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12. The liquid sample was contained in an Al_2O_3 ceramic crucible penetrated by, and cemented to, a lower buffer rod made out of dense, polycrystalline Al_2O_3. The crucible was enclosed in a graphite sleeve that acted as an inductive load for the radio-frequency (rf) induction heater. A ZrO_2 radiation shield provided insulation for the graphite sleeve. The sample T was monitored by an Al_2O_3-sheathed W-Re thermocouple located in the melt. Another protected thermocouple, also located in the melt, was connected to a temperature controller that controlled the furnace power supply. A clear-fused quartz tube was placed around the high-temperature assembly and was sealed at both ends by water-cooled flanges. The movable upper buffer rod was a 20-cm-long by 1.27-cm-diameter single-crystal sapphire rod. In preparation, both ends of both rods were polished to achieve a 1-μm surface finish and flatness and a mutual perpendicularity to the rod axis of ±0.01° (1.2 min). Before each experiment, a trial run was undertaken at room conditions with distilled water (H_2O) or glycerol ($C_3H_8O_3$) to identify misalignment of the buffer rods (asymmetry in the resonance peaks), poor transducer bonding or electronic tuning (low overall transmitted amplitude), and lack of parallelism or flatness of the rod faces (ripples in the decay pattern).
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Inducible Expression and Phosphorylation of Coactivator BOB.1/OBF.1 in T Cells

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BOB.1/OBF.1 is a transcriptional coactivator that is constitutively expressed in B cells and interacts with the Oct1 and Oct2 transcription factors. Upon activation of Jurkat T cells and primary murine thymocytes with phorbol esters and ionomycin, BOB.1/OBF.1 expression and transactivation function were induced. BOB.1/OBF.1 was phosphorylated at Ser^{184} both in vivo and in vitro, and this modification was required for inducible activation. Mutation of Ser^{184} also diminished transactivation function in B cells, suggesting that the activating phosphorylation that is inducible in T cells is constitutively present in B cells. Thus, BOB.1/OBF.1 is a transcriptional coactivator that is critically regulated by posttranslational modifications to mediate cell type–specific gene expression.

The octamer motif is a critical element for constitutive B cell–specific gene regulation (*1*). Its function is dependent on the combinatorial activity of ubiquitous or lymphoid-specific octamer (Oct) transcription factors and B cell–restricted transcriptional coactivators (*2–7*). One such coactivator named BOB.1, OBF.1, or OCA-B (BOB.1/OBF.1) interacts with the POU domains of the Oct1 and Oct2 transcription factors, even in the absence of DNA, and mediates transcriptional activation from octamer-dependent promoters (*5, 6, 8*). Complexes containing BOB.1/OBF.1 and Oct proteins are selective for a subset of octamer motifs (*9*), and this is at least in part due to direct contacts of the NH_2-terminal domain of BOB.1/OBF.1 with the major groove of the DNA (*10*). Mutation of the gene for BOB.1/OBF.1 results in a severe defect in terminal B cell differentiation, strongly reduced responses to both T cell–independent and T cell–dependent antigens, and a lack of germinal-center formation (*11*).

Several genes that show regulated expression in T cells contain functional octamer motifs within their proximal promoter regions (*12–14*). These elements are required for inducible transcription. In some cases, like the proximal interleukin-2 (IL-2) promoter, octamer motifs are in close proximity and functionally cooperate with bind-

ing sites for other inducible transcription factors, such as AP1 (*15*).However, such an association is not observed in all cases. Therefore, we analyzed whether octamer motifs by themselves are able to mediate inducible transcription in T cells. A reporter gene whose expression is driven by multimerized octamer motifs was tested. The reporter was inactive in unstimulated T cells, and its expression was slightly increased upon treatment of cells with either the phorbol ester phorbol 12-myristate 13-acetate (PMA) or ionomycin. Upon stimulation with both agents, strong activation of the reporter construct was observed, which could be largely reverted by inclusion of the immunosuppressive agent cyclosporin A (CsA). Identical results were obtained with different octamer-dependent minimal promoters, and point mutations in the octamer motif abolished this inducible activity (Fig. 1A) (*16*).

T cells contain a single octamer-binding transcription factor, Oct1, which cannot drive activity of these reporters on its own. The cell type–specific activity of octamer elements requires the presence of coactivators in addition to Oct1 or Oct2 in B cells (*2–7*). We therefore assumed that these coactivators might also be important for the inducible activity in T cells. To determine whether coactivator BOB.1/OBF.1 is involved in the inducible activity of T cells, we analyzed its expression as a consequence of T cell activation. Untreated Jurkat T cells did not express detectable amounts of BOB.1/OBF.1 RNA or protein. However, costimulation of T cells with both PMA and ionomycin resulted in the increased production of BOB.1/OBF.1 transcripts (Fig. 1B) and protein (Fig. 1C). This effect was apparently T cell–specific, as it was not observed in HeLa cells (Fig. 1C) or NIH/3T3 fibroblasts (*17*). Increased production of BOB.1/OBF.1 RNA was detected within 2 hours of stimulation (Fig. 1D) and appeared to depend on protein synthesis because it was sensitive to the protein synthesis inhibitor anisomycin (*17*). Induction of BOB.1/OBF.1 expression not only occurred in the Jurkat T cell line. It was also observed in primary murine thymocytes upon stimulation with PMA and ionomycin (Fig. 1E) and was inhibited by treatment with the immunosuppressive agents CsA (Fig. 1) or FK506 (*17*).

When we attempted to bypass the requirement for costimulation of T cells by cotransfecting the octamer-dependent re-

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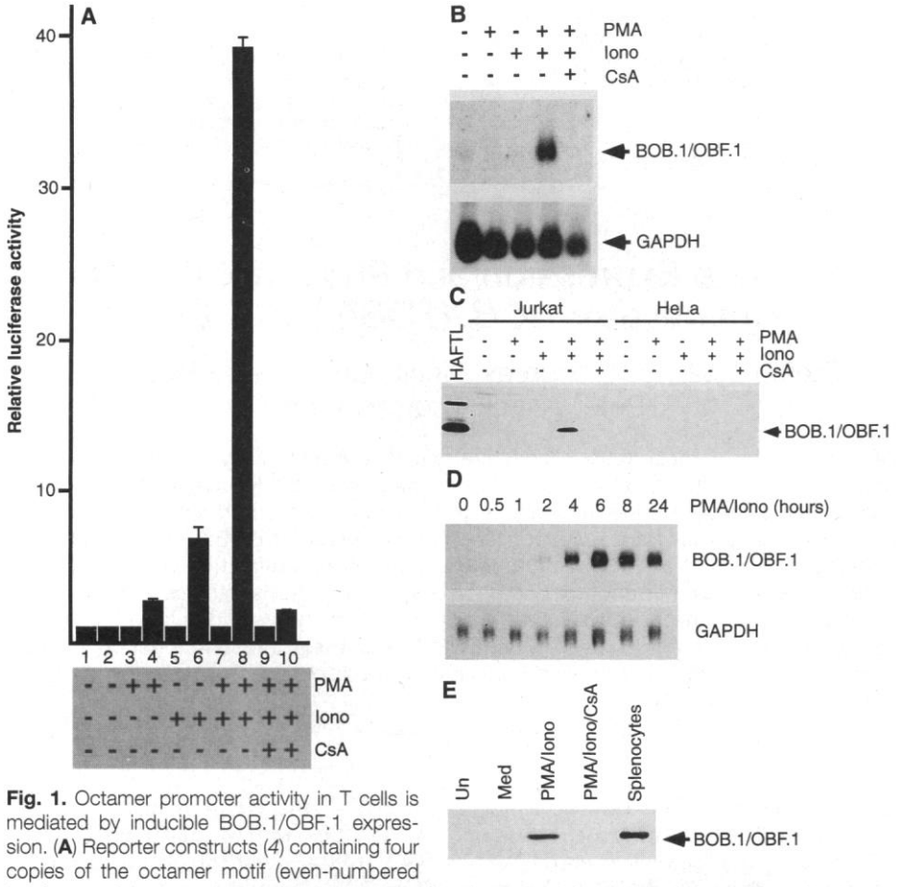


Fig. 1. Octamer promoter activity in T cells is mediated by inducible BOB.1/OBF.1 expression. (**A**) Reporter constructs (*4*) containing four copies of the octamer motif (even-numbered bars) or a point-mutated version (odd-numbered bars) were transfected into Jurkat cells, and luciferase activity was measured in uninduced T cells, and T cells induced with PMA or ionomycin (Iono), or both, as indicated. The activity of the mutant reporter was normalized to 1, standard deviations from a minimum of three independent experiments are included. (**B**) RNA blot analysis of Jurkat cells that were treated as indicated. The probes were specific for BOB.1/OBF.1 and glyceraldehyde phosphate dehydrogenase (GAPDH). (**C**) Protein immunoblot with an affinity-purified BOB.1/OBF.1-specific antibody and extracts from Jurkat cells and HeLa cells that were stimulated as indicated. Extracts from a murine pre–B cell line (HAFTL I) known to express BOB.1/OBF.1 protein was included as a control. (**D**) Time course of BOB.1/OBF.1 RNA induction in Jurkat cells. Cells were costimulated with PMA and ionomycin (PMA/Iono) for the indicated times and RNA was analyzed by RNA blot. The same blot was rehybridized with a GAPDH-specific probe. (**E**) Inducible BOB.1/OBF.1 protein expression in primary murine thymocytes. Protein immunoblots were made of extracts from untreated thymocytes (Un) and thymocytes cultured for 12 hours in medium only (Med), in the presence of PMA and ionomycin, or with PMA, ionomycin, and CsA. Extracts from splenocytes were used as a positive control.

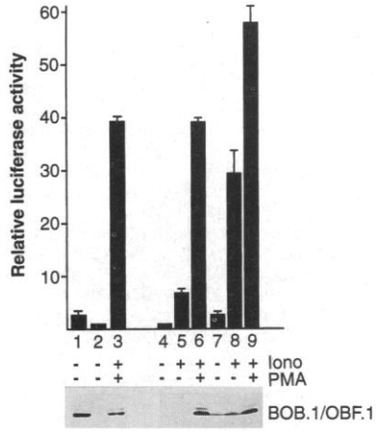
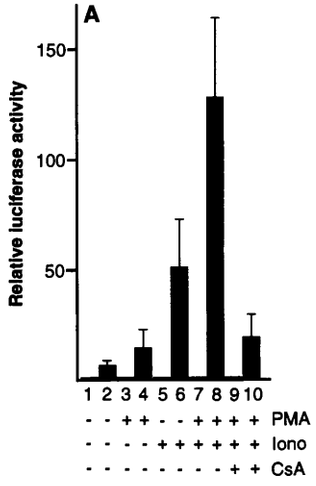


Fig. 2. Lack of transcriptional activation function of ectopically expressed BOB.1/OBF.1 protein in T cells. The left three lanes show activity of an octamer-dependent reporter in uninduced (lane 2) and induced (lane 3) Jurkat cells as well as a stable transfectant of Jurkat cells expressing BOB.1/OBF.1 protein (lane 1). Below is a protein immunoblot of the indicated cells with the use of an antibody specific for BOB.1/OBF.1. On the right, activity of the octamer-dependent reporter in parental (lanes 4, 5, and 6) and stably transfected (lanes 7, 8, and 9) Jurkat T cells stimulated with ionomycin or PMA and ionomycin (as indicated) are shown.

porter with an expression vector for BOB.1/OBF.1, no activity was detected. To determine whether this inactivity was due to low expression of BOB.1/OBF.1 in these transient transfections, we established a Jurkat cell line that was stably transfected with a BOB1./OBF.1 expression vector (*18*). The amount of protein of exogenously expressed BOB.1/OBF.1 was comparable to that observed after costimulation with PMA and ionomycin. Nevertheless, octamer-dependent reporters were still inactive (Fig. 2, lanes 1 and 3). Activity was induced in these stable transfectants by ionomycin, which does not lead to expression of endogenous BOB.1/OBF.1 alone, and which yielded about half-maximal induction (Fig. 2, lanes 8 and 9). Taken together these data implicate posttranscriptional activation of BOB.1/OBF.1 in T cells. On longer exposures, BOB.1/OBF.1 always appeared as a doublet of 34 kD and 35 kD, respectively, both in B cells and in T cells. This doublet had previously been noted by Luo and Roeder, and they suggested that the 35-kD form might represent a modified version of the 34-kD polypeptide (6). Treatment of extracts with phosphatases did not convert the 35 kD band to the 34 kD band, however (*19*).

The BOB.1/OBF.1 protein can be grossly divided into two functional domains. The NH_2-terminus is required for interaction with the POU domain of the Oct1 and Oct2 proteins and also shows weak affinity for the octamer motif, whereas the COOH-terminal domain is largely responsible for the transactivation potential (*8*). To determine whether the postulated posttranscriptional activation targets the transactivation domain of BOB.1/OBF.1, we analyzed the activity of chimeric proteins containing the transactivation domain of BOB.1/OBF.1 fused to the DNA-binding domain of the yeast GAL4 transcription factor (GAL) (*20*). In unstimulated T cells, this fusion protein moderately (6.5-fold) activated transcription from a reporter containing multimerized binding sites for GAL4 (Fig. 3A). PMA and ionomycin stimulated this activity about twofold and eightfold, respectively. Both stimuli together enhanced the stimulation 15- to 20-fold, and this was inhibited by CsA (Fig. 3A). We used a series of deletions of the transactivation domain to map the region of BOB.1/OBF.1 required for inducible activity. A short domain encompassing amino acids 166 to 189 was required for most of the inducible activity. By itself, this domain did not have constitutive or inducible activity, however. A second domain, located at the extreme COOH-terminus of BOB.1/OBF.1 between amino acids 228 and 256, mediated the small amount of constitutive activity in T cells and also a twofold inducible activity (Fig. 3B). Importantly, even when this extreme COOH-terminal domain was deleted, most of the inducibility was still observed.

In vivo phosphate labeling experiments showed that BOB.1/OBF.1 is a phosphoprotein both in B cells and in activated T cells. Furthermore, V8-phosphopeptide mapping (*21*) revealed that the patterns of constitutive and inducible phosphorylation of BOB.1/OBF.1 in B cells and T cells, respectively, are virtually identical (Fig. 4, A and C). All of these phosphorylations occurred on serine and threonine residues, and no evidence for tyrosine phosphorylation was obtained (*22*). To determine whether the increased activity correlated with phospho-



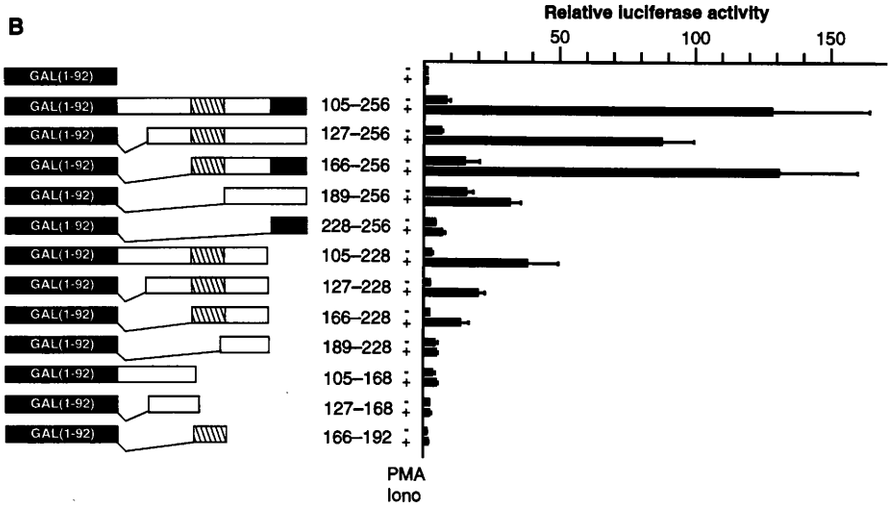


Fig. 3. Mediation of inducible transcriptional activity by the BOB.1/OBF.1 transactivation domain in T cells. (**A**) Activity of the chimeric protein containing the COOH-terminal transactivation domain of BOB.1/OBF.1 fused to the DNA-binding domain of the GAL4 protein (even-numbered bars) was measured after stimulating Jurkat T cells as indicated. Activity of the GAL4 DNA–binding domain alone (odd-numbered bars) was normalized to 1. The reporter construct contained four copies of the GAL4-binding site in front of the minimal herpes simplex virus–thymidine kinase TATA box and the luciferase gene (*26*). (**B**) Analysis of constitutive (–) and PMA- and ionomycin-inducible (+) activity of a series of GAL fusion proteins that contained the indicated deletions of the BOB.1/OBF.1 COOH-terminal transactivation domain. The domains required for constitutive (amino acid 228 to 256; black boxes) and inducible (amino acid 166 to 189; striped boxes) activity are indicated at left. All transfections were normalized to the activity of the GAL4 DNA-binding domain alone. Comparable expression of all fusion proteins was verified by electrophoretic mobility-shift assay with a GAL4-binding site.

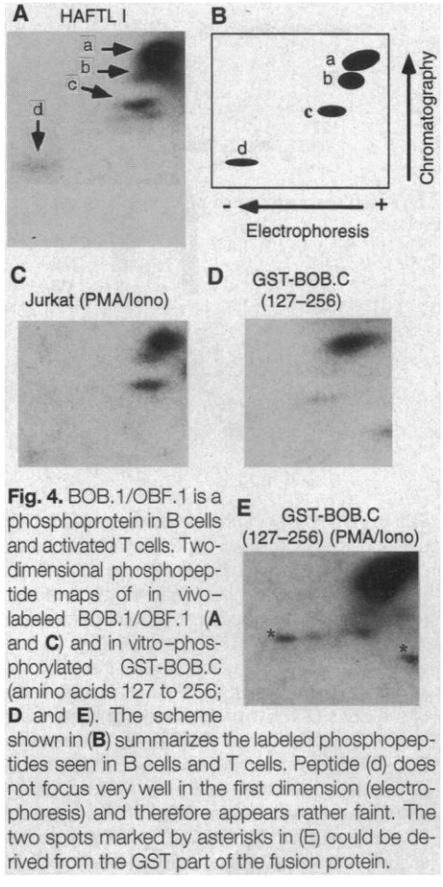


Fig. 4. BOB.1/OBF.1 is a phosphoprotein in B cells and activated T cells. Two-dimensional phosphopeptide maps of in vivo–labeled BOB.1/OBF.1 (**A** and **C**) and in vitro–phosphorylated GST-BOB.C (amino acids 127 to 256; **D** and **E**). The scheme shown in (**B**) summarizes the labeled phosphopeptides seen in B cells and T cells. Peptide (d) does not focus very well in the first dimension (electrophoresis) and therefore appears rather faint. The two spots marked by asterisks in (E) could be derived from the GST part of the fusion protein.

rylation of the COOH-terminal domain, we performed an in vitro phosphorylation assay with the use of purified recombinant BOB.1/OBF.1 COOH-terminal fragment and extracts from unstimulated and stimulated Jurkat T cells (*23*). In extracts from unstimulated cells, low basal phosphorylation of the fragment encompassing the COOH-terminal 130 amino acids of BOB.1/OBF.1 was observed (Fig. 5A). Extracts from ionomycin-treated cells showed enhanced phosphorylation of this fragment. Phosphorylation was further increased in extracts from PMA- and ionomycin-treated cells. When these in vitro phosphorylated proteins were analyzed by V8-phospho-peptide mapping, a very similar pattern to the one observed for the in vivo–labeled proteins was observed (Fig. 4). Exposure of cells to CsA or FK506 inhibited the effect of PMA and ionomycin. CsA and FK506 are inhibitors of the Ser/Thr-phosphatase calcineurin; therefore, calcineurin may be a component of the signal transduction pathway leading to the inducible phosphorylation of BOB.1/OBF.1. However, other potential targets of these drugs cannot be excluded.

To further elucidate the phosphorylation sites, fragments of the COOH-terminus were purified as glutathione-S-transferase (GST) fusion proteins and analyzed in vitro. The COOH-terminus was divided into four fragments, amino acids 127 to 166, 166 to 192, 189 to 228, and 228 to 256. Only two of them, 166 to 192 and 228 to 256, showed inducible phosphorylation (Fig. 5B). These two regions were the ones that were responsible for constitutive and inducible activity of the GAL fusion protein (Fig. 3B). Phospho–amino acid analysis of in vitro phosphorylated GST-BOB.C protein revealed that phosphorylation only occurred at serine residues, suggesting that the phosphorylation that was observed at threonines in full-length BOB.1/OBF.1 protein occurred within the NH_2-terminal part of the protein. The domain from amino acids 166 to 192 contains serine residues at positions 184, 188, and 189. To identify the serine or serines important for inducible phosphorylation and transcriptional activity, serine-to-alanine substitutions were introduced at these positions (Fig. 5C). These mutants were first analyzed for inducible in vitro phosphorylation. A double mutation of serines 188 and 189 (M1) did not affect phosphorylation, whereas a triple mutant (M2) eliminated inducible phosphorylation (Fig. 5C). Likewise, the double mutant was still fully inducible upon transfection into Jurkat cells, whereas the triple mutant failed to show inducibility (Fig. 5D, lanes 3 and 4). When a single point mutation was generated at Ser^{184}, most of the inducible transcriptional activity was eliminated (Fig. 5D, lane 5).

BOB.1/OBF.1 is constitutively expressed and functional in B lymphocytes. We tested whether Ser^{184} is also a critical residue for constitutive activity in B cells. We assayed the transcriptional activity of the GAL fusion proteins containing wild-type or mutant COOH-terminal BOB.1/OBF.1 in the S194 B cell line. Mutation of Ser^{184} abolished activity of the BOB.1/OBF.1 transactivation domain in these cells (Fig. 5D, lanes 9 and 10). This result suggests that the same or a similar pathway addressing Ser^{184} is constitutively operating in B cells and inducible in T cells.

Our results suggest that BOB.1/OBF.1, in addition to playing a role for B cell–specific functions of the octamer motif, also regulates inducible gene expression in T cells. Both the expression of the BOB.1/OBF.1 coactivator as well as its transactivation function are regulated in T cells. Our analysis not only unravels the requirements for inducible activity in T cells, but also leads to the identification of critical domains for function of BOB.1/OBF.1 in B cells.

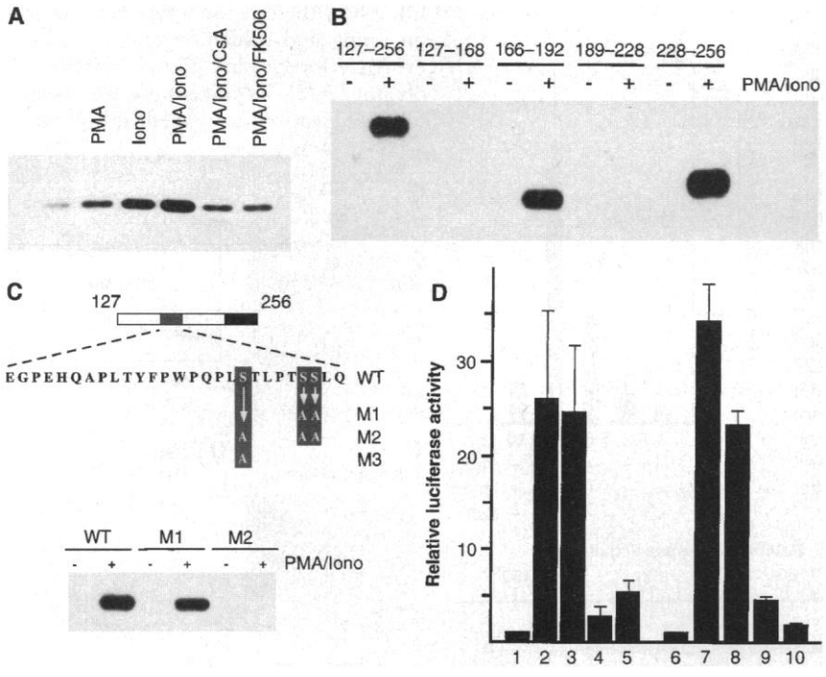


Fig. 5. Inducible phosphorylation of the BOB.1/OBF.1 transactivation domain. (**A**) In vitro kinase assay of uninduced and induced Jurkat cell extracts (as indicated) with the use of recombinant BOB.1/OBF.1 protein. A fusion protein of GST and the COOH-terminal domain (amino acids 127 to 256) of the BOB.1/OBF.1 protein were used for the in vitro phosphorylation experiments. (**B**) In vitro kinase assay of uninduced (–) and induced (+) Jurkat cell extracts and recombinant GST fusion proteins containing the indicated regions of the BOB.1/OBF.1 transactivation domain. (**C**) Analysis of in vitro phosphorylation of wild-type (WT) and mutant forms of the BOB.1/OBF.1 protein bearing serine-to-alanine point mutations in induced (+) or uninduced (–) Jurkat cell extracts. The scheme (top) shows the COOH-terminal domain of the BOB.1/OBF.1 protein and highlights the sequence from amino acid 166 to 192, as well as the specific point mutations. (**D**) GAL4 fusion proteins containing the COOH-terminal 150 amino acids of the BOB.1/OBF.1 protein in its wild-type or mutated forms were assayed for PMA- and ionomycin-inducible activity in Jurkat T cells (lanes 1 to 5) or constitutive activity in S194 B cells (lanes 6 to 10). Lanes 1 and 6 show the activity of just the GAL4 DNA-binding domain, which was set to 1 for both cell lines. Lanes 2 and 7, wild-type BOB.1/OBF.1 COOH-terminal domain; lanes 3 and 8, mutant M1; lanes 4 and 9, mutant M2; and lanes 5 and 10, mutants M3. The same GAL4 reporter plasmid was used in Fig. 3.

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16. Different octamer-dependent reporter constructs (wild-type and mutant versions of 4xwt.TATA and ED.TATA) were used for transient transfections of Jurkat cells (*4*). CMV lacZ was cotransfected to control for differences in transfection efficiencies. Immediately after transfection, cells were split into different dishes and induced with PMA (10 ng/ml), ionomycin (1 μg/ml), CsA (100 ng/ml), or FK 506 (1 ng/ml). Extracts for enzyme assays were prepared 18 hours after transfection.
17. Total cytoplasmic RNA (10 μg) was analyzed as described (*24*). For protein immunoblots, 100 μg of whole-cell extracts were run on 12.5% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with affinity-purified antibodies to BOB.1/OBF.1. Blots were developed with the ECL-system (Amersham) (*8*). When NIH/3T3 cells were analyzed, no increased BOB.1/OBF.1 RNA or protein expression was observed after PMA and ionomycin stimulation. Anisomycin (100 μM) and FK506 (1 ng/ml) inhibited the increase of BOB.1/OBF.1 RNA and protein expression.
18. Stable transfectants of Jurkat cells were generated with the two components of the tet-regulated system (*25*).
19. BOB.1/OBF.1 was immunoprecipitated from B cell extracts or induced Jurkat T cell extracts, and precipitates were treated with calf intestinal alkaline phosphatase or λ phosphatase. Efficient dephosphorylation was confirmed by including an in vitro phosphorylated protein in some of the samples. Subsequent analysis of the dephosphorylated immunoprecipitates did not show a decrease in the 35-kD band or an increase in the 34-kD band.
20. All COOH terminally deleted and point-mutated fragments of BOB.1/OBF.1 used for GAL fusion and GST fusion experiments were generated by polymerase chain reaction (PCR) amplification followed by sequence analysis. Details on the primers used are available upon request. The different subfragments were cloned in frame into either the expression vectors containing the DNA-binding domain of the GAL4 protein (amino acids 1 to 92) (*26*) or the GST gene in the pGEX-3X (Pharmacia) vector. GST fusion proteins were expressed in bacteria and purified by glutathione sepharose chromatography.
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22. HAFTL I B cells and Jurkat T cells were labeled for 14 hours with [^{32}P]orthophosphate (1 mCi/ml) in a total of 10 ml of medium containing dialyzed fetal bovine serum. BOB.1/OBF.1 protein was immunoprecipitated from whole-cell extracts with the use of affinity-purified antibodies (*8*) and separated on 12% SDS-polyacrylamide gels. Wet gels were autoradiographed, and BOB.1/OBF.1 was excised and eluted from the gel. After cleavage with 100 ng of V8 protease (Glu-C endopeptidase, Boehringer Mannheim) for 15 hours at 30°C, phosphopeptides were separated on cellulose plates by electrophoresis at pH 1.9 followed by chromatography (*21*). Phospho–amino acid analysis was performed as described (*21*) for in vivo–labeled proteins, and only phosphoserine and phosphothreonine were identified.
23. Jurkat cells were grown in medium containing low concentrations of serum (0.5% fetal bovine serum) for 16 hours, followed by treatment with PMA (10 ng/ml), ionomycin (1 μg/ml), CsA (100 ng/ml), or FK506 (1 ng/ml) for 15 min. Cells were collected, washed three times with cold phosphate-buffered saline, and lysed in 200 μl of lysis buffer [10 mM tris (pH 7.0), 30 mM sodium pyrophosphate, 100 μM $NaVO_4$, 2 mM iodoacetic acid, 50 mM NaCl, 50 mM NaF, 5 μM $ZnCl_2$, and 1% Triton X-100]. Extracts were clarified by a 20-min spin in the Eppendorf centrifuge at 4°C. We incubated 20 μg of cell extract with 500 ng GST fusion protein in kinase buffer [20 mM tris (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$, 100 μM $NaVO_4$, and 1 mM dithiothreitol] containing 10 μCi [γ-^{32}P]ATP for 30 min at 30°C in a total volume of 40 μl. Subsequently, the fusion protein was purified by affinity chromatography using glutathione agarose beads. Proteins were dissociated from the resin by boiling in SDS sample buffer and then were subjected to 12% SDS–polyacrylamide gel electrophoresis.
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Induction of Cell Migration by Matrix Metalloprotease–2 Cleavage of Laminin-5

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Structural changes in the extracellular matrix are necessary for cell migration during tissue remodeling and tumor invasion. Specific cleavage of laminin-5 (Ln-5) by matrix metalloprotease–2 (MMP2) was shown to induce migration of breast epithelial cells. MMP2 cleaved the Ln-5 γ2 subunit at residue 587, exposing a putative cryptic promigratory site on Ln-5 that triggers cell motility. This altered form of Ln-5 is found in tumors and in tissues undergoing remodeling, but not in quiescent tissues. Cleavage of Ln-5 by MMP2 and the resulting activation of the Ln-5 cryptic site may provide new targets for modulation of tumor cell invasion and tissue remodeling.

Cell migration across extracellular matrix (ECM) tissue boundaries is required in many important biological processes, including tissue remodeling and tumor invasion (*1*, *2*). To overcome ECM barriers, advancing cells may focus proteases such as metalloproteases (*2*) or protease activators such as urokinase (*1*, *2*) at their leading edge, where complex proteolysis (*1*) can direct migration, preserve ECM attachment, or avoid unwanted tissue damage. The precise mechanisms by which proteases alter ECM components remain unresolved, and it is unclear whether proteases simply remove physical barriers to migration or mold ECM components into substrates suitable for migration.

We report that the matrix metalloprotease MMP2 induces the migration of breast epithelial cells by cleaving and regulating the function of a specific ECM component, Ln-5 (also known as kalinin, epiligrin, nicein, and ladsin). Ln-5 is a component of epithelial basement membranes, which also contain collagen type IV (Coll IV), laminin-1 (Ln-1), and, during tissue remodeling, fibronectin (Fn) (*3*). Cells adhere to or migrate on these ECM substrates by means of integrin receptors. For example, Ln-5 interaction with integrins is essential for the adhesion of epithelial cells to basement membranes (*4*) and promotes migration (*5*).

To test whether the effect of these basement membrane components on cell migration is protease-dependent, we studied their interaction with MMP2 (gelatinase A, 72-kD type IV collagenase), a protease that is concentrated along basement membranes at sites of tissue remodeling (*1*) and at the leading edge of invading tumors (*1*, *2*, *6*). In a transwell migration assay (*7*), normal human breast epithelial cells (HUMEC) or the nontumorigenic breast cell line MCF10 (*8*) adhered to the top side of the filter without crossing to the other side (Fig. 1A), which was coated with one or more purified ECM components (Coll IV, Ln-1, Fn, or Ln-5). Addition of MMP2 to the cells caused a dose-dependent induction of migration on Ln-5 (Fig. 1A), but not on any of the other three substrates. This result was surprising because Coll IV, Ln-1, and Fn have each been identified as an MMP2-recognized substrate (*1*) and might be expected to induce protease-dependent migration. Ln-5, on the other hand, has not been previously recognized as a target for MMP2. However, when filters were coated with Ln-5 pretreated in a solution containing different concentrations of MMP2 (Fig.

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