt pathway.

There are no significant differences between three members of Akt in terms of upstream regulators and downstream targets. However, several lines of evidence suggest that the biological/physiological functions of different levels of Akt1, Akt2, and Akt3 are regulated by different mechanisms. Akt1, Akt2, and Akt3 exhibit distinct localization and functions in growth factor signaling, cell growth, survival, differentiation, and metabolism (15–19). Akt1, Akt2, and Akt3 share a pleckstrin homology domain and a kinase domain, but they differ in their expression patterns and functions. Akt1 and Akt2 proteins require membrane localization for specific interaction with other signaling proteins (19). Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different. First, there are different levels of Akt1, Akt2, and Akt3 in different cell types. Second, Akt2 binds to Akt1 and Akt2 but not Akt3, indicating the need to maintain different specificity in interactions with other signaling proteins. Third, Akt1, Akt2, and Akt3 are different.
vide the first evidence that AKTI is a direct Stat3 target and mediates Stat3 survival signal.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, Materials, and Transfection—Human epithelial kidney HEK293, MCF10A, MCF7, mouse fibroblast NIH3T3, Src-transformed NIH3T3, and Stat3−/−MEF were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The plasmids expressing Akt, Stat3, and Src as well as dominant negative Stat3 have been described previously (30, 34). The antibodies to Akt1 and Stat3 were purchased from Cell Signaling and Santa Cruz Biotechnology, respectively. For transfection, the cells were seeded 18–24 h before transfection using Lipofectamine Plus. The sequence for the Stat3 antisense oligonucleotide synthesized using phosphorothioate chemistry is 5′-AAAAATGCCCAGATGCCC-3′. The sequence for control oligonucleotide is identical to the antisense oligonucleotide except for three mismatched bases (underline), 5′-AAAAAGAGCCTTTGATTGCCC-3′. The sequence of Stat3 small interfering (si) RNA is 5′-AA-CAUCUGCUAGAUCCUCGAdTdT-3′; 3′-dTdTGTUAGACGAGGUCUAGCGCAU-5′.

Transcription Start Site Mapping of Human AKTI Gene—For the analysis of the AKTI1 transcription start site, human MCF7 mRNA was reverse transcribed at 55 °C using SuperScript reverse transcriptase (Invitrogen) and a primer from the AKTI1 exon 1–specific reverse complement oligonucleotide, 5′-TGACTTCTTTGACCCAGGCTTG-3′ (31). Synthesized cDNAs were amplified by polymerase chain reaction using a series of forward primers specific for the DNA sequence from the 6,000 bp upstream of the translation start site and reverse from the non-coding region of exon 1, and the amplified products were resolved by agarose gel electrophoresis.

Cloning and Analysis of Human AKTI Promoter—5′-flanking region of the human AKTI gene was obtained by screening a human placenta genomic cosmid library (Stratagene) as template. The amplified DNA fragments, obtained by screening a human placenta genomic cosmid library, were sequenced and compared with the human genome database. They were found to contain 12–28-kb DNA fragments upstream of the translational start site. Two DNA fragments (−4293/+1 and −305/+1888, Fig. 1C) were amplified with the GC-RICH PCR System (Roche) using a cosmid clone as template. The amplified DNA fragments, −4293/+1 and −4293/+1888, were subcloned into the luciferase reporter vector pGL3 (Promega) at the KpnI/BglII site. Progressive deletion mutants of the pGL3-AKT1 promoter were created by PCR. The integrity of constructs was confirmed by DNA sequencing. The primers were used as follows: −4293(5′), 5′-CTTGTGAAATTAACGACAGGCGCTTGG-3′; −3392(5′), 5′-TTTACCGACAACTTCAGACCTCAGG-3′; −3356(5′), 5′-CTGGCCCTTTGAGGCTCTCCGAGG-3′; −2741(5′), 5′-CTCTCGCTCTTCTGATATTCCTCC-3′; −1603(5′), 5′-CAATGTCGAGAACCTCTTACG-3′; −1361(5′), 5′-CAGCAGGCGGCCGCGCTTCCC-3′; −460(5′), 5′-GGCTGTCGACCCAGAGGACTGTTACC-3′; −880(5′), 5′-CCAGAATTGGAAGGACGGAGGACCAGG-3′; −732(5′), 5′-GCGGCCACCTTCCCTGTAC-3′; −305(5′), 5′-GCTTGGCCCTTATGATGAT-3′; −103(5′), 5′-GGAGCTGCTAGTATTCCCCAT-3′; +159(3′), 5′-GCTCTGCAAGCTGCTATCTG-3′; +595(3′), 5′-GGCTGTCGAGGACCCATGTG-3′; +1809(3′), 5′-GCTTCTTTCTGCTCTCACCAGG-3′.

Luciferase Reporter Assay—NIH3T3 or HEK293 cells were cultured in 12-well plates and transiently transfected with AKTI1-Luc, Src, and/or Stat3. The amount of DNA in each transfection was kept constant by the addition of empty vector. After 36 h of transfection, luciferase activity was measured using a luciferase assay reagent (Promega). Transfection efficiency was normalized by co-transfection with β-galactosidase expressing vector. The β-galactosidase activity was measured by using Galato-Light (Tropix). Luciferase activity was expressed as relative luciferase activity.

Northern and Western Blot Analysis—Northern blot analysis of total cellular RNA was performed according to standard procedures. RNA was extracted using the RNeasy purification kits (Qiagen Inc.). Total RNA was electrophoresed in 1.0% formaldehyde-agarose gels, transferred to Duralon-UV membrane (Stratagene), and then hybridized with randomly primed α-32P-labeled cDNA probes for AKTI. Membranes were exposed to autoradiography and the mRNA levels were visualized and quantified using PhosphorImager analysis (Amersham Biosciences). Western blot analysis was performed as described previously (32). Briefly, the cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin), separated in SDS-PAGE, and immunoblotted with appropriate antibodies as indicated in the figure legends.

Chromatin Immunoprecipitation Assay (ChIP) and EMSA Assay—ChIP assay was performed as previously described (33). Solubilized chromatin from cells was cross-linked with 1% formaldehyde, treated with 2 × 105 asynchronously growing wild type Stat3 and Src. The cells were then lysed with ChIP dilution buffer (1% NP-40, 150 mM NaCl, 16.7 mM Tris-HCl, pH 8.1, and 1 mg/ml protease inhibitors), and precleared with protein-A beads blocked with 2 μg/ml of sheared salmon sperm DNA and preimmune serum. The sheared chromatin solution was divided and utilized in parallel ChIP and IP and IP assays with either an anti-Stat3 antibody or an anti-Src antibody. Following wash, the antibody–protein–DNA complex was eluted from the beads by resuspending the pellets in 1% SDS, 0.1 M NaHCO3 at room temperature for 20 min. After cross-link, protein and RNA were removed by incubation with 10 μg of proteinase K and 10 μg of RNase A at 42 °C for 3 h. Purified DNA was subjected to PCR with primers specific for 13 putative Stat3-binding sites within the AKTI1 promoter. The sequences of the PCR primers used are as follows: region 1 forward (−4293), 5′-CTTCTGTAACATTAACGACAGGG-CC-3′, reverse (−305), 5′-AATGCCACCCCTGACTAAGGTG-3′; region 2 forward (−422), 5′-AACCCTCAGTTGTTCTCTCATCC-3′, reverse (−4056), 5′-TGCTGGAAATTCACCAAATCTCAGG-3′; region 3 forward (−4134), 5′-CTTCTTGCAAGCTTCTCTGGTCGTG-3′, reverse (−3840), 5′-CCCTACTTCTCTCTGAGTGGTT-CCAGG-3′; region 4 forward (−223), 5′-ATCCAGAGGTTCTTCTGAGGAGCACC-3′, reverse (−438), 5′-AGCTGGACAGGAGAACAGC-3′; region 5 forward (−413), 5′-AGGTGTGTTCTCTTCGTCCTGTGGC-3′, reverse (−481), 5′-GTCACATTTGAAATGGTGAGCAGG-3′; region 6 forward (−447), 5′-AGGCCATGCGCTCTCTCGCATCC-3′, reverse (−598), 5′-TCCCAACATGGTCTTCTCTCACG-3′; region 7 forward (−831), 5′-GAATCAATAGGTCTCAGTGGTGG-3′, reverse (−1038), 5′-GGCTGCTTACGTTTTGTCCGACGGG-3′; region 8 forward (−899), 5′-TCTTCTTTGTCCGACAGGGGCAC-3′, reverse (−1200), 5′-ATGAGAGAGAGAGGACAGATGC-3′; region 9 forward (−1262), 5′-GATCCTGGTAGTTAGGAAGACA-CCACCC-3′, reverse (−1419), 5′-TCCCAACGCTTCCAACCTAGTGC-3′; region 10 forward (−1440), 5′-AGCCTGGTCAAAAGAAGTCTCA-AGGG-3′, reverse (−1598), 5′-ATCTGAGGTCAGGCGCACCACCC-CC-3′. Amplified PCR products were resolved by 1.2% agarose gel electrophoresis.
FIGURE 1. Human AKT1 promoter contains multiple Stat3-binding sites. A, schematic representation of the human AKT1 genomic locus. The exons are shown as boxes 1–12. B, the AKT1 promoter sequence. Putative transcription factor binding sites are boxed. Transcriptional start site and boundaries of exon 1, intron 1, and exon 2 are indicated. Putative TATA box was boxed within the AP-1-binding site. The translation initiation site, ATG, was shaded. C, a diagram displays the location of 12 putative Stat3-binding sites indicated by asterisk within the 6-kb AKT1 promoter.
electrophoresis and visualized by BioImage. EMSA was performed as previously described (35).

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done following the procedure described previously (36). Briefly, MEF cells (4 x 10^3 cells/well) were plated in 96-well microtiter plates and infected with retrovirus expressing Cre for disrupting Stat3. After infection, wild type Akt1 was transfected into the cells using Lipofectamine (Invitrogen). Cells were serum starved, incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (10 μL/well), and solubilized with 100 μL/well of 20% SDS in 50% N,N-dimethylformamide (pH 4.7) at room temperature for 4 h. Absorbance was determined in a Titertek plate reader at 570 nm. The absorbance is directly related to the viable cell number. The experiment was repeated three times.

RESULTS

Cloning of the Human AKT1 Promoter Revealed Multiple Putative Stat3-binding Sites—To analyze the transcriptional regulation of the serine/threonine kinase AKT1, we cloned the 5′-flanking region of the human AKT1 gene. Sequence analyses revealed that the AKT1 gene consists of 12 exons. The exon 1 is 1.4 kb and is located within the 5′-untranslated region. The translation initiation site ATG resides within exon 2 (Fig. 1A). The transcription start site, which was determined by 5′-rapid amplification of cDNA ends PCR, lies 1,888 bp upstream of the translation start site. A putative TATA box was identified 7 bp upstream of the transcriptional start site. Transcription element analyses of 6,181 bp upstream of the translation initiation site of the AKT1 gene revealed multiple binding sites for Stat3, NFκB, AP1, and FAS. 3 www.motif.genome.ad.jp.
The transcription factor that has the most binding sites in the AKT1 promoter is Stat3 (12 putative Stat3-binding sites: 4230/4223, 4143/4136, 4053/4046, 3287/3278, 2851/2844, 439/448, 483/491, 978/986, 1047/1055, +1324/+1331, and +1443/+1450). Consensus sequences of the Stat3-binding site are TT(N)4–6 AA (37). These observations suggest that the AKT1 gene could be regulated by Stat3 at the transcriptional level.

Stat3 Increases AKT1 Expression at mRNA and Protein Levels—To directly demonstrate whether the AKT1 is transcriptionally regulated by Stat3, MCF-10A cells were infected with adenovirus expressing constitutively active Stat3 (Stat3C) and dominant negative Stat3. The cells infected with adeno-green fluorescent protein vector were used as control. Northern blot analysis showed that constitutively active Stat3C up-regulates the AKT1 (Fig. 2A). Furthermore, immunoblotting studies revealed an elevated protein level of AKT1 in cells treated with constitutively active Stat3C (Fig. 2B). Expression of dominant negative Stat3 slightly inhibited the mRNA and protein levels of AKT1 (Fig. 2, A and B).

As Stat3 is strongly activated by Src kinase (30), we next examined if the expression level of AKT1 is elevated in v-Src-transformed NIH3T3 cells. As shown in Fig. 2, C and D, both protein and mRNA levels of AKT1 were significantly increased in v-Src-transformed NIH3T3 cells. Furthermore, blockage of Stat3 with antisense RNA considerably reduced v-Src-induced AKT1. Moreover, up-regulation of AKT1 was also detected in the human breast cancer cell line MDA-MB-468, which exhibits constitutively active Src and Stat3, but not in MDA-MB453, which does not. Inhibition of Src/Stat3 by Src inhibitor (PD180970) or Stat3-siRNA reduced AKT1 protein level in MDA-MB-468 (Figs. 2, E and F). In addition, conditional knock-out of the Stat3 gene decreased Akt1 expression in mouse embryonic fibroblasts (Fig. 6A). Based on these data, we concluded that Akt1 is a downstream target of Stat3.

Stat3 Transactivates the AKT1 Promoter—We further examined whether the AKT1 promoter is regulated by Stat3. Luciferase reporter assay revealed that pGL3-AKT1−4293/+1, which contains 5 putative Stat3-binding sites, was not stimulated by wild type or constitutively
active Stat3, even in combination with v-Src. However, ectopic expression of constitutively active Stat3 significantly induced the pGL3-AKT1 promoter activity. Furthermore, wild type Stat3 enhances v-Src-induced AKT1 promoter activity in a dose-dependent manner (Fig. 4B). Notably, expression of dominant negative Stat3 considerably reduces the AKT1 promoter activity induced by coexpression of v-Src/Stat3 (Figs. 3A and 4B), indicating that the major Stat3 response elements exist in exon 1/intron 1 regions, where there are 7 putative Stat3 binding motifs, as well as in a distal region of the promoter.

To determine whether Stat3 could directly bind to the Stat3-binding site within the AKT1 promoter in vivo and to further define the Stat3 response elements in the promoter, we carried out ChIP assay, which detects specific genomic DNA sequences that are associated with a particular transcription factors in intact cells. HEK293 cells were transfected with wild type Stat3 and v-Src and immunoprecipitated with a Stat3 antibody. The Stat3 bound chromatin was subjected to PCR using oligonucleotide primers that amplify regions spanning each Stat3-binding site within the AKT1 promoter. As show in Fig. 5C, the anti-Stat3 antibody pulled down four Stat3-binding sites (−4230/−4223, +978/+986, +1324/+1331, and +1443/+1450 (SB1, SB7, SB9, and SB10)). In contrast, immunoprecipitation with an irrelevant antibody (anti-actin) resulted in the absence of bands in these sites. These results indicate that Stat3 directly binds to the AKT1 promoter. By mutation of Stat3 binding consensus sequences (TT → GG) in AKT1 −325/+1888 that is highly

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FIGURE 4. AKT1 promoter is activated by Src through Stat3. A, reporter assay showed that pGL3-AKT1−325/+1888 was significantly induced by coexpression of v-Src/Stat3 (Figs. 3B and 5A), whereas deletion of a region between −4293 and −3172, i.e., pGL3-AKT1−3172/+1888 considerably reduced the promoter activity. Furthermore, pGL3-AKT1−3172/+595 failed to respond to v-Src/Stat3 (Fig. 5A), indicating that the major Stat3 response elements exist in exon 1/intron 1 regions, where there are 7 putative Stat3 binding motifs, as well as in a distal region of the promoter. Notably, the results also suggest the presence of a repression factor binding site(s) within the −3172/−325 region because the promoter activity is significantly increased by deletion of this region as revealed by comparison of the activity between −3172/−325 and −325/+1888 (Fig. 5, A and B).

To determine whether Stat3 could directly bind to the Stat3-binding site within the AKT1 promoter in vivo and to further define the Stat3 response elements in the promoter, we carried out ChIP assay, which detects specific genomic DNA sequences that are associated with a particular transcription factors in intact cells. HEK293 cells were transfected with wild type Stat3 and v-Src and immunoprecipitated with a Stat3 antibody. The Stat3 bound chromatin was subjected to PCR using oligonucleotide primers that amplify regions spanning each Stat3-binding site within the AKT1 promoter. As show in Fig. 5C, the anti-Stat3 antibody pulled down four Stat3-binding sites (−4230/−4223, +978/+986, +1324/+1331, and +1443/+1450 (SB1, SB7, SB9, and SB10)). In contrast, immunoprecipitation with an irrelevant antibody (anti-actin) resulted in the absence of bands in these sites. These results indicate that Stat3 directly binds to the AKT1 promoter. By mutation of Stat3 binding consensus sequences (TT → GG) in AKT1−325/+1888 that is highly...
induced by Src/Stat3 and Stat3C (Fig. 5, A and B), we further demonstrated that the Stat3-binding sites (SB6, SB9, and SB10) within the exon 1 and intron 1 regions were required for Stat3 transactivation of the AKT1 promoter (Fig. 5 D). Moreover, EMSA revealed that Stat3 is capable of binding in vitro to DNA oligonucleotides corresponding to the four Stat3 SIE/GAS binding sites identified in the Akt1 promoter (Fig. 5E). Unlike SIE positive control, however, the supershift was not detected in these four Stat3-binding sites within the promoter (Fig. 5E and data not shown), which is also observed in other Stat3-induced promoters, such as vascular endothelial growth factor, Bcl-xL, and c-Myc (34, 38, 39).

**AKT1 Mediates Stat3 Function**—Both Stat3 and AKT1 play an essential role in cell survival (40–45). Because AKT1 is a direct target of Stat3, we reasoned that AKT1 could mediate Stat3 function. To test this hypothesis, conditional knock-out of Stat3 MEF cells were infected with Cre and adenovirus expressing wild type AKT1 (Fig. 6A). Cell survival was evaluated after serum withdrawal for 24 and 48 h. Triple experiments showed that Cre-infected Stat3 MEF

**FIGURE 5.** Definition of the Stat3 response elements. A and B, major Stat3 response elements located within the exon 1/intron 1 region of the AKT1 promoter. A series of deletion mutants of the AKT1 promoter (left) were introduced into NIH3T3 cells together with or without v-Src/Stat3 (A) or constitutively active Stat3C (B) and then subjected to luciferase reporter assay. C, Stat3 binds to 4 sites of the AKT1 promoter in vivo. ChIP assay was performed as described under “Experimental Procedures.” Triple experiments showed that Stat3 directly binds to 4 sites of the AKT1 promoter. D, mutation of Stat3 DNA-binding sites within the AKT1 promoter abrogates the Src/Stat3-stimulated promoter activity. Luciferase assay was performed in NIH3T3 cells transfected with the AKT1-Luc/−325/+1888 and its mutants as well as v-Src and Stat3. E, Stat3 binds to DNA oligonucleotides corresponding to Stat3 SIE/GAS binding sites in the Akt1 promoter. EMSA of double-stranded oligonucleotides containing Stat3-binding sites are indicated on the top. Equal amounts of 32P-labeled oligonucleotides were incubated with nuclear extract prepared from Stat3C-transfected HEK293 cells in the presence or absence of a 100 μM excess of the unlabeled oligonucleotides (competitor). SIE oligonucleotides were used as positive control (right panel).
increased cell death approximately 30%. Ectopic expression of wild type AKT1 largely rescued knockdown Stat3-induced cell death (Fig. 6B). It is noted that the Akt1 protein level was considerably decreased in Stat3 knock-out MEFs, further suggesting the critical role of Stat3 in transcriptional regulation of the AKT1 gene. To further examine the effects of Akt1 on the Stat3 cell survival signal, constitutively active Stat3-presenting breast cancer cell line MDA-MB-468 was treated with Stat3-siRNA or Akt1-siRNA as well as the co-transfection of siRNA-Stat3 and HA-Akt1 (Fig. 6C). Tunel assay revealed that knockdown of either Stat3 or Akt1 induced cell death about 45–50% in response to serum starvation. However, reintroduction of Akt1 largely inhibits the apoptosis resulted from knockdown of Stat3 (Fig. 6D).

**DISCUSSION**

Alterations of AKT1 at the DNA level have been reported in a single gastric cancer (23). However, a number of tumors exhibit elevated levels of mRNA, protein, and/or kinase of AKT1 (46), implicating that AKT1 is regulated at the transcriptional, translational, and/or post-translational levels. Post-translational regulation of AKT1 has well been doc-
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FIGURE 6. Reintroduction of the AKT1 into Stat3−/− MEFs rescues cell death induced by serum withdraw. A, conditional knock-out of the Stat3 down-regulates Akt1. Stat3 knock-out MEFs were infected with retrovirus expressing Cre or retrovirus vector alone and immunoblotted with anti-Stat3, -Akt1, and -actin antibodies (left panels). Right panels show expression of AKT1 by reintroduction of the AKT1 into Stat3−/− MEFs. B, Stat3-induced cell survival is mediated, at least in part, by AKT1. Stat3−/− MEFs infected with or without retrovirus-Cre and adeno-AKT1 were assayed for cell survival after serum withdraw for the indicated times. C and D, knockdown of either Stat3 or Akt1 induces apoptosis and the reintroduction of Akt1 rescues programmed cell death from knockdown of Stat3. MDA-MB-468 cells were treated with the siRNA of Stat3 (lanes 2 and 3) or Akt1 (lane 5) as well as scramble siRNA (lanes 1 and 4). HA-Akt1 was simultaneously introduced into the Stat3-siRNA cells (lane 3). Following 48 h of transfection and 16 h of serum starvation, apoptotic cells were assessed by Tunel assay and quantified. Data are representative of three independent experiments. E, schematic illustration of the regulation of Akt1 by the Src/Stat3 pathway.

It has been shown that Src is a key molecule for activation of the Akt pathway. We and others (11, 56) have previously demonstrated that constitutively active Src induces Akt kinase activity through phosphatidylinositol 3-kinase. There is also evidence showing that Src directly binds to Akt and activates Akt through phosphorylation of Akt at tyrosine 315 (57). Inhibition of Src reduces Akt activation by growth factor(s). In addition, Src mediates estrogen/estrogen receptor and androgen/androgen receptor activation of Akt (58–61). In the present report, we present evidence of Src regulation of Akt at the transcriptional level through activation of Stat3 (Fig. 6F). It is noted that the Akt1 promoter activity induced by Src is considerably lower than that by expression of constitutively active Stat3C (Figs. 3–5), suggesting that a repressive molecule(s) toward the Akt1 promoter is regulated by Src kinase.

In summary, we have isolated and characterized the AKT1 promoter. The promoter sequence analysis demonstrates Stat3 transcriptional regulation of the AKT1. Furthermore, we have shown that Src/Stat3 induces Akt1 expression through directly binding to the promoter. Blocking Stat3 by antisense or genetic knock-out significantly decreases Akt1 expression. Ectopic expression of AKT1 rescues the cell survival phenotype from Stat3−/− MEF cells. These findings are important for several reasons. First, they provide a mechanistic understanding of regulation of the AKT1 at the transcriptional level. Second, a direct link between Akt1 and Stat3 pathways has now been established. Finally, pharmacological inhibition of the AKT pathway may have anti-growth effects in tumor cells with activation of Stat3 or vice versa.
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