The EMBO Journal Vol.17 No.15 pp.4442–4455, 1998

TLS/FUS, a pro-oncogene involved in multiple chromosomal translocations, is a novel regulator of BCR/ABL-mediated leukemogenesis

Danilo Perrotti[1], Silvia Bonatti[2], Rossana Trotta, Robert Martinez, Tomasz Skorski, Paolo Salomoni, Emanuela Grassilli, Renato V.Iozzo[3], Denise R.Cooper[4] and Bruno Calabretta[1]

Department of Microbiology and Immunology, Kimmel Cancer Center, [3]Department of Pathology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107, [4]Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine and the James A.Haley Veterans Hospital, Tampa, FL 33612, USA and [2]Department of Biomedical Sciences, University of Modena, Italy

[1]Corresponding authors
e-mail: perrott1@jeflin.tjn.edu or B_Calabretta@hendrix.jci.tju.edu

The leukemogenic potential of BCR/ABL oncoproteins depends on their tyrosine kinase activity and involves the activation of several downstream effectors, some of which are essential for cell transformation. Using electrophoretic mobility shift assays and Southwestern blot analyses with a double-stranded oligonucleotide containing a zinc finger consensus sequence, we identified a 68 kDa DNA-binding protein specifically induced by BCR/ABL. The peptide sequence of the affinity-purified protein was identical to that of the RNA-binding protein FUS (also called TLS). Binding activity of FUS required a functional BCR/ABL tyrosine kinase necessary to induce PKCβII-dependent FUS phosphorylation. Moreover, suppression of PKCβII activity in BCR/ABL-expressing cells by treatment with the PKCβII inhibitor CGP53353, or by expression of a dominant-negative PKCβII, markedly impaired the ability of FUS to bind DNA. Suppression of FUS expression in myeloid precursor 32Dcl3 cells transfected with a FUS antisense construct was associated with upregulation of the granulocyte-colony stimulating factor receptor (G-CSFR) and downregulation of interleukin-3 receptor (IL-3R) β-chain expression, and accelerated G-CSF-stimulated differentiation. Downregulation of FUS expression in BCR/ABL-expressing 32Dcl3 cells was associated with suppression of growth factor-independent colony formation, restoration of G-CSF-induced granulocytic differentiation and reduced tumorigenic potential *in vivo*. Together, these results suggest that FUS might function as a regulator of BCR/ABL leukemogenesis, promoting growth factor independence and preventing differentiation via modulation of cytokine receptor expression.

Keywords: BCR/ABL/leukemogenesis/TLS/FUS/zinc finger protein

Introduction

The BCR/ABL fusion genes of the Philadelphia chromosome (Ph[1]), encode the *p210*$^{bcr/abl}$ or *p185*$^{bcr/abl}$ oncoproteins that transform immature hematopoietic cells *in vitro* (McLaughlin *et al*., 1987) and cause chronic myelogenous leukemia (CML)-like syndromes in mice (Daley *et al*., 1990; Heisterkamp *et al*., 1990). The ability of BCR/ABL oncoproteins to transform hematopoietic cells depends on their tyrosine kinase activity (Lugo *et al*., 1990), which is essential to recruit and activate multiple biochemical pathways that transduce oncogenic signals (Cortez *et al*., 1995). Thus, the identification of signaling molecules regulated by BCR/ABL proteins is essential to elucidate the mechanism(s) underlying the leukemogenic process. While the role in leukemogenesis of certain cytoplasmic downstream effectors, such as RAS and PI-3K (Goga *et al*., 1995; Sawyers *et al*., 1995; Skorski *et al*., 1997), is understood in some detail, much less is known about the nuclear effectors activated by BCR/ABL and the mechanisms by which they contribute to the phenotype of BCR/ABL-transformed cells.

Nuclear effectors of BCR/ABL might include myeloid-specific zinc finger proteins, because of their involvement in hematopoietic differentiation and leukemogenesis (Tenen *et al*., 1997). Using electrophoretic mobility shift assay (EMSA) on lysates from BCR/ABL-expressing cells and a double-stranded oligonucleotide containing a zinc finger consensus sequence recognized by fingers 1–4 of the myeloid zinc finger protein 1 (MZF-1) transcription factor (Morris *et al*., 1994), we detected a DNA–protein complex whose formation depends on the tyrosine kinase activity of the p210$^{BCR/ABL}$ oncoprotein. Additional studies led to the identification of FUS as the only protein of this BCR/ABL-regulated DNA–protein complex. FUS was first discovered as the N-terminal part of a fusion gene with CHOP in myxoid liposarcoma carrying the translocation t(12;16) (Crozat *et al*., 1993; Rabbitts *et al*., 1993). Homologies were found with the EWS oncogene, which is rearranged in Ewing sarcomas and other neoplasia (Aman *et al*., 1996). In the t(16;21) translocation, detected in different types of human myeloid leukemia (Shimizu *et al*., 1993), the C-terminal region of FUS is replaced by the DNA-binding domain of ERG (Panagopoulos *et al*., 1995). In other chromosomal translocations, the N-terminal region of FUS or of the homologous EWS gene is fused to the DNA-binding domain of one of several transcription factors such as FLI1, ERG, ATF1, CHOP and WT1, to generate oncogenes with high transforming potential (Ladanyi, 1995; Ron, 1997). While the oncogenic forms of FUS (fused to CHOP or ERG) have been the subject of intense investigation since their identification in myxoid liposarcoma and myeloid leukemias, much less is known about the function of its normal cellular form (Ron, 1997).

The C-terminus of FUS contains a highly conserved region of 80 amino acids, the ribonucleoprotein consensus sequence (RNP-CS) (Crozat *et al*., 1993), an RNA recogni-

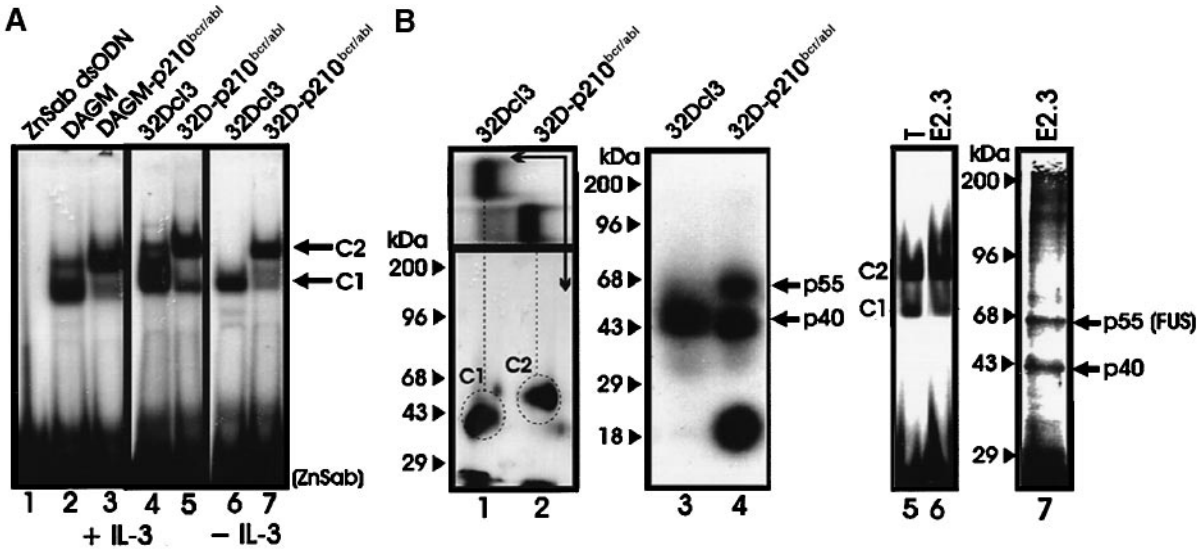

Fig. 1. Characterization and purification of the dsODN ZnSab binding proteins. (**A**) EMSA performed with ^{32}P-labeled dsODN ZnSab and extracts from parental and BCR/ABL-expressing DAGM and 32Dcl3 cells. (**B**) Left panel, *in situ* UV cross-linking analysis of C1 and C2 complexes; middle panel, Southwestern assay with ^{32}P-labeled dsODN ZnSab on extracts from IL-3-deprived parental and BCR/ABL-expressing 32Dcl3 cells; right panels, EMSA with ^{32}P-labeled dsODN ZnSab and 30 μg cell extracts (T) from 32Dp210$^{BCR/ABL}$ cells (lane 5) or 25 ng of ZnSab affinity-purified proteins (E2.3) (lane 6); silver staining of the affinity-purified fraction E2.3 (lane 7).

tion motif (RRM) flanked by Arg-Gly-Gly (RGG) repeats and a C2-C2 zinc finger domain homologous in structure to that found in the RNA binding proteins snRNP-associated protein 69KD and RBP56 (Hackl and Luhrmann, 1996; Morohoshi *et al*., 1996). The N-terminal domain of FUS contains instead a sequence of glutamine- and proline-rich degenerate hexapeptide repeats, which resembles the transcriptional activation domain of Sp-1 (Courey and Tjian, 1988). The C-terminal region is required, both *in vitro* and *in vivo*, for FUS binding to pre-mRNA and mRNA (Crozat *et al*., 1993; Zinszner *et al*., 1997a), while the FUS N-terminus functions as a potent transcriptional activation domain necessary for the oncogenic potential of FUS–CHOP or FUS–ERG proteins (Crozat *et al*., 1993; Prasad *et al*., 1994).

FUS is expressed at high levels in hematopoietic and non-hematopoietic tissues (Aman *et al*., 1996; Morohoshi *et al*., 1996) and is primarily localized in the nucleus (Crozat *et al*., 1993), where it might be involved in nucleo-cytoplasmic shuttling (Zinszner *et al*., 1997b). Like the homologues hTAF$_{II}$68, EWS and *Drososphila* SARFH protein, FUS might function as a basal transcription regulator, as suggested by its presence in RNA pol II transcription complexes (Zinszner *et al*., 1994; Immanuel *et al*., 1995; Bertolotti *et al*., 1996). The association of FUS with products of RNA pol II transcription is dependent on ongoing transcription and leads to the formation of large ternary complexes with other heterogeneous nuclear RNA-binding proteins (hnRNPs) such as hnRNP A1 and C1/C2 (Zinszner *et al*., 1994). Consistent with its involvement in pre-mRNA processing and mRNA export, FUS has been independently identified as the hnRNP P2 protein (Calvio *et al*., 1995). Despite the wealth of information on structure, RNA-binding or single-stranded (ss) DNA-binding specificity, and cellular distribution (Ron, 1997), little is known about the mechanisms regulating FUS expression and function.

In this study, following the identification of FUS in the BCR/ABL-regulated DNA–protein complex, we have analyzed the BCR/ABL-dependent pathway(s) leading to FUS activation and assessed the functional consequences of interfering with FUS expression. We report here that a PKCβII-dependent pathway is required for the activation of FUS and that FUS activity is important for the growth factor independence and reduced propensity for differentiation of BCR/ABL-transformed cells.

Results

Characterization of two BCR/ABL-regulated DNA binding proteins (p55 and p40)

The phenotype of hematopoietic cells expressing the BCR/ABL oncoproteins includes reduced susceptibility to apoptosis, growth factor-independent proliferation and differentiation arrest. To assess whether BCR/ABL regulates the activity of zinc finger transcription factors with a potential role in hematopoietic cell differentiation, a double-stranded oligodeoxynucleotide (dsODN ZnSab: 5′-ttttctccccactttagatc-3′) containing a canonical zinc finger consensus sequence recognized by the MZF-1 transcription factor (Morris *et al*., 1994; Perrotti *et al*., 1995) was tested in EMSA for its ability to bind proteins in cell extracts from two interleukin-3 (IL-3)-dependent murine myeloid cell lines, 32Dcl3 and DAGM, expressing wild-type p210$^{BCR/ABL}$ or not. Two complexes, C1 and C2, were detected in lysate of both cell lines cultured with IL-3 or IL-3-starved 32Dcl3 cell lysate (Figure 1A, lanes 2–5 and lanes 6 and 7, respectively). Formation of the C2 complex was specifically induced in BCR/ABL-expressing cells, while the C1 complex was more clearly detectable in parental cells. Both complexes were resistant to sodium deoxycholate concentrations (0.2–0.8%) that disrupt protein–protein interaction or to formamide treatment (20–30%) (not shown), suggesting that a complex of multiple proteins in which only one interacts with the DNA is not involved in the binding to dsODN ZnSab.

Moreover, the presence of bivalent cations in the binding reaction was required for C2 complex formation (not shown).

In *in situ* UV cross-linking experiments using the ZnSab probe and cell extracts from parental and BCR/ABL-expressing 32Dcl3 cells, the two complexes resolved as two distinct species migrating with apparent molecular masses of 40 and 55 kDa, respectively (Figure 1B, lanes 1 and 2). Southwestern analysis indicated that the two complexes derived from the interaction of the dsODN ZnSab with proteins migrating in SDS–PAGE at ~40 and 68 kDa, respectively (Figure 1B, lanes 3 and 4).

Purification of total lysate from 32Dp210$^{BCR/ABL}$ cells by size-fractionation and DNA-affinity chromatography using a column prepared with the multimerized dsODN ZnSab, but not with a 35mer unrelated double-stranded ODN (5′-ggggtccccccttactggactcaggttgccccctg-3′), yielded two DNA binding proteins which were enzymatically cleaved and sequenced by mass spectrometry (Figure 1B, lanes 5–7). Computer search for homology to the peptide sequences p55(1): LKGEATVSFDDPPSAK; p55(2): AAIDWFDGK; and p40(1): IFVGGLSPDTPEEK, identified p55 as the murine homologue of FUS, while p40 was identical to the hnRNP C1/C2. Direct proof that the C2 complex detected in EMSA with lysates of parental and BCR/ABL-expressing 32Dcl3 cells (Figure 2A, lanes 1 and 2) was due to the interaction of FUS with dsODN ZnSab was obtained by observing a supershift with a polyclonal FUS antiserum (Figure 2A, lanes 3 and 4), but not with an unrelated polyclonal antiserum (Figure 2A, lane 5).

To investigate sequence requirements for the dsODN ZnSab–FUS interaction, EMSA were performed with dsODN ZnSab mutated in the zinc finger motif (ZnMut1) or in the nucleotides flanking it (ZnMut2 and ZnMut3) used as probes or as competitor of ^{32}P-labeled wild-type dsODN ZnSab. The C2 complex was competed by ZnMut1, but not by ZnMut2 or ZnMut3 (Figure 2B); conversely, only the ^{32}P-labeled ZnMut1 formed the C2 complex upon incubation with lysate from BCR/ABL-expressing 32Dcl3 cells (Figure 2B).

Since FUS reportedly binds mRNA and ssDNA (Prasad *et al.*, 1994; Zinszner *et al.*, 1997b), we investigated whether FUS binding to ssDNA is also sequence-dependent. By EMSA, a complex similar to that formed with dsODN ZnSab was detected in extracts of BCR/ABL-expressing 32Dcl3 cells with the ^{32}P-labeled ssODN ZnSb (Figure 2C, lane 4), but not with the complementary ZnSa (Figure 2C, lane 2). Specificity of the ZnSb–FUS interaction was confirmed in competition experiments in which an excess of ssODN ZnSb, but not ssODN ZnSa, abrogated the detection of the ZnSab–FUS–FUSAb or the ZnSab–FUS complex (Figure 2A, lanes 6–9 and 2C, lanes 7–15, respectively). The relative affinities of FUS for ZnSb and ZnSab were investigated by EMSA in which the ZnSab or the ZnSb probes were incubated with increasing amounts of lysate from BCR/ABL-expressing 32Dcl3 cells (Figure 2D, lanes 1–5 and 10–14, respectively), or with increasing amounts of cold ssODN ZnSb or dsODN ZnSab used as competitor (Figure 2D, lanes 6–9 and 15–18, respectively). The results of these experiments indicate that FUS has a higher affinity for ssODN ZnSb than for dsODN ZnSab.

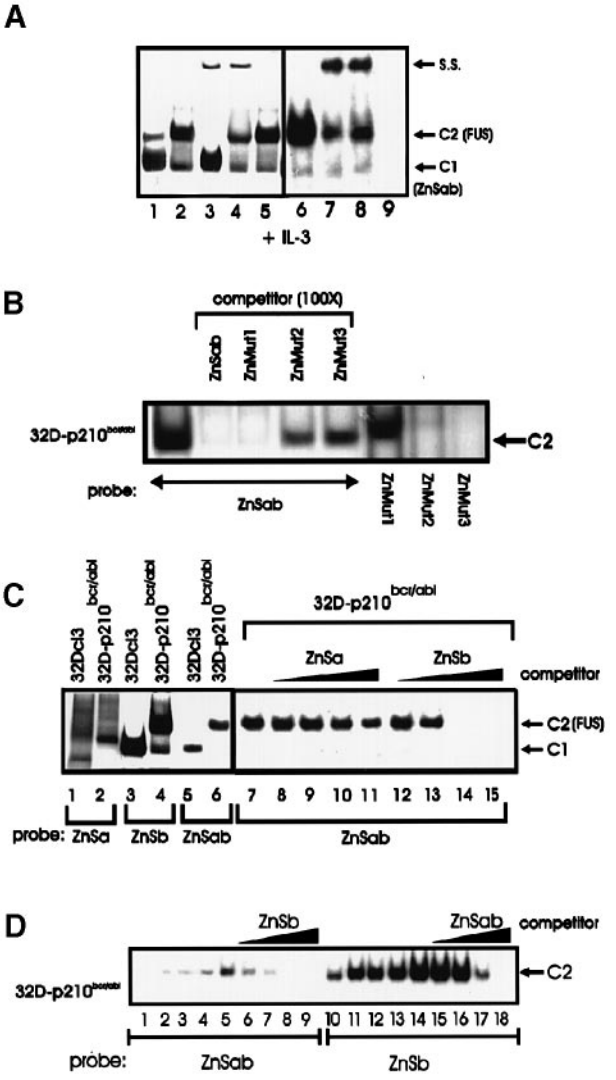

Fig. 2. Sequence specificity of FUS DNA-binding activity. (**A**) EMSA with ^{32}P-labeled dsODN ZnSab and lysates from parental (lanes 1 and 3) and BCR/ABL-expressing 32Dcl3 cells (lanes 2 and 4–9). Assays were performed in the presence of a polyclonal FUS antiserum (lanes 3, 4 and 7–9) or in the presence of an irrelevant polyclonal antiserum (lane 5). Cold ssODN ZnSa (lane 8) or ssODN ZnSb (lane 9) was added (100-fold molar excess) to the reaction to determine binding specificity. s.s. indicates the supershifted complex. (**B**) EMSA performed on extracts from BCR/ABL-expressing 32Dcl3 cells and wild-type ^{32}P-labeled ZnSab or mutated (ZnMut1, ZnMut2 and ZnMut3) dsODNs used as probes or as competitors. Representative of three different experiments with similar results. (**C**) EMSA performed on extracts from parental and BCR/ABL-expressing 32Dcl3 cells with ^{32}P-labeled ZnSa or ZnSb, or with ^{32}P-labeled ZnSab in the absence (lane 7) or in the presence of increasing concentration (5-, 10-, 50- and 100-fold molar excess) of cold ZnSa or ZnSb used as competitor. (**D**) EMSA performed with ^{32}P-labeled dsODN ZnSab or ssODN ZnSb, and increasing amount of lysates (1, 2.5, 5, 7.5 and 15 μg) from BCR/ABL-expressing 32Dcl3 cells (lanes 1–5 and 10–14, respectively). Assays using 15 μg of lysates were also performed in the presence of increasing concentrations (5-, 10-, 50- and 100-fold molar excess) of cold ZnSb (lanes 6–9) or ZnSab (lanes 15–18).

FUS expression and DNA binding activity in BCR/ABL-expressing cells

To assess whether induction of FUS binding activity in BCR/ABL-expressing 32Dcl3 cells was associated with enhanced expression of FUS, Western blot analysis was

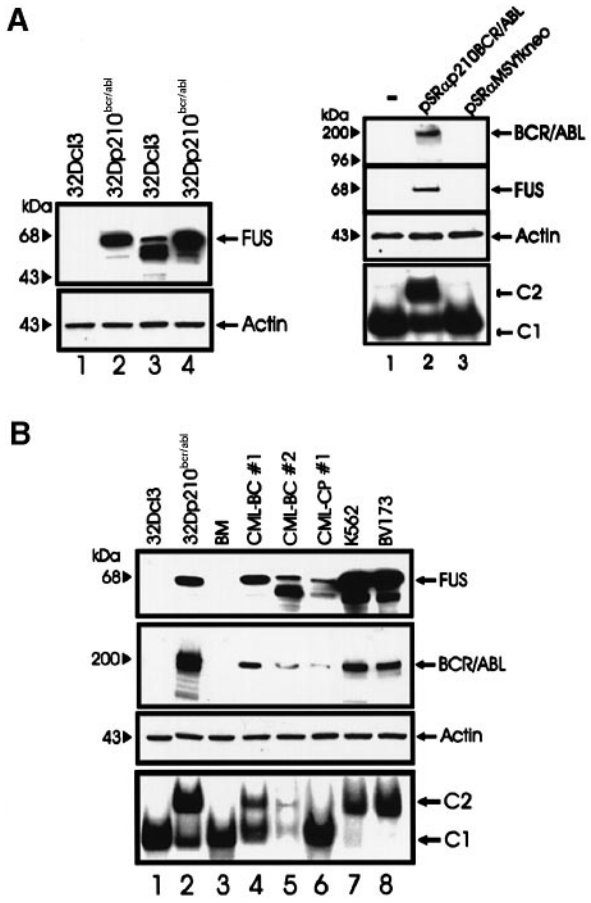

Fig. 3. FUS expression and binding activity in murine and human BCR/ABL-expressing cells. (**A**) Left panel, Western blot shows FUS expression in total cell lysate (25 μg) from parental and BCR/ABL-expressing 32Dcl3 cells cultured in IL-3-containing medium (lanes 3 and 4) or after IL-3 starvation (lanes 1 and 2); right panel, FUS expression (second row) and binding activity (bottom row) in non-infected parental 32Dcl3 cells (lane 1), and in cells infected with wild-type BCR/ABL (lane 2) or an insert-less retrovirus (lane 3). (**B**) FUS expression and binding activity in lysates from IL-3-starved 32Dcl3 cells, normal and CML marrow cells, and from the Ph^1-positive K562 and BV173 cell lines, as indicated. Actin and BCR/ABL were detected using an anti-Actin (Santa Cruz) or an anti-ABL (Ab3, Oncogene Science) antibody. Representative of three different experiments.

performed on lysates from parental 32Dcl3 cells and from cells stably or transiently expressing $p210^{BCR/ABL}$. Transient expression of $p210^{BCR/ABL}$ was achieved in retrovirus-infected cells (72 h post-infection) in experiments designed to determine whether enhanced FUS expression is an early change in BCR/ABL-expressing cells. FUS was readily detectable in IL-3-starved BCR/ABL-expressing 32Dcl3 cells, while in parental 32Dcl3 cells it was detected exclusively when cells were maintained in the presence of IL-3 (Figure 3A), suggesting that BCR/ABL expression circumvents the requirement for signals generated in non-transformed cells by IL-3–interleukin-3 receptor (IL-3R) interaction.

FUS expression and its ability to bind the dsODN ZnSab correlated with BCR/ABL levels also in the Ph^1-positive cell lines K562 and BV173 and in primary cells from patients with chronic phase- or blast crisis-CML (Figure 3B).

Phosphorylation-dependence of FUS DNA binding activity in cells expressing a functional BCR/ABL tyrosine kinase

EMSA of cell lysates from IL-3-starved 32Dcl3 cells stably or transiently expressing wild-type or kinase-deficient (p210K1172R) BCR/ABL revealed the C2 complex only in lysates from cells expressing wild-type $p210^{BCR/ABL}$ (Figure 4A, lanes 2 and 4). The absence of C2 complex in EMSA of 32Dp210K1172R extracts was not due to lack of BCR/ABL expression (Figure 4A, lanes 3 and 5), indicating that a tyrosine kinase-dependent pathway is required to maintain elevated levels of FUS expression and activity (Figure 4A). Indeed, FUS binding to dsZnSab was identical in IL-3-deprived 32Dcl3 cells expressing any of several BCR/ABL mutants (32Dp210ΔSH3, 32Dp210ΔSH2 and 32Dp210ΔBCR) lacking different portions of the BCR/ABL chimeric protein but retaining the tyrosine kinase activity and in cells transfected with wild-type BCR/ABL (not shown).

Binding activity of FUS to dsODN ZnSab was abolished by treating the cell extracts with alkaline phosphatase (AP), and was restored by adding phosphatase inhibitors (see Materials and methods) to the binding reaction prior to AP treatment (Figure 4B). In agreement with these data, Southwestern analysis with the ^{32}P-labeled dsODN ZnSab as probe on phosphoproteins immunoprecipitated from $32Dp210^{BCR/ABL}$ cells using anti-Ptyr, anti-PSer and anti-PThr monoclonal antibodies detected the ~68 kDa protein identified as FUS, which is overexpressed in $32Dp210^{BCR/ABL}$ cells (Figure 4C, compare lanes 1 and 2). FUS binding activity was readily detected in the anti-phosphoserine immunoprecipitates (Figure 4C, lane 5), present at lower levels in the anti-phosphotyrosine and anti-phosphothreonine immunoprecipitates (Figure 4C, lanes 4 and 6), and undetectable in immunoprecipitates using an isotype-matched irrelevant antibody (Figure 4C, lane 3). Western blots with anti-phosphoserine/threonine antibody mixtures on FUS immunoprecipitates from IL-3-deprived parental and BCR/ABL (wild-type or kinase deficient)-expressing 32Dcl3 cells revealed a phosphorylated FUS protein (~68 kDa) in $32Dp210^{BCR/ABL}$ cells (Figure 4D, lane 5) but not in parental 32Dcl3 or 32Dp210K1172R cells (Figure 4D, lanes 4 and 6, respectively). In addition to FUS, a phosphoprotein of ~80 kDa was detected in the anti-phosphoserine/threonine blot (Figure 4D, lane 5, lower panel).

Dependence of FUS activation on a BCR/ABL-regulated PKCβII-kinase pathway

To investigate mechanism(s) whereby BCR/ABL regulates FUS DNA-binding activity, EMSA were performed using dsODN ZnSab as probe and whole-cell extracts from IL-3-starved $32Dp210^{BCR/ABL}$ cells treated with kinase or phosphatase inhibitors, some of which interfere with BCR/ABL-dependent pathways involved in cell survival and proliferation. The levels of the C2 complex (Figure 5A, lane 1) were markedly reduced after treatment with the serine-threonine kinase inhibitor staurosporine (1 μM) (Figure 5A, lane 6), or the specific protein kinase C (PKC) inhibitor calphostin C (200 ng/ml) (Tamaoki *et al*., 1991) (Figure 5A, lane 5), suggesting that a PKC-dependent pathway is involved in the regulation of FUS binding activity. The DNA binding activity of FUS was suppressed

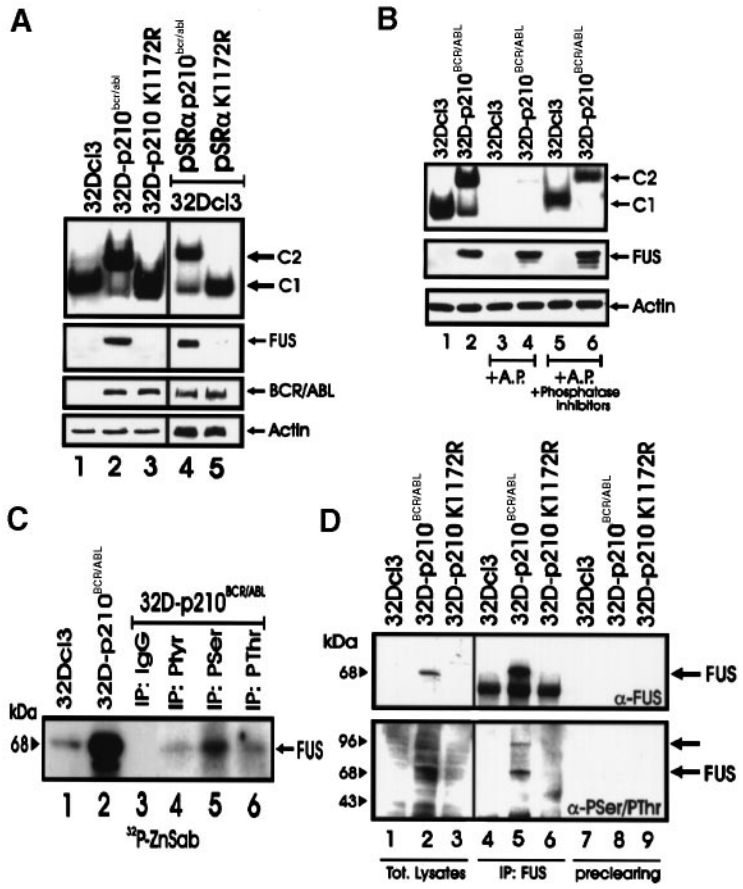

Fig. 4. Phosphorylation-dependent DNA binding activity of FUS. (**A**) EMSA with ^{32}P-labeled dsODN ZnSab (top) and Western blots with anti-FUS, anti-ABL, and anti-Actin antibodies, on lysates from parental 32Dcl3 cells (lane 1), cells stably expressing wild-type p210$^{BCR/ABL}$ (lane 2) or kinase-deficient BCR/ABL protein (K1172R) (lane 3), and from 32Dcl3 cells transiently expressing wild-type p210$^{BCR/ABL}$ (lane 4) or the kinase-deficient BCR/ABL (K1172R) protein (lane 5). Cells were IL-3-starved for 8 h prior to lysis. (**B**) EMSA (upper panel) with ^{32}P-labeled dsODN ZnSab and Western blot (middle panel) with the anti-FUS serum on IL-3-starved parental and BCR/ABL-expressing 32Dcl3 cells. When indicated, lysates were treated with AP in the presence or absence of phosphatase inhibitors. (**C**) Southwestern analysis with ^{32}P-labeled dsODN ZnSab on 50 μg lysate from parental (lane 1) and BCR/ABL-expressing cells (lane 2), and on immunoprecipitates from BCR/ABL-expressing 32Dcl3 cells using the indicated anti-phosphoprotein Abs (lanes 4–6). Immunoprecipitates with an unrelated IgG were used as control (lane 3). (**D**) Lower panel, Western blots with anti-phosphoserine/ threonine (PSR-45 and PTR-8; Sigma Chemical Co.) antibody mixtures on total lysates and on FUS immunoprecipitates from IL-3-starved parental and BCR/ABL (wild-type or kinase deficient)-expressing 32Dcl3 cells; upper panel, Western blots with FUS antiserum on total lysates, FUS immunoprecipitates and protein G-preclearing performed as control. Immunoprecipitates with an irrelevant IgG were also used as control (not shown). Autoradiograms are representative of three experiments.

by treatment of BCR/ABL-expressing cells with the specific phospholipase C-γ (PLC-γ) inhibitor U73122 (1 μM), but not with its inactive U73343 (1 μM) derivative (Chen *et al*., 1996) (Figure 5A, lanes 4 and 3, respectively), and upon treatment with the Ca^{2+} chelator EGTA (1 mM), (Figure 5A, lane 8). Since PLC-γ activates certain PKC isoforms via an increase in the intracellular levels of Ca^{2+} and diacylglycerol (DAG), Western blots were performed on subcellular extracts from IL-3-cultured parental and BCR/ABL-expressing 32Dcl3 cells with an anti-cPKC antibody that recognizes the DAG- and Ca^{2+}-dependent α, βI, βII and γ isoforms. Compared with parental cells or cells expressing the kinase-deficient BCR/ABL, expression of the nuclear PKC isoforms was enhanced in wild-type BCR/ABL-expressing cells, upon culture in the absence of IL-3 (not shown). In contrast, the levels of other conventional PKC isoforms present in the cytosolic/membrane cell fraction were unchanged (not shown). In light of these observations and the fact that FUS is primarily localized in the nucleus, 32Dcl3 cells expressing wild-type BCR/ABL were treated with CGP53353 (10 μM), a specific inhibitor of the nuclear PKCβII isoform (Chalfant *et al*., 1996), to determine whether this kinase regulates the DNA binding activity of FUS. Indeed, EMSA assays revealed that the levels of the C2 complex were reduced after treatment of BCR/ABL-expressing cells with CGP53353 (Figure 5A, lane 7); moreover, a marked inhibition of C2 complex formation was also observed in EMSA performed with extracts from 32Dp210$^{BCR/ABL}$ cells transiently expressing a PKCβII dominant negative mutant (Chalfant *et al*., 1996) (Figure 5A, lane 10). Instead, FUS binding to dsODN ZnSab was not affected by treatment of 32Dp210$^{BCR/ABL}$ cells with other inhibitors such as the serine-threonine phosphatase inhibitor okadaic acid (50 nM), the p70^{S6kinase} inhibitor rapamycin (15 nM) (Downward, 1994), the mitogen-activated protein (MAP) kinase inhibitor PD098059 (50 μM) (Dudley *et al*., 1995) and the phosphatidylinositol-3 kinase (PI-3K) inhibitor wortmannin (50 nM) (Powis *et al*., 1994) (Figure 4A, lanes 2 and 11–13). As expected, the levels of FUS binding to dsODN ZnSab in extracts from cells treated with each of these compounds correlated

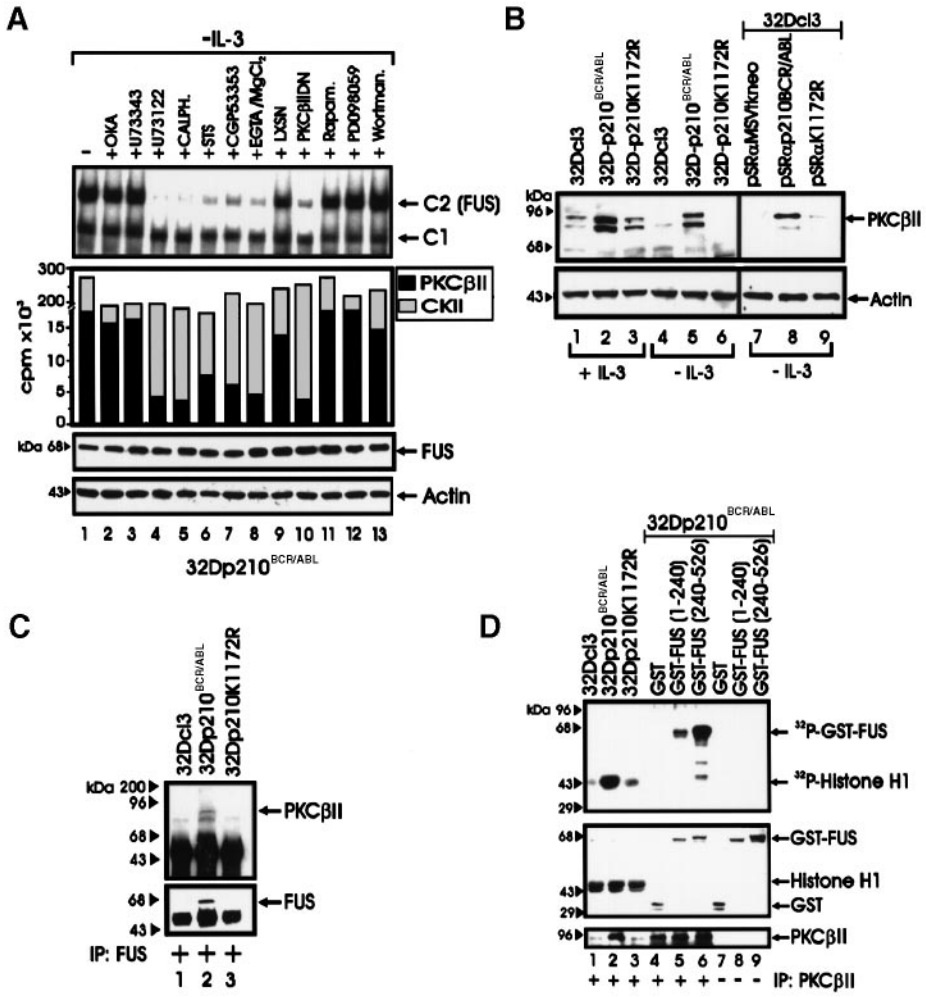

Fig. 5. Identification of a BCR/ABL-dependent PKCβII pathway involved in FUS activation. (**A**) Upper panel, FUS DNA binding activity in IL-3-deprived, untreated BCR/ABL-expressing 32Dcl3 cells (lane 1), or cells treated with okadaic acid (5 h) (lane 2), U73343 (3.5 h) (lane 3), U73122 (3.5 h) (lane 4), calphostin (5 h) (lane 5), staurosporine (5 h) (lane 6), CGP53353 (8 h) (lane 7), EGTA/$MgCl_2$ (8 h) (lane 8), rapamicyn (5 h) (lane 11), PD098059 (5 h) (lane 12) and wortmannin (5 h) (lane 13). FUS binding activity was also assessed in IL-3-deprived BCR/ABL-expressing 32Dcl3 cells infected with an insert-less retrovirus (lane 9) or with a retrovirus carrying a dominant negative PKCβII (lane 10). Middle panel, PKCβII (black bars) and casein kinase II (CKII) (gray bars) activity in untreated or inhibitor-treated BCR/ABL-expressing 32Dcl3 cells. PKCβII was immunoprecipitated using a specific anti-PKCβII polyclonal serum (Santa Cruz). Representative of two experiments. Lower panels, FUS and actin expression (Western blotting) in untreated or inhibitor-treated BCR/ABL-expressing 32Dcl3 cells. (**B**) Left, PKCβII expression in parental, wild-type and kinase-deficient BCR/ABL-expressing 32Dcl3 cells. Right, PKCβII expression (Western blotting) in 32Dcl3 cells retrovirally infected with the insert-less vector or with wild-type (lane 8) or kinase deficient (K1172R) BCR/ABL. Seventy-two hours post-infection, cells were IL-3-deprived for 8 h and lysates were prepared as described (Perrotti *et al*., 1996). (**C**) Upper panel, detection of PKCβII (Western blotting) in FUS-immunoprecipitates (IP) from IL-3-deprived parental, wild-type, or kinase-deficient (K1172R) BCR/ABL-expressing 32Dcl3 cells; lower panel, FUS was detected (Western blotting) as control. (**D**) Upper panel, PKCβII kinase assay performed on PKCβII-immunoprecipitates from parental and BCR/ABL-expressing (wild-type and kinase-deficient) 32Dcl3 cells using histone H1 as substrate (lanes 1–3), or from wild-type BCR/ABL-expressing 32Dcl3 cells using N-terminal (1–240) and C-terminal (240–526) GST–FUS protein as substrate (lanes 5 and 6). GST protein was used as control (lane 4); middle panel, substrates were detected by Ponceau red-staining of the membrane; lower panel, immunoprecipitated PKCβII was detected by Western blot. Lanes 7–9, kinase assays (negative controls) using an irrelevant isotype-matched IgG and GST or GST–FUS proteins as substrates.

with that of PKCβII (Figure 5A, black bars), but not casein kinase II (CKII) (Figure 5A, gray bars) activity.

Upon IL-3 withdrawal, PKCβII levels were markedly reduced in parental and in p210K1172R-expressing 32Dcl3 cells (Figure 5B, compare lanes 1 and 3 with 4 and 6), but remained constant in wild-type BCR/ABL-transfectants (Figure 5B, compare lanes 2 and 5). Moreover, induction of PKCβII expression was also observed upon infection of parental 32Dcl3 cells with the wild-type BCR/ABL, but not after infection with the insert-less retrovirus or with the retrovirus carrying the kinase-deficient (K1172R) $p210^{BCR/ABL}$ (Figure 5B, lanes 7–9).

Sequence analysis of FUS revealed the presence of multiple putative PKC phosphorylation sites clustered in the C-terminal region (amino acids 240–526). Because of this, and the co-immunoprecipitation of an ~80 kDa phosphoprotein reminiscent of PKCβII with FUS (Figure 4D), we further assessed the role of PKCβII in BCR/ABL-dependent activation of FUS.

PKCβII was expressed at high levels in 32Dcl3 cells expressing wild-type BCR/ABL (Figure 5B), and was also active, as indicated by the ability of PKCβII immunoprecipitates to phosphorylate histone H1 substrate in an *in vitro* kinase assay (Figure 5D, lane 2, upper panel). In

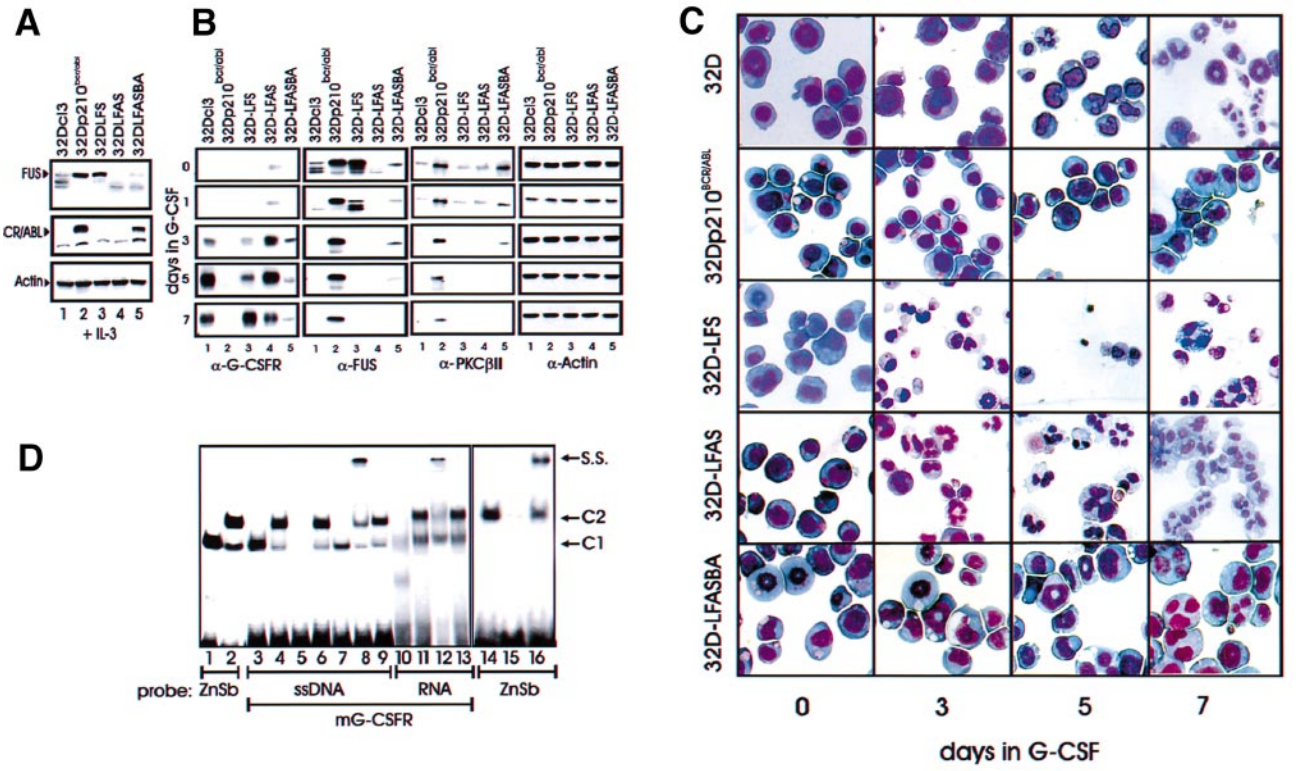

Fig. 6. Effects of FUS on granulocytic differentiation of parental and BCR/ABL-expressing 32Dcl3 cells. (**A**) FUS, BCR/ABL and actin expression (Western blotting) in parental and representative clones of transfected 32Dcl3 cells. (**B**) Kinetics of FUS, PKCβII and G-CSFR expression in parental and representative (of three for each transfectant) derivative lines cultured in the presence of G-CSF. G-CSFR was detected using a specific anti-murine G-CSFR antibody (Santa Cruz). (**C**) G-CSF-induced differentiation of parental and representative (of three for each transfectant) 32Dcl3-derived cell lines. Representative microphotographs of May-Grunwald/Giemsa-stained cytospins. (**D**) EMSA performed with ^{32}P-labeled ssODN ZnSb, mG-CSFR (nucleotides 2630–2658) ssODN, or mG-CSFR RNA oligonucleotide on extracts from IL-3-starved parental (lanes 3, 7, and 10) and BCR/ABL-expressing 32Dcl3 cells (lanes 4–6, 8, 9 and 11–16). Specificity of the binding was determined using an anti-FUS serum (lanes 7, 8, 12, 15 and 16), or an unrelated polyclonal rabbit antiserum (lanes 9, 13 and 14). Binding reactions were also performed in the presence of a 100-fold molar excess of ZnSb, ZnSa or mGCSFR ssODN used as competitor (lanes 5, 6 and 15, respectively).

32Dcl3 cells expressing wild-type BCR/ABL, PKCβII was detected in anti-FUS immunoprecipitates (Figure 5C, lane 2), raising the possibility that FUS is a direct target of PKCβII. Indeed, the C-terminus (but not the N-terminus) of FUS was phosphorylated by PKCβII in *in vitro* kinase assays with an immunoprecipitated PKCβII (Figure 5D, lanes 5 and 6).

Role of FUS in in vitro differentiation of normal and BCR/ABL-expressing hematopoietic cells

To assess the consequences of altered FUS expression for differentiation and proliferation of 32Dcl3 cells, we generated 32Dcl3-derived cell lines ectopically expressing the full-length FUS cDNA (32DLFS), a FUS construct in the antisense orientation (32DLFAS), or both wild-type BCR/ABL and the FUS antisense construct (32DLFASBA) (Figure 6A).

FUS levels were rapidly downmodulated upon granulocyte-colony stimulating factor (G-CSF)-induced granulocytic differentiation of parental and 32DLFS cells (Figure 6B, lanes 1 and 3); instead, the expression of endogenous FUS was not altered by G-CSF-treatment of BCR/ABL-expressing 32Dcl3 cells (Figure 6B, lane 2). 32DLFSBA cells showed low levels of FUS expression (probably reflecting the inability of the antisense FUS construct to completely suppress the enhanced expression of FUS induced by wild-type BCR/ABL) that remained constant for the first 3 days of G-CSF treatment and decreased slightly thereafter (Figure 6B, lane 5). As expected, FUS was undetectable in 32DLFAS cells (Figure 6B, lane 4). Like FUS, PKCβII levels decreased after G-CSF treatment of parental and 32DLFS cells whereas they were essentially unaffected in 32Dp210$^{BCR/ABL}$ cells (Figure 6B, lane 2). In 32DLFASBA cells, PKCβII expression was still detectable on the third day of culture in G-CSF-containing medium, but was below the detection level on the fifth day of culture (Figure 6B, lane 5), probably reflecting partial differentiation (Figure 6C) of 32Dcl3 cells expressing both wild-type BCR/ABL and antisense FUS transcripts.

After 3 days of G-CSF-stimulation, 32DLFAS cell cultures revealed evident features of differentiation, as demonstrated by the detection of polymorphonuclear cells (Figure 6C, fourth row). Cytoplasmic shrinkage, nuclear condensation and presence of apoptotic bodies were the predominant features of 32DLFS cells cultured with G-CSF (Figure 6C, third row); surviving 32DLFS cells retained the capacity to differentiate into mature granulocytes, but the kinetics of this process was slower than in parental 32Dcl3 cell cultures, which showed terminally differentiated cells after 7 days in G-CSF (Figure 6C, first row). Ectopic expression of wild-type BCR/ABL in 32Dcl3 cells prevented G-CSF-induced differentiation (Figure 6C, second row). To determine whether FUS downmodulation

in BCR/ABL-expressing cells restores the capacity of these cells to undergo differentiation in response to G-CSF, 32DLFASBA cells were cultured for 7 days in the presence of G-CSF: an ~35% increase in the number of intermediate and segmented cells and a relative decrease in blast cells was observed (Figure 6C, fifth row), indicating that inhibition of FUS expression induces a more mature phenotype in these cells.

The G-CSF receptor (G-CSFR) is a marker of granulocytic differentiation whose expression is induced upon G-CSF treatment of parental 32Dcl3 cells and is abolished by ABL oncoproteins (Steinman and Tweardy, 1994; Tkatch *et al*., 1995). Anti-murine G-CSFR immunoblots of lysates from cells growing in the presence of IL-3 showed detectable levels of G-CSFR only in 32DLFAS cells (Figure 6B, lane 4); upon removal of IL-3 and addition of G-CSF, G-CSFR expression was also induced in parental and, with slower kinetics, in 32DLF cells (Figure 6B, lanes 1 and 3). Expression of the G-CSFR was undetectable in 32Dp210$^{BCR/ABL}$ cells (Figure 6B, lane 2), but it became detectable in 32DLFASBA cells cultured for 3–7 days in the presence of G-CSF (Figure 6B, lane 5). These data and similar results from additional experiments assessing G-CSFR mRNA levels in 32Dcl3-derived cell lines (not shown) are consistent with the possibility that FUS is a negative regulator of G-CSFR expression. Interestingly, a region highly homologous to ssZnSb was found in the murine G-CSFR cDNA sequence. To determine whether FUS binds this region, EMSAs were performed with whole-cell extracts from IL-3-starved parental or BCR/ABL-expressing cells and a ^{32}P-labeled ssDNA or RNA corresponding to nucleotides 2630–2658 of the murine G-CSFR cDNA (Fukunaga *et al*., 1990). As a control, EMSAs were performed with the ssODN ZnSb. A complex comigrating with C2 was detected using 32Dp210$^{BCR/ABL}$ cell extracts (Figure 6D, lanes 4 and 11), but not those from parental cells (Figure 6D, lanes 3 and 10). Binding was inhibited by a 100-fold molar excess of ssODN ZnSb (Figure 6D, lane 5) but not by the same amount of ssODN ZnSa (Figure 6D, lane 6), and supershifted by a polyclonal FUS antiserum (Figure 6D, lanes 8, 12 and 16), but not by an unrelated polyclonal antiserum (Figure 6D, lanes 9, 13 and 14). Detection of the ZnSb–FUS–FUSAb supershifted complex was abolished by a 100-fold excess of cold mGCSFR ssODN (Figure 6D, lane 15).

FUS requirement for growth factor-independent proliferation and tumorigenesis of BCR/ABL-expressing cells

In IL-3-containing medium, proliferation of 32DLFS cells was slightly faster than that of parental 32Dcl3 cells (~20% increase in cell numbers at days 3 and 6), while 32DLFAS cells exhibited a moderate decrease in proliferation (not shown). Compared with 32Dp210$^{BCR/ABL}$ cells, 32DLFASBA cells showed a modest, but reproducible, decrease in their proliferation rate (not shown).

After 16 h in IL-3-deprived culture, a higher frequency of dead cells was observed in 32DLFAS than in 32DLFS cells (~70 versus ~40%) (not shown). As expected, 32Dp210$^{BCR/ABL}$ cells were resistant to apoptosis induced by growth factor deprivation and suppressing FUS expression only marginally enhanced their susceptibility to apoptosis (not shown).

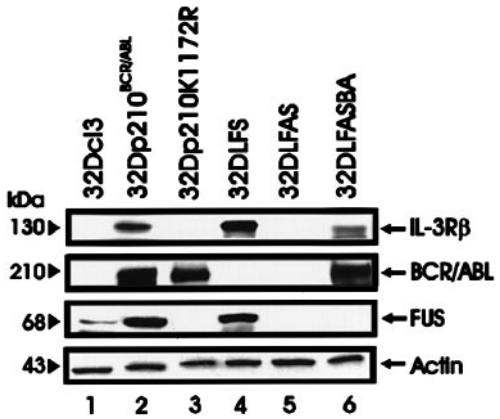

Fig. 7. Expression of the murine IL-3Rβ in parental and transfected 32Dcl3 cells. Western blots were performed using (top to bottom) anti-IL-3R β chain (T-20, Santa Cruz), anti-ABL, anti-FUS and anti-actin antibodies.

Table I. Role of FUS on colony-formation of parental and BCR/ABL-expressing 32Dcl3 cells

IL-3 (% v/v)[a]	0	0.1	1	15
32Dcl3	0	102 ± 10[b]	337 ± 24	526 ± 18
32Dp210$^{BCR/ABL}$	799 ± 11	792 ± 40	819 ± 22	797 ± 23
32DLFS	0	171 ± 20	433 ± 11	707 ± 13
32DLFAS	0	93 ± 12	249 ± 16	494 ± 26
32DLFASBA	27 ± 2	169 ± 7	374 ± 10	461 ± 42

[a]Methylcellulose colony formation, in the absence or in the presence of different concentrations of WEHI-3B conditioned medium used as source of IL-3, from parental and 32Dcl3-derived cell lines (10^3 cells/plate).
[b]Values are mean ± SD of duplicate cultures from two independent experiments.

32Dp210$^{BCR/ABL}$ cells formed a high number of colonies in methylcellulose (~800/10^3 plated cells), both in the absence and in the presence of increasing concentrations of IL-3 (0.1, 1 and 15% of WEHI-3B conditioned medium) (Table I). In contrast, the colony-forming ability of 32DLFASBA cells was diminished significantly (~95% inhibition) in the absence of IL-3 (Table I). This was less evident in the presence of increasing concentrations of IL-3, with only 40% inhibition in cell cultures maintained in 15% WEHI-CM (Table I). Compared with parental 32Dcl3 cells, 32DLFS cells showed enhanced colony-forming ability (~30–70%) in the presence of suboptimal concentrations of IL-3-containing medium, but failed to generate colonies in the absence of IL-3 (Table I). As expected, 32DLFAS did not form colonies in the absence of IL-3 and only few colonies developed when semi-solid cultures were supplemented with 0.1% WEHI-CM; however, the number of colonies increased with higher concentrations of WEHI-CM (Table I).

The reduced clonogenic efficiency of 32DLFASBA cells correlated with decreased expression of IL-3R β-chain (Figure 7); 32Dp210$^{BCR/ABL}$ and 32DLFASBA cells expressed similar levels of p210$^{BCR/ABL}$ protein (Figure 7, lanes 2 and 6), and yet FUS downregulation was associated with decreased levels of IL-3R β-chain (IL-3Rβ) (Figure 7, lane 6). This suggests that a functional BCR/ABL tyrosine

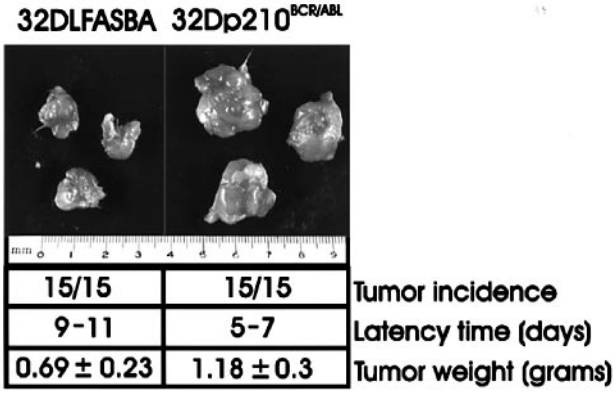

Fig. 8. Requirement for FUS in tumorigenesis of BCR/ABL-expressing 32Dcl3 cells. Subcutaneous tumors in SCID mice injected with 32Dp210 BCR/ABL and 32DLFASBA cells. Latency time (day), and tumor weight (mean ± SD) were calculated. Significance, $P < 0.011$. Representative of two independent experiments.

kinase (Figure 7, compare lanes 2 and 3), regulates IL-3Rβ expression, at least in part, via FUS. Indeed, IL-3Rβ expression was clearly enhanced in 32DLFS cells (Figure 7, lane 4), but detectable at low levels and after long exposure in parental and 32DLFAS cells (not shown).

To assess whether FUS plays a role in BCR/ABL-dependent tumorigenesis, SCID mice (15 per group) were injected subcutaneously with 32Dcl3 cells expressing both BCR/ABL and antisense FUS transcripts (32DLFASBA), with cells expressing wild-type $p210^{BCR/ABL}$ only (32D $p210^{BCR/ABL}$), with cells transfected with the FUS antisense construct (32DLFAS), or with the FUS full-length cDNA (32DLFS) and monitored for the presence of palpable tumors at the injection site. While 32DLFS and 32DLFAS cells did not form tumors, $32Dp210^{BCR/ABL}$ and 32DLFASBA cells formed tumors after 5–7 and 9–11 days, respectively (Figure 8). Tumors were excised for macroscopic and histopathologic analysis on day 20 after cell injection. On average, the weight of the tumors formed in mice injected with BCR/ABL-expressing 32Dcl3 cells was twice that of those from cells expressing BCR/ABL and the FUS antisense construct (Figure 8). Histopathological analysis revealed no major differences, except for higher frequency of cells with signs of advanced granulocytic maturation in tumors from mice injected with 32DLFASBA cells (not shown).

Discussion

Upon transformation by BCR/ABL, hematopoietic cells exhibit growth factor-independent proliferation and survival, altered responses to differentiative stimuli, and enhanced motility and trafficking. However, the regulatory mechanisms underlying such phenotypes are only partially understood, especially in the case of nuclear regulators of BCR/ABL activity. For example, functional c-Myc and c-Jun appear to be required in BCR/ABL-dependent transformation (Sawyers *et al*., 1992; Raitano *et al*., 1995), but the mechanisms of their activation or their role in BCR/ABL leukemogenesis are still unclear. Increasing evidence indicates that zinc finger proteins are involved in the control of normal hematopoiesis, and that the structure, expression and function of several of them is altered in patients with hematopoietic malignancies, often as a result of genetic alterations (Tenen *et al*., 1997).

In EMSA and Southwestern analyses using a double-stranded oligonucleotide containing a zinc finger consensus sequence (dsODN ZnSab) identified as the binding motif for fingers 1–4 of the MZF-1 transcription factor, we detected two complexes, C1 and C2, whose formation was regulated by a functional BCR/ABL tyrosine kinase. MZF-1 interacts with DNA through two different zinc finger domains (fingers 1–4 and 5–13) recognizing two specific DNA consensus sequences (Morris *et al*., 1994). The presence of MZF-1 in the C1 or C2 complex was excluded because the consensus binding site for the 5–13 zinc fingers did not form a complex comigrating with C1 or C2 (not shown). Moreover, neither the C1 nor the C2 complex was altered by an anti-MZF-1 antibody (not shown). Sequence analysis of the DNA-affinity-purified proteins identified them as hnRNP-C1/C2 and FUS, which are associated in intact cells, and bind mRNA and ssDNA (Görlach *et al*., 1994; Prasad *et al*., 1994; Zinszner *et al*., 1997). FUS, the only protein apparently present in the C2 complex, bound DNA (ss and ds) and RNA in a sequence-specific manner. Such dual function is not unprecedented, as the sn-RNP associated protein 69kD which is structurally related and highly homologous to FUS also binds both RNA and double-stranded DNA (Hackl and Luhrmann, 1996).

In its C-terminal region, FUS contains a conserved C2-C2 finger motif similar to that found in other RNA binding proteins (Hackl and Luhrmann, 1996). FUS-binding activity was abrogated by the presence of EDTA in the zinc ion-containing binding buffer (not shown), consistent with the importance of this C2-C2 finger for the function of the protein. However, integrity of the zinc finger consensus sequence in the dsODN ZnSab was not essential for FUS DNA binding, as indicated by EMSA performed with the dsODN ZnSab mutated in the zinc finger consensus sequence, used as probe or as competitor (Figure 2B). Thus, the C2-C2 finger might maintain FUS in a conformation required for entry in the RNA pol II transcription complex where, as a specific TAF_{II} element, it might function to determine promoter selectivity (Zinszner *et al*., 1994; Bertolotti *et al*., 1996). An analogous zinc-dependent mechanism has been reported for the transcription factor IIE (TFIIE) (Maxon and Tjian, 1994). On the other hand, the sequence-specific interaction of FUS with ssDNA and RNA, and its reported capacity to shuttle from the nucleus to the cytoplasm in large complexes with pre-mRNA or mRNA molecules (Zinszner *et al*., 1997b) are also consistent with a role for FUS as a post-transcriptional regulator of gene expression.

A functional BCR/ABL tyrosine kinase is essential for FUS expression and binding activity

In Ph^1 cell lines and in marrow cells from CML patients, expression and binding activity of FUS correlated with BCR/ABL levels, suggesting that FUS functions as a BCR/ABL-downstream effector. Previous studies have shown that BCR/ABL exerts its leukemogenic potential, in part, by circumventing the cytokine-dependent regulation of proliferation and survival (Matulonis *et al*., 1993). Indeed, expression and DNA-binding activity of FUS were not affected by growth factor deprivation in BCR/ABL-

expressing 32Dcl3, whereas the presence of IL-3 in the culture medium was essential for expression and binding activity of FUS in parental 32Dcl3 cells. Thus, BCR/ABL-dependent signals might prevent the activity of specific proteases and/or induce post-translational modifications of FUS that maintain the protein in a stable and active state. Degradation of FUS in the absence of IL-3 was prevented by the trypsin-like serine-proteases inhibitor TLCK (not shown), a finding reminiscent of the TLCK-dependent rescue of the hnRNP C1/C2 proteins specific cleavage that occurs upon induction of apoptosis (Waterhouse *et al*., 1996).

The BCR/ABL tyrosine kinase activity is required to maintain elevated levels of functional FUS, since kinase-deficient BCR/ABL (K1172R), but not other BCR/ABL mutants retaining tyrosine kinase activity, failed to induce FUS expression and binding activity. Moreover, the C2 complex did not form using cell lysates treated with alkaline phosphatase (Figure 4), an observation consistent with the notion that FUS binding activity not only depends on its expression but also requires BCR/ABL-induced phosphorylation. Phosphorylation-dependent binding activity has been demonstrated for several transcriptional and post-transcriptional regulators of gene expression (Karin and Hunter, 1995; Fung *et al*., 1997); in particular, the RNA binding activity of different hnRNP proteins is regulated by cell-cycle-dependent phosphorylation and dephosphorylation (Mayrand *et al*., 1993; Pinol-Roma and Dreyfuss, 1993).

BCR/ABL-dependent induction of PKCβII serine-threonine kinase is essential for FUS-binding activity

In BCR/ABL-expressing 32Dcl3 cells, FUS binding activity was suppressed by the specific PKC inhibitor calphostin C and the serine-threonine kinase inhibitor staurosporine, suggesting that BCR/ABL-dependent activation of PKC is necessary for FUS function. The serine/threonine kinase PKC family includes at least 12 isoforms differing in their structure, tissue distribution, subcellular localization, mode of activation and substrate specificity (Dekker and Parker, 1994). Activation of PLC-γ by $p210^{BCR/ABL}$ (Gotoh *et al*., 1994) is expected to enhance the levels of DAG and inositol triphosphate with subsequent increase in intracellular $[Ca^{2+}]$ and activation of conventional (α, βI, βII and γ) PKC isoforms. Thus, inhibition of an upstream regulator of conventional PKC isoforms using the PLC-γ inhibitor U73122 would be expected to lead to a marked decrease in FUS binding activity, as we have shown here (Figure 5). Moreover, treatment of BCR/ABL-expressing cells with the PKCβII inhibitor CGP53353 or infection of these cells with a retrovirus carrying a PKCβII dominant-negative mutant resulted in a decrease of FUS DNA-binding activity (Figure 5). Consistent with an important role of PKCβII in FUS activation, wild-type BCR/ABL, but not a kinase-deficient mutant, induced a marked increase in the expression and activity of the nuclear PKCβII isoform. Moreover, FUS was detected in complex with PKCβII, and the BCR/ABL-activated PKCβII serine-threonine kinase specifically phosphorylated the C-terminal, but not the N-terminal region of FUS (Figure 5). Notably, expression of the oncogenic v-ABL in the IL-3-dependent IC.DP cell line induced activation and nuclear translocation of PKCβII (Evans *et al*., 1995).

FUS activation by BCR/ABL might depend on multisite FUS phosphorylation. Accordingly, signals activated upon IL-3R triggering or constitutively induced by BCR/ABL may lead, sequentially, to PLC-γ and PKCβII activation. After BCR/ABL-dependent activation, PKCβII translocates into the nucleus where it associates with phosphorylates, and induces the binding activity of FUS. Phosphorylated FUS might be intrinsically less susceptible to degradation by trypsin-like serine proteases. Alternatively, BCR/ABL-generated signals might inhibit the activity of these proteases, thus preventing FUS proteolysis.

FUS regulates in vitro differentiation of normal and BCR/ABL-expressing cells

The ability of BCR/ABL to maintain expression and activity of FUS contrasts with the rapid downregulation of FUS expression and activity observed upon culture of parental 32Dcl3 cells in the absence of IL-3 or in G-CSF-supplemented medium. Thus, FUS might function as one of the nuclear effectors of BCR/ABL to generate proliferative and/or survival signals. Upon G-CSF treatment, the majority of 32Dcl3 cells ectopically expressing FUS showed morphological features of apoptosis, while the surviving cells differentiated into mature granulocytes with kinetics slower than that in parental cells. The apoptosis-promoting effect of ectopic FUS expression in parental 32Dcl3 cells might be the consequence of the conflict in growth signals generated by inappropriate expression of FUS under conditions that normally promote growth arrest and differentiation. This interpretation is consistent with the observation that 32Dcl3 cells overexpressing the transcription factors c-Myc, Evi-1 or CHOP, or the G_1 cyclins D2 and D3 were unable to differentiate and died in G-CSF (Askew *et al*., 1991; Morishita *et al*., 1992; Kato and Sherr, 1993; Friedman, 1996). In parental 32Dcl3 cells, suppression of FUS expression resulted in accelerated differentiation. Similarly, FUS downregulation in BCR/ABL-expressing cells, which exhibited high levels of FUS and remained undifferentiated during the course of G-CSF treatment, restored, at least in part, their ability to differentiate in response to G-CSF. FUS might regulate granulocytic differentiation of normal and BCR/ABL-expressing 32Dcl3 cells by affecting G-CSFR expression. G-CSFR mRNA and protein expression was upregulated in cells in which FUS levels were suppressed. In contrast, G-CSFR levels remained undetectable in G-CSF-treated BCR/ABL-expressing cells. This is consistent with the observation of Steinman and Tweardy (1994) that expression of v-ABL in 32Dcl3 cells suppressed G-CSFR mRNA expression. Interestingly, expression of FUS antisense transcripts in BCR/ABL-expressing cells partially restored the ability of these cells to express G-CSFR in response to G-CSF-stimulation. The enhanced G-CSFR expression in cells transfected with the FUS antisense construct, which correlated with the early detection of differentiated cells in G-CSF-treated cultures, suggests that FUS may affect hematopoietic differentiation by blocking the expression of the G-CSFR receptor. In support of this hypothesis, expression of G-CSFR mRNA was not detected in 32Dcl3 cells ectopically expressing FUS, and

the G-CSF-induced increase in G-CSFR mRNA expression was markedly lower than that in parental 32Dcl3 cells (not shown). Since FUS binds *in vitro* to a sequence (nucleotides 2630–2658) contained in the murine G-CSFR mRNA, it is possible that FUS also binds to G-CSFR mRNA in intact cells and induces its rapid degradation, one possible mechanism whereby BCR/ABL suppresses myeloid cell differentiation.

FUS activation is required in vivo and in vitro for BCR/ABL oncogenic potential

Ectopic FUS expression slightly enhanced proliferation of 32Dcl3 cells in the presence of IL-3 and reduced susceptibility to apoptosis upon IL-3 deprivation. In contrast, FUS downregulation was associated with reduced proliferation in the presence of IL-3 and with accelerated kinetics of cell death upon IL-3 withdrawal. The role of FUS as a potential transducer of anti-apoptotic signals has been previously postulated. Indeed, 3T3 fibroblasts ectopically expressing the oncogenic EWS–Fli-1 or FUS–ERG proteins were resistant to apoptosis induced by the calcium ionophore A23187, while apoptosis protection by the N-terminal domain of the fusion proteins EWS or FUS was only partial (Yi *et al*., 1997). Downregulation of FUS expression did not significantly enhance the susceptibility of BCR/ABL-expressing cells to apoptosis induced by IL-3 deprivation, whereas their factor-independent colony formation was drastically reduced (Table I). This is consistent with the inability of wortmannin, an inhibitor of PI-3K-dependent survival signals in many cell types including BCR/ABL-expressing marrow cells (Skorski *et al*., 1997), to suppress BCR/ABL-dependent FUS activation (Figure 5). The reduced colony-forming ability of 32Dcl3 cells cotransfected with wild type BCR/ABL and the FUS antisense construct was evident in the absence of IL-3, but was restored by increasing concentrations of the growth factor (Table I). Consistent with these observations, expression of IL-3Rβ was readily detected in cells overexpressing BCR/ABL or the exogenous FUS, whereas it was diminished in cells expressing both BCR/ABL and the antisense FUS construct (Figure 7). Thus, a positive regulatory loop, whose mechanisms are presently unclear, links the expression of FUS with that of the IL-3R. Downregulation of FUS expression also impaired the tumorigenic potential of BCR/ABL-expressing cells in mice (Figure 8), further suggesting the importance of FUS in transducing mitogenic signals required for the oncogenic potential of BCR/ABL.

In conclusion, we have provided evidence for a novel role of FUS as a regulator of normal hematopoiesis and BCR/ABL-mediated leukemogenesis. FUS regulation of hematopoiesis is probably dependent on its RNA–DNA binding function which, via post-transcriptional and transcriptional regulatory mechanisms, may induce opposite effects on the expression of cytokine receptors that control hematopoietic proliferation and differentiation.

Materials and methods

Cell cultures and primary cells

The murine IL-3-dependent 32Dcl3 myeloid precursor and its derivative cell lines were maintained in culture or induced to differentiate as previously described (Bellon *et al*., 1997). Morphologic differentiation was monitored by May-Grunwald/Giemsa staining of cytospin preparations. For assays requiring cell starvation, cells were washed three times in phosphate-buffered saline (PBS) and incubated for 8–9 h in IMDM supplemented with 10% FBS or 0.1% bovine serum albumin (BSA) and 2 mM L-glutamine, as indicated. The murine IL-3-dependent DAGM myeloid cell line and a BCR/ABL-expressing DAGM-derived cell line (kind gift of Dr C.Sawyers, UCLA, Los Angeles, CA), were maintained in IMDM supplemented with WEHI-conditioned medium as a source of natural IL-3.

Hematopoietic progenitor cells from bone marrow of a normal donor and from patients with CML in chronic phase (CML-CP) or blast crisis (CML-BC) were obtained as described (Skorski *et al*., 1995). The Philadelphia-positive cell lines BV173 and K562 were kept in culture at 37°C in IMDM supplemented with 10% FBS and 2 mM L-glutamine.

Transfection and retroviral infection of 32Dcl3 cells and 32Dcl3-derived cell lines

32Dcl3-derived cell lines were generated by electroporation (GenPulser, Bio-Rad: 200 mV/960 μF) of the following retroviral constructs: LXSN/FUS-S (32DLFS), LXSN/FUS-AS (32DLFAS), pSRαWT-$p210^{BCR/ABL}$ ($32Dp210^{BCR/ABL}$) and pSRαK1172R $p210^{BCR/ABL}$ (32Dp210K1172R). 32DLFAS cells were subsequently transfected with pSRαWT$p210^{BCR/ABL}$ and the LXSP vector used for selection in puromycin-containing medium (32DLFASBA). Mixed populations and single cell clones, obtained after G418 (1 mg/ml) and/or puromycin (2 μg/ml) selection, were maintained in culture as described (Bellon *et al*., 1997). 32Dcl3 cells transfected with empty vectors (LXSN and pSRαMSV-tkneo) were morphologically identical to the parental cells. Methylcellulose colony formation assays of parental and 32Dcl3-derived lines were carried out as described (Bellon *et al*., 1997). Retroviral infection of parental and BCR/ABL-expressing 32Dcl3 cells were carried out as described (Pear *et al*., 1993). After 72 h, lysates were prepared from IL-3-starved (8 h) cells, as described (Perrotti *et al*., 1996).

Plasmids

LXSN-FUS-S and LXSN-FUS-AS. A *Bgl*II–*Sac*II fragment of the FUS cDNA [nucleotides +71 to 717 of the published sequence (Rabbitts *et al*., 1993)], obtained by digestion of plasmid pSG5FUS (kind gift from Dr S.Reddy, Allegheny University, Philadelphia, PA) was ligated in-frame to a *Sac*II–*Eco*RI (nucleotides 717–1141) and an *Eco*RI (nucleotides 1141–1822) fragment obtained by RT–PCR amplification of total RNA from the human KG-1 myeloblastic cell line, followed by cloning the amplified segment into the pCRII vector (Invitrogen Corp., San Diego, CA). These three segments were subcloned in the *Bam*HI–*Eco*RI-digested pBlueScript SK^+. The resulting pBSFUS (1–1822) was digested with *Spe*I and *Hin*dIII, Klenow-blunted and subcloned in the sense or antisense orientation into the blunted *Eco*RI site of the LXSN retroviral vector.

pGEX-FUS (1–240). A FUS cDNA fragment encoding the N-terminus of FUS (amino acids 1–240) was generated by PCR performed on the pBSFUS plasmid using an upstream primer containing a *Bam*HI restriction site at the 5′ end and a downstream primer carrying a stop codon at the 3′ end. The gel-purified fragment was phosphorylated, digested with *Bam*HI and directionally subcloned into the *Bam*HI–*Eco*RI-blunted sites of the pGEX-2TK vector (Pharmacia Biotech).

pGEX-FUS (240–526). A FUS cDNA fragment encoding the C-terminus of FUS (amino acids 240–526) was generated by PCR performed on pBSFUS using an upstream primer containing an ATG codon flanked at the 5′ end by a *Bam*HI restriction site and a downstream oligodeoxynucleotide primer overlapping the FUS stop codon. The gel-purified fragment was phosphorylated, *Bam*HI-digested and directionally subcloned into the *Bam*HI–*Eco*RI-blunted sites of pGEX-2TK vector (Pharmacia Biotech).

LXSN PKCβII (DN). The M217 dominant-negative form of PKCβII (Chalfant *et al*., 1996) was subcloned in the sense orientation into the *Xho*I site of the LXSN retroviral vector.

The plasmid pSRαMSVtkneo-$p210^{BCR/ABL}$ has been described previously (Skorski *et al*., 1997). The $p210^{BCR/ABL}$ kinase-deficient (K1172R) mutant was obtained from Dr C.Sawyers (UCLA, Los Angeles, CA). The LXSP retroviral vector containing the puromycin resistance gene was a kind gift from Dr A.Sacchi (Regina Elena Cancer Institute, Rome, Italy).

Enzyme inhibitors

Where indicated, cells were IL-3 starved in the presence of kinase- or phosphatase-inhibitors used at the following concentration: rapamycin, 15 nM (Calbiochem, San Diego, CA); calphostin C 200 ng/ml (Calbiochem); PD098059 50 μM, (Calbiochem); wortmannin 50 nM; staurosporine 1 μM, (Sigma Chemical Co., St Louis, MO); okadaic acid, 50 nM (Calbiochem); U73122, 1 μM (Biomol); U73343, 1 μM (Biomol); CGP53353, 1 μM (Novartis).

Electrophoretic mobility shift assay

Whole-cell extracts from parental and 32Dcl3-derived cell lines were prepared and used in EMSAs as previously described (Perrotti *et al*., 1996), except for some modifications in the binding buffer [25 mM HEPES–KOH pH 7.5, 50 mM KCl, 10 μM $ZnSO_4$, 10% glycerol, 0.1% Nonidet P-40 (NP-40), 1 mM phenylmethylsulfonylfluoride (PMSF), 25 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml pepstatin A, 5 mM benzamidine, 1 mM Na_3VO_4, 50 mM NaF and 10 mM β-glycerolphosphate]. When indicated, EMSAs were performed in the presence of a 100-fold molar excess of ds- or ssODNs used as specific or non-specific competitors. Either 1 μl of anti-FUS rabbit polyclonal antiserum raised against the N-terminus FUS (Zinszner *et al*., 1994) or of irrelevant rabbit polyclonal antiserum were used to determine binding specificity. When indicated, extracts were pre-incubated (4°C, 20 min) with 2U of AP (Boehringer Mannheim) in the absence or in the presence of phosphatase inhibitors (10 mM Na_3VO_4, 250 mM NaF and 100 mM β-glycerolphosphate).

The following ds- and ssODNs were used in EMSAs either as probes or as competitors: dsODN ZnSab (5′-ttttctccccactttagatc-3′) containing a canonical MZF-1 consensus sequence (Perrotti *et al*., 1995); dsODNs ZnMut1 (5′-tttccacgtcattttgatc-3′), dsODN ZnMut2 (5′-ttttctccccgctcgactgcg-3′) and dsODN ZnMut3 (5′-tccgatccccgcttttagatc-3′) containing mutations (underlined bases) in the zinc finger consensus sequence (ZnMut1) or in the nucleotides flanking it (ZnMut2 and ZnMut3); ssODNs ZnSa (5′-gatctaaaagtggggagaaaa-3′) and ZnSb (5′-ttttctccccacttttagatc-3′) representing the ZnSab strands used separately in EMSA; the ssODN mG-CSFR (5′-atttgattttcccctctttcaggggctcc-3′), and the RNA oligonucleotide (5′-auuugauuuuccccucuuucaggggcucc-3′), both corresponding to nucleotides 2630–2658 of the murine G-CSFR cDNA sequence (Fukunaga *et al*., 1990); the 35mer dsODN (5′-ggggtcccccttactggactcaggttgccccctg-3′) was used as non-specific competitor.

UV cross-linking and Southwestern blotting

End-labeled ODN ZnSab and cell lysates from parental and BCR/ABL-expressing 32Dcl3 cells were used in UV cross-linking and Southwestern experiments as previously described (Perrotti *et al*. 1996).

Protein purification and peptide microsequencing

A three-step purification procedure (Teale *et al*., 1992) was used to characterize the proteins forming a complex with dsODN ZnSab. Lysates (in 20 mM HEPES pH 7.9, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 10 μM $ZnSO_4$, 10% glycerol and 1 mM PMSF) from $32Dp210^{BCR/ABL}$ cells (2.5×10^9) growing in IL-3-containing medium were size-fractioned using a Sephacryl S-200 column (Pharmacia Biotech) and the fractions retaining the ability to bind dsODN ZnSab in EMSA were applied to a HiTrap heparin column (Pharmacia Biotech) pre-equilibrated in the same buffer. The flow-through, but not the eluted fractions, contained the proteins (p40 and p55) which interact with dsODN ZnSab. p40 and p55 were purified by DNA-affinity chromatography as described (Kadonaga and Tjian, 1986). Fractions interacting with dsODN ZnSab in EMSA were subjected to a second round of DNA affinity purification after which an ~100-fold enrichment in DNA-binding activity was obtained (25 ng of the eluted fraction E2.3 showed the same binding activity observed with 30 μg of total lysate). Purified p40 (~1.4 μg) and p55 (~2 μg) proteins were enzymatically digested, and the resulting peptides were analyzed and sequenced by mass-spectrometry (performed at the Harvard Microchemistry Facility, Harvard University, Cambridge, MA). Computer analyses of the derived peptide sequences identified p55 and p40 as TLS/FUS and hnRNP C1/C2 protein, respectively.

Immunoprecipitation and Western blot analyses

Cells (10^7) were harvested, washed twice with ice-cold PBS and lysed in 100 μl HEPES buffer [10 mM HEPES pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM DDT] containing 0.5% (v/v) Nonidet (NP-40) in the presence of the protease inhibitors indicated above. Lysates were processed as described (Bellon *et al*., 1997). Immunoprecipitated proteins were obtained and processed as described (Skorski *et al*., 1997).

Recombinant protein purification

BL-21 (DH3) cells were transformed with plasmid pGEX-2TK (Pharmacia Biotech), or with pGEXFUS 1–240, or pGEXFUS 240–526 encoding glutathione *S*-transferase (GST) fused in-frame with the N-terminus (amino acids 1–240) or the C-terminus (amino acids 240–526) of FUS. Purified proteins were obtained according to the manufacturer's protocol (Pharmacia Biotech).

Kinase assays

Lysates were prepared from serum- and growth factor-starved (8 h) parental and BCR/ABL-expressing 32Dcl3 cells as described above.

PKCβII serine-threonine kinase activity was assayed in anti-PKCβII immunoprecipitates using an *in vitro* PKC kinase assay kit (Upstate Biotechnology, Inc.), and 5 μg of histone H1, or 1 μg of GST–FUS (1–240) or GST–FUS (240–526) as substrate. Briefly, immune complexes containing PKCβII were resuspended in 30 μl kinase buffer [50 mM Tris pH 7.5, 5 mM magnesium acetate, 200 μM $CaCl_2$, 50 μM ATP, 2 μCi [γ-^{32}P]ATP, 25 μg/ml phosphatidyl serine (Avanti Polar Lipids) and 1 μM 12-0-tetradecanoylphorbol-13-acetate (TPA)]. Phosphorylation reaction mixtures were incubated at 30°C for 20 min.

CKII enzymatic activity was examined using an *in vitro* kinase assay kit according to the manufacturer's protocol (Upstate Biotechnology Inc.).

Tumorigenesis in SCID mice

$32Dp210^{BCR/ABL}$, 32DLFS, 32DLFAS and 32DLFASBA cells (5×10^6 cells/mouse) were injected subcutaneously into 5- to 7-week old ICR SCID outbred mice (Taconic, Germantown, NY). Before injection, cells were washed and resuspended (2.5×10^7 cells/ml) in PBS. Tumor growth was monitored every other day. Mice were sacrificed 20 days post-injection and sections of the excised tumors were fixed in phosphate-buffered formalin.

Acknowledgements

We thank Andrew Engelhard and Bice Perussia for critical reading of the manuscript, David Ron (New York University School of Medicine, New York, NY) for the kind gift of the anti-TLS (FUS) antibody, and Malgorzata Nieborowska-Skorska for establishing the BCR/ABL-expressing cell lines. D.P. is the recipient of a fellowship from the American-Italian Cancer Foundation (New York, NY). This work was supported in part by NIH and ACS grants to B.C.

References

Aman,P. *et al.* (1996) Expression patterns of the human sarcoma-associated genes FUS and EWS and the genomic structure of FUS. *Genomics*, **37**, 1–8.

Askew,D.S., Ashmun,R.A., Simmons,B.C. and Cleveland,J.L. (1991) Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene*, **6**, 1915–1922.

Bellon,T., Perrotti,D. and Calabretta,B. (1997) Granulocytic differentiation of normal hematopoietic precursor cells induced by transcription factor PU.1 correlates with negative regulation of the c-myb promoter. *Blood*, **90**, 1828–1839.

Bertolotti,A., Lutz,Y., Heard,D.J., Chambon,P. and Tora,L. (1996) hTAF (II)68, a novel RNA/ssDNA-binding protein with homology to the pro-oncoproteins FUS and EWS is associated with both TFIID and RNA polymerase II. *EMBO J.*, **15**, 5022–5031.

Calvio,C., Neubauer,G., Mann,M. and Lamond,A.I. (1995) Identification of hnRNP P2 as FUS using electrospray mass spectrometry. *RNA*, **1**, 724–733.

Chalfant,C.E., Ohno,S., Konno,Y., Fisher,A.R., Bisnant,L.D., Watson,J.E. and Cooper,D.R. (1996) A carboxy-terminal deletion mutant of protein kinase CβII inhibits insulin-stimulated 2-deoxyglucose uptake in L6 rat skeletal muscle cells. *Mol. Endocrinol.*, **10**, 1273–1281.

Chen,P., Xie,H. and Wells,A. (1996) Mitogenic signaling from the EGF receptor is attenuated by a phospholipase C-γ/protein kinase C feedback mechanism. *Mol. Biol. Cell*, **7**, 871–881.

Cortez,D., Kadlec,L. and Pendergast,A.M. (1995) Structural and signaling requirements for BCR/ABL-mediated transformation and inhibition of apoptosis. *Mol. Cell. Biol.*, **15**, 5531–5541.

Courey,A.J. and Tjian,R. (1988) Analysis of Sp1 *in vivo* reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell*, **55**, 887–898.

Crozat,A., Aman,P., Mandahl,N. and Ron,D. (1993) Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. *Nature*, **363**, 640–644.

Daley,G.R., Van Etten,R.A. and Baltimore,D. (1990) Induction of chronic myelogenous leukemia in mice by the $p210^{BCR/ABL}$ gene of the Philadelphia chromosome. *Science*, **247**, 824–830.

Dekker,L.V. and Parker,P.J. (1994) Protein kinase C: a question of specificity. *Trends Biol. Sci.*, **19**, 73–77.

Downward,J. (1994) Signal transduction. Regulating S6 kinase. *Nature*, **371**, 378–379.

Dudley,D.T., Pang,L., Decker,S.J., Bridges,A.J. and Saltiel,A. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl Acad. Sci. USA*, **92**, 7686–7689.

Evans,C.A., Lord,J.M., Owen-Lynch,P.J., Johnson,G., Dive,C. and Whetton,A.D. (1995) Suppression of apoptosis by v-ABL protein tyrosine kinase is associated with nuclear translocation and activation of protein kinase C in an interleukin-3-dependent haemopoietic cell line. *J. Cell Sci.*, **108**, 2591–2598.

Friedman,A.D. (1996) GADD153/CHOP, a DNA damage-inducible protein, reduced CAAT/enhancer binding protein activities and increased apoptosis in 32D cl3 myeloid cells. *Cancer Res.*, **56**, 3250–3256.

Fukunaga,R., Ishizaka-Ikeda,E., Seto,Y. and Nagata,S. (1990) Expression cloning of a receptor for murine granulocyte colony-stimulating factor. *Cell*, **61**, 341–350.

Fung,P.A., Labrecque,R. and Pederson,T. (1997) RNA-dependent phosphorylation of a nuclear RNA binding protein. *Proc. Natl Acad. Sci. USA*, **94**, 1064–1068.

Goga,A., McLaughlin,J., Afar,D.E.H., Saffran,D.C. and Witte,O.N. (1995) Alternative signals to RAS for hematopoietic transformation by the BCR/ABL oncogene. *Cell*, **82**, 981–988.

Görlach,M., Burd,C.G. and Dreyfuss,G. (1994) The determinants of RNA-binding specificity of the heterogeneous nuclear ribonucleoprotein C proteins. *J. Biol. Chem.*, **269**, 23074–23078.

Gotoh,A., Miyazawa,K., Onyashiki,K. and Toyama,K. (1994) Potential molecules implicated in downstream signaling pathways of $p185^{BCR/ABL}$ in Ph^{+}ALL involve GTPase-activating protein, phospholipase C-γ1 and phosphatidylinositol 3′-kinase. *Leukemia*, **8**, 115–120.

Hackl,W. and Luhrmann,R. (1996) Molecular cloning and subcellular localisation of the snRNP-associated protein 69KD, a structural homologue of the proto-oncoproteins TLS and EWS with RNA and DNA-binding properties. *J. Mol. Biol.*, **264**, 843–851.

Heisterkamp,N., Jenster,G., ten Hoeve,J., Zovich,D., Pattengale,P.K.X. and Groffen,J. (1990) Acute leukaemia in bcr/abl transgenic mice. *Nature*, **344**, 251–253.

Immanuel,D., Zinszner,H. and Ron,D. (1995) Association of SARFH (sarcoma-associated RNA-binding fly homologue) with regions of chromatin transcribed by RNA polymerase II. *Mol. Cell. Biol.*, **15**, 4562–4571.

Kadonaga,J.T. and Tjian,R. (1986) Affinity purification of sequence-specific DNA binding proteins. *Proc. Natl Acad. Sci. USA*, **83**, 5889–5893.

Karin,M. and Hunter,T. (1995) Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr. Biol.*, **5**, 747–757.

Kato,J.Y. and Sherr,C.J. (1993) Inhibition of granulocyte differentiation by G1 cyclins D2 and D3 but not D1. *Proc. Natl Acad. Sci. USA*, **90**, 11513–11517.

Ladanyi,M. (1995) The emerging molecular genetics of sarcoma translocations. *Diagn. Mol. Pathol.*, **4**, 162–173.

Lugo,T.G., Pendergast,A.M., Muller,A.J. and Witte,O.N. (1990) Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science*, **247**, 1079–1082.

Matulonis,U., Salgia,R., Okuda,K., Druker,B. and Griffin,J.D. (1993) Interleukin-3 and p210 BCR/ABL activate both unique and overlapping pathways of signal transduction in a factor-dependent myeloid cell line. *Exp. Hematol.*, **21**, 1460–1466.

Maxon,M.E. and Tjian,R. (1994) Transcriptional activity of transcription factor IIE is dependent on zinc binding. *Proc. Natl Acad. Sci. USA*, **91**, 9529–9533.

Mayrand,S.H., Dwen,P. and Pederson,T. (1993) Serine/threonine phosphorylation regulates binding of C hnRNP proteins to pre-mRNA. *Proc. Natl Acad. Sci. USA*, **90**, 7764–7768.

McLaughlin,J., Chianese,E. and Witte,O.N. (1987) *In vitro* transformation of immature hematopoietic cells by the P210 BCR/ABL oncogene product of the Philadelphia chromosome. *Proc. Natl Acad. Sci. USA*, **84**, 6558–6562.

Morohoshi,F., Arai,K., Takahashi,E.I., Tanigami,A. and Ohki,M. (1996) Cloning and mapping of a human RBP56 gene encoding a putative RNA binding protein similar to FUS/TLS and EWS proteins. *Genomics*, **38**, 51–57.

Morishita,K., Parganas,E., Matsugi,T. and Ihle,J.N. (1992) Expression of the Evi-1 zinc finger gene in 32Dc13 myeloid cells blocks granulocytic differentiation in response to granulocyte colony-stimulating factor. *Mol. Cell. Biol.*, **12**, 183–189.

Morris,J.F., Hromas,R. and Rausher,T.J.,III (1994) Characterization of the DNA-binding properties of the myeloid zinc finger protein MZF1: two independent DNA-binding domains recognize two DNA consensus sequences with a common G-rich core. *Mol. Cell. Biol.*, **11**, 6166–6176.

Panagopoulos,I., Mandahl,N., Mitelman,F. and Aman,P. (1995) Two distinct FUS breakpoint clusters in myxoid liposarcoma and acute myeloid leukemia with the translocations t (12;16) and t (16; 21). *Oncogene*, **11**, 1133–1137.

Pear,W.S., Nolan,G.P., Scott,M.L. and Baltimore,D. (1993) Production of high-titer helper free retroviruses by transient transfection. *Proc. Natl Acad. Sci. USA*, **90**, 8392–8396.

Perrotti,D., Melotti,P., Skorski,T., Casella,I., Peschle,C. and Calabretta,B. (1995) Overexpression of the zinc finger protein MZF1 inhibits hematopoietic development from embryonic stem cells: correlation with negative regulation of CD34 and c-myb promoter activity. *Mol. Cell. Biol.*, **15**, 6075–6087.

Perrotti,D., Bellon,T., Trotta,R., Martinez,R. and Calabretta,B. (1996) A cell proliferation-dependent multiprotein complex NC-3A positively regulates the CD34 promoter via a TCATTT-containing element. *Blood*, **88**, 3336–3348.

Pinol-Roma,S. and Dreyfuss,G. (1993) Cell cycle-regulated phosphorylation of the pre-mRNA-binding (heterogeneous nuclear ribonucleoprotein) C proteins. *Mol. Cell. Biol.*, **13**, 5762–5770.

Powis,G. *et al.* (1994) Wortmannin, a patent and specific inhibitor of phosphatidylinositol-2-kinase. *Cancer Res.*, **54**, 2419–2423.

Prasad,D.D., Ouchida,M., Lee,L., Rao,V.N. and Reddy,E.S. (1994) FUS fusion domain of FUS-erg chimeric protein resulting from the t (16;21) chromosomal translocation in human myeloid leukemia functions as a transcriptional activation domain. *Oncogene*, **9**, 3717–3729.

Rabbitts,T.H., Forster,A., Larson,R. and Nathan,P. (1993) Fusion of the dominant negative transcription regulator CHOP with a novel gene FUS by translocation t (12;16) in malignant liposarcoma. *Nature Genet.*, **4**, 175–180.

Raitano,A.B., Halpern,J.R., Hambuch,T.M. and Sawyers,C.L. (1995) The Bcr-Abl leukemia oncogene activates Jun kinase and requires Jun for transformation. *Proc. Natl Acad. Sci. USA*, **92**, 11746–11750.

Ron,D. (1997) TLS-CHOP and the role of RNA-binding proteins in oncogenic transformation. *Curr. Top. Microbiol. Immunol.*, **220**, 131–142.

Sawyers,C.L., Callahan,W. and Witte,O.N. (1992) Dominant negative MYC blocks transformation by ABL oncogenes. *Cell*, **70**, 901–910.

Sawyers,C.L., McLaughlin,J. and Witte,O.N. (1995) Genetic requirement of Ras in the transformation of fibroblasts and hematopoietic cells by the BCR/ABL oncogene. *J. Exp. Med.*, **181**, 307–313.

Shimizu,K., Ichikawa,H., Tojo,A., Kaneko,Y., Maseki,N., Hayashi,Y., Ohira,M., Asano,S. and Ohki,M. (1993) An ets-related gene, ERG, is rearranged in human myeloid leukemia with t (16;21) chromosomal translocation. *Proc. Natl Acad. Sci. USA*, **90**, 10280–10284.

Skorski,T., Nieborowska-Skorska,M., Szczylik,C., Kanakaraj,P., Perrotti,D., Zon,G., Gewirtz,A., Perussia,B. and Calabretta,B. (1995) C-RAF-1 serine/threonine kinase is required in BCR/ABL-dependent and normal hematopoiesis. *Cancer Res.*, **55**, 2275–2278.

Skorski,T. *et al.* (1997) Transformation of hematopoietic cells by bcr/abl requires activation of a PI-3k/Akt-dependent pathway. *EMBO J.*, **16**, 6151–6161.

Steinman,R.A. and Tweardy,D.J. (1994) Granulocyte colony-stimulating factor receptor mRNA upregulation is an immediate early marker of myeloid differentiation and exhibits dysfunctional regulation in leukemic cells. *Blood*, **83**, 119–127.

Tamaoki,T. (1991) Use and specificity of staurosporine, UCN-01 and calphostin C as protein kinase inhibitors. *Methods Enzymol.*, **201**, 340–347.

Teale,B., Singh,S., Khanna,K.K., Findik,D. and Lavin,M.F. (1992) Purification and characterization of a DNA-binding protein activated by ionizing radiation. *J. Biol. Chem.*, **267**, 10295–10301.

Tenen,D.G., Hromas,R., Licht,J.D. and Zhang,D.E. (1997) Transcription factors, normal and myeloid development and leukemia. *Blood*, **90**, 489–519.

Tkatch,L.S., Rubin,K.A., Ziegler,S.F. and Tweardy,D.J. (1995) Modulation of human G-CSF receptor mRNA and protein in normal and leukemic myeloid cells by G-CSF and retinoic acid. *J. Leuk. Biol*., **57**, 964–971.

Waterhouse,N. *et al*. (1996) Heteronuclear ribonucleoproteins c1 and c2, components of the spliceosome, are specific targets of interleukin 1-beta-converting enzyme-like proteases in apoptosis. *J. Biol. Chem*., **271**, 29335–29341.

Yi,H., Fujimura,Y., Ouchida,M., Prasad,D.D., Rao,V.N. and Reddy,E.S. (1997) Inhibition of apoptosis by normal and aberrant Fli-1 and ERG proteins involved in human solid tumors and leukemias. *Oncogene*, **14**, 1259–1268.

Zinszner,H., Albalat,R. and Ron,D. (1994) A novel effector domain from the RNA-binding protein TLS or EWS is required for oncogenic transformation by CHOP. *Genes Dev.*, **8**, 2513–2526.

Zinszner,H., Immanuel,D., Yin,Y., Liang,F.X. and Ron,D. (1997a) A topogenic role for the oncogenic N-terminus of TLS: nucleolar localization when transcription is inhibited. *Oncogene*, **14**, 451–461.

Zinszner,H., Sok,J., Immanuel,D., Yin,Y. and Ron,D. (1997b) TLS (FUS) binds RNA *in vivo* and engages in nucleo-cytoplasmic shuttling. *J. Cell Sci*., **110**, 1741–1750.

Received March 30, 1998; revised June 8, 1998; accepted June 12, 1998