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The PKC targeting protein RACK1 interacts with the Epstein–Barr virus activator protein BZLF1

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Phorbol esters reactivate Epstein–Barr virus (EBV) from latently infected cells via transcriptional activation of the viral immediate-early gene BZLF1. BZLF1 is a member of the extended AP-1 family of transcription factors that binds to specific BZLF1-binding motifs within early EBV promoters and to consensus AP-1 sites. Regulation of BZLF1's activity is achieved at the transcriptional level as well as through post-translational modifications. Recently, we reported that the transcriptional activity of BZLF1 is augmented by TPA [Baumann, M., Mischak, H., Dammeier, S., Kolch, W., Gires, O., Pich, D., Zeidler, R., Delecluse, H. J. & Hammerschmidt, W., (1998) *J. Virol.* **72**, 8105–8114]. The increase of BZLF1's activity depends on a single serine residue (S186) that is phosphorylated by protein kinase C (PKC) *in vitro* and *in vivo* after stimulation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Here, we identified RACK1 as a binding partner of BZLF1 in a yeast interaction trap assay. RACK stands for receptor of activated C-kinase and is involved in targeting activated PKCs and other signaling proteins. *In vivo*, RACK1 binds directly to the transactivation domain of BZLF1. Although a functional relationship between BZLF1 and PKC could be mediated by RACKs, RACK1 did not have a detectable effect on the phosphorylation status of BZLF1 in *in vitro* or *in vivo* phosphorylation assays. We suggest that RACK1 may act as a scaffolding protein on BZLF1 independently of activated PKCs.

Keywords: BZLF1; interaction; phosphorylation; PKC; RACK1.

Epstein–Barr virus (EBV) is a human herpesvirus associated with different lymphoid and epithelial cell malignancies. EBV infection is generally latent in B-cells (reviewed in [1]). In latently infected cells, induction of the lytic phase of EBV can be achieved by treatment with compounds such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or cross-linking of surface immunoglobulin [2,3] These reagents activate the expression of the viral immediate-early protein BZLF1, also termed EB1, Zta, Z, or ZEBRA [4–6].

BZLF1 is a sequence-specific DNA-binding protein related to the bZip family of transcription factors that transactivates several early lytic promoters via *cis*-acting ZRE (*Z*ta-responsive elements) DNA-binding motifs [7]. BZLF1 contains a C-terminal domain that mediates homodimerization through a coiled-coil interaction [8,9] and a basic region sharing sequence homology with the DNA-binding domain of members of the AP-1 family of transcription factors [7,10]. As a consequence, BZLF1 binds to TPA-responsive elements (TREs) or AP-1 sequence motifs with high affinity. The amino-terminal domain of BZLF1 plays a role in activation of transcription [11,12] and is also directly involved in the activation of the EBV lytic origin of DNA replication, *oriLyt* [13–15].

The key role of BZLF1 in maintaining a balance between EBV latent and lytic replication is reflected by transcriptional control of the *BZLF1* gene and by post-translational modification, namely phosphorylation, of the BZLF1 protein [16–18]. BZLF1 is phosphorylated by protein kinase C (PKC) *in vitro* and *in vivo* following TPA treatment resulting in enhanced transcriptional activity and enhanced DNA-binding of the BZLF1 protein [18].

PKC is a serine/threonine protein kinase that is crucially implicated in the transduction of a variety of signals that alter cellular functions and proliferation. Upon activation by diacylglycerol (DAG) or phorbol esters like phorbol-12-myristate13-acetate (PMA) or TPA, PKCs associate with the particulate or membrane cellular fraction whereas the inactive kinases are preferentially found in the soluble cytosolic compartment [19]. This phenomenon is called translocation and several proteins have been identified that might be targets of activated and translocated PKCs (reviewed in [20]). Among these is a group of proteins collectively termed RACKs, for receptors for activated C-kinase (reviewed in [21,22]). RACKs are thought to interact only with activated PKC and appear to serve as anchor proteins that recruit PKC isoforms into a

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Abbreviations: 3-AT, 3-amino-1,2,4-triazole; BL, Burkitt's lymphoma; EBV, Epstein–Barr virus; GST, glutathione S-transferase; MBP, maltose-binding protein; PDE4, cAMP-specific phosphodiesterase; PKC, protein kinase C; PMA, phorbol-12-myristate13-acetate; PtdSer, phosphatidylserine; RACK, receptor of activated C-kinase; TAD, transactivation domain; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRE, TPA-responsive element.

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signaling complex with other proteins [23–25]. Recently, it has been reported that PKC activation caused the association and coordinated movement of RACK1 and PKCβII to the same intracellular sites [26].

The purpose of this study was to isolate and characterize BZLF1-interacting proteins that potentially control the activity of BZLF1. We focused on protein–protein interactions that involved the transactivation domain (TAD) of BZLF1. Using the yeast two-hybrid assay to screen a B-cell cDNA library, RACK1 was identified as a BZLF1 interactor. Since BZLF1 has been identified as a substrate for PKC recently [18], we addressed the question of whether the activation of BZLF1 by PKC-mediated phosphorylation is influenced by RACK1. As RACK1 does not affect the phosphorylation or activation status of BZLF1 *in vitro* and *in vivo*, we suggest that the binding of BZLF1 to RACK1 is unrelated to the interaction of RACK1 and PKCβ.

MATERIALS AND METHODS

Yeast two-hybrid assay

Saccharomyces cerevisiae strain Y190 (MATa, *ura*3–52, *his*3-Δ200, *ade*2-101, *trp*1-901, *leu*2-3,112, *gal*4Δ*gal*80Δ, URA3::GAL-*lac*Z, *cyh*^{r}2, LYS2::GAL-HIS3) [27] was transformed with a protocol using lithium acetate [28]. As a bait in the two-hybrid screen, a partial cDNA of BZLF1 of the B95.8 strain of EBV coding for amino acids 3–196 was fused to a cDNA encoding the GAL4 DNA binding domain in the centromer expression vector pGBT9 [29] to generate pADH-GAL4:BZLF1(amino acids 3–196). Two deletion mutants of this expression vector were constructed encoding deletions in the TAD of BZLF1 from amino acids 29–53 [pADH-GAL4: BZLF1(Δ29–53)] and amino acids 54–78 [pADH-GAL4: BZLF1(Δ54–78)]. For library screening, Y190 was transformed with pADH-GAL4:BZLF1(amino acids 3–196) to tryptophan prototrophy. A single colony was grown in synthetic minimal carbon source (2%, v/v, glucose) medium lacking tryptophan and transformed with a cDNA library in the expression vector pACT [30]. The cDNA library was obtained from an EBV-immortalized lymphoblastoid cell line [30]. The transformed yeast was plated on SC medium lacking tryptophan, histidine, and leucine in the presence of 25 mM 3-amino-1,2,4-triazole (3-AT), incubated at 30 °C for 5 days, and colonies were screened for β-galactosidase activity as described [30].

Plasmids for protein expression in *Escherichia coli*

All proteins were expressed in *E. coli* DH5α [31] as glutathione-S-transferase (GST) or maltose-binding protein (MBP) fusions in pGEX-1λT (Pharmacia) or pMALc2 (New England Biolabs) vectors, respectively. The BZLF1-encoding cDNA sequence was derived from the EBV strain B95–8 [4]. Full length BZLF1 cDNA (amino acids 1–245) was inserted into a *Bam*HI/*Eco*RI-cut pGEX-1λT plasmid that provides a thrombin cleavage site between the two encoded protein domains. This recombinant plasmid was termed pGST: BZLF1(wt). pGST:BZLF1(amino acids 3–166) is also based on pGEX-1λT but encodes a C-terminally truncated form of BZLF1 encoding the transactivation domain only. RACK1 full-length cDNA was obtained by ligation of two overlapping partial cDNAs encoding N-terminal and C-terminal portions of RACK1. The complete RACK1 cDNA was inserted into *Hin*dIII/*Eco*RI-cut pMALc2 (pMBP:RACK1) encoding a fusion of MBP and RACK1. All plasmids were subjected to DNA sequence analysis of the relevant parts.

Recombinant eukaryotic plasmids

The reporter plasmid pBHRF1:luc was constructed by ligating a 7.2-kb *Bam*HI–*Sal*I fragment from the EBV strain B95.8 [32] carrying the wild type *oriLyt* into a *Bam*HI/*Sal*I-cut pUC 18 vector plasmid. To perform the transient transcription assays (see below), the 1.7-kb *Bgl*II–*Sal*I fragment carrying the BHRF1 open reading frame was replaced by the luciferase reporter gene (luc) [33] to yield pBHRF1:luc. Construction of the plasmid pCMV:BZLF1(wt) has been described in detail elsewhere [34]. pCMV:BZLF1(Δ26–88) was generated by excision of an internal *Hin*dIII fragment in the transactivation domain of BZLF1. BZLF1 cDNA derived from the EBV strain B95.8 was cloned into the eukaryotic vector pRC/CMVΔNeo (Invitrogen) using a *Eco*RI restriction site and the plasmid was designated pT7:BZLF1. Full-length RACK1 cDNA was inserted into the *Bam*HI-cut eukaryotic cloning vector pcDNAI/Amp (Invitrogen) and the plasmid was termed pCMV:RACK1. Alternatively, the RACK1 fragment was ligated with a Flag-epitope in a *Bgl*II-cut CMV-driven expression vector designated pCMV:Flag-RACK1. In these constructs, BZLF1, RACK1 or Flag-RACK1 can be either expressed from the CMV promoter *in vivo* or the T7 promoter *in vitro* using T7 polymerase (see below).

Cell lines and transformation of primary B-cells

Human embryonic kidney 293 cells, the EBV-negative Burkitt's lymphoma cell line BL41, the EBV-positive Burkitt's lymphoma cell line RAJI and the lymphoblastoid cell line B95.8 were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Human primary B-lymphocytes were prepared and purified from routine tonsillectomies by generating single cell suspensions and depletion of T-lymphocytes by rosetting with sheep red blood cells as described [35]. The B-cells were infected with filtered B95.8 virus stocks obtained from B95.8 cell line supernatants and plated at a dilution of 3.5×10^5 cells per well in 96-well cluster plates in RPMI 1640 supplemented with 10% fetal bovine serum on a lethally irradiated (50 Gy) human feeder cell layer (WI38) previously described (33).

RNA isolation and Northern-blot analysis

Total RNA was extracted from exponentially grown yeast cells, cultured B-cell lines or primary B-cells by the guanidine isothiocyanate method [36]. Total RNA (10 μg) were fractionated by electrophoresis on a denaturing agarose gel, transferred to a nylon membrane, and hybridized with a DNA probe specific for BZLF1 or RACK1. In the latter case, the DNA probe was generated by PCR amplification of the RACK1 cDNA in pMBP:RACK1 using the RACK1-specific primers RACK1 forward (5′-CGACACACGCTCTCGCCG-3′) and RACK1 backward (5′-GCTCTGCCATAAACTTCTAGC-3′) followed by random primed ^{32}P-labeling (Boehringer Mannheim). The BZLF1-specific DNA probe was generated by random primed ^{32}P-labeling of a 371-bp internal *Bam*HI/*Xho*I fragment present in BZLF1(amino acids 3–196) as well as in the deletional BZLF1 mutants (Δ29–53 and Δ54–78).

Nuclear extraction

The 293 cells were transfected with 10 μg of the plasmid pCMV-BZLF1-wt or a control vector using Lipofectamine (GibcoBRL Life Technologies). 30 h after transfection, cells were harvested and resuspended in 300 μL of hypotonic buffer A [10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, protease inhibitors (0.5 $\mu g \cdot mL^{-1}$ leupeptin, 1 $\mu g \cdot mL^{-1}$ pepstatin, 1 mM phenylmethanesulfonyl fluoride, 50 mM benzamidin, 1 mM pefabloc, 0.5 $\mu g \cdot mL^{-1}$ aprotinin] and incubated on ice for 30 min. Permeablized nuclei were recovered by brief centrifugation. The supernatant represents the cytoplasmic extract. To obtain the nuclear fraction, the crude nuclear pellet was resuspended in 200 μL of hypertonic buffer B (20 mM Hepes, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 25%, v/v, glycerol) followed by incubation on ice for 30 min. After centrifugation, the supernatant was used for immunoblot analysis with the anti-BZLF1 or the anti-RACK1 Ig.

Western blot analysis

To run equal amounts of protein per sample, cellular extracts were measured for their protein content using the BCA protein assay reagent (Pierce). Protein samples were boiled in Laemmli buffer, subjected to SDS/PAGE on 12.5% PAGE gels and electrotransfered onto nitrocellulose membranes. BZLF1 was detected by Western blotting using either a monoclonal antibody recognizing an epitope between amino acids 59 and 93 (Z125) or a monoclonal antibody recognizing an epitope between amino acids 177 and 196 (Z130) of BZLF1 [37], a horseradish peroxidase-conjugated goat anti-(mouse IgG) Ig (Promega) and ECL detection reagent (Amersham). Detection of RACK1 was performed with a monoclonal mouse IgM antibody (Transduction Laboratories) in combination with a horseradish peroxidase-conjugated rabbit anti-(mouse IgM) Ig (Dianova).

Bacterial protein synthesis and purification

Escherichia coli DH5α strains transformed with pGST:BZLF1 and pMBP:RACK1 were grown in 400 mL of Luria–Bertani medium to an D_{600} of 0.5 and induced for 2 h with 1 mM isopropyl thio-β-D-galactoside at 30 °C. Cells were harvested and the bacterial pellet was resuspended in 5 mL of cold lysis buffer (1× Tris/EDTA, 1% Triton X-100, and protease inhibitors as mentioned above) and sonified for 5 min. After ultracentrifugation at 110 000 ***g*** for 1 h, the cleared extracts were bound to Sepharose beads coupled with either glutathione (Pharmacia) or amylose (New England Biolabs). Where indicated, BZLF1 was released by enzymatic cleavage with 1 U of thrombin (Sigma) for 30 min from its fusion with GST. MBP-RACK1 was eluted with 10 mM maltose from the amylose matrix. Proteins were analyzed by SDS/PAGE [38] and Coomassie staining. Protein content was determined by standard procedures.

In vitro translation

pT7:BZLF1 and pCMV:RACK1 were used to produce BZLF1 and RACK1 polypeptides by *in vitro* transcription/translation using a T7 polymerase (Promega) and rabbit reticulocyte lysate in the presence of 40 μCi [^{35}S]methionine according to the procedure suggested by the manufacturer.

Protein-binding assays

In vitro *binding assays.* Bacterial lysates (0.5 mL) containing either GST, GST:BZLF1(3–166), MBP, or MBP:RACK1 was mixed with 20 μL of glutathione-Sepharose or amylose-Sepharose, respectively. Following incubation for at least 1 h, the resin was washed with binding buffer (20 mM Hepes pH 7.7, 75 mM KCl, 0.1 mM EDTA, 25 mM $MgCl_2$, 10 mM dithiothreitol, 0.15% (v/v) NP40, and protease inhibitors as mentioned above) and *in vitro* translated ^{35}S-labeled RACK1 or BZLF1 were added. After incubation for 1 h at 4 °C, the resin was washed with binding buffer and bound fractions were eluted by boiling in Laemmli buffer, fractionated by SDS/PAGE, and visualized by autoradiography.

In vivo *interaction assays.* For the assays 5×10^6 293 cells were transiently transfected with 10 μg of pCMV:BZLF1(wt) or pCMV:BZLF1(Δ26–88). Where indicated, cells were treated with 100 $ng \cdot mL^{-1}$ TPA for 12 h. Sixteen hours after transfection, cells were lysed in NaCl/Tris (25 mM Tris/OH pH 7.5, 137 mM NaCl, 2.7 mM KCl) containing 1% Triton X-100 and protease inhibitors as mentioned above. The cell lysate was incubated with protein A–Sepharose beads (Pharmacia) coupled with rabbit anti-(mouse IgM) Ig and the RACK1-specific monoclonal mouse IgM antibody. As a negative control, an aliquot of the cell lysate was incubated with protein A–Sepharose beads coupled only with rabbit anti-(mouse IgM) Ig. Incubation was carried out overnight at 4 °C. After washing with lysis buffer (NaCl/Tris plus 1% Triton X-100), the beads were aliquotated and washed twice either in lysis buffer, RIPA buffer (150 mM NaCl, 20 mM Tris/HCl pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5%, v/v, deoxycholic acid), or Tris/EDTA (10 mM Tris/OH pH 8.0, 1 mM EDTA) containing 0.5 M NaCl. Bound proteins were released by boiling in Laemmli buffer, separated by SDS/PAGE and blotted onto a nitrocellulose membrane. Precipitated RACK1 and BZLF1 were visualized by Western blotting techniques as described above.

Transient transcription assay

For the assay 5×10^6 BL41 cells were transfected by electroporation using 5 μg of pBHRF1:luc together with 2 μg of pCMV:BZLF1(wt) and 2 μg of pCMV:RACK1. Eight hours after transfection, cells were treated with 100 $ng \cdot mL^{-1}$ TPA for 12 h. The luciferase activity was measured in cell extracts as described in detail [13].

In vitro phosphorylation

Purified BZLF1 or histone proteins isolated from bovine thymus (Sigma) were phosphorylated in a reaction mixture (15 μL) containing kinase buffer (25 mM Tris/HCl pH 7.5, 1.32 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM EDTA, 1.25 mM EGTA, 1 mM dithiothreitol) supplemented with 10 nM TPA, 5 $\mu g \cdot mL^{-1}$ phosphatidylserine (PtdSer), 5 μM ATP and 0.5 μCi [γ-^{32}P]ATP. Reactions were incubated at room temperature with purified PKC isoforms for 15 min. Products were subsequently separated by electrophoresis on 12.5% SDS/PAGE and visualized by Coomassie staining and autoradiography. Signal intensities were quantified by a phosphorimager (Fuji).

In vivo labeling

The 293 cells were transfected with 10 μg of pCMV:BZLF1(wt) or pCMV:BZLF1(Δ26–88) as described

above. 12 h after transfection 293 cell cultures (in 90-mm dishes) were rinsed twice with NaCl/P_i and incubated in 5 mL of phosphate-free Dulbecco's modified Eagle's medium (Sigma) for 3 h; 2.5 mCi [^{32}P]orthophosphate (Amersham) was then added. After a 4 h labeling period (the last hour in the presence or absence of 100 ng·mL^{-1} TPA), the cells were washed twice with ice-cold NaCl/P_i and labeled proteins were immunoprecipitated from cell lysates with the Z130 monoclonal antibody directed against BZLF1 coupled to protein G–Sepharose (Pharmacia). Bound proteins were eluted by boiling in Laemmli buffer and analyzed by SDS/PAGE. The SDS gel was blotted onto a nitrocellulose membrane and the labeled proteins were detected by autoradiography (data not shown). The amount of immunoprecipitated BZLF1 was evaluated by immunodetection with the biotinylated Z125 (data not shown).

Phosphopeptide mapping

BZLF1 (wt or Δ26–88) was radiolabeled *in vivo,* immunoprecipitated and subjected to SDS/PAGE as described above. After autoradiography the BZLF1 band was excised from the corresponding area of the gel, washed extensively with water, and digested to completion with trypsin in 50 mM NH_4HCO_3 at 37 °C overnight. After trypsin treatment, the released peptides were subjected to lyophilization, resuspended in water, and applied to HPLC or TLC sheets for two-dimensional peptide mapping. Separation of the labeled phosphopeptides was performed in the horizontal dimension by electrophoresis at pH 1.9 for 20 min at 1000 V and in the vertical dimension by ascending chromatography.

RESULTS

Isolation of RACK1 as a BZLF1-binding protein

The interaction of BZLF1 with different cellular factors has been reported by several groups. These include the p65 subunit of NF-κB [39], the retinoic acid receptors RAR and RXR [40,41], and the tumor suppressor protein p53 [42]. To identify other cellular partners interacting with BZLF1, we performed a two-hybrid screen in *S. cerevisiae* with the transcriptional activation domain of BZLF1 as a bait (Fig. 1A). Among several positive pACT plasmids, the clone X28.1 contained a partial human cDNA of RACK1. RACK1 is a 36-kDa protein consisting of 317 amino acids that form seven repeating units of Trp-Asp (WD) motifs [43]. The cDNA insert in X28.1 codes for a peptide from amino acids 70–317 of the highly homologous rat protein [44] and corresponds to nucleotide coordinates from 313 to 1075 of the human cDNA, which has also been described as guanine nucleotide-binding protein β-subunit-like protein on the basis of sequence homology searches [45]. The missing 5′ part of the human cDNA of X28.1 was obtained via RT-PCR to assemble the complete coding sequence for human RACK1.

Mutational analysis was performed in yeast to identify the RACK1-binding domain. Two consecutive deletion mutants of the BZLF1 bait, GAL4:BZLF1(Δ29–53) and GAL4:BZLF1(Δ54–78), were used in the yeast two-hybrid experiment (Fig. 1A). These mutants lack parts of the transactivation domain from amino acids 29–78, which are relevant for transcriptional activation and DNA replication of *oriLyt* [14,46]. Both BZLF1 mutants are expressed in *S. cerevisiae* (Fig. 1B) but failed to interact with X28.1, indicating that residues 29–78 are necessary to bind RACK1 in yeast (Fig. 1C).

Specificity of the RACK1–BZLF1 interaction

To further analyse the interaction between RACK1 and BZLF1, we first assured the expression of RACK1 in different B-cells by Northern-blot analysis. As shown in Fig. 2A, RNAs obtained from B-cells including EBV-immortalized B-cell lines contained readily detectable RACK1 transcripts suggesting that RACK1 is available for a putative interaction with BZLF1 in EBV-infectable cells. Next,

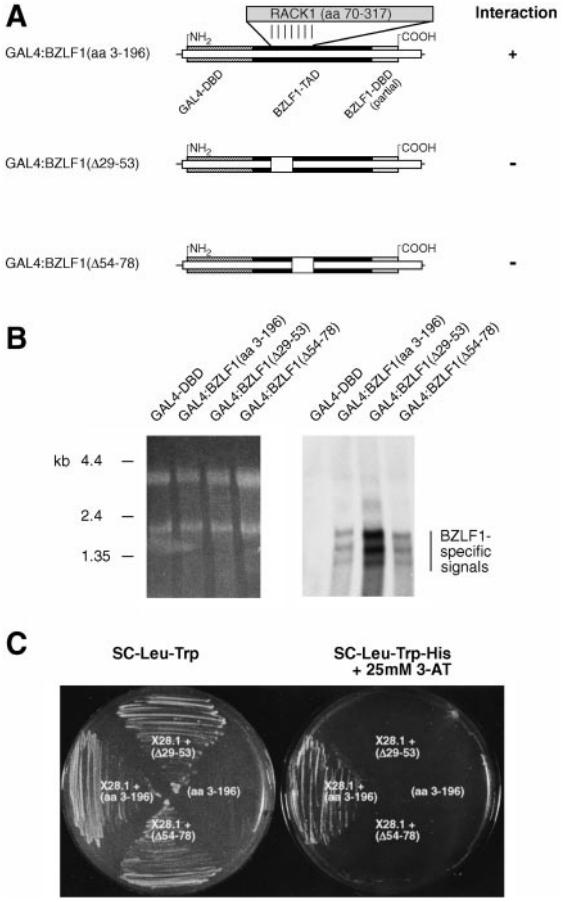


Fig. 1. Identificaton of RACK1 as a protein interacting with BZLF1 in *S. cerevisiae*. (A) Scheme of the yeast two-hybrid screen. A chimeric bait consisting of the GAL4-DBD fused to the TAD and a small part of the DNA-binding domain of BZLF1 was used to identify potential interacting partners of BZLF1 in the yeast strain Y190. A partial cDNA of RACK1 was isolated that encodes amino acids 70–317 but lacks the N-terminal part of RACK1. This isolate X28.1 was unable to interact with the two mutant bait plasmids derived from GAL4:BZLF1(amino acids 3–196) that encode deletions in the TAD of BZLF1 (Δ29–53 and Δ54–78). (B) Northern blot analysis of wild-type and mutant GAL4:BZLF1 transcripts. Total RNA prepared from the indicated yeast clones used for two-hybrid screening [GAL4-DBD or GAL4:BZLF1(amino acids 3–196, Δ29–53, and Δ54–78)] was separated on an agarose gel (left panel) and visualized by autoradiography (right panel) after hybridization with a labeled BZLF1-specific DNA probe. (C) Growth of yeast transformants under selective conditions. Y190 was transformed with expression plasmids for GAL4:BZLF1(amino acids 3–196, Δ29–53, and Δ54–78) and X28.1 or GAL4:BZLF1(amino acids 3–196) alone and streaked on SC medium plates without tryptophan and leucine. Likewise, transformants were streaked on a minimal medium lacking leucine, tryptophan, and histidine in the presence of 25 mM 3-amino-1,2,4-triazole (3-AT). Growth is shown after five days of incubation at 30 °C.

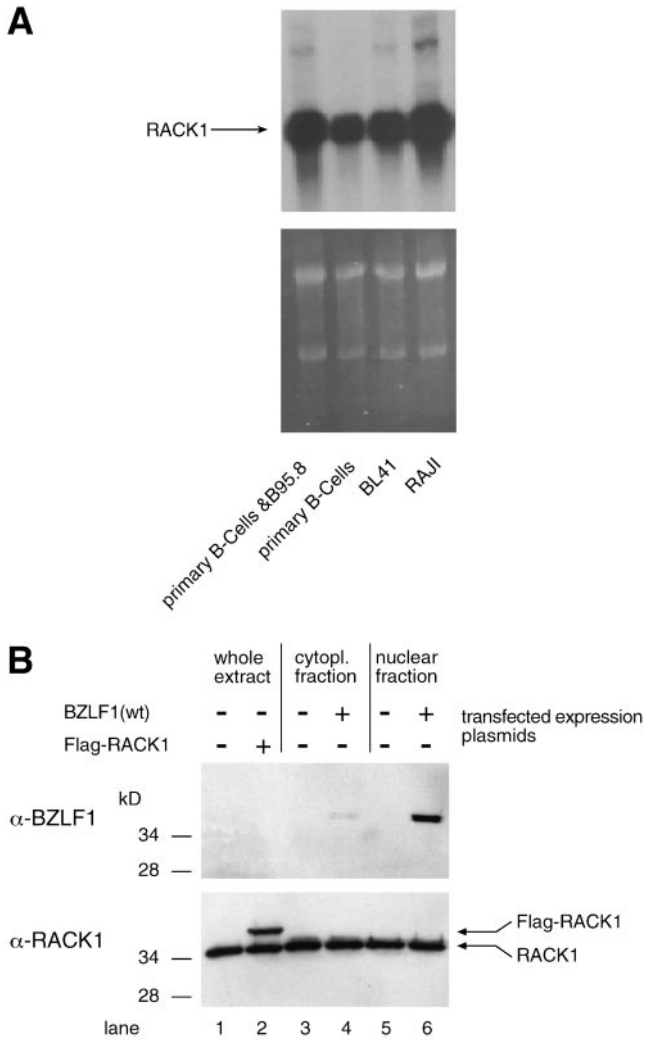


Fig. 2. Detection of RACK1 in EBV-positive and EBV-negative cell lines. (A) Northern blot analysis of RACK1 in primary and EBV-immortalized B-cells. RNA probes obtained from B-cells immortalized with the EBV strain B95.5, primary B-cells, the EBV-negative Burkitt's lymphoma cell line BL41, and the EBV-positive Burkitt's lymphoma cell line RAJI were separated on an agarose gel (lower panel) and visualized by autoradiography (upper panel) after hybridization with a labeled RACK1-specific DNA probe. (B) Western blot analysis of RACK1 and BZLF1 in 293 cells. Lanes 1 and 2: 293 cells were transfected with pCMV:Flag-RACK1 or a control vector prior to preparation of crude extracts from transfected cells. Lane 3–6: 293 cells were transfected with pCMV:BZLF1(wt) or a control vector followed by preparation of cytoplasmic and nuclear fractions. BZLF1, endogenous RACK1 and Flag-RACK1 were detected by immunoblotting as described in Materials and methods.

to determine if BZLF1 and RACK1 are localized in the same cellular compartment, cytoplasmic and nuclear fractions were prepared from BZLF1 transfected cells and analysed for the presence of RACK1 and BZLF1, respectively. Whereas RACK1 was nearly equally expressed in the nucleus and in the cytoplasm, BZLF1 was only detectable in the nuclear fraction (Fig. 2B, lanes 3–6). This finding suggests that the interaction between BZLF1 and RACK1 takes place within or at the nuclear border where RACK1 is preferentially found in unstimulated cells (reviewed in [47]).

We then examined the interaction of RACK1 and BZLF1 *in vitro* by pull-down assays with recombinant proteins synthesized in *E. coli*. For this purpose, a GST fusion protein containing the transactivation domain of BZLF1, GST:BZLF1(amino acids 3–166), was immobilized on glutathione-Sepharose and incubated with ^{35}S-labeled *in vitro* translated full-length RACK1. The BZLF1 transactivation domain efficiently interacted with RACK1 in this assay (Fig. 3A). The same held true when immobilized MBP:RACK1 was incubated with ^{35}S-labeled BZLF1, indicating specific and direct protein–protein interactions between BZLF1 and RACK1 *in vitro*.

To investigate an *in vivo* interaction between BZLF1 and RACK1, we transiently transfected 293 cells with pCMV:BZLF1(wt). The cell lysate was incubated with a RACK1-specific antibody coupled to protein A–Sepharose beads and precipitates were obtained under different conditions and tested for the presence of RACK1 and BZLF1 (Fig. 3B). BZLF1 and RACK1 coprecipitated in the presence of 140 mM NaCl (NaCl/Tris) and 1% Triton. Increasing the salt concentration to 500 mM NaCl severely impaired binding of BZLF1 to RACK1 whereas an incubation under stringent conditions in RIPA buffer completely abolished interaction between these proteins. This finding demonstrates an interaction of both proteins under conditions that prevail in the cell. Furthermore, mutant BZLF1 harboring a deletion from amino acids 26–88 was unable to interact with RACK1 (Fig. 3B). These results confirmed the yeast two-hybrid experiment showing that the transactivation domain of BZLF1 is involved in the binding to RACK1.

In addition to PKC, RACK1 has been reported to interact with several other cellular proteins including the integrin β subunit [24], the Src tyrosine kinase [23], and the cAMP-specific phosphodiesterase (PDE4) isoform PDE4D5 [25]. Phorbol ester treatment is critical for the interaction of RACK1 with both PKC [26,44] and the integrin β subunit [24]. As shown in Fig. 3(C), coimmunoprecipitation of RACK1 and BZLF1 from cell lysates did not require phorbol ester treatment as has been demonstrated for the interaction of RACK1 with Src [23], PDE4D5 [25], and the common β-chain of the IL-5/IL-3/GM-CSF receptors [48].

Interaction between RACK1 and PDE4D5 is confined to the very first 88 N-terminal amino acids in PDE4D5 [25]. We explored whether BZLF1 and PDE4D5 share a structural similarity by aligning the corresponding domains of both proteins responsible for RACK1-binding. PDE4D5 shows an overall identity of 15% and additional similarity of 13% with BZLF1 within their RACK1-interacting domains (Fig. 3C), suggesting a rather conserved structure/function relationship.

RACK1 does not influence the transcriptional activity of BZLF1

Binding of PKC to RACKs has been suggested to play a role in PKC-mediated signal transduction [49–51]. As phorbol esters also enhance the transcriptional activity of BZLF1 via PKC-mediated phosphorylation of BZLF1 [18], we reasoned that RACK1 might facilitate this phosphorylation by linking activated PKC with its substrate BZLF1. To test this hypothesis, we cotransfected expression plasmids for RACK1 and BZLF1 and measured the transcriptional activity of BZLF1 after stimulation with TPA. As shown in Fig. 4, BZLF1 expression led to a 10-fold upregulation of a BZLF1-responsive promoter that could be increased by TPA 200-fold in the presence of BZLF1 as previously reported [18]. RACK1 coexpression did not affect the promoter activity in these

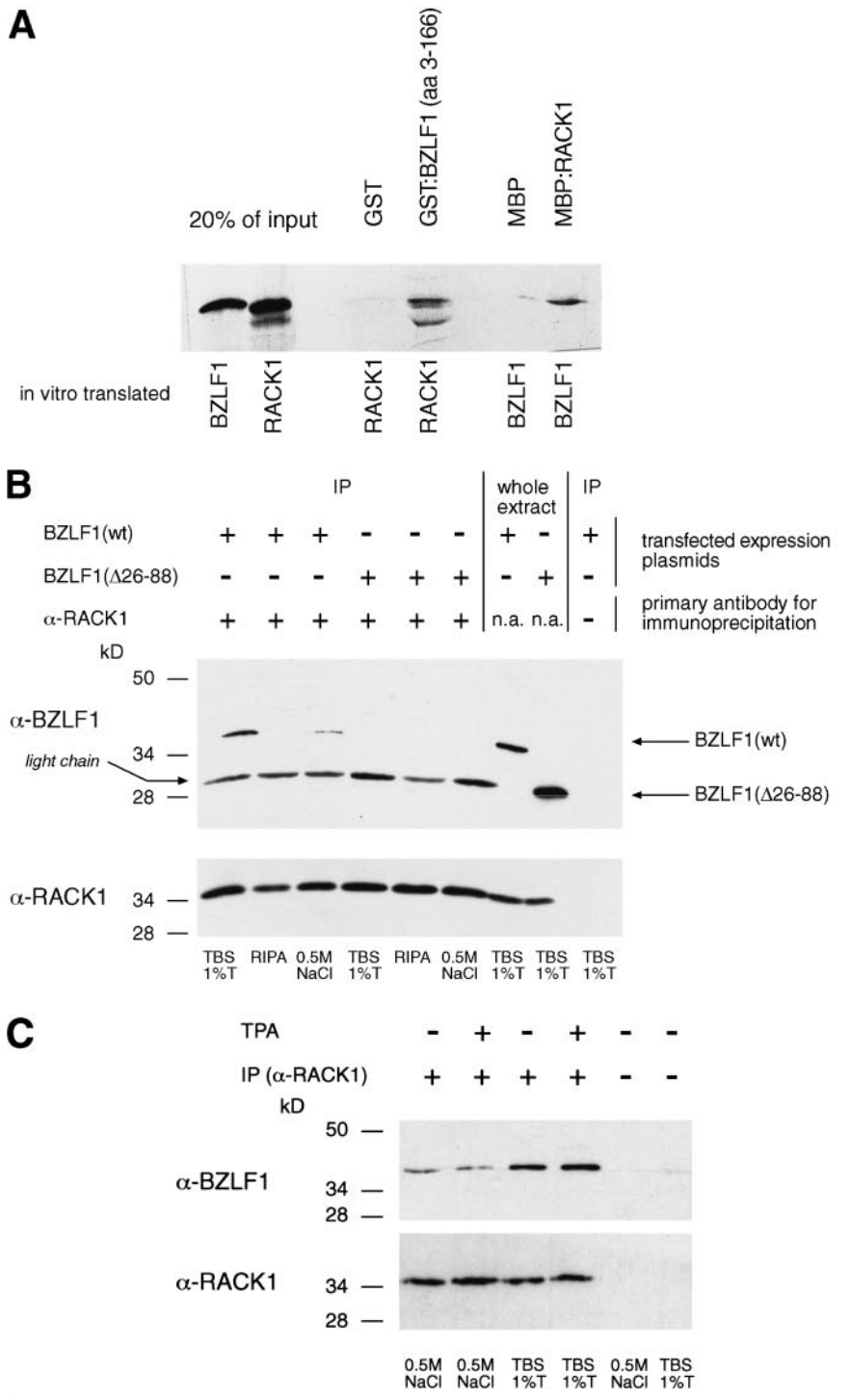


Fig. 3. Analysis of the interaction between RACK1 and BZLF1A. (A) GST pull-down assay. BZLF1 was transcribed and translated *in vitro* in the presence of [^{35}S]methionine and incubated with bacterially expressed MBP or MBP:RACK1 coupled to amylose–Sepharose beads. Vice versa, RACK1 was *in vitro* labeled and incubated with bacterially expressed GST or GST:BZLF1 (amino acids 3–166) coupled to glutathione-Sepharose beads. Bound fractions were analyzed by autoradiography. The input of labeled proteins used in the binding reaction is shown in the two leftmost lanes. The other lanes show the results of the binding experiments. (B) *In vivo* coimmunoprecipitation. 293 cells were transiently transfected with pCMV:BZLF1(wt) or BZLF1(Δ26–88). Cell lysates were incubated with a RACK1-specific antibody coupled to protein A–Sepharose beads. As a control, the RACK1-specific antibody was omitted. Following binding and washes in NaCl / Tris buffer containing 1% Triton-X100 (T), beads were aliquotated and washed with either NaCl / Tris plus 1% Triton-X100, RIPA buffer, or Tris/EDTA buffer containing 0.5 M NaCl. Precipitated RACK1 and BZLF1 were visualized by Western blotting. (n.a., not applicable). (C) *In vivo* coimmunoprecipitation. 293 cells were transiently transfected with pCMV:BZLF1(wt). Where indicated, cells were treated with TPA prior to lysis and incubation with protein A–Sepharose beads coupled or not with a RACK1-specific antibody. Beads were washed in Tris/EDTA buffer containing 0.5 M NaCl or in NaCl / Tris buffer containing 1% Triton-X100 following fractionation by SDS/PAGE. Precipitated RACK1 and BZLF1 were visualized by Western blotting. (D) Amino-acid sequence comparisons of the RACK1-binding region of PDE4D5 and BZLF1. The alignment was performed with the CLUSTAL W algorithm revealing 14 identical and 12 similar residues within 88 amino acids with three gaps inserted. Identical and related amino acids are shaded.

reporter assays suggesting that the transcriptional activity of BZLF1 in the presence of TPA is independent of RACK1.

Since RACK1 seems to be expressed ubiquitously at high levels (Figs 2 and 3B), endogenous amounts of RACK1 may be too high to permit exogenous overexpression having a detectable effect. We therefore tested the ratio of native and overexpressed recombinant RACK1 by transfecting an expression plasmid for Flag-tagged RACK1. As shown in Fig. 2B, lane 2, Flag-RACK1 could be distinguished from endogenous RACK1 due to retarded migration in SDS gels. The amount of recombinant Flag-RACK1 in the cellular extracts was almost comparable to the native protein (Fig. 2B, lane 1). Although coexpression of Flag-RACK1 increased the level of RACK1 twofold, no differences in the transcriptional activity of BZLF1 were apparent.

RACK1 does not influence PKC-mediated phosphorylation of BZLF1

A peptide derived from the pseudo-RACK1 site of PKC has been suggested to enhance the kinase activity of PKC when histone III was used as a PKC substrate in phosphorylation experiments *in vitro* [51]. As BZLF1 is readily phosphorylated by different PKC isozymes *in vitro* [18], we tested recombinant RACK1 in such phosphorylation assays to investigate whether it influences the rate of phosphate incorporation into BZLF1. According to a previous report [52], RACK1 binds PKC only in the presence of Ca^{2+} and lipid and may require activation of PKC. Therefore, we performed our assays in the presence of the PKC activators TPA and PtdSer. As shown in Fig. 5A, lanes 8 and 9, the presence of these reagents stimulated PKCα activity significantly, indicating that the kinase was conditionally activated. Constant amounts of recombinant BZLF1 were incubated with purified MBP:RACK1 in the presence of PKCα. The phosphorylation status of BZLF1 was not significantly altered with increasing levels of recombinant MBP:RACK1 (Fig. 5A, lanes 2–6). Similar results were obtained with different amounts of BZLF1 substrate, PKCα (or other isoforms), or purified RACK1 cleaved off from MBP (data not shown). To exclude the possibility that the failure of RACK1 to affect the phosphorylation of BZLF1 by PKC was due to the substrate BZLF1, we performed *in vitro* phosphorylation assays with different amounts of histone as substrate (data not shown). Again, we were not able to detect significant effects.

To examine a consequence of the RACK1–BZLF1 interaction for the phosphorylation of BZLF1 *in vivo*, 293 cells were transfected with pCMV:BZLF1(Δ26–88) and subjected to phosphopeptide mapping as described for BZLF1(wt) [18]. As shown in Fig. 5B, TPA-induced signals with BZLF1(Δ26–88) resemble those obtained with BZLF1(wt), indicating that the BZLF1(Δ26–88) mutant is still a substrate for PKC-mediated phosphorylation *in vivo* although BZLF1(Δ26–88) and RACK1 do not interact (Fig. 3B).

Together, these data suggest that RACK1-binding to BZLF1 is not functionally relevant for PKC-mediated phosphorylation and activation of BZLF1 *in vitro* and *in vivo*.

RACK1 does not affect the nuclear localization of BZLF1

RACK1 has also been implicated recently in the shuttling of PKCβII from one intracellular site to another [26]. Accordingly, one possible function of the interaction between BZLF1 and RACK1 may be that RACK1 acts as a shuttling protein on BZLF1 for its translocation from the cytoplasm into the nucleus. We tested this possibility looking for the presence of wild-type BZLF1(wt) or mutant BZLF1(Δ26–88) deficient for RACK1-binding in nuclear extracts of transiently transfected 293 cells. As shown in Fig. 6, wild-type and mutant BZLF1 were detectable at almost equal amounts in nuclear fractions of transfected cells arguing against an influence of RACK1 on the translocation of BZLF1 into the nuclear compartment.

DISCUSSION

Infection of primary B-cells by EBV is predominately latent with only a subset of viral genes being expressed. The viral immediate-early gene BZLF1 is repressed in EBV's latent phase to prevent the onset of the lytic phase and the eventual death of the host cell. Phorbol ester treatment induces EBV to enter productive lytic infection by activation of the BZLF1 promoter (reviewed in [53]). TPA treatment also directly activates the BZLF1 protein via PKC-mediated phosphorylation [18].

In the present study, we report the isolation of RACK1 as a BZLF1-binding protein. Several independent experimental settings provide evidence for the interaction of BZLF1 and RACK1, including yeast two-hybrid screening, the interaction of *in vitro* translated RACK1 with a bacterially expressed GST fusion protein containing the BZLF1 transactivation domain, and coimmunoprecipitation assays, demonstrating that endogenous RACK1 forms a complex with BZLF1 in cells. All protein binding assays presented here confirmed that the interaction between RACK1 and BZLF1 requires a crucial region in the activation domain of BZLF1 with regard to its activity. Particularly, genetic data indicate that the RACK1-binding motif within BZLF1 is indispensable for transcriptional

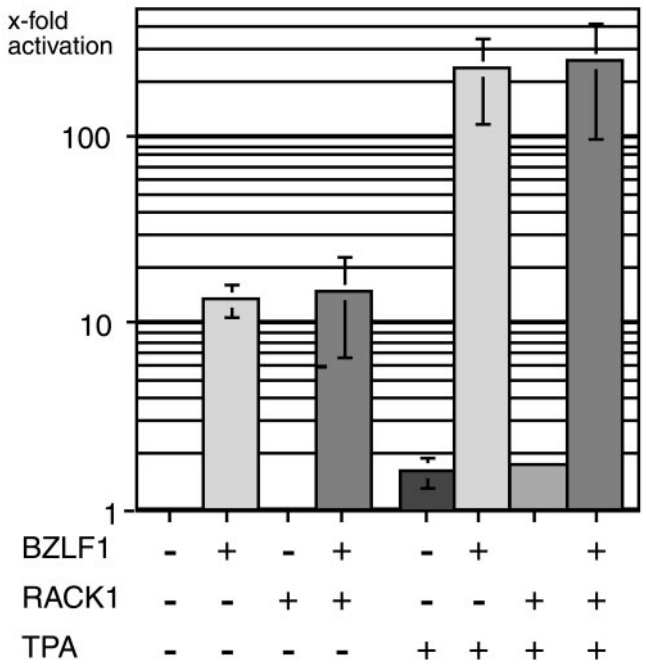
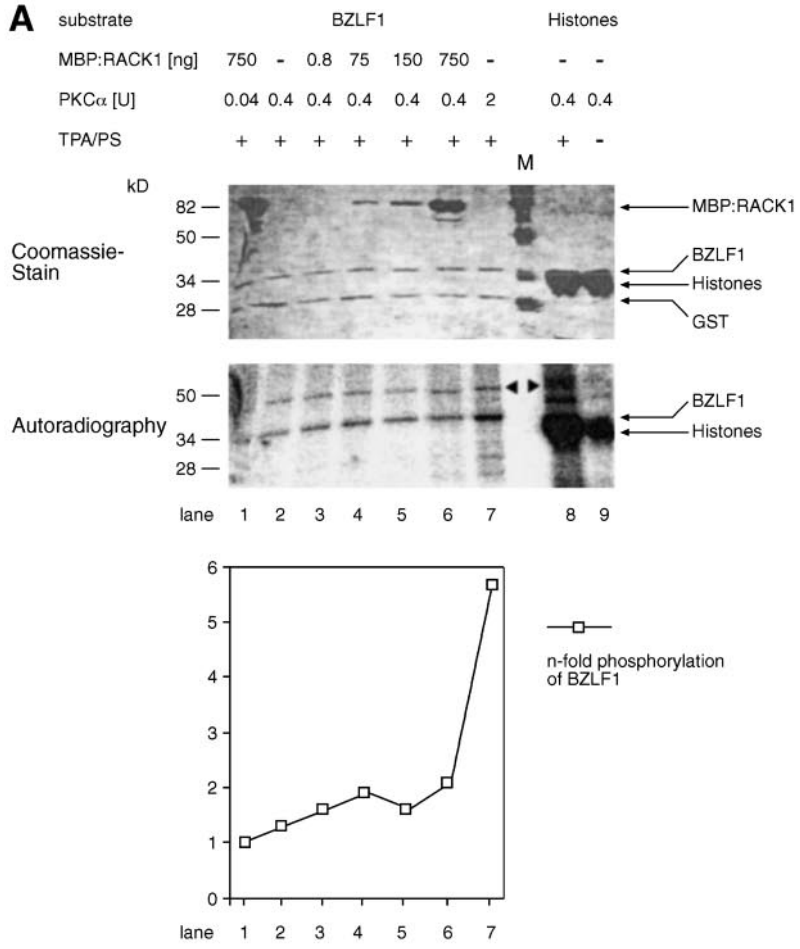


Fig. 4. Influence of RACK1 overexpression on activation of the BZLF1-responsive promoter BHRF1. Expression vectors for BZLF1 and RACK1 were transfected into BL41 cells together with pBHRF1:luc and treated with TPA as indicated. Activation of the BHRF1 promoter was calculated on the basis of the luciferase activity in cells transfected with the reporter plasmid, only, which was set to one.



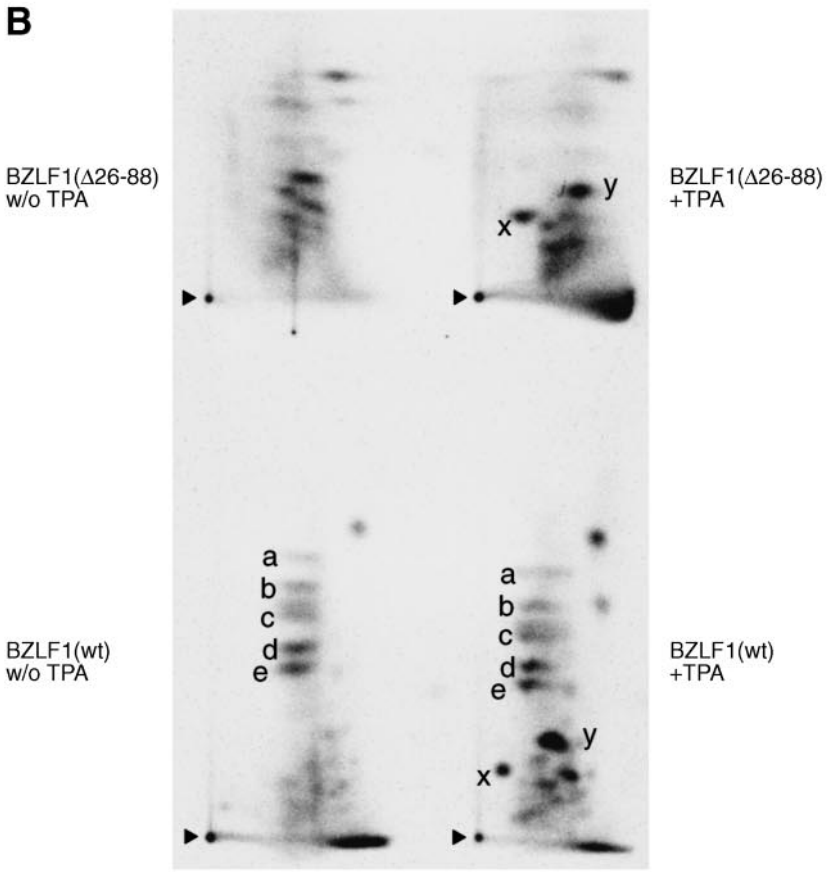


Fig. 5. Phosphorylation of BZLF1 by PKC is independent of the presence of RACK1. (A) *In vitro* phosphorylation of BZLF1 in the presence of RACK1. Recombinant BZLF1 was incubated with increasing amounts of PKCα and MBP:RACK1 in the presence of [γ-^{32}P]ATP, TPA, and PtdSer as indicated. As a control, histones were incubated with PKCα in the presence or absence of TPA and PtdSer. After separation by SDS/PAGE, proteins were visualized by Coomassie staining (upper panel) prior to autoradiography (lower panel). Signal intensities were evaluated with a phosphorimager and graphically depicted as a diagram. The black arrowheads mark signals resulting from the autophosphorylation of PKCα. One unit of PKC is defined as incorporation of 1 pmol orthophosphate into histone as a substrate per min (M, protein standard marker). (B) Two-dimensional tryptic phosphopeptide mapping of BZLF1(Δ26–88) and BZLF1(wt) proteins from transiently transfected 293 cells that were metabolically labeled with [^{32}P] orthophosphate *in vivo*. The cells were left untreated or incubated with TPA for 1 h prior to harvest. BZLF1 was immunoprecipitated and run on SDS/PAGE. The band was excised and subjected to tryptic digestion and two-dimensional phosphopeptide chromatography. BZLF1(wt) but not BZLF1(Δ26–88) is constitutively phosphorylated as indicated by five spots labeled a through e. After TPA stimulation, both BZLF1-wt and BZLF1(Δ26–88) reveal two additional signals labeled *x* and y corresponding to phosphorylated serine 186 as has been reported previously [18]. Black arrowheads indicate the positions where the peptide samples were applied.

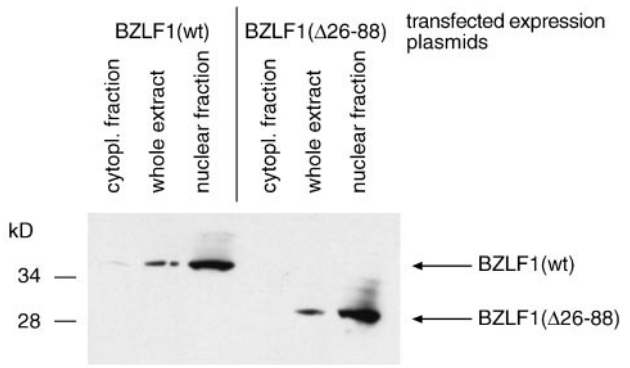


Fig. 6. Detection of wild-type and mutant BZLF1 in various cellular compartments. 293 cells were transfected with pCMV:BZLF1(wt) or pCMV:BZLF1(Δ26–88). Following preparation of whole cell extracts and cytoplasmic and nuclear fractions, respectively, BZLF1 was detected by immunostaining with a BZLF1-specific antibody.

activation and induction of DNA replication via *oriLyt* [11,14,15,46,54–56] suggesting that RACK1 might be involved in these functions of BZLF1.

Furthermore, interaction between RACK1 and BZLF1 seems to be similar to the association of RACK1 with the cAMP phosphodiesterase isoform PDE4D5 [25], as both PDE4D5 and BZLF1 bind directly to RACK1 in a phorbol ester-independent manner (Fig. 3A,C) and exhibit partial sequence homology within the protein domains involved in RACK1-binding (Fig. 3D). A connection of PDE4D5 and BZLF1 via RACK1 might therefore be a possible option for the functional significance of the BZLF1–RACK1 interaction, which may be independent of PKC.

RACK1 and G-protein β subunit are both members of an ancient family of regulatory proteins made up of highly conserved repeating units usually ending with Trp-Asp (WD) [43]. WD repeats represent structural motifs formed by four antiparallel β-strands and have been implicated in protein–protein interaction. For instance, the $G_s\beta$ subunit serves as an anchor for the β-adrenergic receptor kinase [57]. It has been proposed that RACK1 binds to PKC in the presence of Ca^{2+} and lipid *in vitro* and acts as an intracellular receptor for activated protein kinase C that targets PKC to the particulate fraction [44]. The regulatory domain of PKC contains a short sequence of homology to RACK1 termed the pseudo-RACK1 binding site. This region is supposed to be another auto-regulatory sequence in PKC similar to the pseudosubstrate autoregulatory sequence [51]. A peptide derived from the pseudo-RACK1 binding site of PKC enhanced the kinase activity in histone phosphorylation experiments *in vitro* [49]. Presumably, the peptide competes with the RACK1-binding site in PKC, thereby inducing a conformational activating change.

According to these findings, it is tempting to speculate that RACK1 might function as a scaffold protein to recruit BZLF1 into a signaling complex with PKC. However, all attempts to verify the existence of a ternary complex between RACK1, BZLF1, and PKC isozymes [α, β, γ, δ, ε, η, μ, θ, ζ and λ (data not shown)] failed in our hands. Recently, Ron and colleagues could show complex formation of RACK1 and PKCβII *in vivo* [26], but, historically, the detection of the RACK1–PKC complex by coimmunoprecipitation has been arduous, and overlay assays have been used instead to circumvent the problem [44,49,50,52,58]. In addition, we could not find any functional consequence of RACK1-binding for the activity or cellular localization of BZLF1 despite many different experimental approaches including transient transactivation assays (Fig. 4), phosphorylation experiments *in vitro* and *in vivo* (Fig. 5), and cellular extract fractionation (Fig. 6). As PKC is a family of 10 different isozymes of which only PKCβII interacts with RACK1, the fact that RACK1 is not involved in the phosphorylation of BZLF1 may indicate that PKCβII is not likely to be the isozyme mediating the phosphorylation *in vivo*.

RACK1 has also been shown to physically interact with the integrin β-subunit [24], the β-subunit of γ-aminobutyric acid type A receptors [58], the common β-chain of the IL-5/IL-3/GM-CSF receptors [48], and the tyrosine kinase Src [23]. Whereas Src kinase activity and growth of NIH-3T3 cells is inhibited by RACK1 overexpression [23], the physiologic role of the interaction between RACK1 and other cellular proteins remains enigmatic. The putative function of RACK1 as a bridging protein for activated PKC is an intriguing possibility to explain the cellular mechanisms underlying PKC-mediated signal transduction. Since most of the protein interactions between RACK1 and other cellular factors have not been linked yet to any physiologic function, further attempts are necessary to confirm the relevance of RACK1 as a mediator of PKC signaling.

However, the fact that RACK1 binds to several signaling proteins might be indicative of additional PKC-unrelated functions for RACK1. Certain authors, for instance, favor the idea that RACK1 may act as a scaffold protein to recruit other proteins into a signaling complex [24,25,48]. This might hold true for BZLF1 as well and, hence, the identification of PKC-independent roles for the interaction of RACK1 and BZLF1 should be subject to further investigations.

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