CD40 stimulation induces Pax5/BSAP and EBF activation through a APE/Ref-1 dependent redox mechanism

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Running title: APE/Ref-1 mediated CD40 activation of Pax5 and EBF

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SUMMARY

CD40 is a member of the growing tumor necrosis factor receptor (TNFR) family that has been shown to play important roles in T cell-mediated B lymphocyte activation. Ligation of B cell CD40 by CD154, mainly expressed on activated T cells, stimulates B cell proliferation, differentiation, isotype switching, upregulation of surface molecules contributing to antigen presentation, development of the germinal center, and the humoral memory response.

In this study we demonstrate that the redox factor APE/Ref-1 acts as a key signaling intermediate in response to CD40 mediated B cell activation. The transcription factors Pax5a or BSAP (B cell lineage-specific activator protein) and EBF (Early B cell factor) are constitutively expressed in spleen B cells and CD40 cross-linking induces increases in Pax5a and EBF binding activity compared with non stimulated B cells. We show that upon CD40 antibody mediated cross-linking, APE/Ref-1 translocates from the cytoplasm to the nucleus of activated B cells, where it modulates the DNA-binding activity of both Pax5a and EBF. Moreover, we show that the repression of APE/Ref-1 protein production is able to block CD40 mediated Pax5a activation. We also provide evidence that APE/Ref-1 can modulate the cooperative activation of the blk promoter operated by Pax5a and EBF and that APE/Ref-1 might directly regulate EBF functional activity. Finally, we show that the interaction between Pax5a and EBF enhances EBF binding activity to its consensus sequence, suggesting that Pax5a can physically interact with EBF and modulate its DNA-binding activity.
INTRODUCTION

CD40 is a surface receptor expressed on B cells and certain accessory cells that belongs to the pleiotropic growing tumor necrosis factor receptor (TNFR) superfamily. The interaction between CD40 and its ligand (CD154), which is mainly expressed on activated CD4+ T cells, is critical in the regulation of immune response. Engagement of CD40 on B lymphocytes promotes proliferation, cytokine production, up-regulation of various surface molecules involved in antigen presentation, formation of germinal center, memory B cell and antibody isotype switching (1,2). CD40 activates the JNK-SAPK, NF-κB (3,4), p38 kinase (5) and ERK-mitogen-activated protein kinase (MAPK) pathways (6). In addition to these serine-threonine kinases, a link between CD40 signaling and PI-3 kinase (PI-3K) has been suggested (7-9). In each of the above pathways, one of the major final outcomes is an alteration in the activity of one or more transcription factors.

Pax5a or BSAP (B cell lineage-specific activator protein) and EBF (Early B cell factor) are two transcription factors essential for B cell development. Both are expressed at all stages of B cell development except in the plasma cell and are involved in the transcription control of several B cell restricted genes.

Pax5a, the product of the Pax5 gene, is a member of a multigene family of transcription factors that share the paired box DNA binding domain and are important regulators of early development (10). The Pax5 gene is alternatively spliced during B cell development: the predominant form Pax5a (full-length Pax5), Pax5b, Pax5d, and Pax5e (11). Only Pax5a and Pax5d posses an intact DNA-binding domain, enabling them to interact with and compete for Pax5-binding sites on DNA. Pax5d and Pax5e do not have transactivation, repression, or partial homeodomain homology regions at the C terminus but present a 42-aa novel sequence with unknown function (11). Pax5a is essential for the development of B cells; Pax5 gene knockout mice have no mature B cells and serum immunoglobulins, and B cell development in such mice is arrested at the pro-B stage (12-14).

Pax5a binding sites have been identified in the regulatory sequences of a number of genes, including mb-1, CD19 (15), the κ light chain (16-18), VpreB1, λ5 (19), the J chain (20), blk (21,22), the mouse engrailed gene (23), the human X-box binding protein-1 (24) and p53 (25), although the functional significance of many of these sites in vivo is unknown. In mature B cells, Pax5a is involved in cell activation, proliferation and Ig class...
switching (16,26-32).

We have previously shown that Pax5a activity is regulated through a redox mechanism. In particular we demonstrated that an oxidized form of Pax5a is unable to interact with DNA, whereas the reduced form binds strongly, and that an intramolecular disulfide bond within the paired domain of Pax5a causes interference with specific DNA binding (37). Moreover, exposure of B cells to \( \text{H}_2\text{O}_2 \) results in rapid transfer of the cytoplasmic redox factor APE/Ref-1 into the nucleus, event correlated with an increase in Pax5a binding activity (33). APE/Ref-1 (also designated HAP-1) is a trifunctional protein involved in apurinic/apyrimidinic endonuclease DNA base repair activity, in proofreading exonuclease activity (34) and in modulating DNA-binding activity of several transcription factors including NF-\( \kappa \)B, Egr-1, p53 and members of Pax family (33,35-37). The redox and repair activities of APE/Ref-1 are localized to distinct, non overlapping domains of the protein that function independently. The N-terminal domain is essential for redox activity and contains the nuclear localization sequence (residues 1–36) (38), while the endonuclease activity resides in the C-terminal region (39). APE/Ref-1 expression is ubiquitous; however, it exhibits a complex and heterogeneous expression pattern that differs among tissue types (40).

Early B-cell factor was identified as a B lymphocyte-specific protein that recognizes a site in the \textit{mb-1} promoter (41,42). This protein was also identified as olfactory factor 1 (Olf-1) in olfactory neurons (43). DNA binding studies showed that EBF binds as an homodimer and recognizes specific nucleotide sequences representing variations of an inverted repeat of a 5'-GGGAA/TT half site separated by 2 bp spacer (42,44). DNA binding activity is mediated by a novel zinc-coordination motif in the amino-terminal half of EBF where there are also sequences that mediate DNA-dependent dimerization and transactivation (45). The C-terminal region of EBF contains a second, serine/threonine-rich activation domain with homology to the second helix of basic-helix-loop-helix (bHLH) proteins (42). The deletion of these \( \alpha \)-helical repeats was found to markedly reduce DNA binding and dimerization in solution. EBF was shown to interact with functional regions in the promoters of \textit{mb-1} (41), \textit{\lambda 5} (42,44,46-48), VpreB (46), B29 (49), and \textit{blk} genes (50). This suggests that EBF has a role in the regulation of several genes encoding proteins of the pre-B and B cell receptor. Pax5a is required also during later stages of B cell development for the maintenance of mature B cell characteristics and
function (51), but the role of EBF in mature B cells is currently unknown.

The aim of this work was to determine if CD40 mediated stimulation of B lymphocytes promotes activation of EBF and Pax5a and to investigate the role of APE/Ref-1 in this process. Our data demonstrate that upon CD40 antibody mediated cross-linking, APE/Ref-1 translocates from the cytoplasm to the nucleus of activated B cells, where is able to control the DNA-binding activity of Pax5a. In addition, we show that APE/Ref-1 modulates EBF activity and the Pax5a and EBF cooperative activation of the \( \text{blk} \) promoter. Moreover, we observed that the interaction between Pax5a and EBF enhances EBF binding activity to its consensus sequence, suggesting that Pax5a can physically interact with EBF and modulate its DNA-binding activity. Together, these data suggest a novel role of APE/Ref-1 in B cells CD40 mediated activation.

EXPERIMENTAL PROCEDURES

Cell preparation and culture conditions

Purified splenic B cells were obtained from 6-12-wk-old female Balb/c mice as follows: splenocyte cell suspension were depleted of RBCs by hypotonic lysis with ACK lysing buffer (BioWhittaker) and of T cells by complement-mediated cytotoxic lysis using anti-Thy 1.2 mAb1 (a gift from K. Hathcock, Experimental Immunology Branch, NIC/NIH, Bethesda, MD USA) in conjunction with rabbit complement (Low- Tox M; Cedar Lane).

Cells were maintained in RPMI 1640 supplemented with 10% FCS (Sigma), 20mM Hepes (Gibco BRL), 2mM L-glutamine (Gibco BRL), 1mM sodium pyruvate (Gibco BRL), 1% non-essential amino acids (Gibco BRL) antibiotics (100 U/ml penicillin, and 100 \( \mu \)g/ml streptomycin) (BioWhittaker) and 50mM \( \beta \)-ME (Sigma) at 37 °C and 5% CO₂.

The resulting cells were checked by cytofluorimetric analysis (Becton Dickinson FACScan) and then were incubated with purified anti-mouse CD40 mAb HM40-3 at 1 \( \mu \)g/ml (Pharmingen) for 72 h. All experiments reported in this paper were performed at this time point, which represent the maximum value of proliferation, assayed by measuring \( [\text{H}] \)thymidine incorporation.
HeLa (human cervical carcinoma) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS (Sigma), 1% non-essential amino acids (Gibco BRL), 2mM L-glutamine (Gibco BRL) and antibiotics (100 U/ml penicillin, and 100 µg/ml streptomycin) (BioWhittaker Europe).

NS-1 (non-secreting mouse myeloma) and Raji (Burkitt lymphoma) cells were grown in RPMI 1640 supplemented with 10% FCS (Sigma), 2mM L-glutamine (Gibco BRL) and antibiotics (200 U/ml penicillin, and 200 µg/ml streptomycin) (BioWhittaker Europe).

Preparation of cell extracts

10^7 cells were washed twice with PBS and then resuspended in 50 µl ice cold buffer A (10mM Hepes pH 7.9, 10mM KCl, 1.5mM MgCl_2, 0.1mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). The samples were centrifuged at 800 x g for 10 min at 4°C and the supernatants were collected as cytoplasmic extracts. Then, the pellets were resuspended in 50 µl buffer B (20mM Hepes pH 7.9, 400mM NaCl, 1.5mM MgCl_2, 0.1mM EDTA, 5% glycerol, 0.5mM PMSF, 0.5mM DTT) to extract nuclear proteins. After incubation for 20 min on ice, the samples were centrifuged at 10,000 x g for 30 min at 4°C and the supernatants were collected as nuclear extracts.

For whole protein NS-1 extracts, 10^7 cells were re-suspended with 100 µl of lysis buffer (10mM Tris-HCl pH 7.5, 50mM NaF, 150mM NaCl, 1mM DTT, 0.5% NP-40, 0.5mM PMSF) and, after incubation for 20 min at 4°C, the extracts were centrifuged at 22,000 x g for 20 min to remove all cell debris. Protein concentrations were determined using the Bradford method (52) and the samples used immediately for western blot or EMSA analysis or kept at –80°C.

Western Blot Analysis

Equal amounts of protein, obtained as described above, were electrophoresed on a 12% SDS-PAGE minigel and transferred to nitrocellulose membranes (Schleicher & Schuell) using a semidry apparatus (Multiphor II, Pharmacia Biotech). The filters were blocked for 1 h at room temperature in blocking solution: 5% non-fat milk.
powder in PBS/0.05% Tween 20 (PBST). After washing with PBST, the membranes were incubated with primary Ab (diluted in blocking solution) overnight at 4°C or 1 h at room temperature. APE/Ref-1 was detected by a rabbit polyclonal specific antibody (Santa Cruz Biotechnology), actin and nucleoporin by the mouse polyclonal specific antibody (Sigma Chemicals Co. and BD Biosciences respectively), and Pax5a by the mouse polyclonal specific antibody D2A8 generated in our lab (33). The filters were then washed with PBST and incubated with the HRP-conjugated secondary antibodies (Sigma Chemicals Co.) in blocking solution for 1 h at room temperature. After washing the immunoreactive bands were visualized using a chemiluminescence substrate (Pierce) and Biomax–Light films (Kodak) according to the manufacturer’s protocol.

**Electrophoretic Mobility Shift Assay (EMSA)**

The sequence of the top strand of double-stranded oligonucleotide probes used in EMSAs were:

- H2A2.2: 5’-TCTGACGCAGCGGTGGGTGACGACT,
- mb-1: 5’-GAGAGAGACTCAAGGGAATTGTGG,
- CREB: 5’-AGAGATTGCCTGACGTCAGAGAGCTAG.

Oligonucleotides were end labeled with [32P] γ-dATP by incubation with T4 polynucleotide kinase (Fermentas), annealed, and purified on column spinX Costar 8160 (Corning Incorporated) filled with Sephadex G50 fine (Pharmacia). Nuclear extracts (8 µg) were incubated with 0.5-1 ng labeled probe (10,000-20,000 cpn) for 30 min at room temperature in binding buffer (100 mM Tris-HCl pH 7.4, 150 mM KCl, 10 mM EDTA, 10 mM DTT) with 1 µg calf thymus and BSA. In the EMSA experiment with the mb-1 probe, the binding buffer contain 0.2 mM ZnCl2. Unlabeled oligonucleotides as DNA competitors were added 10 min before the addition of DNA probe at molar excesses indicated in the respective figures. The samples were separated on a 5% polyacrylamide Tris-Borate-EDTA (TBE) gel, which was dried and then exposed to a Hyperfilm-MP (Amersham Pharmacia Biotech) film at –80°C.

For *in vitro* experiments with recombinant proteins, EMSA were performed with the mb-1 probe incubated with 100 ng GST-EBF oxidized protein, obtained after prolonged air exposure, in presence or absence of 400 ng of APE/Ref-1 recombinant protein or with 10 µg of crude, boiled, or APE/Ref-1 depleted NS-1 cellular extracts.
The immuno-precipitation of APE/Ref-1 was performed as described before (33). In these experiments binding buffer did not contain EDTA and DTT. To test the role of Pax5a on EBF DNA-binding activity, EMSA were performed with mb-1 probe, equal amounts of oxidized GST-EBF and recombinant APE/Ref-1, and 100 ng of recombinant Pax5a. Moreover, 3 µg of NS-1 stable transfected with Pax5a (NS-1/Pax5a) nuclear extracts were incubated with or without 100 ng of GST-EBF.

Oligonucleotide treatment of B cells

APE/Ref-1 antisense oligonucleotide and the complementary sense oligonucleotide were synthesized and HPSF purified by MWG-Biotech AG. The oligonucleotides were phosphorotioated at 3' and 5'ends (at the position marked by *) to confer nuclease resistance (53). The sequence of the APE/Ref-1 antisense probe was 5'-T*T*CCCCGCTTTGGCATC*G*C*-3' and the sense 5'-G*C*GATGCCAAAGCGGGG*A*A*-3' (nucleotides 3 to 16). The probe did not show homology to other known genes according to the GenBank database.

Spleen B cells were washed once with serum free media (OPTIMEM, Life Technologies) and then 1.6 ml of serum free media was added for 4x10⁶ cells in a 60-mm tissue culture plate. 25 µl of Lipofectin (Life Technologies) was diluted in 200 µl of serum free media, incubated for 30 min at room temperature and then mixed with the DNA (10 µg) diluted in 200 µl serum free media. The mixture was incubated for 15 min and the combined volume of 400 µl was added to the cells. The cells were then maintained in 5% CO₂ incubator at 37°C for 12 h, after which 2 ml of growth medium supplemented with 20% FCS was added to each transfection well. Then transfected cells (1x10⁶/ml) were incubated with the appropriate stimulus. At the end of that incubation, culture growth was determined by counting, and viability confirmed with trypan blue exclusion.

Plasmids and construction of the bidirectional expression vector

The pBI vector (Clontech) allows to simultaneously express two genes under control of a bidirectional tetracycline-responsive promoter (Pbi-1) (54). Pbi-1 promoter contains the Tet-responsive element (TRE),
which is repressed in the presence of tetracycline (Tc).

pBI-GL (Clontech) allows the simultaneous regulation of both luciferase and β-galactosidase genes by one central TRE.

The 965 bp BamHI/EcoRI fragment containing hRef-1 cDNA was taken from pGEX-3X-hRef-1 (see below) and filled in with Klenow fragment. The resulting blunt fragment was cloned into the NheI Klenow filled in site of pBI to make pBI-APE/Ref-1.

Primers EBF-NheI-REV (5’-GCAGCTAGCCACTCTGGGACTCATG-3’) and EBF-NheI-FOR (5’-GTAGCTAGCTGGGCAGCGGCATGAA-3’) containing the NheI restriction site were used to amplify the region encoding EBF cDNA, using CMV-EBF as a template. The amplified sequence was cloned into the NheI site of pBI to make pBI-EBF. DNA sequencing confirmed that all PCR products were free of any undesired mutations.

The 1.2-kb and 750 bp NotI fragments containing Pax5a and Pax5d cDNA respectively were taken from pcDNA3.1-Pax5a and pcDNA3.1-Pax5d (11) and cloned into the NotI site of pBI to make pBI-Pax5a and pBI-Pax5d or cloned into the NotI site of pBI-EBF to make pBI-Pax5a-EBF and pBI-Pax5d-EBF respectively or in NotI site of PBI-Ref-1 to make pBI-Pax5a-APE/Ref-1 and pBI-Pax5d-APE/Ref-1 respectively.

**Generation of stable cells expressing various transactivators and quantification of transactivation activity**

HeLa cells were grown in 35mm dishes to 50-60% confluence and transfected via the calcium phosphate procedure with 5 ¼g tTA2/3/4 (Clontech) plasmids. These are three vectors that express at different levels the tetracycline sensitive Pbi-1 transactivator and cause expression of pBI cloned genes at different levels (55).

After 24 h, cells were transferred to 10cm dishes and maintained in medium containing 500 µg/ml G418 (Gibco BRL). After 4 weeks, at least thirty resistant clones were isolated using cloning cylinders (Sigma) expanded separately and frozen as soon as possible. The presence of integrated tTA in neomycin-resistant cell lines were confirmed by PCR on chromosomal DNA using primers ptTA FOR (5’-GTCTGGATCCTTACTTAGTTACCC-3’) and ptTA REV (5’-ATAGAAGACACCGGGACCGATC-3’) that amplify a portion of the appropriate (TA plasmid. Positive clones were screen by transient transfections with PBI-GL reporter vector to identify G418 resistant clones that meet
the criteria for good Tet-Off cell lines: low background and high induction of luciferase and \( \beta \)-galactosidase in response to Tc (2 \( \mu \)g/ml).

A clone from HeLa-tTA2 cells (HeLa cells that constitutively synthesize tTA2) was selected for the highest fold-induction (e.g. showing highest expression with lowest background) for propagation and further testing.

HeLa-tTA2 cells were seeded at a density of 1 \( \times 10^5 \) cell/35mm dish and grown in the presence or absence of Tc (2 \( \mu \)g/ml). The next day, two hours prior to transfection, the culture medium was renewed and the cells were incubated at 37°C and 5% CO\(_2\). The calcium phosphate/DNA precipitate contains 2.5 \( \mu \)g plasmid DNA (consisting of 0.6 \( \mu \)g pBl, 0.8 \( \mu \)g pGLblk, 0.2 \( \mu \)g lacZ expression vector (CMV-\( \beta \)gal), included for normalization of transfection efficiency, and pUC18 as non-specific carrier DNA for equalization the DNA content in each transfection). The precipitate (55 \( \mu \)l/dish) was added to HeLa-tTA2 cells which were further incubated at 37°C and 5% CO\(_2\) for 24 h (56). The next day, the cells were washed twice in PBS and grown in the presence or absence of Tc (2 \( \mu \)g/ml). After 48 h the cells were scraped using TEN buffer (40mM Tris-HCl pH 7.4, 1mM EDTA, 150mM NaCl), centrifuged at 300 x g for 5 min at 4°C and the pellets resuspended in 150 \( \mu \)l of lysis buffer provided by \( \beta \)-gal kit (Roche Diagnostics) plus 1 mM DTT. After incubation for 20 min at room temperature, the samples were subjected to three cycles of freeze-thaw, and after centrifugation at 18,000 x g for 10 min at 4°C, the supernatants were collected and used immediately for assays or kept at 80°C.

Luc activity was determined using a luciferase assay. In brief, assays were conducted in a luminometer tube with 50 \( \mu \)l of the obtained extracts, 350 \( \mu \)l luciferase assay buffer (25mM glycyglycine pH 7.8, 2mM ATP, 10mM MgSO\(_4\)) and 100 \( \mu \)l of injection solution (0.2mM luciferin, 25mM glycyglycine pH 7.8). The data were normalized for transfection efficiency against the activity of 0.2 \( \mu \)g co-transfected CMV controlled-\( \beta \)-galactosidase (\( \beta \)-gal) reporter gene. The \( \beta \)-galactosidase assays were conducted with 25 \( \mu \)l as described in the instruction manual of kit (Roche Diagnostics).

Results of luc assays are shown as fold induction of luc conversion \( \pm \)S.D. Relative-fold induction values for each transfection were calculated by dividing each of normalized luc activity by the activity of the reporter construct alone.
NS-1 cells were stably transfected with pCDNA3.1-Pax5a by electroporation using a Bio-Rad gene pulser (Bio-Rad Inc.). Aliquots of $1 \times 10^7$ cells were resuspended in 350 ul of RPMI 1640 in a Bio-Rad electroporation cuvette (0.4 cm gap) and pulsed once with 340 V and 960 µF. After electroporation, cells were maintained at 4°C for 10 min and then cultured in pre-equilibrated RPMI 1640 supplemented with 10% fetal serum for 24 h. The next day, the cells were washed once in PBS and grown in medium containing 400 µg/ml G418 for one month. The presence of integrated Pax5a in neomycin-resistant NS-1 cell lines was confirmed by western blot using D2A8 antibody (33).

Transient transfections of HeLa cells were performed by calcium phosphate method as above described using 2.5 µg of total DNA.

**Protein expression and purification**

The APE/Ref-1 cDNA was cloned in the prokaryotic expression vector pGEX-3X (Amersham Biosciences) and used to transform BL21(DE3)pLysS (Novagen) bacterial strain (Stratagene). Transformed cells were grown at 37°C to OD$_{600}$ 0.6 and then induced by 100µM IPTG for 3 h. Cells were harvested by centrifugation and resuspended in lysis buffer (200mM Hepes, 150mM NaCl, 5% glycerol, 5mM DTT, pH 7.2). Cells were disrupted by sonication (4 x 30 sec at 250W with 1 min pause) and debris were removed by centrifugation at 23,500 x g for 40 min. The supernatant was loaded onto Glutathione Sepharose 4b resin (Amersham Biosciences) pre-equilibrated with lysis buffer. The resin was washed 3 x 15 min with washing buffer (200mM Hepes, 400mM NaCl, 5mM DTT, pH 7.2) and then equilibrated with 50mM Tris HCl, 100mM NaCl, 1mM CaCl$_2$, pH 8. 10 U/ml of factor Xa were added to the resin and incubated O/N under rotation at RT to obtain the fusion protein APE/Ref-1. The eluted was added to benzamidine resin (Amersham Biosciences) to eliminate factor Xa; the concentration of NaCl was increased to 300mM and 1mM DTT was added.

The EBF cDNA was cloned in the prokaryotic expression vector pGEX-4T1 (Amersham Biosciences) and used to transform BL21(DE3)pLysS bacterial strain. Transformed cells were grown at 25°C to OD$_{600}$ 0.8 and then induced by 100 µM IPTG for 3 h. Cells were harvested by centrifugation and resuspended in lysis buffer.
(200mM Hepes 150mM NaCl, 5% glycerol, 5mM DTT, 2mM ATP, 10mM MgSO₄, pH 7.2 and protease inhibitor EDTA-free) in a volume of 3 ml/g bacterial pellet. After sonication and centrifugation as described above, the supernatant was loaded with peristaltic pump at 4°C onto 1ml pre-equilibrated GSTrap FF column (Amersham Biosciences). The column was washed with wash buffer (50mM Hepes, 5mM DTT, pH 7.2) and the protein was eluted with elution buffer (50mM Hepes, 10mM reduced glutathione, 5mM DTT, pH 7.2). The collected fractions were concentrated with Centricon 30MW (Millipore).

The Pax5a cDNA was cloned in the prokaryotic expression vector pQE-31 (Qiagen) and used to transform BL21(DE3)RIL bacterial strain (Stratagene), previously transformed with pREP4 plasmid (Qiagen). Transformed cells were grown at 37°C to OD₆₀₀ 0.6 and then induced by 1 mM IPTG for 3 h. Cells were harvested by centrifugation and resuspended in 10ml lysis buffer TE 5x (1x TE, 10mM TrisHCl, 1mM EDTA, pH 7.5) for each gram of bacterial pellet. The protein was purified as described by Tell et al (57) and then concentrated with Centricon 30MW (Millipore).

The concentration of the three recombinant proteins was determined by Bradford assay (52). The proteins were then stored at –20°C in 50% glycerol.

**Pull down assay**

GST and GST-EBF proteins, prepared as above described, were incubated 2h 4°C with Glutathione-Sepharose beads (Amersham Pharmacia) in the lysis buffer (see above). After 3 washes in the same buffer, the beads were incubated 2h with nuclear extracts of HeLa cells transiently transfected with Pax5a, in binding buffer (25mM Hepes pH 7.9, 50mM NaCl, 1mM DTT, 0.01% NP40). For detection of bound protein, beads were washed 3 times with binding buffer, boiled in SDS-sample buffer and bound material detected by SDS-PAGE and western blotting.
RESULTS

CD40 crosslinking enhances DNA binding activity of Pax5a and EBF in primary mouse B cells

The nuclear proteins EBF and Pax5a are important regulators of B lymphocyte development. Their co-expression in B cells at the earliest stages of differentiation allows for collaboration on regulatory modules of early B cell-specific genes (58). Thus, EBF and Pax5a work separately and in concert to activate genes required for B cell differentiation. For instance, Pax5a is a transcriptional regulator of B cell-specific genes and is involved in activation and proliferation of B lymphocytes and Ig class switching. EBF interacts with \( k \) promoters and may modify Ig light chain expression (46,59).

To determine whether CD40 signaling influences the functional activity of these two B cell specific transcription factors mouse spleen B cells were activated by CD40 cross-linking with purified anti-mouse CD40 mAb HM40-3. Because maximal cell proliferation was observed after 72 h (data not shown) we choose this time to perform all further experiments. We performed electrophoretic mobility shift assay (EMSA) with H2A2.2 and \( mb-1 \) labeled probes specific for Pax5a and EBF respectively using nuclear extracts of mouse B cells before and after CD40 mediated activation. As shown in Fig. 1 we observed that: Pax5a (panel A) and EBF (panel B) are constitutively active in non-stimulated resting B cells and CD40 cross-linking differentially increases Pax5a and EBF DNA binding activity, as compared to the activity observed in non-stimulated B cells. As control, we used a probe specific for the constitutively expressed transcription factor CREB (panel C).

We investigated if the increase in Pax5a DNA binding activity was due to an increase in the protein levels by western blot. As shown in Fig. 2B, the protein levels of Pax5a remain constant in mouse spleen B cells before and after CD40 cross-linking.

Given that the Pax5a protein levels remain constant upon CD40 stimulation, we investigated if post-transcriptional mechanisms such as redox regulation could account for the increase of Pax5a activity.

Nuclear translocation of APE/Ref-1 in stimulated B cells

APE/Ref-1 is a ubiquitous protein that has the ability to influence DNA binding of several transcription factors
through a redox mechanism (60). We have recently shown that reactive oxygen species (ROS) elicit a nuclear translocation of APE/Ref-1 in B cell line, which on turn modulates Pax5a DNA binding activity through redox regulation (33).

Since a specific role for ROS has been proposed in the activation of B lymphocytes (61), and given the role APE/Ref-1 in ROS mediated signals, we investigated the possibility that APE/Ref-1 could be involved in the signal transduction through surface CD40. APE/Ref-1 protein levels in both nuclear and cytoplasmic compartments were evaluated by western blot analysis using a APE/Ref-1 specific antibody. Fig. 2A shows that engagement of CD40 and activation of B cells induces the translocation of APE/Ref-1 from the cytoplasm to the nucleus, suggesting a role for APE/Ref-1 in CD40 mediated activation of B lymphocytes. We observed that the increase of APE/Ref-1 in the nucleus peaked at 6 h (data not shown) and remained elevated at 72 h when maximal proliferation and activation was observed.

**APE/Ref-1 modulates anti-CD40-induced Pax5a activity**

To investigate the role of APE/Ref-1 in the regulation of Pax5a and EBF DNA binding activity, we transfected mouse B cells with phosphorothioate-substituted that is complementary to APE/Ref-1 mRNA sequence and overlapping the translation initiation site (antisense oligonucleotide) (53). As control, a sense oligonucleotide was used. The cells were pretreated for 12 h with oligonucleotide plus Lipofectin as described in Experimental procedures and then treated with anti-CD40 mAb for 72 h. Fig. 3A shows the ability of antisense oligonucleotide to efficiently downregulate APE/Ref-1 protein production. Next, we examined the effect of such downregulation on Pax5a activity by EMSA. As shown in Fig. 3B, preincubation of anti-CD40 mAb treated spleen B cells with antisense APE/Ref-1 oligonucleotide significantly reduced Pax5a DNA binding activity in nuclear extracts (Fig. 3B, *left panel*), suggesting that nuclear translocation of APE/Ref-1 plays a role in the redox modulation of Pax5a DNA binding activity under CD40 stimulation. This decrease in Pax5a DNA binding activity was not due to a decrease in the protein levels, as shown by western blot for Pax5a protein (Fig. 3A, *lower panel*). As control, we used a probe specific for the constitutively expressed transcription factor CREB (Fig. 3B, *right panel*).
Similar experiments were performed with \textit{mb-1} probe specific for EBF, but this approach did not lead to conclusive evidence for APE/Ref-1 mediated redox modulation of EBF. This prompted us to design different experimental approaches to investigate the involvement of APE/Ref-1 in EBF activity.

**APE/Ref-1 is involved in EBF DNA binding activity**

In order to investigate a possible direct role of the redox factor APE/Ref-1 in the regulation of EBF DNA binding activity, we carried out a series of \textit{in vitro} experiments with recombinant proteins. APE/Ref-1, EBF and Pax5a were produced from prokaryotic expression systems, as described in Experimental procedures, and tested in EMSA assays. First EBF was purified as a GST fusion protein and tested in EMSA using the specific \textit{mb-1} promoter probe. Recombinant GST-EBF (Fig. 4A, \textit{lane 3}) produced a specific band shift of the \textit{mb-1} probe, as assessed by the comparison with the shift produced with a nuclear extract of Raji cells (expressing endogenous EBF, Fig. 4A, \textit{lane 1}). We observed that upon oxidation, recombinant GST-EBF loses its DNA binding activity (Fig. 4B, \textit{lane 1}). Oxidized GST-EBF protein reacquires its DNA-binding activity in presence of recombinant APE/Ref-1 (shown in Fig. 4B, \textit{lane 2}) but not in presence of others reducing agents (e. g. DTT or β-ME) (data not shown).

To confirm the result, we incubated GST-EBF with a total extract of NS-1 cellular line (Fig. 4B, \textit{lane 3}). This cellular line does not express endogenous EBF and Pax5a but expresses APE/Ref-1. As control, the presence of aspecific interactions was tested with cellular extracts alone before (Fig. 4B, \textit{lane 7}) and after boiling (Fig. 4B, \textit{lane 8}). To establish a direct role for APE/Ref-1, we immunodepleted NS-1 cellular extracts with anti-APE/Ref-1 Ab and observed that these extracts were unable to rescue the oxidized EBF DNA-binding activity (Fig. 4B, \textit{lane 4}). Furthermore we performed similar experiments using total extracts of HeLa cell line. In presence of this cellular extracts which does not express EBF but expresses endogenous APE/Ref-1, we observed that recombinant GST-EBF produced a specific band shift of the \textit{mb-1} probe (Fig. 4B, \textit{lane 9}).

Taken together the data presented in Fig. 4B indicate that APE/Ref-1 is able to directly reduce the oxidized form of GST-EBF and rescue its DNA-binding activity suggesting that APE/Ref-1 might directly regulate EBF functional activity.
APE/Ref-1 affects the functional cooperation between Pax5a and EBF on blk promoter activation in a non-lymphoid cell line

Based on earlier in vitro data indicating that EBF plays an important role in the regulation of blk promoter in early B cell development and that Pax5a and EBF are capable to act in cooperation to induce this target gene (50), we sought to investigate in vivo whether such cooperation is sensitive to APE/Ref-1 redox regulation. For this set of experiments, we used a reporter construct containing a portion of the blk promoter with responsive elements for both Pax5 and EBF and the HeLa cell line, which offers a neutral functional background as it does not express endogenous EBF and Pax proteins. Earlier studies on the effects of EBF and Pax5a function, used co-transfections of two expression vectors, one for EBF and one for Pax5a. With the aim to overcome the problem of disparate levels of expression, we used a tetracycline regulated bidirectional expression vector that allowed transcription initiation from the same regulatory element. HeLa cells were stable transfected with an expression vector producing a chimerical protein (tTA2) consisting of the DNA binding domain of the Tc repressor and the transactivation domain of the herpes simplex virus1 VP16. In the absence of Tc the chimerical tTA is able to bind the regulatory element and drive overexpression from two minimal promoters. This system exhibits tight on/off regulation and absence of pleiotropic effects. All experiments in this study were done in the absence of tetracycline.

Bidirectional expression vectors were transiently cotransfected with pGL3-blk luciferase reporter in HeLa-tTA2 cells and transactivation levels were measured. Reporter gene expression from the blk promoter is only detected at background levels when pGL3-blk luciferase is transfected into HeLa-tTA2 cells. As shown in Fig. 5A, the co-transfection of expression vectors containing Pax5a or EBF results in significant transactivation: transfection of pBI-Pax5a and pBI-EBF resulted in a 7-and 5-fold induction respectively, whereas the reporter gene was expressed at high level (29 x) in the presence of both the proteins suggesting that Pax5a and EBF cooperate on the activation of blk promoter. This combined effect was dependent on a whole functional Pax5a protein, because transfection of pBI-Pax5d-EBF resulted in only a 8 x fold induction.

Next, it was tested whether APE/Ref-1 could affect the functional cooperation between Pax5a and EBF. Reporter construct blk-luc was transiently co-transfected into HeLa-tTA2 cells using the same amounts of
pBI-APE/Ref-1 and pBI-Pax5a or pBI-EBF (Fig. 5A).

As expected, the construct that induced co-expression of EBF and Pax5a (pBI-Pax5a-EBF) showed higher activity than EBF or Pax5a alone. Comparison of the values obtained with pBI-EBF versus pBI-EBF-APE/Ref-1 expression constructs showed an APE/Ref-1-dependent increase of EBF activity on the promoter. Similarly, comparison of pBI-Pax5a versus pBI-Pax5a-APE/Ref-1 showed a APE/Ref-1-dependent increase of Pax5a activity on the promoter. Lastly, the combined expression of Pax5a, EBF and APE/Ref-1 lead to significantly higher activity as compared to Pax5a and EBF alone (46-fold versus 29-fold increase), suggesting that APE/Ref-1 is able to enhance the activity of both Pax5a and EBF, leading to a cumulative effect on promoter activity. Transfection of APE/Ref-1 had a minimal effect on the basal transcriptional machinery of the \textit{blk} promoter (data not shown). Based on these results, we wished to explore more quantitatively the observed effect of APE/Ref-1 on the pBI-EBF induced activation. Transient co-transfections were performed using constant amounts of CMV-EBF with increasing amounts of CMV-APE/Ref-1 constructs. As shown in Fig. 5B, EBF activation of the reporter is enhanced by APE/Ref-1 in a dose-dependent manner, and reaches a saturation peak when excess APE/Ref-1 is used. As control we performed the same experiment using constant amounts of Pax5a with increasing amounts of APE/Ref-1 constructs. With this experiment we demonstrated that Pax5a activation of the reporter is enhanced by APE/Ref-1 in a dose-dependent manner, as shown in Fig. 5C.

Taken together these results indicate that APE/Ref-1 modulates the functional activity of both Pax5a and EBF, that these proteins act in cooperation on the \textit{blk} promoter and finally that such cooperation is sensitive to APE/Ref-1 redox regulation.

\textbf{Pax5a physically interacts with EBF and modulates its DNA-binding activity}

The evidence of a functional cooperation between EBF and Pax5a on \textit{blk} promoter (50), prompted us to verify if Pax5a can directly regulate EBF DNA-binding activity \textit{in vitro}. We decided to perform an EMSA assay with the two recombinant proteins incubated with the EBF specific probe from the \textit{mb-1} promoter. As shown before, the oxidized form of GST-EBF did not bind to the DNA (Fig. 6A, \textit{lane 1}), and reacquires its DNA-binding
activity in presence of recombinant APE/Ref-1 (Fig. 6A, lane 2). Surprisingly, we observed that oxidized GST-EBF reacquired DNA-binding activity when the protein was incubated with recombinant protein Pax5a (rPax5a) (Fig. 6A, lane 3). Adding rAPE/Ref-1 to this complex further increased its DNA binding activity (Fig. 6A, lane 4). To rule out that this effect could be artificial to the use of recombinant Pax5a, nuclear extracts obtained from stable transfected Pax5a NS-1 cells (NS-1/Pax5a) were used and, as shown in lane 5, a strong increase in EBF DNA-binding activity was observed. As control, rPax5a (lane 6), NS-1/Pax5a cellular extracts (Fig. 6A, lane 8, 9) were used confirming that rPax5a did not bind mb-1 probe and there are not specific interactions with the NS-1/Pax5a cellular extracts (Fig. 6A, lane 8, 9). These results demonstrate that the observed interaction is specific for GST-EBF and suggest that Pax5a can establish a molecular complex with EBF and enhance its DNA-binding activity.

To confirm this hypothesis we performed a pull down assay using nuclear extracts of HeLa cells transiently transfected with Pax5a (Fig. 6B, lane 1) using GST-EBF(Fig. 6B, lane 2) or GST alone (Fig. 6B, lane 3) proteins as baits. As shown in Fig. 6 B Pax5a was found to be specifically associated with GST-EBF, thus confirming that the two proteins can physically interact.
DISCUSSION

APE/Ref-1 is a tri-functional protein involved in DNA base excision repair, in proofreading exonuclease activity (34) and in modulating DNA–binding activity of several transcription factors (33,35-37). Stress signals such as ROS and others exogenous agents can modify the activity of APE/Ref-1 (33,35,36). For instance, APE/Ref-1 acts as a pivotal signaling factor in the induction of early stress response genes, such as c-Fos and c-Jun. At least one mechanism regulating c-Fos/c-Jun DNA binding is mediated by a conserved cysteine (Cys) located in the basic DNA-binding domain of both proteins (62). In vitro these regulatory cysteines are not permissive for DNA binding under oxidized conditions, whereas reduction to a sulphydryl state promotes DNA binding (35,62). As such, these critical cysteines act as a redox-sensitive “sulphydryl switch” that reversibly modulates DNA binding (36). In the absence of reducing agents, the redox factor-1 (APE/Ref-1) protein regulates c-Fos/c-Jun DNA binding via the same conserved cysteine.

APE/Ref-1 is found exclusively in the cytoplasm of some cells, in the nucleus in others and in some cases it is localized both in the cytoplasm and nucleus (40). The biological relevance of APE/Ref-1 compartmentalization is not understood, but the complexity of staining patterns suggests that localization is regulated. The redox role of APE/Ref-1 may be relevant to its cytoplasmic localization, where it may be required to maintain newly synthesized transcription factors in a reduced state while they are being transported to the nucleus (40). We have previously demonstrated that the DNA binding activity of the Prd domain of Pax proteins is regulated through the oxidation–reduction of conserved cysteine pairs and that Pax5a activity is regulated through a redox mechanism involving APE/Ref-1 (57). Furthermore, we have shown that exposure of B cells to H$_2$O$_2$ results in rapid transfer of the cytoplasmic redox factor APE/Ref-1 into the nucleus and this provide evidence with an increase in Pax5a binding activity (33).

CD40 is involved in different important aspects of B cells activation such as B cell proliferation, differentiation, isotype switching, upregulation of surface molecules, development of the germinal center, and the humoral memory response. CD40 engagement results in the production of reactive oxygen intermediates, which serve as second messengers in cell signaling (63). In this work we investigate if CD40 mediated stimulation of B lymphocytes could promote activation of EBF and Pax5a. We show that both these transcription factors are
constitutively expressed in spleen B cells. Moreover, we provide evidence for the first time that APE/Ref-1 acts as a key signaling intermediate in response to CD40 mediated B cell activation. We observe that APE/Ref-1 translocates from the cytoplasm to the nucleus of activated B cells. We demonstrate that CD40 mediated activation of spleen B cells enhances the DNA-binding activity of Pax5 and EBF. APE/Ref-1 appears to be required for CD40 mediated Pax5 activation, as the repression of APE/Ref-1 protein production is able to block CD40 induced Pax5 DNA binding activity.

Based on earlier in vitro data indicating that Pax5a and EBF are capable to act in cooperation to induce blk gene expression (50) and our current observations that CD40 triggering stimulates DNA binding activity of both these transcription factors, we sought to investigate if APE/Ref-1 could have a direct effect on EBF DNA binding activity. Here we show both in vitro and in vivo that APE/Ref-1 could modulate EBF activity.

In in vitro experiments, we show that recombinant GST-EBF loses its DNA binding activity upon oxidation, which is reacquired upon with recombinant APE/Ref-1 but not in presence of other reducing agents (e. g. DTT or β-ME) or in presence of APE/Ref-1 immunodepleted NS-1 cellular extracts demonstrating that APE/Ref-1 can directly modulate EBF DNA-binding activity.

In in vivo experiments we provided evidence that the cooperative activation of the blk promoter operated by Pax5a and EBF (50) is dependent on a whole functional Pax5a protein and that APE/Ref-1 can modulate this functional cooperation. In fact, EBF does not show cooperative activation of the blk promoter when cotransfected with Pax5d (an isoform of Pax5 which possess an intact DNA-binding domain but does not have a transactivation domain and partial homeodomain homology region). This suggests that the transactivation domain, the partial homeodomain homology region of Pax5 or both play a role in the functional cooperation with EBF. Moreover, for the first time we provide evidence that APE/Ref-1 is able to enhance the cooperative activation of Pax5a and EBF on the blk promoter, because cotransfection of APE/Ref-1 with Pax5a and EBF has more than additive effects on reporter expression. We also show that APE/Ref-1 increases EBF and Pax5a mediated blk promoter activation, confirming our in vitro data. Taken together, these data show that both Pax5a and EBF are sensitive to APE/Ref-1 regulation and also that their functional cooperation on the blk promoter can be modulated by APE/Ref-1.
In addition, in vitro EMSA experiments show a gain of EBF DNA binding activity on its consensus sequence when Pax5a is present and in particular that Pax5a can influence the EBF functional activity establishing a molecular complex. This is confirmed by GST-EBF pull-down assay that show that there is a physical interaction between the two proteins and this interaction appear to be independent from the presence of the DNA.

APE/Ref-1 might contribute to B cell activation linking extracellular generated signals with the regulation of key target genes, as it translocates from the cytoplasm to the nucleus and regulates B cell specific transcription factors following the triggering of CD40 or the exposure to ROS. Given the ability of APE/Ref-1 to control the DNA-binding activity of Pax5a and Pax5a-EBF complexes, we propose that APE/Ref-1 could act as a key signaling intermediate in B cell activation and thus in the regulation immune responses.

FOOTNOTE

1The abbreviation used are: DTT, dithiothreitol; β-ME, β-Mercaptoethanol; PAGE, Polyacrylamide Gel Electrophoresis; mAb, monoclonal antibody; IPTG, Isopropyl β-D-1-Thiogalactopyranoside; BSA, Bovine Serum Albumine; PMSF, Phenyl-Methyl-Sulfonyl Fluoride; CMV, Cytomegalovirus; luc, luciferase; GST, Glutathione S-Transferase; SDS, Sodium Dodecyl Sulfate; β-gal, β-galactosidase; EMSA, Electrophoretic Mobility Shift Assay; PBS, Phosphate Buffered Saline; FCS, Fetal Calf Serum; PCR, Polymerase Chain Reaction; HRP, Horseradish Peroxidase; Tc, tetracycline; ROS, Reactive Oxygen Species.

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FIGURE LEGENDS

Fig. 1. CD40 crosslinking enhances DNA binding activity of Pax5a and EBF in primary mouse B cells
A) EMSA detects Pax5a functional activity in mouse B cells stimulated and not stimulated with anti-CD40 mAb (1µg/ml). Nuclear extracts (8µg) were incubated with 32P labeled probe encompassing the promoter of the sea urchin histon H2A-2.2.
B) EMSA detects EBF functional activity in mouse B cells stimulated and not stimulated with anti-CD40 mAb. Nuclear extracts (8µg) were incubated with 32P labeled probe encompassing the promoter of mb-1.
C) EMSA detects CREB functional activity in the same extracts of panel A and B, as control.
In all the experiments nuclear extracts from Raji cell were used as positive control, the protein-DNA complexes were separated on 5% polyacrylamide gel and competitors were added in molar excess as indicated. F indicates the migration of free probe. The arrows indicate the position of protein-DNA complexes.

Fig. 2. Nuclear translocation of APE/Ref-1 in CD40-stimulated spleen B cells
A) Nuclear (Nuc.) and cytoplasmic (Cyt.) proteins from HeLa cells, mouse B cells stimulated with anti-CD40 mAb (1µg/ml) for 72 h and mouse B cells not stimulated were analyzed by western blot using anti-APE/Ref-1Ab.
B) Western blot analysis with the anti-Pax5a Ab D2A8 on nuclear extracts from HeLa cells, mouse B cells stimulated and not stimulated with anti-CD40 mAb.
The same filter was reprobed with Abs anti-actin and anti nucleoporin as controls for the purity of the nuclear and cytosolic fractionation.

Fig. 3. APE/Ref-1 modulates CD40-induced Pax5a activity
A) Specificity downregulation of APE/Ref-1 by antisense oligonucleotides. Western blot analysis using anti-APE/Ref-1 Ab (upper panel) and anti-Pax5a D2A8 Ab (lower panel) on equal amount (15µg) of nuclear extracts from mouse B cells stimulated with anti-CD40 mAb and from mouse B cells pretreated with APE/Ref-
1 sense (S) or with APE/Ref-1 antisense (AS) and then stimulated with anti-CD40 mAb

B) Mouse B cells unstimulated, mouse B cells stimulated with anti-CD40 mAb or stimulated with anti-CD40 mAb after the transfection with APE/Ref-1 antisense (AS) and sense (S) were analyzed by EMSA. Nuclear extracts (15 µg) were incubated with 32p labeled probes specific for Pax5a and CREB (from left to right). F indicates the migration of free probe. The arrows indicates the position of protein-DNA complexes.

**Fig. 4. APE/Ref-1 is involved in EBF DNA binding activity**

A) EMSA of 32P labeled probe encompassing the promoter of mb-1, specific for EBF using 100 ng of GST-EBF. Nuclear extracts (8µg) from Raji cells were used as positive control. The protein-DNA complexes were separated on 5% polyacrylamide gel. F indicates the migration of free probe. The arrows indicates the position of protein-DNA complexes.

B) EMSA of 32P labeled EBF specific probe mb-1 using 100 ng GST-EBF oxidized protein, obtained by prolonged air exposure, without or with 400 ng of APE/Ref-1 recombinant protein (rAPE/Ref-1) (lane 1 and 2 respectively) or with 10 µg of crude or APE/Ref-1 depleted NS-1 cellular extracts (lane 3 and 4). As control, the presence of no specific interactions was tested with NS-1 cellular extracts alone before (lane 7) and after boiling (lane 8). HeLa (lane 9) total cellular extracts were used as additional positive control because this cell line express endogenous APE/Ref-1. Different size of the EBF complexes can be seen in the two cellular extracts probably reflecting that NS1 cells are a mieloma cell line different from the HeLa cell line and the complex between GST-EBF and mb1 probe will be influenced by the cellular environment. The asterisk (*) indicates the NS-1 cellular extracts boiled and then incubated with the probe. F indicates the migration of free probe.

**Fig. 5. APE/Ref-1 affects the blk promoter activation mediated by Pax5a and EBF.**

A) Transfections of HeLa-tTA2 cells with blk promoter luc construct and the same amounts of the indicated bidirectional expression vectors (pBI) using calcium phosphate method. The DNA content in each transfection
was equalized by the addition of pUC18 plasmid.

B) Transient co-transfections of HeLa-cells with \textit{blk} promoter luc reporter with CMV-EBF plasmid and increasing amounts of CMV-APE/Ref-1 (in ng DNA).

C) Transient co-transfections of HeLa-cells with \textit{blk} promoter luc reporter with CMV-Pax5a plasmid and increasing amounts of CMV-APE/Ref-1 (in ng DNA).

pcDNA3.1 DNA was added to maintain equal amounts of total DNA per sample. The luciferase data, assessed 48 h later, were normalized for transfection efficiency with co-transfected CMV controlled-β-gal plasmid. Results of luc assays are shown as fold induction of luc conversion + S.D. Relative-fold induction values for each transfection were calculated by dividing each of normalized luc activity by the activity of the reporter construct alone. Bars indicate the means + S.D. of at least three independent experiments.

**Fig. 6. Pax5a physically interacts with EBF and modulates its DNA-binding activity**

A) EMSA of $^{32}$P labeled EBF specific probe \textit{mb-1} using 100 ng of oxidized GST-EBF without or with rAPE/Ref-1 (lane 1 and lane 2 respectively), with rPax5a (lane 3), with the two proteins together (lane 4), or with extract from NS-1 Pax5a stable transfected (NS-1/ Pax5a) (lane 5). rPax5a (lane 6) and NS-1/Pax5a cellular extracts (lane 8 and 9) were used as control. The bands observed in presence of NS-1/Pax5a are not specific as confirmed by using the competitor (lane 9). F indicates the migration of free probe.

B) Pull down assay using nuclear extracts of HeLa cells transfected with Pax5a (lane 1) incubated with affinity bound to Sepharose GST-EBF (lane 2) or GST alone (lane 3). Proteins were analyzed in SDS-PAGE and western blot using anti-Pax5a Ab D2A8. The Input represents 25% of the amount of nuclear extracts used in the binding experiment, as described in Experimental procedures.
Figure 3

A

anti-APE/Ref-1

anti-Pax5a

B

Competitor

Pax5a complex

H2A2.2 free probe

CREB complex

CREB free probe
Figure 4

A

B

| Competitor (molar excess) | GST-EBF oxidated | NS1 tot. extracts | HeLa tot. extracts | rAPE/Ref-1 | APE/Ref-1 depleted | Competitor 100x |
|---------------------------|------------------|-------------------|-------------------|------------|-------------------|---------------|
| Rail                      | +                | -                 | -                 | -          | -                 | -             |
| GST-EBF                   | +                | +                 | +                 | +          | +                 | -             |
| Competitor 100x           | -                | -                 | -                 | -          | +                 | -             |

mb-1 free probe

EBF complex
Figure 6

A

|           | 1  | 2  | 3  | 4  | 5  | 6  | F  | 8  | 9  |
|-----------|----|----|----|----|----|----|----|----|----|
| GST-EBFox | +  | +  | +  | +  | +  | -  | -  |    |    |
| rAPE/Ref-1| -  | +  | -  | +  | -  | -  | -  |    |    |
| rPax5a    | -  | -  | +  | +  | -  | -  | -  |    |    |
| NS-1/Pax5a| -  | -  | -  | -  | +  | -  | +  | +  | +  |
| Comp. 100x| -  | -  | -  | -  | -  | -  | -  | -  | +  |

EBF complex

mb-1 free probe

B

|           | 1  | 2  | 3  |
|-----------|----|----|----|
| Input     |    |    |    |
| GST-EBF   |    |    |    |
| GST       |    |    |    |

Pax5a
CD40 stimulation induces PAX5/BSAP and EBF activation through a APE/REF-1 dependent redox mechanism

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