Fusion of OTT to BSAC Results in Aberrant Up-regulation of Transcriptional Activity

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OTT/RBM15-BSAC/MAL/MKL1/MRTF-A was identified as a fusion transcript generated by t(1;22)(p13;q13) in acute megakaryoblastic leukemia. Previous studies have shown that BSAC (basic, SAP, and coiled-coil domain) activates the promoters containing CArG boxes via interaction with serum response factor, and OTT (one twenty-two) negatively regulates the development of megakaryocytes and myeloid cells. However, the mechanism by which OTT-BSAC promotes leukemia is largely unknown. Here we show that OTT-BSAC, but not BSAC or OTT strongly activates several promoters containing a transcription factor Yin Yang 1-binding sequence. In addition, although BSAC predominantly localizes in the cytoplasm and its nuclear translocation is considered to be regulated by the Rho-actin signaling pathway, OTT-BSAC exclusively localizes in the nucleus. Moreover, OTT interacts with histone deacetylase 3, but this interaction is abolished in OTT-BSAC. Collectively, these functional and spatial changes of OTT and BSAC caused by the fusion might perturb their functions, culminating in the development of acute megakaryoblastic leukemia.

Transcriptional activation of many genes depends on activities of the transcriptional factors that recognize specific target sequences but also the chromatin structures. Histone acety-
Yin Yang 1 (YY1) is a ubiquitous zinc finger transcription factor that binds to many different cellular and viral promoters in a sequence-specific manner to regulate transcription (15, 16). The mechanism by which YY1 activates or represses transcription largely depends on the interaction with other transcription factors or histone modification enzymes including DNA-binding domains of both OTT and MAL. However, the molecular mechanism whereby this fusion protein induces leukemogenesis is largely unknown. On the other hand, we and others have independently identified a murine homolog of MAL, referred to as BSAC and MKL1/MRTF-A by functional cloning to inhibit tumor necrosis factor α-induced cell death and bioinformatics to identify related genes to myocardin, respectively (9, 10). Accumulating studies have shown that BSAC/MAL/MKL1/MRTF-A activates the promoters containing CArG boxes (CC(A/T)6GG) through associating with serum response factor (SRF) (9–11). In addition, nuclear translocation of BSAC is tightly regulated by the Rho-actin signaling pathway (12). Although BSAC/MAL/MKL1/MRTF-A and SRF are broadly expressed in various tissues, the defect of MKL1/MRTF-A−/− mice is unexpectedly restricted to the development of the mammary gland (13, 14).

Given that the promoters containing CArG boxes are found in immediate early genes or muscle-specific genes and OTT-BSAC is involved in the development of leukemia, we speculated that OTT-BSAC activates promoter(s) containing a motif other than CArG box. We found that OTT-BSAC strongly activated the promoters of human platelet collagen receptor glycoprotein VI (GPVI) gene. Deletion and mutation analysis revealed that OTT-BSAC-mediated transcriptional activity depended on the YY1-binding sequences. Interestingly, in contrast to BSAC, which predominantly localized in the cytoplasm and the nuclear translocation of which is tightly regulated by the Rho-dependent signals (12), OTT and OTT-BSAC exclusively localized in the nucleus. The constitutive nuclear accumulation of OTT-BSAC may contribute to a significant enhancement of its transcriptional activity. Moreover, OTT interacted with HDAC3, and this interaction was abolished in OTT-BSAC. Collectively, these functional and spatial alterations of OTT and BSAC may culminate in the development of leukemia.

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**Experimental Procedures**

**Reagents and Cell Culture**—Anti-FLAG (Sigma-Aldrich), anti-hemagglutinin (Roche Applied Science), anti-Myc, anti-GAL4, and anti-YY1 (Santa Cruz Biotechnology), anti-HDAC3 (Biomol), anti-β-actin (BioLegend) antibodies, control mouse IgG (BD Biosciences), and control rabbit IgG (Sigma-Aldrich) were purchased from the indicated sources. HEK293 and HEK293T cells were cultured in high glucose Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Megakaryocytic leukemic cell lines, CMS and CMY (T. Sato), and MEG-01 cells (M. Seto) (19) were kindly provided and cultured in RPMI1640 medium containing 10% fetal calf serum. Anti-OTT antibody was generated by immunizing rabbits with GST-OTT (609–730). Anti-BSAC antibody was generated and described previously (9).

**Plasmids**—pBj5-FLAG-HDAC1 (S. Schreiber), pME18S-FLAG-HDAC2 and pCPE4-FLAG-HDAC3 (E. Seto), and pCMX-mSMRTβ-FL (R. M. Evans) were kindly provided from the indicated researchers. pCR-FLAG-YY1 was constructed by PCR using pCR-YY1 as a template (20). pCR-FLAG-HDAC3AN and pCR-FLAG-HDAC3ΔC were constructed by deleting N-terminal 307 and C-terminal 121 amino acids using PCR, respectively. A full-length OTT cDNA was isolated by screening a library derived from human HTLV-1-transformed T cell line HAT109 as a standard procedure. To express full-length and various deletion mutants of OTT as fusion proteins with DNA-binding domain of a yeast transcriptional factor GAL4, PCR products encoding the indicated amino acids were subcloned into pFA vector (Stratagene), designated as pFA-OTT, pFA-OTT(1–677), pFA-OTT(654–957), pFA-OTT(609–730), and pFA-OTT(1–533). pcDNA3-Myc-OTT was constructed by PCR and subcloned into pcDNA3-Myc vector. pcDNA3-Myc-human BSAC and pcCR-FLAG-human HDAC6 were constructed by PCR using KIAA1438 (human BSAC/MAL/MKL1/MRTF-A) and KIAA0901 (human HDAC6) cDNAs derived from the Kazusa DNA Institute as templates, respectively. To make an expression vector for OTT-BSAC, PCR products of OTT and BSAC were connected by creating an additional EcoRI site at the fusion junction and ligated to pcDNA3-Myc vector. The artificially created EcoRI site was subsequently mutated to the originally published sequence of the OTT-BSAC fusion junction using a QuikChange site-directed mutagenesis kit (Stratagene). Expression vectors for C-terminal deletion mutants of OTT-BSAC (−351) and OTT-BSAC (−537) were constructed by using the internal restriction enzyme sites XmnI and BsgI to delete C-terminal fragments, respectively. The numbers indicate deleted amino acids from the C terminus of OTT-BSAC.

**Reporter Assay**—The promoter of the human GPVI gene (−315 to +29) was amplified by PCR using human genomic DNA as a template and subcloned into pGL3-basic vector (Promega), designated as pGL3-GPVI (−315/+29). A series of 5′ deletion mutants, pGL3-GPVI (−207/+29), pGL3-GPVI (−79/+29), and pGL3-GPVI (−39/+29) were generated by PCR using pGL3-GPVI (−315/+29) as a template. pGL3-GPVI (−208/+29M1), pGL3-GPVI (−208/+29M2), and pGL3-GPVI (−79/+29M3) were generated by introducing mutations.
of GATA (AGATAA to CGCTTA), the first YY1 (GATGAG to GCTTAG), and third YY1 (CTCATC to CTAAGC) binding sites, respectively. Reporter plasmids, pGL3-c-fos (−700/+53), pGL3-FcErRα (−605/+29), and mPGV-B-il-6 (−181/+14) were described previously (9, 20, 21). Luciferase assays using HEK293 and HEK293T cells were performed as previously described (22). MEG-01 cells (2 × 10^5) were transfected with the indicated expression vectors along with reporter plasmids using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were harvested, and the luciferase activities were measured on a luminescence detector, FMBIO-100 (Takara Shuzo).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as previously described (20). Briefly, 5 μg of the nuclear extracts were incubated with the rhodamine-labeled wild-type oligonucleotides in the absence or presence of anti-YY1 antibody, wild-type, or mutant cold competitors with a 10–100-fold excess. The oligonucleotides used were as follows: wild-type sense oligonucleotide for GPVI, 5′-AGGAAGGGAGGAGCATTCTCTCACATCATCCTCGGCTCGATGAGTCTC-3′; reverse primer (5′-ATCCTCATCACATCCTG-3′), mutant sense oligonucleotide, 5′-AGGAAGGGAGGAGCATTCTCTCATCCTAAGCGCATTCTCG-3′. Mobility shift of the complexes was analyzed by a fluorescence detector, MFBIO-100 (Takara Shuzo).

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was performed using a ChIP Assay kit (Millipore) as previously described (23). Quantitative PCRs were performed using TaqMan Universal PCR master mix and a 7500 Real-Time PCR system (Applied Biosystems). The primers to amplify the promoter region of GPVI gene (−127/+29) and a TaqMan probe were as follows: Forward primer (5′-GGCTACGGCTCGATGAGTCTC-3′), reverse primer (5′-TCAGGCCTGTCTCTAGAGCTCT-3′), and a TaqMan probe (5′-FAM-TTCATCTCATCATCCAC-GMB-3′). The amount of target DNA bound to YY1 or OTT-BSAC was quantified using immunoprecipitates with control, anti-YY1, or anti-Myc antibodies from the cycle threshold value, which was determined using 7500 SDS software (Applied Biosystems). In brief, the ratio of the amount of a specific DNA fragment in each immunoprecipitate to the amount of that fragment in the DNA before immunoprecipitation (input DNA) was calculated from each cycle threshold value.

Subcellular Fractionation, Immunoprecipitation, and Immunoblotting—HEK293 cells (4 × 10^6) were transiently transfected with the indicated expression vectors using Lipofectamine (Invitrogen). MEG-01 cells (4 × 10^6) were untreated or transfected with the indicated expression vectors using a nuclease according to the manufacturer’s instructions (Amaxa). For subcellular fractionation, the cells were harvested at 24–36 h after transfection and washed with 1 ml of a buffer A (10 mM Hapes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and then resuspended in 500 μl of the buffer A. After incubation for 30 min, the cells were passed with a 30-gauge syringe 10 times, followed by centrifugation at 700 × g. The supernatants were further centrifuged at 15,000 × g to remove insoluble pellets, and the resulting supernatants were collected as the cytoplasmic fractions. The pellets were resuspended in 100 μl of buffer B (20 mM Hapes, pH 7.9, 450 mM NaCl, 1.5 mM MgCl₂, 25% glycerol, 0.2 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) for 60 min. After centrifugation at 15,000 × g for 10 min, the supernatants were collected as the nuclear fractions. Equal amounts of proteins from each fraction were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were incubated with the indicated antibodies followed by the corresponding secondary antibodies. The membranes were then developed with the ECL Western Blotting Detection System Plus (GE Healthcare).

Small Interfering RNAs (siRNAs)—HEK293T cells (2.5 × 10^5) were transfected with siRNAs targeting green fluorescent protein (control) or YY1 (ON-TARGETplus SMARTpool siRNA, Dharmacon) and pGL3-GPVI (−315/+29) along with Myc-OTT-BSAC using Lipofectamine 2000 (Invitrogen). After 48 h, knockdown of YY1 was evaluated by immunoblotting with anti-YY1 antibody using total lysates, and luciferase assay was performed as previously described (22).

Immunostaining—MEG-01 cells (4 × 10^6) were untreated or transfected with the indicated expression vectors and plated on glass slides. After 24 h, the cells were washed with phosphate-buffered saline, fixed with 2% paraformaldehyde, and then incubated with anti-OTT, anti-BSAC, and anti-Myc antibodies. The primary antibodies were detected by secondary antibodies conjugated with Alexa 488 or Alexa 594 (Invitrogen). To visualize the nuclei, the cells were incubated with Hoechst 33258 (Invitrogen). Stained cells were mounted in SlowFade (Invitrogen) and analyzed on a laser scanning confocal microscope (Zeiss).

RESULTS

Identification of a Novel Target Gene Activated by the Fusion Protein OTT-BSAC—OTT-MAL encodes a fusion protein containing complete domain structures of both OTT and MAL (Fig. 1A). However, the molecular mechanism whereby this fusion protein induces leukemia is largely unknown. Given that CARG boxes are found in the promoters of immediate early genes or muscle-specific genes, we speculated that OTT-BSAC should control gene(s) that might be responsible for the development of leukemia. Given that OTT-BSAC might impair the differentiation of megakaryocytes, we first tested whether OTT-BSAC could affect the promoter activity of megakaryocyte-specific genes. A previous study has shown that GPVI is specifically expressed in megakaryocytes and platelets (24). Then we generated a reporter plasmid containing the GPVI promoter upstream of a luciferase gene. We transfected megakaryocytic leukemic cell line MEG-01 cells with expression vectors for BSAC, OTT, and OTT-BSAC along with a reporter plasmid, pGL3-GPVI (−315/+29), and tested the effect of each protein on this promoter activity. Expression of BSAC or OTT did not significantly increase this promoter activity (Fig. 1B), which is consistent with the fact that this promoter does not contain a CARG box. Surprisingly, expression of OTT-BSAC strongly activated this promoter in a dosedependent fashion (Fig. 1B). This OTT-BSAC-mediated transcriptional activation of the GPVI promoter was also observed in HEK293 cells (Fig. 1B). To investigate whether OTT-BSAC-dependent transcriptional activity on the GPVI promoter is
FIGURE 1. OTT-BSAC strongly activates the GPVI and c-fos promoters. A, a diagram of domain structures of OTT/RBM15, BSAC/MAL/MKL1/MRTF-A, and OTT-BSAC. RNA recognition motif, SPOC, RPEL, basic (B), glutamine-rich (Q), SAP, coiled-coil (CC), and TA domains are shown. B, MEG-01 and HEK293 cells were transfected with increasing amounts of the indicated expression vectors along with pGL3-GPVI (−315/+29). Luciferase activities are expressed as fold increases above that with control vector. Each experiment was performed in triplicate, and the results are expressed as the means ± SE of three experiments. C, MEG-01 cells were transfected with increasing amounts of the indicated expression vectors along with pGL3-c-fos (−700/+53). Luciferase activities are expressed as in B.

Transcriptional Activation by OTT-BSAC Depends on the YY1-binding Sequences—To identify a target sequence recognized by OTT-BSAC, we constructed a series of 5′ deletion mutants of the GPVI promoter (Fig. 2A). Although deletion up to the position −208 did not impair the transcriptional activity induced by OTT-BSAC, further deletion up to −80 reduced the transcriptional activity to −50% (Fig. 2B). Moreover, deletion up to −40 resulted in complete loss of the transcriptional activation. These results indicate that the regions spanning −207 to −80 and −79 to −40 are essential for OTT-BSAC-mediated actually binds to the promoter by EMSA. We prepared the nuclear extracts from MEG-01 cells and performed EMSA using double-stranded oligonucleotides (oligonucleotides) containing a region spanning −79 to −40 as a probe. As shown in Fig. 2C, two major retarded bands (designated the complex I and II hereafter) were detected in this assay, and these two bands disappeared by the addition of the nonlabeled wild-type oligonucleotides. In contrast, the addition of the mutant probe, in which YY1-binding core sequence (TCAT) was mutated to TAAG, did not abolish the binding of the two complexes to the labeled oligonucleotides, indicating that these complexes specifically bound to TCAT sequence. Moreover, complex II but not complex I disappeared in the presence of anti-YY1 antibody, suggesting that complex II contains YY1. Collectively, YY1 binds to the GPVI promoter via TCAT sequence. However, we could not detect direct interaction of YY1 with BSAC or OTT in cotransfection experiments or a ternary complex containing YY1 and BSAC or OTT-BSAC in the presence of YY1-binding sequence in EMSAs (data not shown).

To directly show that endogenous YY1 is recruited to the GPVI promoter under more physiological conditions, we performed ChIP assays. Consistent with EMSAs, anti-YY1 but not control antibody efficiently immunoprecipitated the GPVI promoter from HEK293T and MEG-01 cells (Fig. 2D). However, the relative intensities of the GPVI promoter using immunoprecipitates with anti-Myc antibody were not different in between mock and Myc-OTT-BSAC-transfected HEK293T cells (Fig. 2E). This suggests that the recruitment of transfected transactivation. A previous study has shown that the GPVI promoter activity is regulated by Sp1, GATA, and Ets motifs (24). In addition, we found three putative YY1-binding sequences (designated as YY1-I, YY1-II, and YY1-III) in the GPVI promoter (Fig. 2A). We tested whether the mutation of these sites impairs OTT-BSAC-mediated transactivation. The mutation of the GATA-binding sequence did not reduce but rather enhanced the transcriptional activity. Unexpectedly, mutation of YY1-I reduced the transcriptional activity comparable with a deletion mutant (−79/+29) (Fig. 2B). Furthermore, the mutation of YY1-III substantially reduced the transcriptional activity 5-fold. Notably, combined mutations of YY1-III along with Ets motifs or YY1-II did not further reduce the transcriptional activity compared with YY1-III mutation alone (data not shown). Combining these data together indicates that YY1-I and YY1-III sequences are essential for OTT-BSAC-dependent transcriptional activation.

We next tested whether YY1 mediated by the transcriptional activation (TA) domain of BSAC, we transfected MEG-01 cells with deletion mutants of OTT-BSAC lacking the TA domain. Although the expression levels of two OTT-BSAC mutants lacking the TA domain were comparable with OTT-BSAC (supplemental Fig. S1), two mutants failed to activate this promoter (Fig. 1C). This indicates that the TA domain of BSAC mediates transcriptional activity of OTT-BSAC. Given that protein expression levels of OTT-BSAC were consistently lower than BSAC, possibly because of its large molecular mass of OTT-BSAC (supplemental Fig. S1), increased transcriptional activity of OTT-BSAC is not due to the increased protein expression levels. Consistent with a previous study (11), we also observed significant enhancement of transcriptional activity of OTT-BSAC on the c-fos promoter compared with BSAC (Fig. 1D). Given that the GPVI but not c-fos promoter does not contain CArG box, these results suggest that OTT may directly or indirectly recruit BSAC to the GPVI promoter, resulting in up-regulation of the GPVI promoter activity.
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FIGURE 2. OTT-BSAC activates the GPVI promoter via the YY1-binding motifs. A, the promoter region of human GPVI gene. The putative transcriptional factor binding sites are underlined by the arrows showing its orientation (sense or antisense orientation). +1 indicates the transcription start site. The putative YY1-binding sequences are indicated by bold characters. B, delineation of the regions required for OTT-BSAC-dependent transactivation. MEG-01 cells were transfected with OTT-BSAC along with the indicated mutants of pGL3-GPVI reporter vector. Luciferase activities are expressed as in Fig. 1B. M1, M2, and M3 are the mutants, in which GATA1, the first YY1, and third YY1 motifs were mutated, respectively. C, YY1 specifically binds to the promoter of GPVI. The nuclear extracts were incubated with the rhodamine-labeled wild-type oligonucleotides containing the GPVI promoter (+79 to −39) in the absence or presence of increasing amounts of the nonlabeled wild-type (WT) or mutant (MT) oligonucleotides or anti-YY1 or control antibodies. The retarded bands are indicated by arrows. The asterisk indicates nonspecific bands. D, in vivo binding of YY1 to the GPVI promoter in HEK293T and MEG-01 cells. The binding of YY1 to the GPVI promoter region (−127/+22) was quantified using ChIP assays. The results are expressed as the means ± S.D. of three independent PCRs with duplicates samples. E, OTT-BSAC does not bind to the GPVI promoter in vivo. Mock or Myc-OTT-BSAC-transfected HEK293T cells were subjected to ChIP assays using anti-YY1, anti-Myc, or control antibodies. The results are expressed as in D.

OTT-BSAC to the GPVI promoter could not be detected, at least under our experimental conditions.

YY Is Not Essential for OTT-BSAC-dependent Transcriptional Activation—Previous studies have shown that myocardin and BSAC do not directly bind to the promoters containing CArG boxes but activates them through interaction with SRF (9–11). Under these conditions, transcriptional activities by myocardin and BSAC are extremely sensitive to the levels of SRF, because high concentration of SRF does not enhance but rather attenuates myocardin- or BSAC-dependent transcriptional activation (25). To test whether similar interplay between OTT-BSAC and YY1 is also observed on the GPVI promoter, we examined whether expression of YY1 attenuates OTT-BSAC-dependent transactivation. Although expression of YY1 alone weakly activated this promoter, expression of YY1 substantially inhibited OTT-BSAC-mediated transcriptional activation in a dose-dependent fashion (Fig. 3A). Notably, this inhibitory effect of YY1 was promoter-specific, because expression of YY1 only weakly inhibited OTT-BSAC-dependent transactivation on the c-fos promoter (Fig. 3B). We confirmed that the expression levels of transfected YY1 in the GPVI promoter-transfected MEG-01 cells were nearly identical to those of c-fos promoter-transfected MEG-01 cells (Fig. 3C).

The fact that YY1 substantially suppressed OTT-BSAC-mediated transcriptional activation raises two possibilities. One is that YY1 may recruit OTT-BSAC to the GPVI promoter, although the recruitment of transfected OTT-BSAC to the GPVI promoter was not detected under our experimental conditions (Fig. 2E). The other is that a transcription factor other than YY1 recruits OTT-BSAC to the YY1-binding sequences and activates the GPVI promoter; therefore YY1 appears to suppress OTT-BSAC-dependent transcriptional activation through competitive binding inhibition (Fig. 3A). To discriminate these two possibilities, we knocked down endogenous YY1 using siRNA and tested whether OTT-BSAC-dependent transcriptional activation is abolished in YY1-knockdown HEK293T cells. Although YY1 siRNA efficiently knocked down expression of YY1, OTT-BSAC-mediated transcriptional activation was not impaired (Fig. 3D). Collectively, OTT-BSAC activates transcription on the GPVI promoter through the YY1-binding sequences, but YY1 is not essential for OTT-BSAC-mediated transcriptional activation.

We finally investigated whether OTT-BSAC activates other promoters containing the YY1-binding sequences. We have previously shown that the human FceRla subunit promoter contains the YY1-binding sequences and is activated by YY1 (20). Thus, we tested whether expression of OTT-BSAC activates this promoter. As expected, OTT-BSAC substantially increased this promoter activity (Fig. 3E). We also found that OTT-BSAC activates the murine il-6 promoter, which also contains the YY1-binding sequences (Fig. 3E).

The Signal-independent Nuclear Accumulation of OTT-BSAC—To elucidate the mechanism whereby transcriptional activity of OTT-BSAC is enhanced compared with BSAC, we speculated that OTT fusion to BSAC could affect the subcellular localization of BSAC. We first examined the subcellular
localization of endogenous BSAC and OTT in MEG-01 cells. Consistent with a previous study (12), endogenous BSAC predominantly localized in the cytoplasm with a minor population in the nucleus (Fig. 4A). To investigate the subcellular localization of endogenous OTT, we generated anti-OTT antibody. This antibody recognized endogenous OTT with a molecular mass of 120 kDa in the whole cell lysates from MEG-01, HeLa, HEK293, and Jurkat T, but not CMS or CMY cells (Fig. 4C). Consistent with a very recent study, in which ectopically expressed OTT localizes in the nucleus (26), endogenous OTT localized in the nucleus (Fig. 4A). Similarly, transfected BSAC and OTT showed identical subcellular distribution patterns to endogenous BSAC and OTT, respectively (Fig. 4B). We finally investigated the localization of OTT-BSAC. Because leukemia cell line(s) from patients with acute megakaryoblastic leukemia are not currently available, we transiently transfected MEG-01 cells with Myc-OTT-BSAC. Interestingly, OTT-BSAC exclusively localized in the nucleus (Fig. 4B).

To evaluate the subcellular localization of OTT, BSAC, and OTT-BSAC more quantitatively, we separated the cells into the cytoplasmic and nuclear fractions and detected each protein in the fractions by using Western blotting. Consistent with the results using a confocal microscopy, endogenous and transfected OTT mainly localized in the nucleus (Fig. 4, D and E). Although endogenous and transfected BSAC predominantly localized in the cytoplasm and the small population of BSAC localized in the nucleus, transfected OTT-BSAC exclusively localized in the nucleus. Collectively, OTT fusion to BSAC drastically changed the subcellular localization of BSAC. This might be one of the molecular mechanisms underlying the aberrant up-regulation of OTT-BSAC-dependent transcriptional activity.

**OTT Interacts with HDAC3**—A previous study has shown that SHARP interacts with HDACs, SMRT, and N-CoR and acts as a transcriptional repressor (5). OTT has a structural similarity to SHARP (5, 6), prompting us to test whether OTT interacts with HDACs. We transiently transfected HEK293 cells with Myc-OTT along with FLAG-HDAC1, -2, or -3. The lysates were immunoprecipitated with anti-Myc antibody, and co-immunoprecipitated HDACs were detected by anti-FLAG antibody. HDAC3, but not HDAC1 or HDAC2, was specifically co-immunoprecipitated with OTT (Fig. 5A). In contrast, BSAC could not bind to HDAC3. A reciprocal immunoprecipitation experiment showed that OTT was also co-immunoprecipitated with HDAC3 (Fig. 5A). On the other hand, OTT did not interact with HDAC6, a member of the class II HDAC family (data not shown).

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FIGURE 4. Signal-independent nuclear accumulation of OTT-BSAC. A and B, MEG-01 cells were untransfected (A) or transfected (B) with Myc-BSAC, Myc-OTT, or Myc-OTT-BSAC. Then the cells were stained with anti-BSAC (A), anti-OTT (A), or anti-Myc (B) antibodies and analyzed by a confocal microscope. The nuclei were stained with Hoechst 33258 (blue) and the merged images are represented at the right. C, expression of OTT in various cell lines. Expression levels of endogenous OTT were analyzed by immunoblotting (IB) with anti-OTT antibody. The relative molecular mass (kDa) is indicated at the left. D, subcellular fractionation of endogenous OTT and BSAC in MEG-01 cells. Subcellular fractionation was performed as described under “Experimental Procedures,” and equal amounts of proteins were subjected to SDS-PAGE. The expression levels of OTT and BSAC were analyzed by immunoblotting with anti-OTT and anti-BSAC antibodies, respectively. C and N indicate the cytosolic and nuclear fractions, respectively. E, subcellular localization of transfected Myc-OTT, Myc-BSAC, and Myc-OTT-BSAC. MEG-01 cells were transfected with the indicated vectors, and the subcellular fractionation and SDS-PAGE were performed as in D. Expression levels of transfected proteins in each fraction were analyzed by immunoblotting with anti-Myc antibody (top panel). The equal loading of the nuclear extracts was verified by immunoblotting with anti-YY1 antibody (bottom panel).

FIGURE 5. OTT physically interacts with HDAC3. A, OTT interacts with HDAC3, but not HDAC1 or HDAC2. HEK293 cells were transfected with the indicated expression vectors. After immunoprecipitation (IP) with control (lane C), anti-Myc, or anti-FLAG antibodies, co-immunoprecipitated proteins were detected by immunoblotting (IB) with anti-FLAG or anti-Myc antibodies (top panel). Expression levels of the transfected proteins in the total lysates (TL) were analyzed by immunoblotting with anti-Myc or anti-FLAG antibodies, respectively (middle and bottom panels). The numbers 1, 2, and 3 indicate HDAC1, HDAC2, and HDAC3, respectively. B, endogenous interaction of OTT with HDAC3 in HEK293 and MEG-01 cells. After immunoprecipitation with control (lane C) or anti-HDAC3 antibodies, co-immunoprecipitated OTT was detected by immunoblotting with anti-OTT antibody (top panels). Expression levels of endogenous OTT and HDAC3 in the total lysates were analyzed by immunoblotting with anti-OTT and HDAC3 antibodies, respectively (middle and bottom panels).

not shown). To confirm the interaction of OTT with HDAC3 under more physiological conditions, we immunoprecipitated the lysates from HEK293 and MEG-01 cells with anti-HDAC3 antibody, and then co-immunoprecipitated OTT was detected with anti-OTT antibody. Anti-HDAC3, but not control antibody efficiently co-immunoprecipitated endogenous OTT (Fig. 5B). Collectively, these results indicate that OTT physically interacts with HDAC3 in vivo.

It is well known that HDAC3 is a component of a large nuclear corepressor complex including Sin3A, N-CoR, and SMRT. We next tried to detect interaction of OTT with Sin3A, N-CoR, and SMRT; however, under our experimental conditions, we could not detect direct interaction of OTT with either of them (data not shown). Therefore, future study will be required to address whether OTT might be a component of the nuclear corepressor complex.

Domain Mapping of OTT and HDAC3 for Their Interaction—We next delineated the regions of HDAC3 and OTT responsible for their interaction. Co-immunoprecipitation experiments using deletion mutants of HDAC3 revealed that N-terminal 307 amino acids were sufficient for binding to OTT (Fig. 6, A and B). Because the expression levels of HDAC3ΔN were consistently very low because of an increase in sensitivity to degradation of the transfected HDAC3ΔN in the cells, we could not formally exclude the possibility that HDAC3ΔN may also mediate the interaction of HDAC3 with OTT.

To determine the binding region of OTT to HDAC3, we constructed a series of deletion mutants of OTT and expressed them as fusion proteins with the DNA-binding domain of GAL4 (Fig. 6C). Although a region containing 609–730 amino acids could not bind to HDAC3, the N-terminal region containing three RNA recognition motifs and the C-terminal SPOC domains independently bound to HDAC3 (Fig. 6D), indicating that OTT interacts with HDAC3 via multiple regions.

OTT-BSAC Does Not Interact with HDAC3—We next investigated whether BSAC fusion to OTT could affect the ability to
OTT-BSAC restored the binding to HDAC3, suggesting that the C-terminal TA domain inhibits the binding of HDAC3 to OTT.

**DISCUSSION**

In the present study, we have shown that a fusion protein OTT-BSAC exhibited strong transcriptional activity to various promoters containing the YY1-binding sequences. Although BSAC predominantly localized in the cytoplasm, OTT-BSAC exclusively localized in the nucleus. This signal-independent nuclear accumulation of OTT-BSAC might contribute to a significant enhancement of transcriptional activity. Moreover, OTT interacted with HDAC3, but this interaction was abolished in OTT-BSAC. Given that OTT negatively regulates the myeloid and megakaryocyte expansion (26, 27), the loss of suppressor function of OTT along with aberrant up-regulation of BSAC-dependent transcription caused by the fusion may culminate in the development of leukemia.

We and others have previously reported that BSAC activates the promoters containing CArG boxes through association with SRF (9–11). However, given that CArG boxes are found in the promoters of many immediate early genes or muscle-specific genes, it is unlikely that CArG box-dependent transcriptional activity of BSAC directly links to the development of leukemia. Thus, we surmised that OTT-BSAC activates a promoter containing a sequence other than the CArG box(es). We found that OTT-BSAC activated the GPVI promoter through YY1-binding sequences. This conclusion is supported by the following results. First, OTT-BSAC-mediated transcriptional activity was abolished on the GPVI promoters, in which the YY1-binding sequences were mutated (Fig. 2B). Second, YY1 bound to this site using EMSAs (Fig. 2C). Third, ChIP assays revealed that endogenous YY1 bound to the GPVI promoter (Fig. 2D). However, we could not detect the recruitment of transfected OTT-BSAC to the promoter of GPVI using ChIP assays under our experimental conditions (Fig. 2E). It is reasonable to speculate that only small populations of transfected OTT-BSAC might be recruited to the promoter; therefore such recruitment might be under the detection levels.

Although the present study has shown that OTT-BSAC activates the GPVI and other promoters containing the YY1-binding sequences, it remains unclear which transcriptional factor(s) is essential for OTT-BSAC-mediated transcriptional activation. Although overexpression of YY1 attenuated OTT-BSAC-dependent transcriptional activation (Fig. 3A), knockdown of YY1 using siRNA did not impair its transcriptional activity (Fig. 3D). Given that we could not detect the interaction of YY1 with OTT-BSAC (data not shown), OTT-BSAC might interact with HDAC3. Interestingly, OTT-BSAC lost the ability to interact with HDAC3 (Fig. 7A). Given that the domain structure of OTT is preserved in OTT-BSAC, this suggests that some region of BSAC could inhibit the interaction of OTT with HDAC3. To determine the inhibitory region, we constructed C-terminal deletion mutants of OTT-BSAC (Fig. 7B). Deletion of C-terminal 351 amino acids containing the TA domain of OTT-BSAC restored the binding to HDAC3, suggesting that the C-terminal TA domain inhibits the binding of HDAC3 to OTT.
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be recruited to the YY1-binding motif through interaction with a transcription factor other than YY1. Intriguingly, YY2, a member of the YY1 family, has been shown to bind to the consensus binding sequences similar to YY1 (28); YY2 may recruit OTT-BSAC to the GPVI promoter. Further study will be required to address this possibility.

A previous study has shown that persistent expression of YY1 in 3DO cells after differentiation signal could perturb the granulocyte differentiation (18). Moreover, up-regulation of YY1 mRNA is frequently observed in some patients of acute myeloid leukemia (18). These results indicate an intimate link between deregulation of YY1 and leukemia. Together, OTT-BSAC might modulate YY1- and/or YY1-related transcriptional factor-dependent transcription, culminating in the development of leukemia.

Another important finding of this study is that OTT fusion to the N terminus of BSAC resulted in signal-independent nuclear accumulation of OTT-BSAC. A previous study has shown that nuclear translocation of BSAC is tightly regulated by the Rho-actin signaling pathway (12). Consistently, under unstimulated conditions, endogenous BSAC predominantly localized in the cytoplasm (Fig. 4). These results suggest that transcriptional activity of BSAC is at least partly regulated by its subcellular localization. This might well explain the reason why OTT-BSAC shows stronger transcriptional activity than BSAC. However, the mechanism by which OTT-BSAC constitutively accumulates in the nucleus remains to be solved in this study. One possible scenario is that the nuclear localization signal(s) of OTT might dominate over the cytoplasmic retention signal(s) of BSAC. Although BSAC has its own nuclear localization signals in the basic domain, N-terminal RPEL motifs are considered to sequestrate BSAC to the cytoplasm via interaction with G actin under unstimulated conditions (12). Therefore, OTT might disrupt such inhibition, resulting in constitutive nuclear translocation of OTT-BSAC.

A recent study has shown that deletion of OTT gene results in megakaryocytic expansion (27). In addition, knockdown of OTT/RBM15 gene using RNA interference promotes myeloid differentiation (26), suggesting an inhibitory role for OTT in myeloid and megakaryocyte development. These results are consistent with our present study, in which OTT might act as a transcriptional repressor via interaction with HDAC3 (Fig. 6). Given that this transcriptional repressor activity of OTT might be abolished in OTT-BSAC, the loss of OTT-mediated suppressor function of OTT-BSAC along with aberrant up-regulation of BSAC-dependent transcriptional activity might synergistically contribute to the development of leukemia.

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