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Immunology

Growth stimulation of primary B cell precursors by the anti-phosphatase Sbf1

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ABSTRACT **SET binding factor 1 (Sbf1) was originally discovered by virtue of its interaction with a highly conserved motif (the SET domain) of unknown function in the protooncoprotein homolog of *Drosophila trithorax,* Hrx. Sbf1 shares extensive sequence similarity with myotubularin, a dual specificity phosphatase (dsPTPase) that is mutated in a subset of patients with inherited myopathies. Both Sbf1 and myotubularin interact with the SET domains of Hrx and other epigenetic regulatory proteins, but Sbf1 lacks phosphatase activity due to several evolutionarily conserved amino acid changes in its structurally preserved catalytic pocket. Thus, Sbf1 has features of an anti-phosphatase that could competitively antagonize dsPTPases; however the *in vivo* role for such factors remains unknown. Given its ability to physically interact with Hrx, a developmental regulator subject to translocation-induced mutations in B cell precursor leukemias, the current studies were undertaken to assess the effects of Sbf1 on lymphopoiesis. After infection with recombinant Sbf1 retroviruses, bone marrow cells were plated under Whitlock-Witte conditions for long-term culture of B lineage cells. Sbf1-expressing cells rapidly dominated the cultures resulting in clonal outgrowths of B cell progenitors that retained a dependence on their primary bone marrow-derived stroma for continuous growth *in vitro*. Structure/function analyses demonstrated that the SET interaction domain of Sbf1 was necessary and sufficient for growth alterations of B cell progenitors. These observations support a model in which Sbf1 functions as a SET domain-dependent positive regulator of growth-inducing kinase signaling pathways that impinge on SET domain proteins. SET domain-dsPTPase interactions appear to be critically important for regulating the growth properties of B cell progenitors.**

Chromosomal translocations are common features of leukemias and lymphomas and have been shown to activate specific cellular protooncogenes (1–3). A subset of chromosomal translocations in acute leukemias results in the fusion of *HRX*, a human homolog of *Drosophila trithorax,* with a variety of heterologous proteins (reviewed in ref. 4). Chromosomal abnormalities involving HRX (also known as MLL, ALL1, or Htrx) are seen in both acute myeloid as well as B cell precursor acute lymphoblastic leukemias (5–7). *HRX* is an essential gene (8) that when haplo-insufficient results in B lineage lymphopenias (9) suggesting a role in normal B cell development. Both *HRX* and *trithorax* are implicated by genetic studies to function in the maintenance of *Hox* gene expression profiles during embryonic development (8, 10). Consistent with this role, the HRX and trithorax proteins contain C-terminal motifs known as Su(var)3–9/enhancer-of-zeste/trithorax (SET) domains, which are present in several additional proteins implicated in the maintenance of specifically altered chromatin states as cells differentiate during development (11–13).

By yeast two-hybrid screening, we previously identified a protein, called Sbf1 [(*S*ET *b*inding *f*actor 1), that interacts with the SET domain of Hrx (14). Sbf1 displays extensive similarity to myotubularin, the product of *MTM1*, a candidate gene for X-linked myotubular myopathy (15). Both myotubularin and Sbf1 interact with the SET domain of HRX and related proteins (14). Based on sequence similarities, myotubularin was originally suggested to be a protein tyrosine phosphatase (15), however recent biochemical analyses demonstrate that myotubularin is a dual-specificity phosphatase (dsPTPase) capable of hydrolyzing synthetic phosphoserine as well as phosphotyrosine substrates (14). Sbf1 differs from all other members of the myotubularin phosphatase family by the absence of several conserved residues in the catalytic pocket that are critical for phosphatase activity (16–19). Therefore, Sbf1 is unlikely to be a functional phosphatase, a prediction confirmed by *in vitro* biochemical analysis (14). However, the backbone structure of Sbf1 is sufficiently conserved such that dsPTPase activity could be restored by limited site-directed mutagenesis of the catalytic pocket (14). These observations suggested that Sbf1 may function as a so-called "protective factor" similar to the role proposed for STYX (20), another catalytically inactive dsPTPase whose activity can also be restored by back mutation of the catalytic pocket. Sbf1 homologs are also present in mouse, *Drosophila* and *Caenorhabditis elegans* (A. Miyamoto, R. Firestein, and M.L.C., unpublished observations) and share similar alterations in catalytic pocket residues relative to known phosphatases.

Myotubularin-family proteins appear to play critical roles in the regulation of cellular growth and differentiation. This was first suggested by myotubularin loss-of-function mutations in a subset of myopathies that display impaired terminal differentiation of myoblasts (15). Consistent with the disease phenotypes, overexpression of Sbf1 prevents myoblast differentiation *in vitro* (14). Forced expression of Sbf1 also induces oncogenic changes in NIH 3T3 fibroblasts (14). These observations have led to the proposal that myotubularin-family phosphatases may normally serve to down-regulate mitogenic signals that impinge on SET domain proteins, whereas Sbf1 may enhance such signals.

The current studies were undertaken to assess the effects of Sbf1 over-expression on lymphopoiesis given its ability to physically interact with HRX, a developmental regulator subject to translocation-induced mutations in B cell precursor leukemias. Using a Whitlock-Witte culture system (21, 22), we demonstrate that forced expression of Sbf1 allows continuous self-renewal of primary B cell progenitors *in vitro*. Structure/function analyses demonstrated that the SET interaction do-

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Abbreviations: Sbf1, SET binding factor 1; SET, Su(var)3–9/enhancer-of-zeste/trithorax; SID, *S*ET *i*nteraction *d*omain; dsPTPase, dual-specificity phosphatase; Hrx, homolog of *Drosophila trithorax*; $Sbf1^{\Delta N}$, amino truncated mutant of Sbf1; IL, interleukin.
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main of Sbf1 was necessary and sufficient for growth alterations of B cell progenitors. These observations suggest that myotubularin-family phosphatases are components of signaling pathways that normally regulate the growth and differentiation of B cell progenitors, and that disruption of their interactions with SET domain proteins results in loss of growth control.

MATERIALS AND METHODS

Viral Stocks and Cell Culture Conditions. A 5.2-kb SBF1 cDNA (14) was inserted into the *Not*I site of pSRαMSVtk-neo (23) downstream from the retroviral long-terminal repeat. All other Sbf1 mutant cDNAs (14) were cloned into the *Eco*RI site, downstream of the long-terminal repeat in the same vector. Bosc23 packaging cells were transfected with retroviral vectors as described (24). Viral stocks were collected at 48 and 72 hr posttransfection, filtered (0.45 μM) and used for infection of freshly harvested whole bone-marrow cells isolated from the femurs of 3-to 4-week old BALB/c mice as described (25) with the addition that hematopoietic cells (2×10^6 cells/ml) were centrifuged with the virus mixture supplemented with 8 μg/ml polybrene for 30 min at $2,500 \times g$ at 33°C (26). Cells were then incubated with virus for 3 hr at 37°C in a 5% CO_2 environment, the medium was then discarded and replaced with fresh medium containing RPMI 1640 medium (GIBCO) supplemented with 5% fetal bovine serum and 50 μM of 2-mercaptoethanol (Sigma). Cultures were fed twice weekly as described (25). The numbers of nonadherent cells were determined 16 days postinfection and every 3 days thereafter. A culture was scored positive for growth if the cell count exceeded 1×10^5 cells/ml.

Protein and DNA Analyses. Expression of Sbf1 proteins was verified by standard Western blot analysis. Lysates from both transiently transfected Bosc23 cells as well as Sbf1-infected bone marrow cells were prepared in 1× SDS buffer (4% SDS/10% 2-mercaptoethanol/100 mM Tris, pH 6.8/20% glycerol/0.2% bromophenol blue) containing protease inhibitors. Proteins (50 μg) were separated by an SDS/10% polyacrylamide gel and immobilized on nitrocellulose filters by electrophoretic transfer. Sbf1 proteins were detected with an anti-Sbf1 mAb (clone 12.1) followed by a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Sigma) in a standard chemiluminescent protocol (enhanced chemiluminescence, Amersham). The human t(17;19)-bearing cell line HAL-01 (27) and the mouse pre-B cell line PM1001 were employed as controls. High-molecular-weight DNA was prepared from nonadherent cells harvested from bone marrow cultures and determined by Southern blot analysis using established procedures. A 1-kb *Bam*HI-*Cla*I fragment of pSRαMSV-SBF1 was used to detect viral integration sites for assessment of clonality.

Morphological Analysis and Phenotyping. Cytocentrifuged cells were stained with Wright-Giemsa to assess cell morphology. Immunophenotypic analysis was performed on cells collected in Hanks' balanced salt solution containing 2% fetal calf serum and 0.02% sodium azide (HFN medium). The conjugated antibodies 6B2-APC (anti-B220) and 2B8-APC (anti-c-Kit) were prepared from hybridoma clones. S7-fluorescein isothiocyanate (anti-CD43), BP-1-PE (clone BP-1), HSA-biotin (clone M1–69) and Mac-1 (clone M1–70) were purchased from PharMingen. Secondary antibodies were obtained from Caltag (San Francisco). Analysis for cytoplasmic μ employed a goat anti-mouse IgM-fluorescein isothiocyanate antibody on cells that were permeabilized with 0.05% saponin in staining medium, after staining with surface marker antibodies. Labeled cells were analyzed with a single laser FACScan or a dual laser FACS Vantage (Becton Dickinson). Dead cells were excluded from analysis based on propidium iodide staining characteristics. Two parameter data are presented as 5% probability plots.

Cell Proliferation Assays. Cells were seeded in triplicate in 96-well plates (10^4/well) in 100 μl of supplemented RPMI 1640 medium containing various combinations of recombinant murine interleukin 7 (IL-7), IL-3, IL-6, granulocyte-colony-stimulating factor, granulocyte/macrophage-colony-stimulating factor, and Flk2L (all at 10 ng/ml) and stem cell factor (100 ng/ml) (R&D Systems). Cell proliferation was measured 5 days later by counting viable cells.

RESULTS

Growth Alterations of Primary Hematopoietic Cells by Retroviral Transduction of Sbf1. *Sbf1* cDNAs were expressed under the transcriptional control of the long-terminal repeat of the SRαMSVtk-neo retroviral vector (23) (Fig. 1). This vector was used because the murine sarcoma virus long-terminal repeat had previously been shown to direct expression of several genes in hematopoietic cells including immature B cells (21, 25) under bone marrow culture conditions identical to those used for our studies. High-titer retroviral stocks were produced by transient transfection into the ecotropic retroviral packaging cell line Bosc23 (24). Viral stocks were used to infect fresh femoral bone marrow cells from 3- to 4-week old BALB/c mice and infected cells were then plated (10^6 per ml) in long-term B-lineage liquid culture conditions (21).

The bone marrow culture assay supports establishment of an adherent layer of stromal cells during the first 2 weeks of culture that is accompanied by the subsequent appearance and growth of nonadherent B-cells and their precursors. A diverse B lymphoid population is maintained, ranging from stem elements to pre-B cells to more mature B cells (21). This typical progression was observed in our cultures that were mock-infected with either vector alone or with Sbf1 in antisense orientation. In 40 of 55 control cultures, nonadherent cells grew to densities between $1–3 \times 10^4$ cells per ml. The infection of primary bone marrow cells with Sbf1 or an amino truncated mutant ($Sbf1^{\Delta N}$) also resulted in the appearance of nonadherent cells by 2 weeks, but the densities were substantially higher (1×10^5/ml observable by day 16 and reaching peak levels of 4×10^5/ml by day 28) (Fig. 2*B*). By week 4 and thereafter, Sbf1-infected cultures maintained their high-density growth pattern. These results were reproducible in 37/55 cultures, consistent with the transfection/infection variability often encountered in such assays as reported by others (28). The time course and magnitude of nonadherent cell outgrowths observed were similar to those obtained with bone marrow cultures that were initiated after infection with $p185^{BCR-ABL}$ in the same vector (Fig. 2*B*). Occasionally (3 of 40 cultures), outgrowths of adherent cells with features of macrophages were observed in the Sbf1 cultures, a phenom-

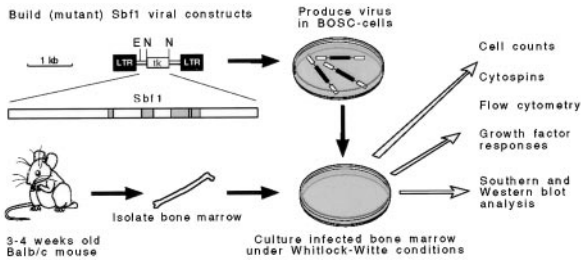

FIG. 1. Experimental strategy for transduction of primary hematopoietic cells with Sbf1 retroviral constructs. Sbf1 cDNAs were cloned into pSRαMSVtk-neo. Recombinant retroviral stocks were produced by using Bosc23 packaging cells and used for infection of primary bone marrow cells isolated from the femurs of BALB/c mice. Nonadherent cells growing *in vitro* under Whitlock-Witte conditions were characterized by using the indicated techniques.

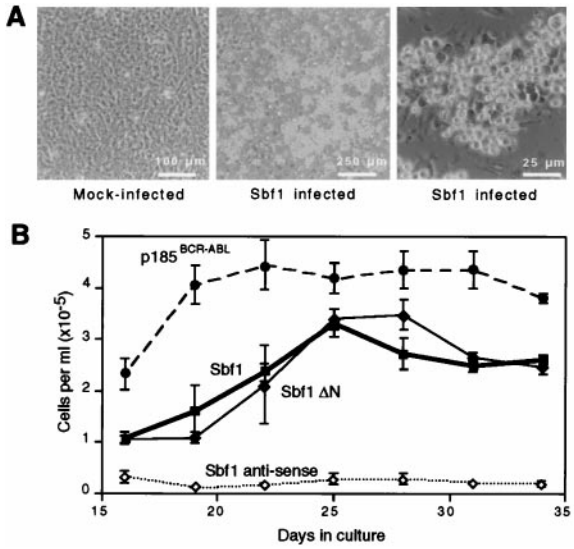

FIG. 2. Sbf1 induces continuous growth of primary bone marrow cells *in vitro*. (*A*) Outgrowth of nonadherent cells in Sbf1-infected bone marrow cultures at 16 days postinfection contrasts with a mock-infected culture that shows confluent stromal layer but only rare nonadherent cells. (*B*) Time course of growth in culture. Nonadherent cells were counted on the indicated days after initiation of cultures after infection with retroviruses expressing the indicated genes. Data points are shown for a typical experiment and represent the average of five determinations from independent cultures with brackets denoting standard deviations.

enon previously reported by others studying the v-myc oncogene (29).

The observed rapid out-growth of nonadherent cells in the majority of cultures suggested that it was a primary effect of forced Sbf1 expression by the retroviral constructs. To confirm this, the nonadherent cells were assessed for their expression of exogenous Sbf1 proteins by Western blot analysis by using a mAb specific for Sbf1. High level expression of proteins with the expected molecular mass of 130 kDa was detected in nonadherent cells harvested from Sbf1$^{\Delta N}$ cultures (Fig. 3). The 190 kDa wild-type Sbf1 protein was also detected in these cells and those harvested from the p185$^{BCR\text{-}ABL}$ cultures. The latter is consistent with widespread expression of Sbf1 in most, if not all cell types, including B cell progenitors as typified by the human t(17;19)-bearing cell line HAL-01 (lane 4) and the mouse pre-B cell line PM1001 (lane 5).

Sbf1 Induces Clonal Outgrowth of Early B Cell Progenitors *in Vitro*. The Sbf1-infected, nonadherent cell populations were composed of large blast-like cells with high nuclear-to-cytoplasmic ratios (Fig. 4*A*). Flow cytometric analysis demonstrated that the cells were uniformly B220$^+$, CD43$^+$, HSA$^+$, and c-kit$^+$, all of which are expressed on primitive hematopoietic precursors, and negative for the myeloid surface antigen Mac-1 (Fig. 4*B*). There was variation in BP-1 staining, with some cultures being negative to low and others positive. This phenotypic profile, particularly the expression of B220, indicated derivation from the B lymphoid lineage. Staining for cytoplasmic and surface Ig μ chains was negative (Fig. 4*C*), consistent with an origin from early B lymphoid progenitor cells.

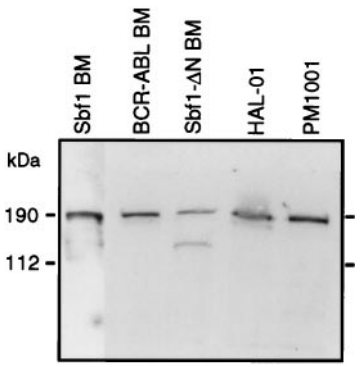

FIG. 3. Western blot analysis of wild-type and mutant Sbf1 proteins. Proteins prepared from whole-cell extracts were analyzed by immunoblotting by using an anti-Sbf1 mAb. Sources of proteins are indicated above the gel lanes. Transfected bone marrow cells were analyzed 4–6 weeks postinfection. Exogenous Sbf1$^{\Delta N}$ displays a migration different from endogenous Sbf1.

The lineage derivation was further assessed by analyses of antigen receptor gene configurations. DNA was prepared from Sbf1-infected cultures at 4–6 weeks postinfection, digested with appropriate restriction enzymes and determined by Southern blot analysis by using probes for the Ig and T cell receptor loci. The Ig heavy and kappa light chain genes as well as the beta T cell receptor loci contained no detectable rearrangements (Fig. 5*A*). These features are typical of very immature B-lineage cells, comparable to results obtained after transformation of susceptible target cells in the bone marrow with Abelson-murine leukemia virus, or with ras or src oncogenes expressed from murine retroviral vectors (30–33). The clonality of the nonadherent cell proliferations was assessed on the basis of retroviral integration sites by using a probe specific for the retroviral construct. Each culture displayed one or two bands of slightly varying size (Fig. 5*B*) indicating that a

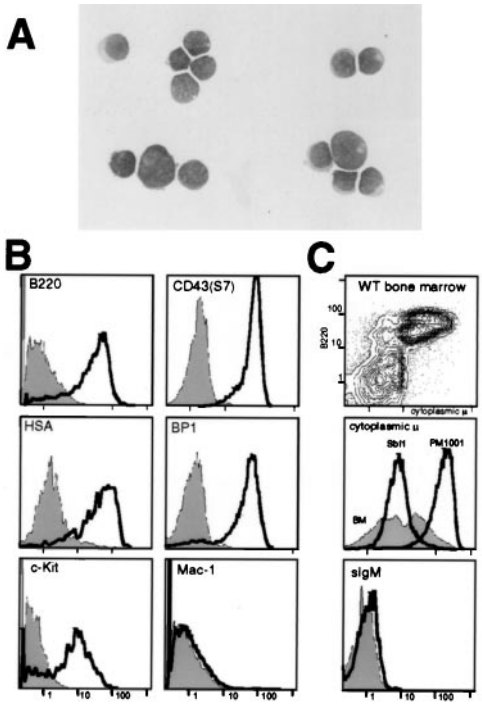

FIG. 4. Morphology and phenotype of cells expressing Sbf1. (*A*) Wright-Giemsa-stained cytospin preparation of Whitlock-Witte culture-derived cells. (*B*) Flow cytometric analysis of surface antigen expression. Solid lines represent staining obtained with conjugated antibodies specific for the indicated hematopoietic cell surface antigens (exception of HSA that employed unconjugated primary). Gray histograms represent background fluorescence on unstained cells (staining with secondary antibody only for HSA). (*C*) Expression of Ig in Sbf1 immortalized B cells. (*Top*) Staining for cytoplasmic μ and B220 in uninfected bone marrow. (*Middle*) Staining for cytoplasmic μ in uninfected bone marrow (same analysis as *Top*, gray histogram), a positive cell line (PM1001, thin line) and Sbf1-immortalized cells (thick line). (*Bottom*) Absence of surface IgM staining in Sbf1-immortalized cells (thick line) and unstained control (gray histogram).

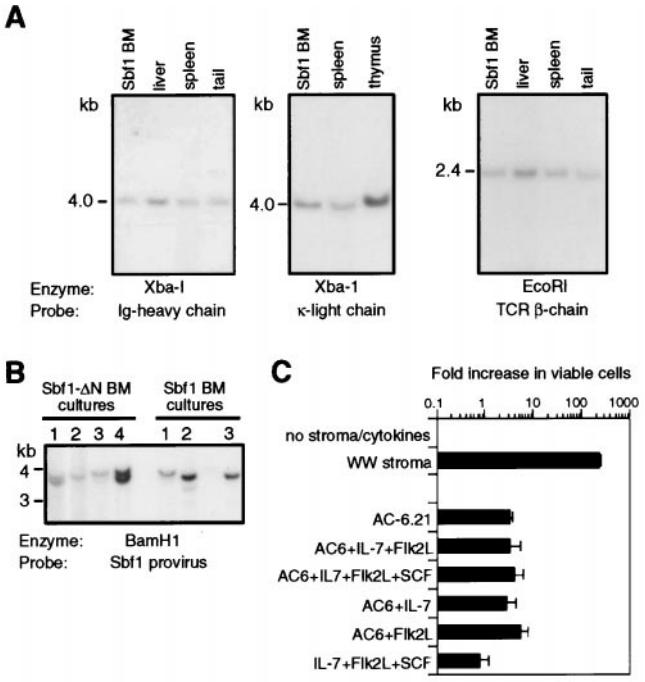

FIG. 5. Proviral integration, antigen receptor gene configurations, and growth requirements of Sbf1-expressing cells. (*A*) Southern blot analysis was performed by using enzymes and probes indicated beneath the respective panels. (*B*) DNA was isolated from bone marrow cultures initiated by either Sbf1 or Sbf1$^{\Delta N}$ retroviruses and subjected to Southern blot analysis using a probe specific for the pSRαMSV-Sbf1 vector to assess the configurations of retroviral integration sites. Each of the several detected bands represent clonal expansions of cells with integrated Sbf1 proviruses. (*C*) Growth responses of Sbf1-expressing cells are displayed as the fold increase in viable cells after 5 days in media containing the indicated cytokines with or without bone marrow-derived stroma or the AC-6.21 stromal cell line.

dominant clone of cells had established itself at this early stage in each of the cultures.

The growth properties of Sbf1-infected cells were characterized by assessing their cytokine and stromal layer requirements. Sbf1-immortalized cells stopped proliferating and died when removed from their stromal layer and transferred to liquid culture for 5 days. However, they continued to grow after transfer to stromal layers from primary bone marrow explants that had been mock-infected (Fig. 5*C*). Therefore, the observed lymphoid growth was cell autonomous and not dependent on Sbf1-induced alterations of the explanted stromal cells. Combinations of various cytokines (IL-7, IL-3, granulocyte-colony-stimulating factor, stem cell factor, and Flk2L) in the absence of a stromal layer sustained the survival but not the proliferation of Sbf1-infected cells (Fig. 5*C* and data not shown). This distinguishes Sbf1-infected hematopoietic cells from v-abl, src, or myc/ras-expressing cells (21, 31, 32, 34), which become growth-factor independent after several weeks in culture. When plated on a clonal stroma cell line (AC-6.21) (35) the Sbf1-immortalized cells displayed minimal expansion, and this was not measurably affected by the inclusion of various cytokines (Fig. 5*C*). Therefore, the Sbf1 population requires the presence of a primary bone marrow stromal layer for continous growth *in vitro*. A similar dependence on bone-marrow stroma was observed for p210$^{BCR\text{-}ABL}$ immortalized cells that remained dependent on their stroma for at least 3 months (25, 36).

The SET Interaction Domain of Sbf1 is Necessary and Sufficient for Growth Alterations of B Cell Precursors. Sbf1 contains an altered, but structurally conserved, dsPTPase catalytic pocket as well as an adjacent motif that mediates interactions with SET domains (referred to as the SID for *S*ET *i*nteraction *d*omain) (Fig. 6). A mutational analysis was carried out to investigate which of these features are necessary for growth-inducing effects of Sbf1 on primary B cell precursors *in vitro*. In each case, appropriate expression of mutated Sbf1 proteins was confirmed in transiently transfected BOSC23 cells or stably transfected fibrobasts. As shown above (Fig. 2*B*), the N-terminal 527 amino acids of Sbf1 were dispensible because their deletion in Sbf1$^{\Delta N}$ did not impair growth alterations (Fig. 6). The role of the defective catalytic domain was addressed by the construct Sbf1^{HCS} that was identical to Sbf1$^{\Delta N}$ except that the sequence of its catalytic pocket was mutated to match that of myotubularin, thereby conferring phosphatase activity (14). Restoration of catalytic activity in Sbf1^{HCS} completely abrogated its ability to induce hematopoietic cell growth even though Sbf1^{HCS} retained the ability to interact with SET domain proteins (Fig. 6). These data suggested a role for Sbf1 as a competitive antagonist of SET-interacting phosphatases. This mechanism was further supported by the inability of construct Sbf1$^{\Delta SID}$, which no longer interacted with SET domains due to deletion of the SID, to induce unrestrained growth of B cell precursors (Fig. 6). Most informative was construct Sbf1^{D4}, consisting almost exclusively of the SID, which induced the growth of primary hematopoietic cells. Sbf1^{D4} is capable of interacting with SET domains, but does not span the catalytic pocket (and lacks phosphatase activity). Therefore, the SET interaction domain of Sbf1 was necessary and sufficient for its effects on the growth properties of B cell precursors, suggesting that competitive interference with endogenous SET domain-binding phosphatases is the mechanism of action.

DISCUSSION

The current studies demonstrate that Sbf1, a catalytically inactive dsPTPase homolog, has potent growth-altering effects on primary B cell progenitors *in vitro*. Forced expression of Sbf1 after retroviral gene transfer resulted in the rapid outgrowth of clonal populations of early B lineage cells. However, Sbf1 did not appear to abrogate their cytokine and/or stromal layer requirements because Sbf1-infected bone marrow cells remained dependent on primary bone marrow stroma for optimal growth. This dependence was partially replaced by AC-6.21, a stromal cell line that has been shown to support

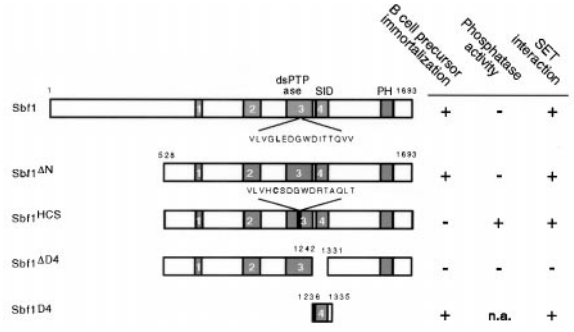

FIG. 6. Schematic illustrations of Sbf1 proteins with a summary of their growth-altering and biochemical properties. ▨, Protein domains conserved among myotubularin family members. Domain 3 displays similarity with the catalytic domains of tyrosine and dual-specificity phosphatases (dsPTPase); domain 4 (SID) mediates interactions with SET domain proteins. Sbf1 lacks several catalytically essential residues in domain 3 that were restored in construct Sbf1^{HCS}. The growth-altering properties of Sbf1 constructs are based on their ability (+) or inability (−) to induce growth of nonadherent bone marrow cells exceeding 10^5 cells per ml after 16 days of culture under Whitlock-Witte conditions. The phosphatase and SET domain interaction properties of the various Sbf1 proteins have been reported elsewhere (14). n.a., not applicable.

B-lymphopoiesis *in vitro* (35). Plating on AC-6.21 or with exogenous cytokines such as IL-7 plus Flk2L or SF facilitated survival but failed to restore the robust growth of Sbf1-immortalized cells that was observed on primary stroma. Given their early B lymphoid phenotype, we expected that IL-7 in combination with SF or Flk2L may be suitable cytokine combinations to support their growth as these cytokines are known to have important roles in lymphopoiesis. In particular, Flk2 [(the mouse homolog of human Flt3 (37)] with its ligand Flk2L is thought to regulate pluripotent stem cells, early progenitor cells, and immature lymphocytes (38). Moreover, Flk2 has been shown to synergize *in vitro* with IL-7 in inducing the proliferation of B cell progenitors (39) and later stage pro-B cells (40). Similar observations have been reported for combined stimulation with SF and IL-7 (41, 42).

In our studies, both Sbf1 and $p185^{BCR\text{-}ABL}$ resulted in the rapid outgrowth of B cell precursors *in vitro* with similar kinetics. These results are consistent with the previously reported effects of $p210^{BCR\text{-}ABL}$ on B lineage cells under similar bone marrow culture conditions (25). The previous studies also showed that p210-expressing cells, although clonal, retained their dependence on a stromal layer for continuous (12 weeks) growth *in vitro*. Similarly, when plated on the S17 stromal line, p210-expressing cells remained dependent on this line, and IL-7 did not appear to be the primary factor required for progenitor cell growth (36). Expression of p210 was growth stimulatory but not sufficient for full oncogenic behavior although some lines progressed to a malignant phenotype (25). Though we did not assess the leukemogenic potentials of Sbf1-expressing cells, the expectation is that, at least initially, they would not efficiently induce leukemias in recipient mice given their stromal layer dependence. Even after 15 weeks of continuous culture, p210-transformed lymphoid cells displayed considerable variation in their oncogenic behavior in spite of uniformly high expression of p210 and monoclonal dominance in cultures (25) presumably caused by a requirement for secondary events. The observation that Sbf1 has effects similar to BCR-ABL raises the intriguing possibility that one or more of the multiple signaling pathways initiated by BCR-ABL may intersect with SET domain proteins in the nucleus, a possibility currently under investigation.

The growth-altering effects of Sbf1 on B cell precursors are directly correlated with its ability to bind SET domain proteins *in vitro*. Restoration of its phosphatase activity, however, abrogated the effects of Sbf1 in our assays. These observations strongly suggest that the growth properties of B cell progenitors are regulated, in part, by the phosphorylation state of SET domain (or other) proteins that interact with Sbf1. Sbf1 shares extensive sequence similarity with myotubularin, a dsPTPase that is a target of mutations in a subset of myopathies displaying impaired differentiation (X-linked myotubular myopathy). Myotubularin displays an ability to interact with SET domain proteins because it contains a paired amphipathic helical motif (SID) highly conserved with that in Sbf1 that is responsible for interactions with SET domains (14). Thus, it is likely that SET-interacting dsPTPases such as myotubularin function normally to oppose growth-inducing signaling pathways by dephosphorylating SET domain proteins. Hyperexpression of Sbf1 or its SID, which is not only necessary but sufficient for immortalization of B cell progenitors [and transformation of fibroblasts (14)], likely results in competitive interference with endogenous SET-interacting dsPTPases. This is consistent with a normal role for Sbf1 as a SET domain-dependent positive regulator of signaling pathways. Because Sbf1 contains a structurally preserved but nonfunctional catalytic domain, it would presumably protect phosphorylated signaling proteins from dephosphorylation. A "protective factor" role was originally proposed for STYX, which also contains a naturally occurring mutation in its dsPTPase core domain whose activity can be similarly restored by backmutation (20).

Protective factors other than Sbf1 have not previously been implicated in oncogenic pathways. However, the oncogenic potential of Sbf1 as a positive regulator of kinase signaling pathways is consistent with the involvement of various kinases in oncogenesis. Both tyrosine-specific and dual specificity kinases play key roles in positive signaling for proliferation and are targets for activating oncogenic mutations. Similarly, a proliferative role for Sbf1, a catalytically inactive dsPTPase homolog, is consistent with the general anti-proliferative activity of protein phosphatases. Many dsPTPases are nuclear and some have been shown to specifically dephosphorylate MAP kinases to oppose mitogenic signaling pathways. The anti-oncogenic functions of protein phosphatases are evidenced by the ability of phosphatase inhibitor sodium orthovanadate to transform fibroblasts *in vitro* (43, 44). Recently, the dsPTPase PTEN (also known as MMAC1) has been shown to be mutated or deleted in several human cancers (45–47). In addition, germ-line mutations of PTEN have been reported in several related genetic disorders with increased susceptibility to benign and malignant tumors (47). The detection of germline mutations verified that PTEN functions as a tumor suppressor and also suggests a normal role in proper development and formation of certain tissues (45, 47, 48). Acquired and inherited PTEN mutations found in cancer generally result in the loss of its phosphatase activity, suggesting that the enzymatic activity of PTEN is necessary for its ability to function as a tumor suppressor. Determination of the mechanism of PTEN tumor suppressor activity will require identification of its physiological substrates.

Our current studies indicate that SET domain-dependent signaling pathways serve an important role in regulating the growth responses of early B cell progenitors. It remains unclear, however, which of the several currently known SET domain proteins are critical targets. A candidate is Hrx, a protooncoprotein that is activated by chromosomal translocations in a subset of acute lymphoid leukemias, because Sbf1 was originally discovered by virtue of its interaction with the SET domain of Hrx in yeast two-hybrid screens (14). As a consequence of chromosomal translocations, the SET domain of Hrx is deleted and replaced by sequences from a wide variety of heterologous proteins, some of which have defined roles in transcriptional regulation. For the Hrx-Enl fusion protein, it has been shown that oncogenic activity results from a gain-of-function after acquisition of constitutive transcriptional activation motifs contributed by Enl (49, 50). Our current studies raise the possibility that the normal effector functions of Hrx, and possibly other SET domain proteins, are responsive to upstream signaling pathways that impinge on the SET domain. This normal regulatory mechanism could be circumvented by heterologous protein fusions or interference with endogenous SET-interacting dsPTPase functions, in both cases inducing inappropriate expression of subordinate genes. Given that Sbf1 can induce growth perturbations in fibroblasts (loss of contact inhibition, anchorage-independent growth and tumorigenicity) and primary hematopoietic cells, SET domain proteins are likely to be critical for growth responses of many if not all cell types.

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