Members of the DTX (Deltex) family act as Notch signaling modifiers and may also regulate transcription through interactions with specific transcription factors. DTX proteins have a basic N terminus; a central proline-rich region; and a C-terminal RING finger domain, a motif often found in ubiquitin-protein isopeptide ligases (E3). Recently, we identified and characterized a unique diffuse large B-cell lymphoma risk-related gene named BAL (B aggressive lymphoma). Using a yeast two-hybrid screen for BAL-binding partners, we have now identified a novel protein termed BBAP (B-lymphoma- and BAL-associated protein). Although BBAP has a unique N terminus, the C-terminal region is highly homologous to that of DTX family members. Herein, we report that BBAP and the human family of DTX proteins (DTX1, DTX2, and DTX3) function as E3 ligases based on their capacity for self-ubiquitination. DTX family members homodimerize and heterodimerize in vivo, suggesting that physical interactions between various DTX family members modify E3 activity and/or substrate availability. Consistent with this idea, BBAP and DTX1 associate via their unique N termini, resulting in enhanced self-ubiquitination.

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid malignancy in adults and a clinically heterogeneous disorder (1). As part of an effort to identify genes associated with the unique behavior of curable versus fatal DLBCLs, we recently isolated and characterized a gene termed BAL (B aggressive lymphoma) that is more highly expressed in fatal tumors (2). BAL is a nuclear protein with a unique C terminus and a duplicated N-terminal domain homologous to the non-histone region of histone macro-H2A (2), a polypeptide that associates with the inactive X chromosome (3) and that may play a role in gene silencing (4).

We employed yeast two-hybrid screening to identify a BAL-binding protein termed BBAP (B-lymphoma- and BAL-associated protein), described herein. Although BBAP contains a unique N terminus, the C terminus is highly homologous to the conserved C termini of DTX (Deltex) family members (5–7), all of which include a classic RING finger domain.

DTX polypeptides participate in the Notch signaling pathway, which controls cell fate determination in multicellular animals (8, 9). In the fly, DTX augments Notch signals that are dependent on the downstream transcription factor Su(H) (10) and also participates in less well understood Su(H)-independent Notch signaling (10). Four murine DTX proteins, DTX1, DTX2, DTX2ΔE (a splice variant), and DTX3, have been described previously (6). In mammalian systems, ectopic expression of DTX perturbs myogenesis and neurogenesis (6) and lymphogenesis (11) (cell fates also influenced by Notch) and alters the transcriptional activity of E2A (6, 7), the general coactivator p300 (12), and the Su(H) homolog CSL (7). Of particular interest, DTX1 appears to promote B-cell development at the expense of T-cell development (11), and its close homolog, DTX2, has been identified recently as a site of recurrent retroviral integration in murine B-cell lymphomas (13). How DTX influences B-cell development and transformation, as well as other diverse phenotypes, is not understood.

Drosophila DTX and human DTX1 include a basic N terminus, which binds the ankyrin repeats of intracellular Notch; a central proline-rich region; and a C-terminal RING finger domain (5, 7). The RING finger consensus sequence has eight conserved cysteines and histidines that coordinate two zinc ions in a “cross-braced” fashion (14). Recently, certain RING finger proteins were found to be ubiquitin-protein isopeptide ligases (E3) that mediate the transfer of ubiquitin to themselves and specific heterologous substrates (15–17). The multistep process of ubiquitination is hierarchical, beginning with an initial activation and transfer of ubiquitin from a common ubiquitin-activating enzyme (E1) to ubiquitin-conjugating enzymes (E2). Thereafter, a given E2 interacts with a specific substrate and E3, which catalyzes the transfer of ubiquitin to the substrate (18). Although polyubiquitinated proteins are often targeted for proteasomal degradation, ubiquitination regulates additional cellular functions that are not directly related to proteasomal degradation (19).

Given the possible roles of DTX family members in B-lineage commitment and transformation, we systematically evaluated BBAP and human DTX family members for E3 activity and heterodimerization. We found that all members of this group are E3 ligases and that heterodimerization of BBAP and DTX1 enhances this activity.
**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screening.—**The C-terminal half (aa 486–854) (2) of BAL was used as bait in a yeast two-hybrid screen. Plasmids encoding candidate BAL-binding proteins were rescued and confirmed by retransformation. Inserts from positive clones were sequenced, leading to identification of a partial cDNA encoding a novel BAL-binding protein, BBAP. A full-length BBAP cDNA was identified in an activated B-cell plasmid library (20) with a 5′ BBAP 400-bp biotinylated probe using the Clont capture library selection kit (Clontech).

**Cloning of Human DTX2A, DTX2B, and DTX3—**Human DTX2A, DTX2B, and DTX3 were cloned by reverse transcription-PCR. Human DTX2 PCR primers were designed from the sequences of mouse dtx2 (NCBI accession number AB015423), mouse dtx2ΔE (NCBI accession number AB015424), and human KIAA1528 (NCBI accession number AB040961) and human genome sequence (NCBI accession number NT_007834). Human DTX3 PCR primers were designed according to the mouse dtx3 sequence (NCBI accession number AB015425) and human genome sequence (NCBI accession number NT_009410). All human DTX PCR products (DTX2A, DTX2B, and DTX3) were cloned into the pCR4-Blunt-TOPO vector (Invitrogen) and verified by sequencing.

**Cell Culture—**Human DLBCL cell lines (DHL-4, -6, -7, -8, and -10), were cultured in RPMI 1640 medium (Invitrogen) containing 10% fetal calf serum (Invitrogen). Human embryonic kidney 293 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum.

**Plasmids and Transfection—**Full-length BBAP, N-terminal BBAP (aa 1–560), C-terminal BBAP (aa 528–740), and full-length human DTX2A and DTX2B cDNAs were subcloned into pFLAG-CMV2 (Sigma) and HA-tagged pcDNA3 (Invitrogen) vectors. Full-length human DTX1 cDNA with a C-terminal Myc tag (a gift from Dr. Spyros Artavanis-Tsakonas) was subcloned into the pcDNA3 vector (Invitrogen). Full-length human DTX3 was subcloned into pFLAG-CMV2 and HA-tagged pHM6 (Roche Applied Science, Mannheim, Germany) vectors. Full-length BAL, N-terminal BAL (aa 1–464), and C-terminal BAL (aa 465–854) were subcloned into pFLAG-CMV2 vectors. All constructs were verified by sequencing. HA-tagged Ubc4; Myc-tagged UbcH5a, UbcH5b, and UbcH5c; and FLAG-tagged UbcH6 and UbcH7 were gifts from Dr. Keiji Tanaka (Tokyo Metropolitan Institute of Medical Science and Core Research for Evolutional Science and Technology, Tokyo, Japan) (21). Transient transfections of 293 cells were performed using the above-mentioned expression plasmids and FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions.

**Immunoprecipitation and Western Blotting—**After ~48 h of transfection, 293 cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 1% Nonidet P-40, 1 mM sodium orthovanadate, and protease inhibitor mixture (Complete, Roche Applied Science). Following centrifugation and precleaving of cell lysates with protein G-Sepharose, the supernatants were subsequently incubated with the appropriate antibodies and protein G-Sepharose. Immune complexes were washed, resuspended in SDS-PAGE sample buffer, heated at 95 °C, size-fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Membranes were immunostained with the appropriate primary and secondary antibodies and developed using a chemiluminescent method (ECL, Amersham Biosciences).

**Antibodies—**The murine anti-BAL monoclonal antibody (clone 28F-3E1) was generated following repetitive immunization with purified BBAP-GST fusion protein according to standard protocols. The previously described rabbit anti-BAL polyclonal antibody (2) was affinity-purified according to standard protocols. The following antibodies were purchased from the indicated commercial sources: mouse anti-FLAG monoclonal antibody M2 (Sigma), mouse anti-Myc monoclonal antibody 9E10 and mouse anti-GST monoclonal antibody B-14 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-HA monoclonal antibody HA.11 (Covance Research Products, Richmond, CA), and horseradish peroxidase-conjugated sheep anti-mouse IgG antibody and horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (Amersham Biosciences).
BBAP and Related Deltex Proteins Are E3 Ligases

In Vivo Ubiquitination Assay—HA-tagged ubiquitin plasmids were cotransfected with FLAG-tagged BBAP or DTX plasmid into 293 cells. Forty hours later, cells were treated with the proteasome inhibitor MG-132 (Calbiochem) at a final concentration of 25 μM for an additional 6 h. FLAG-tagged proteins were immunoprecipitated, size-fractionated by SDS-PAGE, and immunostained using anti-HA or anti-FLAG antibody.

Recombinant Protein Preparation—FLAG-tagged wild-type BBAP and Myc-tagged DTX1, DTX2A, DTX2B, and DTX3 cDNAs were subcloned into pGEX-4T-2 vectors (Amersham Biosciences). A FLAG-tagged RING finger deletion mutant of BBAP, BBAPΔR, was generated by two-step PCR. In the first step, full-length BBAP cDNA was used as a template to generate two separate products, BBAP(1–560) and BBAP(600–740), using the appropriate primers (the BBAP N-terminal primer and 5'-cggattctggctttgagtttgctggctggctgggctggctggcc-3' or the BBAP C-terminal primer with FLAG sequence and 5'-gaccagaag- gaaagggcttagctggctttgagtttgctggctggcc-3') with overlapping ends (underlined). Two PCR products were purified, mixed, and used as templates in the second step, in which BBAP N- and C-terminal FLAG primers were used to amplify BBAPΔR. The final product was subcloned into the pGEX-4T-2 vector. GST fusion proteins were expressed in Escherichia coli BL21 cells (Amersham Biosciences) and purified from bacterial cell lysates using standard protocols.

In Vitro Ubiquitination Assays—Ubiquitination assays were performed as described previously (22) with minor modifications. The standard reaction mixture (50 μl) contained biotinylated ubiquitin (10 μg), rabbit E1 (0.3 μg; Calbiochem), E2 (1 μg), and recombinant BBAP-GST or DTX-GST fusion protein (100–500 ng) in 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 2 mM ATP, and 2 mM dithiothreitol. Specific E2 enzymes, including HbcH5a, UbcH5b, and UbcH5c (Boston Biochem, Cambridge, MA), were used in individual assays. After incubation for 30 min at 30 °C, the ubiquitination assay was stopped by adding 2× sample buffer, and samples were size-fractionated by SDS-PAGE and then Western-blotted. Primary antibodies included anti-BBAP, anti-FLAG, anti-Myc, and anti-GST. Following incubation with horseradish peroxidase-conjugated anti-mouse IgG antibody, proteins were detected using ECL. Peroxidase-conjugated ExtrAvidin (Sigma) was used for detection of biotinylated ubiquitin.

RESULTS

Identification of the BAL-binding Protein BBAP—In a yeast two-hybrid screen using a C-terminal portion of BAL as bait, we identified a novel interacting protein termed BBAP (B-lymphoma- and BAL-associated protein). The full-length BBAP cDNA encodes a 740-aa protein with a unique 560-aa N terminus containing two potential nuclear localization signals (aa 20–26 and 462–478) (23) and a possible nuclear export signal (aa 325–334) (24) (Fig. 1A). The BBAP C terminus is highly homologous to the conserved C termini of human DTX1 and the related murine proteins DTX1, DTX2, and DTX3, including a classic RING finger domain (BBAP aa 561–599) (Fig. 1A). BBAP is expressed as a single major transcript of 6.5 kb (data not shown).
FIG. 3. E3 activity of BBAP. A, in vivo ubiquitination assay of BBAP. FLAG-tagged BBAP and HA-tagged ubiquitin constructs were cotransfected into 293 cells, which were subsequently treated with the proteasome inhibitor MG-132. Thereafter, cell lysates were immunoprecipitated with anti-FLAG antibody, size-fractionated, Western-blotted (WB), and analyzed with either anti-HA antibody (left panel) or anti-FLAG antibody (right panel). B, self-ubiquitination and E3 activity of BBAP in vitro. Recombinant BBAP protein was evaluated for E3 activity and self-ubiquitination in the presence (+) and absence (−) of E1, E2 and biotinylated ubiquitin. The individual samples were size-fractionated, Western-blotted (WB), and analyzed with anti-BBAP antibody (left panel) or peroxidase-conjugated avidin to detect biotinylated ubiquitin (right panel). The arrowhead indicates the original size of the BBAP-GST fusion proteins. C, self-ubiquitination and E3 activity of wild-type BBAP and its RING finger deletion mutant. Recombinant wild-type BBAP or RING finger deletion mutant proteins were evaluated for E3 activity and self-ubiquitination in standard in vitro assays in the presence (+) and absence (−) of E1, E2, and biotinylated ubiquitin. The individual samples were size-fractionated, Western-blotted (WB), and analyzed with anti-FLAG (BBAP) antibody (left panel) or peroxidase-conjugated avidin (right panel).

FIG. 4. Identification of the preferred E2 for BBAP. A, physical interaction between the BBAP E3 and specific E2 enzymes in vivo. FLAG- or HA-tagged BBAP plasmids were cotransfected with selected tagged E2 constructs (Ubc4, UbcH5a, UbcH5b, UbcH5c, UbcH6, and UbcH7) into 293 cells and analyzed thereafter for physical interaction (left panels) and expression (right panels). Left panels, BBAP and E2 co-association. Cell lysates from the individual transfectants were immunoprecipitated (IP) with anti-FLAG or anti-HA (BBAP) antibody, size-fractionated, Western-blotted (WB), and analyzed with antibodies directed against the tagged E2 proteins (anti-HA, anti-Myc, and anti-FLAG). Right panels, BBAP and E2 expression in BBAP/E2 transfectants. Whole cell lysates were Western-blotted with antibodies directed against the tagged E2 proteins (anti-HA, anti-Myc, and anti-FLAG; upper panels) or BBAP (anti-FLAG and anti-HA; lower panels). Specific bands are indicated by arrowheads.

B, in vitro self-ubiquitination of BBAP in conjunction with the UbcH5 family of E2 enzymes. Recombinant E2 enzymes (UbcH5a, UbcH5b, and UbcH5c) were included in separate in vitro ubiquitination assays with the BBAP E3, E1, and biotinylated ubiquitin. The individual samples were size-fractionated, Western-blotted (WB), and analyzed with anti-BBAP antibody. The arrowhead indicates the original size of the BBAP-GST fusion proteins.
Confirmation of BAL/BBAP Binding and Identification of the Interaction Domains—To confirm the association between BAL and BBAP, we immunoprecipitated BAL and associated proteins from a panel of DLBCL cell lines. Thereafter, these proteins were size-fractionated and immunoblotted with the relevant antibodies. In separate assays, FLAG-tagged full-length, N-terminal, and C-terminal BAL proteins were assessed for association with HA-tagged full-length, N-terminal, and C-terminal BBAP proteins (Fig. 1C). When either FLAG-tagged full-length or C-terminal BAL was immunoprecipitated, full-length or N-terminal BBAP was co-associated with the BAL protein (Fig. 1C, upper left panel, lanes 1, 2, and 5). Similarly, when either HA-tagged full-length or N-terminal BBAP was immunoprecipitated, full-length or C-terminal BAL was co-associated with BBAP (Fig. 1C, lower left panel, lanes 1, 2, and 5). In contrast, neither the N-terminal BAL nor the C-terminal BBAP polypeptide coprecipitated with their respective partner proteins (Fig. 1C, upper and lower left panels, lanes 3 and 4), although these proteins were readily detectable in transfected cells (upper right panel, lane 8; and lower right panel, lane 9). Taken together, these data confirm that BAL and BBAP co-associate in vivo and identify the BBAP N terminus (Fig. 1A) and the BAL C terminus (2) as the interaction domains.

DTX Family Members and BBAP—Although the N-terminal BBAP interaction domain is unique, the C terminus of BBAP is highly homologous to those of human DTX1 (7) and the recently described murine family members DTX1, DTX2, DTX2ε, and DTX3 (6). To analyze the function of the conserved C-terminal BBAP/DTX domain, we first cloned and characterized the human homologs of murine DTX2, DTX2ε, and DTX3, hereafter termed DTX2A, DTX2B, and DTX3, respectively (Fig. 2).

Human DTX family members have three distinct domains based on the analogous functional motifs in Drosophila (5, 7) (Fig. 2A). The N-terminal domain 1 can associate with Notch ankyrin repeats, whereas domain 2 contains a proline-rich region with potential SH3 (Src homology-3) domain-binding sites. The C-terminal domain 3 includes a classic RING finger domain and the region of BBAP homology (Fig. 2A).

Human DTX1, DTX2A, and DTX2B are similarly organized, although DTX2B lacks the fourth of nine predicted DTX2 exons (NCBI accession number NT_007833.10) within the proline-rich domain 2. The N-terminal regions of human DTX1, DTX2A, and DTX2B and Drosophila DTX all contain two WEE motifs, a postulated protein/protein interaction domain (25). DTX3 has a truncated domain 1 that lacks homology to other DTX family members, but retains a proline-rich region (Fig. 2A). As noted, the unique BBAP N terminus does not contain the WEE and proline-rich domains of DTX family members (Fig. 2A).

The amino acid sequences of the highly conserved C termini of BBAP, human DTX family members, and Drosophila DTX are aligned in Fig. 2B. Each of these proteins includes a classic RING finger domain of either the RING-H2 or RING-HC subtype (26). DTX1, DTX2, and Drosophila DTX have RING-H2 fingers with histidines in the fourth and fifth positions; in contrast, DTX3 and BBAP contain RING-HC fingers with a single histidine in the fourth position (Fig. 2B). Of interest, the additional C-terminal sequences of the RING-HC-containing DTX3 and BBAP proteins are also more similar to each other than to those of the other RING-H2-containing DTX family members (DTX1, DTX2, and Drosophila DTX) (Fig. 2B).

E3 Activity of BBAP—Certain RING finger proteins function as E3 ligases, mediating the transfer of ubiquitin to heterologous substrates and to the RING finger proteins themselves (17). To determine whether BBAP functions as an E3, we performed ubiquitination assays in cultured cells. In brief,
FLAG-tagged BBAP and HA-tagged ubiquitin were cotransfected into 293 cells, and proteasome activity was inhibited with MG-132. Cell lysates were immunoprecipitated with anti-FLAG (BBAP) antibody, size-fractionated, and immunoblotted with anti-HA (ubiquitin) antibody (Fig. 3A, left panel) or anti-FLAG antibody (right panel). The cotransfection of BBAP and ubiquitin resulted in a dramatic increase in high molecular mass polyubiquitinated proteins (Fig. 3A, left panel), suggesting that BBAP increased ubiquitination and was a possible target for self-ubiquitination.

To directly assess the E3 activity of BBAP, we performed in vitro assays in which self-ubiquitination of recombinant BBAP protein was determined. In the presence of E1, E2, and ubiquitin, recombinant BBAP was heavily polyubiquitinated, confirming that it functions as an E3 capable of self-ubiquitination in vitro (Fig. 3B).

To determine the role of the RING finger domain in BBAP E3 activity, we performed additional in vitro ubiquitination assays using either the wild-type recombinant BBAP protein or a RING finger deletion mutant (BBAPRfdel) and the required additional components (E1, E2, and biotinylated ubiquitin) (Fig. 3C). Whereas wild-type BBAP exhibited obvious E3 activity, BBAPRfdel was inactive (Fig. 3C), confirming that the RING finger domain is required for BBAP E3 function.

Each E3 recognizes specific substrates in cooperation with a common E1 and a smaller subset of E2 enzymes (18). To identify the preferred E2 for BBAP, we first analyzed the coprecipitation of BBAP and individual E2 enzymes (Ubc4, UbcH5a, UbcH5b, UbcH5c, UbcH6, and UbcH7) in transiently transfected 293 cells (Fig. 4A, left panels). Although each of these E2 proteins was expressed at readily detectable levels in whole cell lysates from transfected cells (Fig. 4A, upper right panels), BBAP co-immunoprecipitated only with UbcH5 family members (left panels).

Thereafter, we evaluated the E2 activity of UbcH5 family members for BBAP using recombinant enzymes in an in vitro ubiquitination assay (Fig. 4B). Although each UbcH5 enzyme was active in this assay, the most striking high molecular mass polyubiquitination occurred when the E2 enzyme UbcH5a was used with BBAP (Fig. 4B).

E3 Activities of DTX1, DTX2, DTX2B, and DTX3—Given the high degree of C-terminal homology between BBAP and human DTX family members, we next investigated whether DTX1, DTX2, and DTX3 also function as E3 ligases using tagged recombinant DTX-GST fusion proteins in the in vitro ubiquitination assay (Fig. 5). As indicated, DTX1, DTX2A, DTX2B, and DTX3 all exhibited E3 activity as assessed by self-ubiquitination (Fig. 5, A–C). Like BBAP, the DTX1, DTX2, and DTX3 E3 ligases also functioned most efficiently in conjunction with the UbcH5a E2 enzyme (data not shown).

BBAP Homodimerizes and Interacts with DTX through Its Unique N Terminus—The BBAP RING finger E3 protein was initially identified on the basis of its N-terminal interaction with BAL (Fig. 1C). Additional RING finger proteins, including DTX family members (6, 12, 27), have been reported to dimerize in ways that alter their biological activity. For these reasons, we investigated whether BBAP might homodimerize or heterodimerize with other DTX family members.

In initial experiments, FLAG-tagged wild-type BBAP and HA-tagged wild-type, N-terminal, and C-terminal BBAP polypeptides were coexpressed in 293 cells (Fig. 6A, upper and lower panels). FLAG-tagged wild-type BBAP coprecipitated with HA-tagged wild-type and N-terminal BBAP proteins (Fig. 6A, left panels).
BBAP and Related Deltex Proteins Are E3 Ligases

Myc-tagged wild-type DTX1-GST protein was added to FLAG-tagged wild-type BBAP-GST or BBAP<sub>Δ<sub>PRO</sub></sub>-GST protein; wild-type BBAP protein was also incubated separately. After incubation with the additional required components (E1, E2, and biotinylated ubiquitin), samples were size-fractionated and serially Western-immunoblotted (WB) with anti-FLAG (BBAP) antibody (left panel) and peroxidase-conjugated avidin (biotinylated ubiquitin) (right panel) with anti-FLAG (BBAP) antibody (left panel) and peroxidase-conjugated avidin (biotinylated ubiquitin) (Fig. 7). In this assay, wild-type BBAP and BBAP<sub>Δ<sub>PRO</sub></sub> exhibited increased BBAP polyubiquitination (Fig. 7, lanes 1 versus lanes 3). In contrast, C-terminal BBAP did not (Fig. 7, lanes 1 versus lanes 3). Similarly, Myc-tagged DTX1 coprecipitated with wild-type and N-terminal BBAP proteins (Fig. 6A, upper left panel); similarly, Myc-tagged DTX1 coprecipitated with wild-type and N-terminal BBAP proteins (Fig. 6B, upper left panel). Taken together, these data demonstrate that BBAP/DTX1 heterodimerization also requires the unique N terminus.

DTX1 Enhances BBAP Ubiquitination—BBAP functions as an E3 via its DTX-homologous C-terminal RING finger domain and interacts with DTX1 via its unique N terminus, suggesting that BBAP/DTX1 binding may have functional consequences. To address this possibility, we compared the self-ubiquitination activity of BBAP in the presence and absence of DTX1 (Fig. 7). Myc-tagged DTX1-GST protein was added to FLAG-tagged wild-type BBAP-GST or BBAP<sub>Δ<sub>PRO</sub></sub>-GST protein (Fig. 7, upper left panel); similarly, Myc-tagged DTX1 coprecipitated with wild-type and N-terminal BBAP proteins (Fig. 6A, upper left panel). Taken together, these data demonstrate that BBAP/DTX1 heterodimerization also requires the unique N terminus.

In additional binding studies, DTX1 and BBAP formed heterodimers via their respective N termini in a manner that enhanced BBAP-mediated self-ubiquitination. Therefore, the additional required components (E1, E2, and biotinylated ubiquitin) were added, and samples were size-fractionated and serially immunoblotted with anti-FLAG (BBAP) antibody and peroxidase-conjugated avidin (Fig. 7, left panel, lanes 1 and 4). In comparison with the wild-type BBAP homodimer, the wild-type BBAP/DTX1 heterodimer exhibited increased BBAP polyubiquitination (Fig. 7, left and right panels, lanes 1 versus lanes 3). Taken together, these data indicate that the BBAP/DTX1 heterodimer is a better E3 and substrate for self-ubiquitination compared with the BBAP homodimer.

We have cloned and characterized the BAL-binding protein BBAP, which has highly significant C-terminal homology to DTX family members. However, in contrast to DTX family members, BBAP includes a unique N terminus and lacks a central proline-rich region. The BBAP and DTX C termini contain classic RING-HC or RING-H2 finger domains. Using in vivo and in vitro ubiquitination assays, we have shown that BBAP functions as an E3 and promotes its self-ubiquitination in a RING finger-dependent manner. In analogous ubiquitination assays, we demonstrated that DTX1, DTX2, and DTX3 also function as E3 ligases. In additional binding studies, DTX1 and BBAP formed heterodimers via their respective N termini in a manner that enhanced BBAP-mediated self-ubiquitination.

In lymphoid progenitors, DTX acts as a negative modulator of Notch signaling (11). However, the observed differences in DTX activity in certain in vitro and in vivo assays suggest that DTX function may be dose- and cell type-dependent and tightly regulated. For these reasons, the identification of DTX family members as E3 ligases and targets for self-ubiquitination is of particular interest. Recent studies highlight the pivotal role of ubiquitination in regulating Notch-mediated signaling (28). Four known E3 ligases regulate Notch itself, Notch ligands, or known Notch antagonists (28). That DTX family members also function as E3 ligases further emphasizes the role of ubiquitination in regulating Notch signaling.

The striking C-terminal homology between BBAP and human DTX family members prompted our analysis of their common functions. However, the BBAP C terminus is more closely related to the DTX3 C terminus than to the DTX1 and DTX2 C termini. Of interest, neither BBAP nor DTX3 contains the DTX1/2 N terminus, which physically interacts with Notch ankyrin repeats, suggesting that BBAP and DTX3 have unique functions that are not shared by DTX1 and DTX2. Given the demonstrated role of the BBAP N terminus as an interaction domain (Figs. 1C and 6), BBAP may have additional unique binding partners.

**FIG. 7.** In vitro E3 activity and self-ubiquitination in BBAP/DTX1 heterodimers. Myc-tagged wild-type DTX1-GST protein was added to FLAG-tagged wild-type BBAP-GST or BBAP<sub>Δ<sub>PRO</sub></sub>-GST protein; wild-type BBAP protein was also incubated separately. After incubation with the additional required components (E1, E2, and biotinylated ubiquitin), samples were size-fractionated and serially Western-immunoblotted (WB) with anti-FLAG (BBAP) antibody (left panel) and peroxidase-conjugated avidin (biotinylated ubiquitin) (right panel).
Nevertheless, previous murine studies indicate that DTX1, DTX2, and DTX3 form homo- and heterodimers (6). Recently, heterodimeric RING finger protein complexes have been associated with increased functional activity. For example, the heterodimeric complex containing the BRCA1 tumor suppressor and BARD1 exhibits increased E3 activity (29, 30). Similarly, the complex containing two different negative regulators of p53, Mdm2 and MdmX, has increased E3 activity (31). That BBAP/DTX1 heterodimers exhibit increased E3 activity is consistent with these observations.

However, unlike the Mdm2 and MdmX proteins, which interact via their respective RING finger domains (32), BBAP binds to DTX1 via its unique N terminus. In our studies, a C-terminal BBAP construct containing the RING finger did not bind full-length DTX1 or BBAP. Therefore, other protein interaction domains could be responsible for the heterodimerization of certain RING finger-containing proteins and the consequent increase in E3 activity.

Because BBAP also interacts with the BAL protein via its unique N terminus, the functional consequences of this interaction are of great interest. Although BBAP is clearly an E3, BAL has not yet been identified as a substrate for BBAP-mediated ubiquitination.

Given the relevance of the DTX family of proteins in the control of several critical cellular mechanisms, the demonstration of their E3 activity is important. This newly identified control of several critical cellular mechanisms, the demonstration of certain RING finger-containing proteins and the consequent action domains could be responsible for the heterodimerization of certain RING finger-containing proteins and the consequent increase in E3 activity.

Because BBAP also interacts with the BAL protein via its unique N terminus, the functional consequences of this interaction are of great interest. Although BBAP is clearly an E3, BAL has not yet been identified as a substrate for BBAP-mediated ubiquitination.

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**REFERENCES**

1. Shipp, M. A., Harris, N., and Mauch, P. (1997) in Cancer Principles & Practices of Oncology (DeVita, V. T., Hellman, S., and Rosenberg, S. A., eds) pp. 2165–2226, Lippincott Williams & Wilkins, Philadelphia
2. Aguilar, R. C., Yakushijin, Y., Kharbanda, S., Salgia, R., Fletcher, J. A., and Shipp, M. A. (2000) *Blood* 96, 4328–4334
3. Costanzi, C., and Pehrson, J. R. (1998) *Nature* 393, 599–601
4. Perche, P. Y., Younch, C., Konoe, L., Soucier, C., Robert-Nicoud, M., Dimitrov, S., and Khochbin, S. (2000) *Curr. Biol.* 10, 1531–1534
5. Matsuno, K., Diederich, R. J., Go, M. J., Blauemarkeller, C. M., and Artavanis-Tsakonas, S. (1995) *Development* 121, 2633–2644
6. Kishi, N., Tani, N., Matsuoka, Y., Hira, H., D, M., T., Suzuki, S., Nakao, K., Kinoshiha, T., Kadesch, T., Hui, C., Artavanis-Tsakonas, S., Okano, H., and Matsuno, K. (2001) *Int. J. Dev. Neurosci.* 19, 21–35
7. Matsuno, K., Eastman, D., Mitaides, T., Quinn, A. M., Carcanciu, M. L., Ordentlich, P., Radesch, T., and Artavanis-Tsakonas, S. (1998) *Nat. Genet.* 19, 74–78
8. Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999) *Science* 284, 770–776
9. Greenwald, I. (1998) *Genes Dev.* 12, 1751–1762
10. Matsuno, K., Go, M. J., Sun, X., Eastman, D. S., and Artavanis-Tsakonas, S. (1997) *Development* 124, 4265–4273
11. Leen, D. J., Ater, J. C., He, Y., Weng, A., Karnell, P. G., Patriub, V., Xu, L., Bakkour, S., Rodriguez, C., Allman, D., and Pear, W. S. (2002) *Immunity* 16, 231–243
12. Yamamoto, N., Yamamoto, S., Inagaki, F., Kawaihi, M., Fukamizu, A., Kishi, N., Matsuno, K., Nakamura, K., Weinmaster, G., Okano, H., and Naka, M. (2001) *J. Biol. Chem.* 276, 44501–44504
13. Suzuki, T., Shon, H., Akagi, K., Morse, H. C., Malley, J. D., Naiman, D. Q., Jenkins, N. A., and Copeland, N. G. (2002) *Nat. Genet.* 32, 166–174
14. Saurin, A. J., Borden, K. L., Boddy, M. N., and Freemont, P. S. (1996) *Trends Biochem. Sci.* 21, 258–264
15. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 11364–11369
16. Joazeiro, C. A., and Weissman, A. M. (2000) *Cell* 102, 549–552
17. Jackson, P. K., Eldridge, A. G., Freed, E., Furestenthal, L., Hsu, J. Y., Kaiser, B. K., and Reimmann, J. D. (2000) *Trends Cell Biol.* 10, 429–439
18. Pickart, C. M. (2001) *Annu. Rev. Biochem.* 70, 503–533
19. Pickart, C. M. (2001) *Mol. Cell* 8, 499–504
20. Freedman, A. S., Freeman, G., Horowitz, J. C., Daley, J., and Nadler, L. M. (1987) *J. Immunol.* 139, 3260–3267
21. Shimura, H., Hattori, N., Kudo, S., Muruzo, Y., Asakawa, Y., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., and Suzuki, T. (2000) *Nat. Genet.* 23, 302–305
22. Honda, R., and Yasuda, H. (2000) *Oncogene* 19, 1473–1476
23. Nakai, K., and Horton, P. (1999) *Trends Biochem. Sci.* 24, 34–36
24. Bogerd, H. P., Fridell, R. A., Benson, R. E., Hua, J., and Cullen, B. R. (2000) *Science* 22085, 275–276
25. Lee, E. C. (2002) *Curr. Biol.* 12, R74–R78
26. Hashizume, R., Fukuda, M., Maeda, I., Nishikawa, H., Oyake, D., Yabuki, Y., Ogata, H., and Ohita, T. (2001) *J. Biol. Chem.* 276, 14537–14540
27. Chen, A., Kleiman, F. E., Manley, J. L., Ouchi, T., and Pan, Z. Q. (2002) *J. Biol. Chem.* 277, 22085–22092
28. Badciong, J. C., and Haas, A. L. (2002) *J. Biol. Chem.* 277, 49668–49675
29. Tanimura, S., Ohtsuka, S., Mitsu, K., Shiraizu, A., and Ohshino, M. (1999) *FEBS Lett.* 447, 5–9
30. Jeangnoins, F., Thompson, J. D., Gouy, M., Higgins, D. G., and Gibson, T. J. (1998) *Trends Biochem. Sci.* 23, 403–405
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