Reactivation of HHV-8 lytic replication by β-adrenoreceptors: PKA-dependent control of RTA expression and trans-activating capacity.

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Abstract

Reactivation of lytic gene expression by Human Herpesvirus 8 (HHV-8, or Kaposi's Sarcoma-associated herepesvirus) contributes to several human malignancies, but the physiologic signals controlling reactivation remain poorly understood. Links between autonomic nervous system activity and accelerated AIDS onset in HIV-1 infection led us to examine whether catecholamines might play a role. Physiologic concentrations of norepinephrine induced immediate early, early, and late lytic genes in latently infected B cells. Effects were mediated by β_1-adrenoreceptors and activation of the cAMP/PKA second messenger system, which in turn activated the viral transcription factor *RTA* (ORF50). *RTA* was modulated via induction of its promoter and post-translational modification of its trans-activating capacity. Mutation of putative phosphorylation targets at RTA serines 525 and 526 inhibited PKA-mediated nuclear localization and trans-activation of RTA-responsive promoters. Given high catecholamine levels at HHV-8 latency sites such as the vasculature and lymphoid organs, β-adrenergic control of RTA could constitute a significant physiologic regulator of this oncogenic γ-herpesvirus.

Human Herpesvirus 8 (HHV-8) is a lymphotropic γ-herpesvirus originally identified in the context of Kaposi's Sarcoma (KS) [1] and subsequently found to play a role in primary effusion lymphomas (PEL) and multi-centric Castleman's disease [2,3]. At the genomic level, HHV-8 most closely resembles Herpesvirus Siamiri [4], but the virus also bears more distant structural and functional similarities to Epstein-Barr Virus (EBV) [5,6]. Like all herpesviruses, HHV-8 displays a bifurcating gene-expression program that allows it to defer lytic replication and enter a protracted state of latency in which only a small minority of viral genes are expressed [7-10]. HHV-8 establishes latency in B lymphocytes and vascular endothelial cells where it is largely invisible to the host immune system. However, the virus must resume lytic replication if it is to disseminate throughout the body or colonize a new host. Discovery of the physiologic signals that control HHV-8 reactivation is thus key to controlling its pathogenic potential. Pharmacologic suppression of those signals could block dissemination, and pharmacologic stimulators might be used to flush latently infected cells into lytic replication where they would be vulnerable to nucleoside analogue therapies [11].

Early in the HIV-1 epidemic, it became clear that impaired cellular immunity permits HHV-8 replication but the physiologic stimuli that positively induce lytic gene expression remain poorly understood. Chemical agents such as phorbol esters or N-butyrate can reactivate HHV-8 *in vitro* [12-14], and supra-physiologic concentrations of pro-inflammatory cytokines have similar, though weaker effects [15-17]. At the level of the viral genome, lytic reactivation is mediated by the HHV-8-encoded transcription factor RTA. Over-expression of *RTA* is sufficient to trigger lytic replication in latently infected B cell lines [18,19], and *RTA* mutations can block HHV-8 reactivation *in vitro* [20]. RTA protein can also auto-activate the *RTA* promoter [21]. However, the cellular signals that control RTA activity have not been well defined.

A series of recent studies indicate that high levels of autonomic nervous system (ANS) activity can accelerate the onset of AIDS-defining conditions during HIV-1 infection [22-24]. These effects have

been attributed to HIV-induced immunosuppression following the discovery that the ANS neuroeffector molecule norepinephrine (NE) can accelerate HIV-1 replication via effects on host cell cytokine production and co-receptor expression [23,25,26]. However, direct effects of NE on viral replication raise the possibility that ANS activity might also directly activate opportunistic pathogens such as HHV-8. The present studies examined the effects of catecholamines on HHV-8 lytic gene expression, with an emphasis on cellular signaling mediators and their target in the HHV-8 genome.

Results

Effects of catecholamines on HHV-8 latency

To determine whether ANS activity might reactivate lytic replication of HHV-8, we treated two latently infected B lymphocyte lines with physiologic concentrations of NE and assayed lytic gene expression 48 hrs. later by Western blot. In both KS-1 and BCBL-1 cells, NE concentrations ranging from 10 nM to 10 μM efficiently induced expression of the major lytic antigens (Fig. 1a-b). Lytic protein expression reached peak levels at NE concentrations of 100 nM, with 1000-fold higher concentrations producing no further increase and no high-range inhibition. The catecholamine epinephrine produced similar results (data not shown). To define the scope of NE's effect on the lytic gene cascade, we assayed mRNA for the immediate early gene ORF50 (RTA) and the late gene ORF29 by real-time RT-PCR (Fig. 1c-d). Both transcripts were up-regulated 10- to 30-fold within 12 hrs, and similar effects were observed for representative early genes (e.g., ORF59; data not shown). Thus NE induces genes characteristics of all phases of the lytic replication cycle in HHV-8-infected lymphoid cells.

Mediating receptors

Most effects of catecholamines on leukocytes are mediated by β-adrenergic receptors [27] (βAR), but α-adrenoreceptors can also be expressed under certain conditions [28]. To identify the specific receptors mediating catecholamine induction of HHV-8 lytic genes, we pre-treated KS-1 or BCBL-1 cells with either the β antagonist propranalol or the α antagonist phentolamine for 1 hr. prior to NE exposure. β-blockade strongly inhibited HHV-8 response to NE, but α-blockade did not (Fig. 2a-b). Neither

inhibitor had any effect on HHV-8 lytic replication in the absence of exogenous NE (data not shown). Propranalol inhibits all three subtypes of βAR, and we sought to define the subtype(s) involved by assaying mRNA for β_1, β_2, and β_3 adrenoreceptors. In both KS-1 and BCBL-1 cells, only β_1 adrenoreceptor mRNA was detectable in appreciable quantities by RT-PCR (Fig. 2c).

Role of the cAMP/PKA signaling pathway

In lymphoid cells, βARs signal primarily through $G_{\alpha s}$-mediated activation of the adenylyl cyclase / cAMP / PKA signaling pathway [27,29]. However, other signaling pathways can potentially be activated [30-32]. To determine whether PKA activity mediates adrenergic reactivation of HHV-8, we pre-treated cells with the PKA antagonist KT5720 for 1 hr. prior to NE stimulation and monitored subsequent induction of the viral late gene ORF29 by real time RT-PCR. KT5720 inhibited NE-induced upregulation of ORF29 by more than 10-fold (Fig. 3a), whereas the myristoylated protein kinase C (PKC) antagonist PKC 19-27 was only 1/5 as potent even at 10-fold higher concentrations. Direct activation of cAMP signaling with the cell-permeable analogue db-cAMP also induced HHV-8 lytic genes (Fig. 3b).

Activation of PKA is the most prominent mechanism by which cAMP regulates cell function, but other cAMP targets have recently been identified [33]. To determine whether PKA activity alone is sufficient to account for the effects of cAMP signaling on HHV-8 reactivation, we expressed a constitutively active form of PKA in KS-1 and BCBL-1 cells using a self-inactivating lentiviral vector. The α catalytic subunit of PKA (PKA_c) was expressed in the context of a bi-cistronic mRNA that included a C-terminal EGFP reporter sequence independently translated via a synthetic internal ribosome entry site. This vector transduced more then 50% of KS-1 and BCBL-1 cells (Fig. 4a). Expression of the reporter gene alone had no effect on HHV-8 lytic genes, but expression of the reporter gene in conjunction with PKA_c up-regulated expression of both immediate early (ORF50/RTA) and late lytic transcripts (ORF29) (Fig. 4b). Thus PKA activity alone is sufficient to reactivate HHV-8 lytic genes, and this effect is associated with increased *RTA* expression.

Effect of PKA signaling on RTA expression and activity

Because RTA-mediated *trans*-activation is the key event in HHV-8 reactivation, we sought to determine how βAR/PKA signaling modulates the *RTA* gene. To define direct effects on the *RTA* promoter in the absence of any contribution from viral gene products [21], uninfected DG75 cells were electroporated with an *RTA* promoter-luciferase reporter construct and treated with the PKA activator db-cAMP. Reporter gene activity increased by 2- to 3-fold following PKA activation (Fig. 5a), indicating that cellular transcription factors can directly enhance *RTA* transcription. However, significantly larger effects were observed in HHV-8-infected cells (Fig. 5b), with db-cAMP up-regulating RTA promoter activity by approximately 6-fold in BCBL-1 cells (difference, $p < .0001$ as assessed by the cell type x db-cAMP interaction term from a factorial analysis of variance, $F(1,12) = 30.31$). NE had similar effects, with *RTA* promoter activity increasing by an average 9.37-fold in BCBL-1 cells (standard error $\pm$ 1.6-fold, $p = .0092$ by *t*-test), and significantly less in DG75 cells (difference from BCBL-1, $p = .0012$, $F(1,4) = 56.47$). Thus βAR/PKA signaling appears to interact with viral gene products to activate the *RTA* promoter.

One of the most powerful inducers of the *RTA* promoter is the RTA transcription factor itself [21]. To determine whether PKA signaling might enhance RTA *trans*-activating capacity independently of effects on RTA protein levels, we expressed RTA in *trans* and analyzed the effects of db-cAMP on *RTA* promoter-driven luciferase activity. As in previous studies [21], RTA expression significantly up-regulated *RTA* promoter activity (Fig. 5c). Addition of db-cAMP increased that effect by ~4-fold, from 7-fold above basal activity to 30-fold (Fig. 5c). Similar effects were observed in latently infected KS-1 and BCBL-1 cells, with *RTA* promoter activity increasing from ~16-fold above basal activity levels in the presence of RTA alone to ~125-fold above basal activity following addition of db-cAMP (difference $p = .007$ by t-test). NE had similar effects, with RTA promoter activity increasing from 15.5 $\pm$ 2.9-fold above basal levels with RTA alone to 40.8 $\pm$ 1.4-fold above basal levels when RTA was supplemented by 10 μM NE. Thus βAR/PKA signaling appears to have some post-translational effect on RTA's *trans*-activating capacity.

To determine whether PKA enhancement of RTA trans-activating capacity might impact other HHV-8 lytic genes, we conducted reporter assays using the promoter for HHV-8 polyadenylated nuclear (PAN) RNA gene [34,35]. In cells free of HHV-8 gene products, db-cAMP had a negligible effect on *PAN* promoter activity (Fig. 5d). Expression of *RTA* alone significantly increased *PAN* promoter activity, as previously described [19,20,34,35]. PKA activation during *RTA* expression led to a further 5-fold enhancement of reporter gene activity, from ~700-fold above basal activity to ~4,500-fold (Fig. 5d). Similar effects were observed in PEL cell lines latently infected with HHV-8 (Fig. 5e). NE produced similar effects, with *RTA*-mediated induction of the *PAN* promoter increasing from 150.4 $\pm$ 6.0-fold above basal activity in BCBL-1 cells to 1,518.0 $\pm$ 333.0-fold in the presence of NE (difference $p = .0097$ by t-test). Thus βAR/PKA signaling can up-regulate RTA-mediated expression of HHV-8 lytic gene products even when the cellular signaling pathway has no direct effect on the viral promoter.

Post-translational effects on RTA

RTA protein is extensively phosphorylated [20], but it is unclear what role this plays in RTA's control of lytic replication. Bioinformatic analysis of the RTA coding sequence identified 10 potential PKA phosphorylation sites [36,37]. One candidate, serine 526 (S526), is immediately adjacent to a predicted PKC phosphorylation target (S525), suggesting that this region of RTA could represent a generalized target for serine/threonine kinases. To evaluate the functional significance of these tandem amino acids, both serine codons were converted to alanine via site-directed mutagenesis, and the resulting mutant RTA (S525A-S526A) was tested for PKA-mediated enhancement of PAN promoter activity. Both mutant and wild-type RTA showed similar basal induction of the PAN promoter (Fig. 6a). However, the S525A-S526A mutations significantly inhibited the capacity of PKA to up-regulate RTA's trans-activating capacity (difference $p < .0001$, as assessed by the interaction term from a factorial analysis of variance, $F(1,4) = 343.96$). Serines 525 and 526 reside within a predicted nuclear localization signal [20], and immunofluorescence-based tracking of FLAG-tagged RTA showed that the S525A-S526A mutations also inhibited translocation of RTA to the nucleus following PKA activation (Fig. 6b).

Discussion

The present data show that the ANS effector molecule NE can efficiently reactivate HHV-8 lytic gene expression in latently infected lymphoid cells. These effects occur at physiologic NE concentrations and are mediated by activation of β_1 adrenoreceptors and the cAMP/PKA signaling pathway. β-adrenergic stimulation enhances expression of immediate early (ORF50/RTA), early (ORF59), and late lytic genes (ORF29), and these effects appear to be mediated by PKA-induced actiavtion of the viral transcription factor RTA [18,19]. Both NE and PKA enhance *RTA* gene expression via cellular transcription control pathways, and both signals also modify the capacity of RTA protein to trans-activate viral lytic genes (including the RTA promoter). β-adrenergic modulation of RTA via the cAMP/PKA signaling pathway suggests that ANS activity could constitute an important physiologic determinant of HHV-8 activity *in vivo*. Adrenergic reactivity also suggests several potential therapeutic approaches, including β-blockade to limit reactivation or β-agonists to flush the latent virus reservoir during nucleoside analogue therapy [11].

PKA signaling alone is sufficient to reactivate HHV-8 lytic gene expression. Both transient pharmacologic activation of PKA and persistent over-expression of its catalytic subunit enhanced lytic gene expression. These findings are consistent with results from a recent genome-wide over-expression scan that identified the PKA catalytic subunit as a powerful inducer of RTA-mediated trans-activation (J. Harada, The Genomics Institute of the Novartis Foundation). Such findings imply that HHV-8 cold reactivate in response to a broad spectrum of extracellular signals that converge on PKA, including histamine and prostaglandins [29,38]. Most previous studies of HHV-8 reactivation have relied on PKC activators to induce lytic replication (e.g., PMA/TPA) [20]. Like PKA, PKC is a ubiquitous serine/threonine kinase mediating cellular responses to a diverse array of extracellular stimuli [39,40]. Biological effects of these two kinases are often antagonistic, but they target similar amino acid motifs [37,39]. PKC inhibitors failed to block NE induction of lytic gene expression, so it is clear that the PKA-mediated pathway is functionally independent of the PKC pathway. The present data also show that PKA

activators can re-activate HHV-8 lytic replication even under circumstances where PKC activators fail (Fig. 1a). However, the functional parallels between PKA- and PKC-mediated reactivation suggest a major role for inducible phosphorylation in controlling HHV-8 replication.

The viral transcription factor RTA represents the key "switch" protein controlling HHV-8 reactivation [18,19], and the present data identify two distinct mechanisms by which the βAR/cAMP/PKA signaling pathway can modulate its activity. Cellular signaling pathways can directly activate the RTA promoter in the absence of herpesvirus gene products (e.g., in uninfected DG75 cells). However, these effects are substantially enhanced in the presence of a viral genome, suggesting that some process downstream of *RTA* gene expression may also be modulated. RTA protein can auto-induce the *RTA* promoter, and the present data show that PKA can modulate RTA's trans-activating capacity independently of RTA protein expression. The primary amino acid sequence of RTA includes 10 consensus PKA phosphorylation motifs (R-X-S-X), including one immediately adjacent to a putative PKC phosphorylation site (X-S-X-K). Both of those serines (525-526) fall within an activation domain known to be critical for the induction of lytic promoters [20]. Substitution of alanine residues at both sites maintained the basal trans-activating capacity of RTA, but it largely blocked the ability of PKA to enhance that effect. We were not able to directly measure changes in the phosphorylation of either wild-type serine due to the high background levels of phosphorylation previously described [20]. However, the presence of adjacent PKA and PKC targets within a putative nuclear localization signal [20] suggests a potential functional role for cellular kinases in targeting RTA to the HHV-8 genome. Consistent with that hypothesis, confocal microscopy documented a substantial reduction in the ability of PKA to induce nuclear localization of the S525A-S526A mutant. The mechanisms by which PKA modulates RTA function require further definition, but the present data clearly suggest that inducible phosphorylation of HHV-8 RTA by cellular kinases may play a critical role in regulating viral latency. Similar results have emerged in analyses of PKC-mediated control of the EBV BZLF protein [41-43], suggesting that γ-herpesvirus switch proteins may have evolved cellular phosphorylation targets as a generalized

mechanism for reactivation during favorable cellular conditions. If so, pharmacologic manipulation of cellular kinases might represent a potential strategy for controlling γ-herpesvirues replication.

Adrenergic reactivity is a common characteristic of α- and β-herpesviruses [44-48], and may explain the propensity of those viruses to reactivate in response to environmental stress [45-47,49-51]. The γ-herpesvirus EBV is sensitive to stress-induced glucocorticoids [52-57], and the present studies suggest that HHV-8 might also reactivate during stress-induced ANS activity. The emergence of stress-reactive biology in all major classes of *Herpesviridae* suggests a potential evolutionary advantage for latent viruses to monitor organismic stress levels, which could be diagnostic of reduced cellular immune threat [50,58,59] or lowered host-transmission hurdles [60,61]. Reactivation of HHV-8 in response to catecholamines may thus represent one example in which herpesviruses monitor their host to detect ecological conditions favoring their survival in much the same way they seek to create those conditions by manipulating the cellular micro-environment [6].

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Methods

Cell culture

Reactivation was analyzed in PEL cell lines bearing latent HHV-8 (KS-1 and BCBL-1). The DG75 Burkitt's lymphoma cell line served as a negative control uninfected with HHV-8 or EBV. Cells were cultured under standard conditions in RPMI + 10% fetal bovine serum or serum-free X-VIVO 15 (Cambrex, East Rutheford NJ) + growth factors (MITO+, BD Biosciences, Bedford MA). Reactivation experiments were performed as described [19], using phorbol-12-myristate-13-acetate (PMA, also known as TPA; Calbiochem, San Diego CA), NE ([-]-arterenol; Sigma-Adrich, St. Louis MO), and other indicated agents acting on the βAR / cAMP / PKA signaling pathway (all from Sigma or Calbiochem).

Lytic gene expression

HHV-8 lytic proteins and cellular β-actin were assayed by Western blot with ECL [19]. mRNA for HHV-8 gene products and human β-adrenoreceptor subtypes were quantified relative to cellular housekeeping genes using semi-quantitative real-time RT-PCR. Reactions utilized 1/10 of the total DNAse-treated (Qiagen, Valencia CA) RNA extracted from 10^6 cells in a one-step thermal cycling protocol (Qiagen One-step RT-PCR) with 30 minutes of reverse transcription at 50°C, 15 min. of RT denaturation at 95°C, and 40 cycles of DNA amplification (15s at 95°C, 60s at 60°C). Reactions utilized established primers and fluorescent detection probes for human GAPDH and HHV-8 ORF29, ORF50, ORF57 [8]. Primers for human β-adrenorecptor subtypes were: β_1 (forward: TCG GAA TCC AAG GTG TAG GG, reverse: TGG CTT TTC TCT TTG CCT CG), β_2 (forward: CAT GTC TCT CAT CGT CCT GGC CA, reverse: CAC GAT GGA AGA GGC AAT GGC A), β_3 (forward: GGC TTC TTG GGG AGT TTC TTA GG, reverse: TTC TGG AGG GTA GAG TGT CAC AGC), derived from GenBank sequences ADRB1: J03019, ADRB2: M15169, ADRB3: X70811.

Overexpression of PKA catalytic subunit

Cells were transduced with a self-inactivating lentiviral vector expressing a constitutively active form of PKA [62] and an enhanced green fluorescent protein (EGFP) reporter gene under control of a

recombinant Rh-MLV promoter [63]. Both sequences were translated from a single transcript bearing the PS3 internal ribosome entry site (IRES) [64]. cDNA for the HIV-1 central polypyrimidine tract [65] was introduced into pSIN-18-Rh [63] at the *Xho*I site upstream of the Rh-MLV promoter to produce pSIN18RhMLV-E-CPPT. cDNA of the PKA catalytic subunit α (*PRKACA*: X07767) was amplified with primers bearing *Age*I and *Sal*I restriction sites and subcloned into pCR-Blunt TOPO (Invitrogen, Carlsbad CA), sequenced, released by digestion, purified, and subcloned into *Age*I and *Sal*I sites of pSIN18RhMLV-E-CPPT to produce pSIN18RhMLV-E-CPPT-PKA. The PS3 IRES-EGFP sequence was excised from pDF-PS3 [64] and subcloned into the *Not*I site of a circularized pCRII-TOPO vector (Invitrogen). The *EcoR*V site upstream of the subcloned fragment was changed into a *Sal*I site, and a *SalI/Xho*I fragment was subcloned into pSIN18RhMLV-E-CPPT-PKA to produce pSIN18RhMLV-E-CPPT-PKA-PS3-EGFP. The negative control vector pSIN18RhMLV-E-CPPT-EGFP included EGFP sequences in the absence of upstream PRKAC-PS3 sequences. Vectors were constructed by transfection into 293T cells [63], and target cells were transduced by 1 hr. incubation in vector-containing supernatants (+10 μg/ml polybrene).

RTA promoter activity

Luciferase reporter assays utilized a 3 kb sequence upstream of the *RTA* start site (pRpluc) [21]. 4 μg of pRpluc was electroporated along with 50 ng of the control pRLCMV (*Renilla* luciferase driven by the CMV promoter) (Promega, Madison WI) and 6 μg of empty pcDNA3 vector (10 μg total) into 10^7 KS-1, BCBL-1, or DG75 cells (240 Volts, 125 ohms, 950 μf, from a BTX ECM 630 pulse generator). Firefly and renilla luciferase activity was assessed by dual luciferase assay (Promega). Assays were performed in duplicate, with firefly luciferase light units normalized to renilla luciferase light units prior to analysis of log-fold change by Student's *t*-test.

RTA trans-activating capacity

RTA protein was expressed by substituting 4 μg of pcDNA3 with pcDNA3/RTA (a pcDNA3-based vector expressing genomic HHV-8 *RTA* under control of the CMV promoter [21]) or pFLAG/RTA (C-terminal FLAG-tagged HHV-8 *RTA* [35,66]). To control for any effect of PKA on the CMV promoter [48], the control renilla luciferase was also expressed under the CMV promoter (pRLCMV). To assess RTA-mediated trans-activation of a heterologous HHV-8 promoter, luciferase reporter assays were conducted as above after replacing pRpluc with pLUC/-69, a pGL3-Basic vector expressing firefly luciferase from a 69 nucleotide fragment of the HHV-8 PAN RNA promoter [34].

RTA phosphorylation

The predicted amino acid sequence of RTA (AF091348) was scanned by Phosphobase 2.0 (http://www.cbs.dtu.dk/databases/PhosphoBase/predict/predform.html)[36] to identify consensus PKA phosphorylation sites [37]. Functional significance of predicted PKA and PKC phosphorylation candidates at serines 526 (S525) and 526 (S526) was assessed by converting both codons to alanines (site-directed mutagenesis of pcDNA3/RTA-FLAG, with PCR primers changing *RTA* nucleotides 1573-1574 from GC to AG to produce S525A; and nucleotide 1576 from G to T to produce S526A). Mutations were verified by sequencing and characterized in *PAN* promoter luciferase assays as above. Nuclear localization was assessed by confocal microscopy, with immunofluorescence detection of FLAG-tagged RTA (primary anti-FLAG IgG from Sigma; secondary Cy3-conjugated anti-mouse IgG from Jackson ImmunoResearch, West Grove PA) relative to DAPI-stained DNA (Molecular Probes, Eugene OR).

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Figure Captions.

Figure 1. Induction of HHV-8 lytic gene expression by NE Lytic replication of B lymphoid PEL cell lines bearing latent HHV-8 was monitored by Western blot (primary antibody: KS patient serum) 48 hrs. following treatment with 20 ng/ml of PMA or indicated quantities of NE. The most prominent lytic antigen during HHV-8 reactivation falls at a molecular weight of ~49,800 (lower band is nonspecific). β-actin was assayed in parallel to confirm equivalent protein loading. Both KS-1 (a) and BCBL-1 cells (b) were readily reactivated by NE, even under conditions where PMA had minimal effect (a). To verify that NE induced the full range HHV-8 lytic gene expression, concentrations of mRNA for the immediate early ORF50 and the late ORF29 genes were assayed by real-time RT-PCR following exposure of KS-1 cells to 10 or 100 μM NE, or to PMA as a positive control. Viral mRNA levels were normalized to cellular GAPDH and then expressed as a ratio relative to normalized values observed in untreated cells. Graphs show mean ± standard error of duplicate measurements. (c) NE significantly up-regulated ORF50 mRNA levels at all time points (12 hr, $p < .0001$; 24 hr, $p = .0011$; 36 hr, $p = .0004$; 48 hr, $p = .0027$; all by t-test on log-transformed induction values). (d) NE-induced up-regulation of ORF29 was not significant by 12 hrs ($p = .2086$), but highly significant thereafter (24 hr, $p = .0003$; 36 hr, $p = .0006$; 48 hr, $p = .0003$). Similar kinetics were observed in BCBL-1 cells (data not shown).

Figure 2. Role of adrenergic receptors in NE-induction of HHV-8 lytic replication. (a) BCBL-1 cells were pre-treated with indicated concentrations of the β-adrenergic antagonist propranalol for 1 hr before exposure to 10 μM NE as indicated, and then assayed for HHV-8 lytic protein expression by Western blot 48 hrs later (primary antibody: KS patient serum). (b) To verify the role of β-adrenergic receptors, KS-1 cells were treated for 1 hr with either the α-adrenergic antagonist phentolamine (10 μM) or the β-adrenergic antagonist propranalol (10 μM) before addition of indicated quantities of NE. 24 hrs later, concentrations of mRNA for the late gene product ORF29 were assessed by real-time RT-PCR (with values normalized to GAPDH and expressed as a ratio to untreated cells). (c) Expression of β_1, β_2, and β_3-adrenergic receptors was assessed in untreated KS-1 and BCBL-1 cells by real-time RT-PCR.

Products were resolved on a 3.5% agarose gel and compared to positive control PCR products (parallel amplification of genomic DNA for β_1 and β_3 adrenergic receptors or a peripheral blood cDNA library for β_2 adrenergic receptors and GAPDH). To verify that RT-PCR results were free of contaminating DNA, BCBL-1 and KS-1 RNA samples were amplified in parallel in the absence of reverse transcriptase. Data are representative of 4 independent experiments in which β_1 adrenergic receptors were consistently detected at high levels, whereas β_2 and β_3 receptors were not significantly expressed.

Figure 3. Role of PKA in β-adrenergic reactivation of HHV-8 lytic replication. KS-1 cells were treated with indicated concentrations of the PKA antagonist KT5720 or the myristoylated PKC inhibitor PKC 19-27 for 1 hr before exposure to indicated quantities of NE. 24 hrs later, expression of the HHV-8 early lytic gene ORF50 and the late lytic gene ORF29 were assayed by real-time RT-PCR. Data represent mean (± standard error) of duplicate determinations, with all values normalized to cellular GAPDH mRNA and expressed as a fold-change relative to untreated cells. To determine whether PKA activation alone was sufficient to re-activate HHV-8 lytic replication, KS-1 cells untreated with any other agents were exposed to indicated concentrations of the pharmacologic PKA activator db-cAMP.

Figure 4. Effect of over-expressing the PKA catalytic subunit on HHV-8 lytic replication. KS-1 cells were transduced with a lentiviral vector [63] expressing the catalytic subunit of PKA and a downstream EGFP reporter sequence translated from a single mRNA bearing the PS3 synthetic internal ribosome entry site [64]. (a) At 48 hrs following transduction, GFP+ cells were quantified by flow cytometry, and (b) mRNA for ORF50 and ORF29 were quantified by real-time RT-PCR (with data normalized to GAPDH and expressed as a ratio to levels in untreated cells). Data represent the mean (± standard error) of three independent experiments, and statistical significance of differences was evaluated by paired *t*-test. Similar effects were observed with BCBL-1 cells (data not shown).

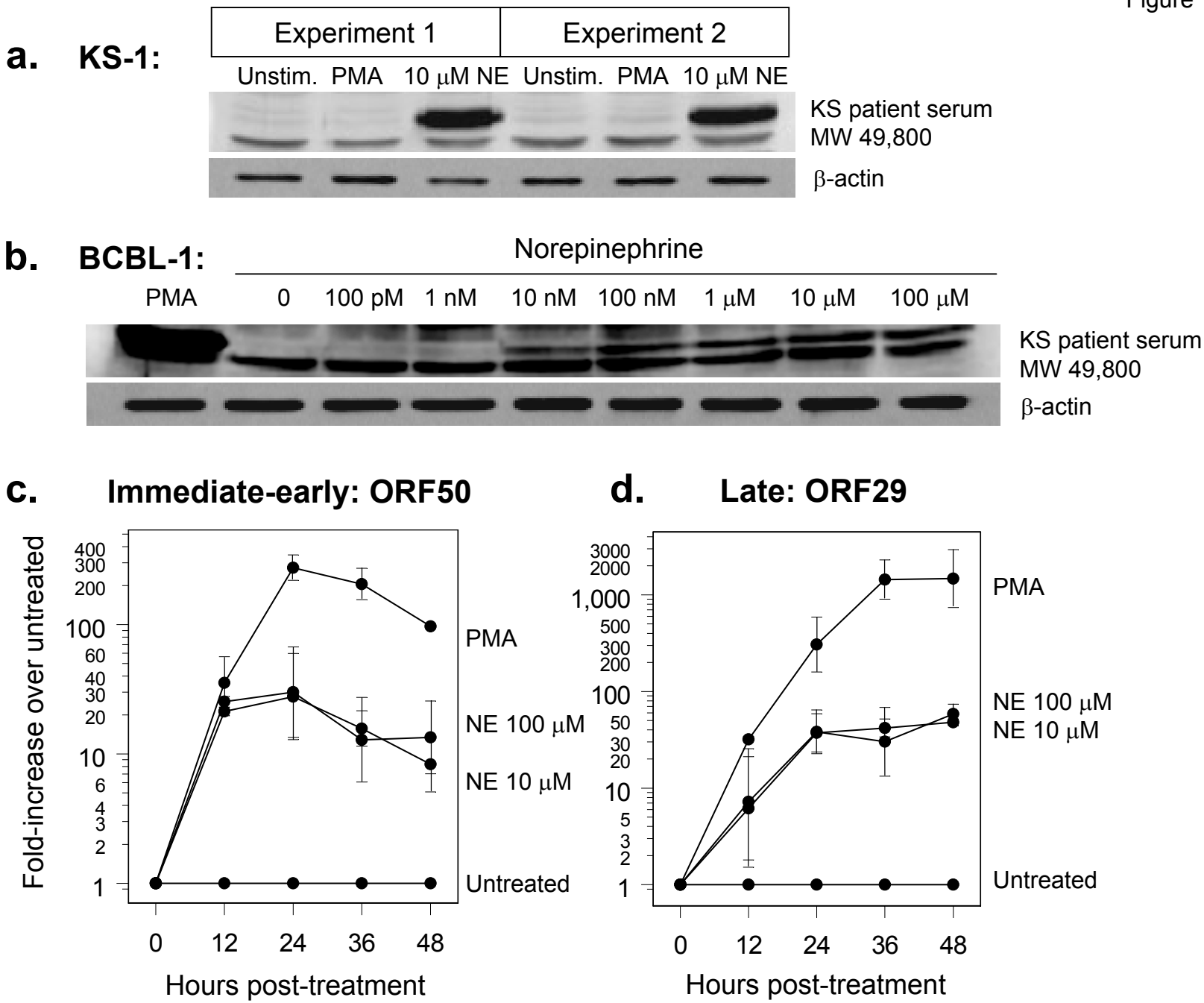
Figure 5. Regulation of RTA promoter activity and trans-activating capacity by NE. (a) Effects of PKA activation on activity of the HHV-8 *RTA* promoter were assessed in luciferase reporter assays in which pRpluc (firefly luciferase coding sequence controlled by ~3 kb of HHV-8 genomic DNA upstream of

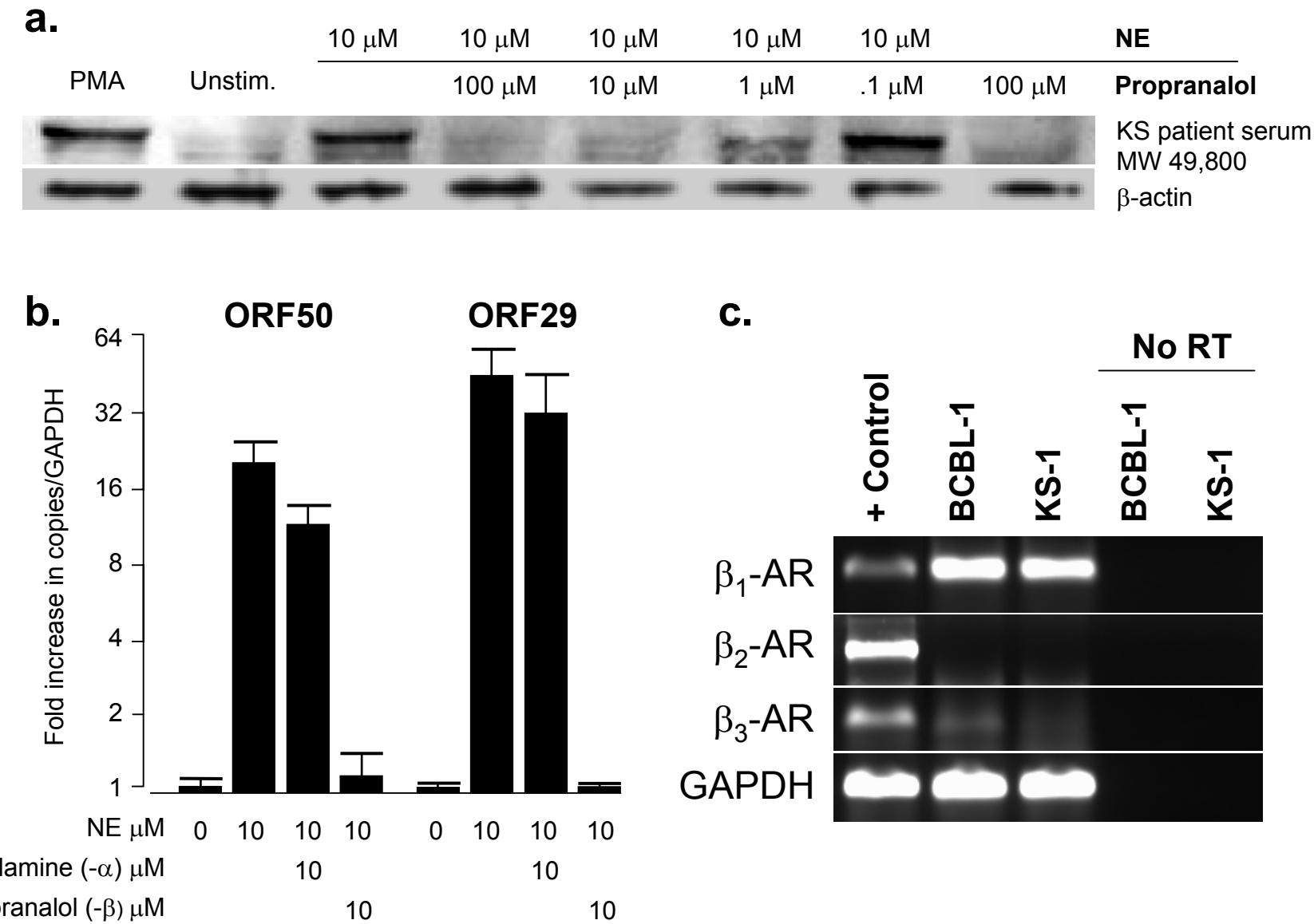
ORF50) was electroporated into the B lymphoid cell line DG75, which is known to be free of HHV-8 and EBV. Following electroporation, cells were incubated for 24 hrs. in medium supplemented as indicated with the PKA activator db-cAMP (300 μM). Firefly luciferase activity was normalized to renilla luciferase activity generated by pRLCMV (renilla luciferase under control of the CMV promoter), and statistical significance of duplicate determinations was evaluated by t-test. (b) When the same experiment was carried in cell lines bearing latent HHV-8 (e.g., BCBL-1), db-cAMP up-regulated RTA promoter activity significantly more strongly than in cell lines free of latent virus (DG-75), as indicated by a Cell Type x db-cAMP interaction term from a factorial analysis of variance ($p < .0001$). Similar effects were observed when PEL cell lines were treated with 10 μM NE (data not shown). (c) To determine whether RTA protein might mediate the enhanced effect of PKA on *RTA* promoter activity in cells bearing latent HHV-8 gene products, FLAG-tagged RTA was expressed in *trans* (vector: pFLAG/RTA) in uninfected DG75 cells and supplemented by 1 mM db-cAMP as indicated. Induction was expressed as a fold-change above basal promoter activity, with statistical significance of differences assessed by t-test. In all studies involving pFLAG/RTA, firefly luciferase activity was normalized to renilla luciferase from pRLCMV, driven by the same CMV immediate-early enhancer-promoter used to drive *RTA* expression. (d) To determine whether PKA-induced enhancement of RTA trans-activating capacity applies to heterologous viral promoters, reporter assays were carried out as in (c) substituting pLUC/-69 (firefly luciferase sequence under control of HHV-8 *PAN* promoter) [34] for the *RTA* reporter construct. Uninfected DG75 cells, and (e) BCBL-1 PEL cells were then tested for synergistic effects of *RTA* expression in *trans* and PKA activation via 1 mM db-cAMP, with statistical significance assessed by t-test.

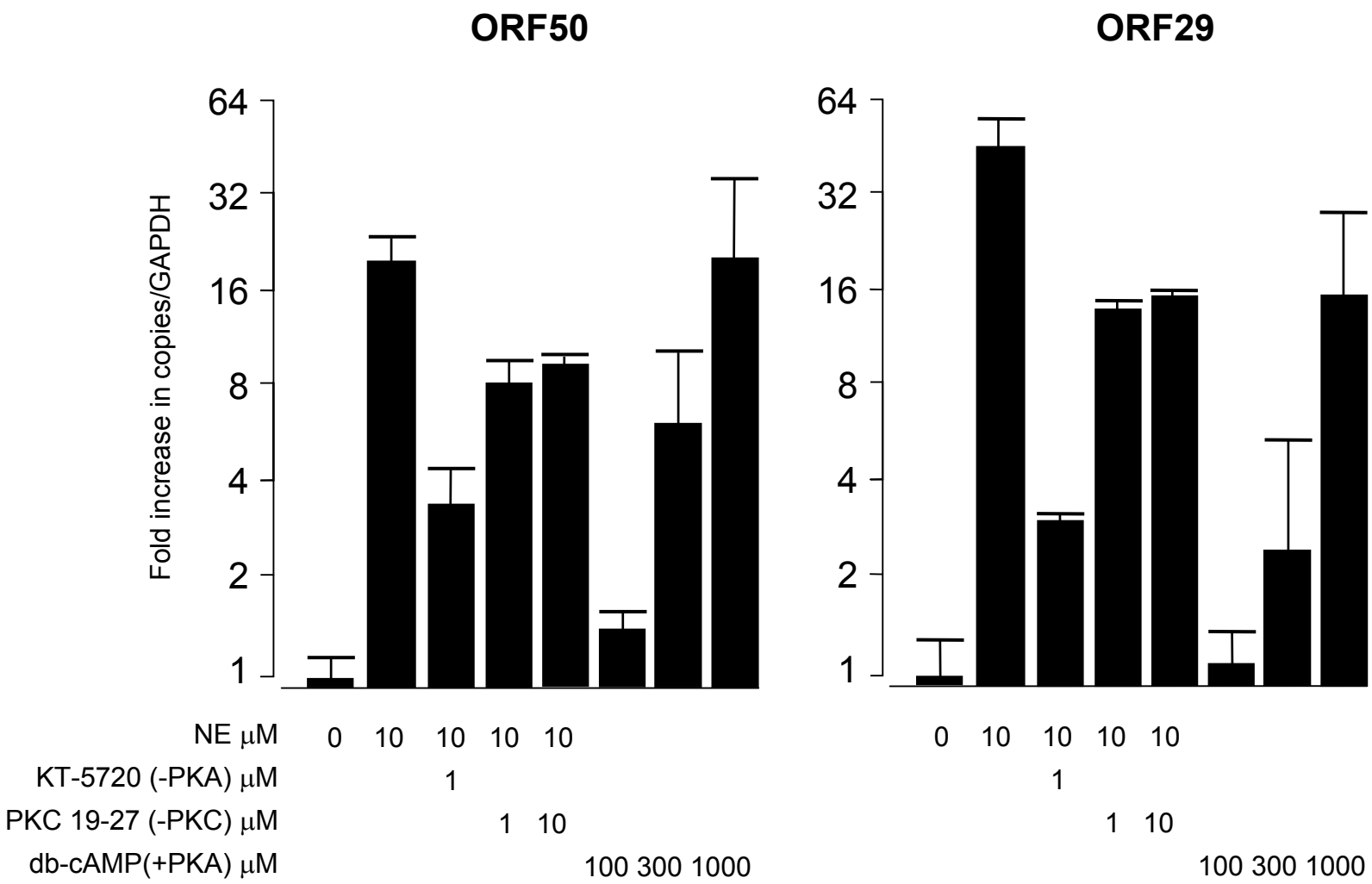
Figure 6. Role of RTA serines 525 and 526 in PKA-mediated enhancement of trans-activation. To evaluate the functional role of predicted phosphorylation targets at S525 and S526 of RTA, both residues were converted to alanines through PCR-based site-directed mutagenesis. (a) Trans-activating capacity was assayed using a luciferase reporter construct driven by the HHV-8 *PAN* promoter as in Figure 6 D-E. Reporter constructs were electroporated into KS-1 cells accompanied by either 1 μg of pFLAG/RTA

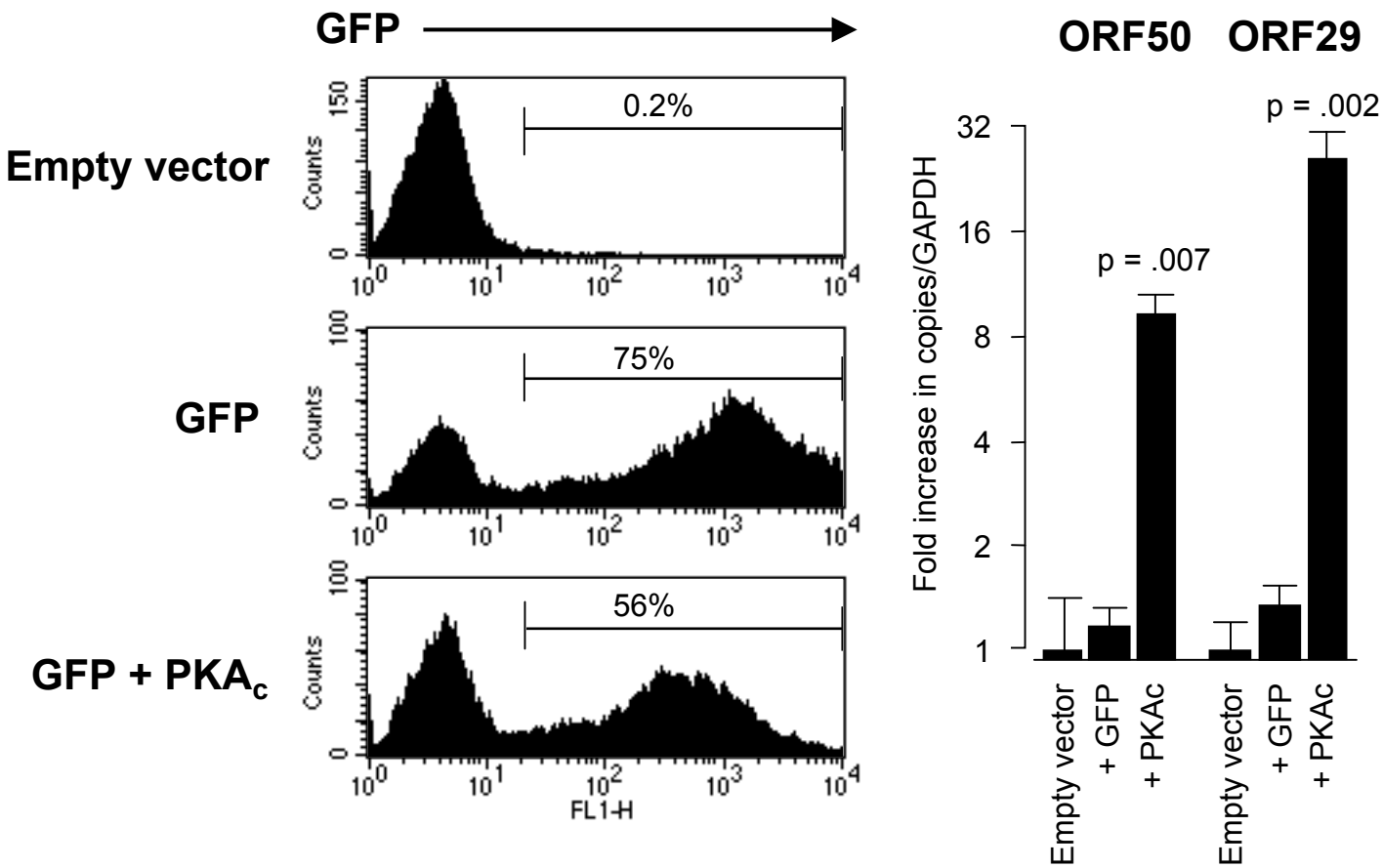
(wild-type RTA), 1 μg of pFLAG/RTA-S525A-S526A (mutant), or 1 μg of pFLAG (vector control). Cells were subsequently cultured in the presence of 300 μM db-cAMP as indicated. Luciferase induction was measured as a fold-change above basal activity, with statistical significance assessed by t-test. A factorial ANOVA was used to test the statistical significance of differences in db-cAMP-mediated enhancement of RTA *trans*-activating capacity in wild-type vs. S525A-S526A mutant RTA. Data are representative of three independent experiments. (b) Nuclear vs. cytoplasmic distribution of RTA was assessed by immunofluorescent detection of FLAG-tagged RTA (orange = Cy3) relative to DNA (purple = DAPI) in KS-1 cells 12 hrs following electroporation as described above. Images were acquired by confocal microscopy and presented in a 50 μ square format. To control for possible nonspecific effects of db-cAMP on generalized protein localization, EGFP was expressed in parallel (bottom row).

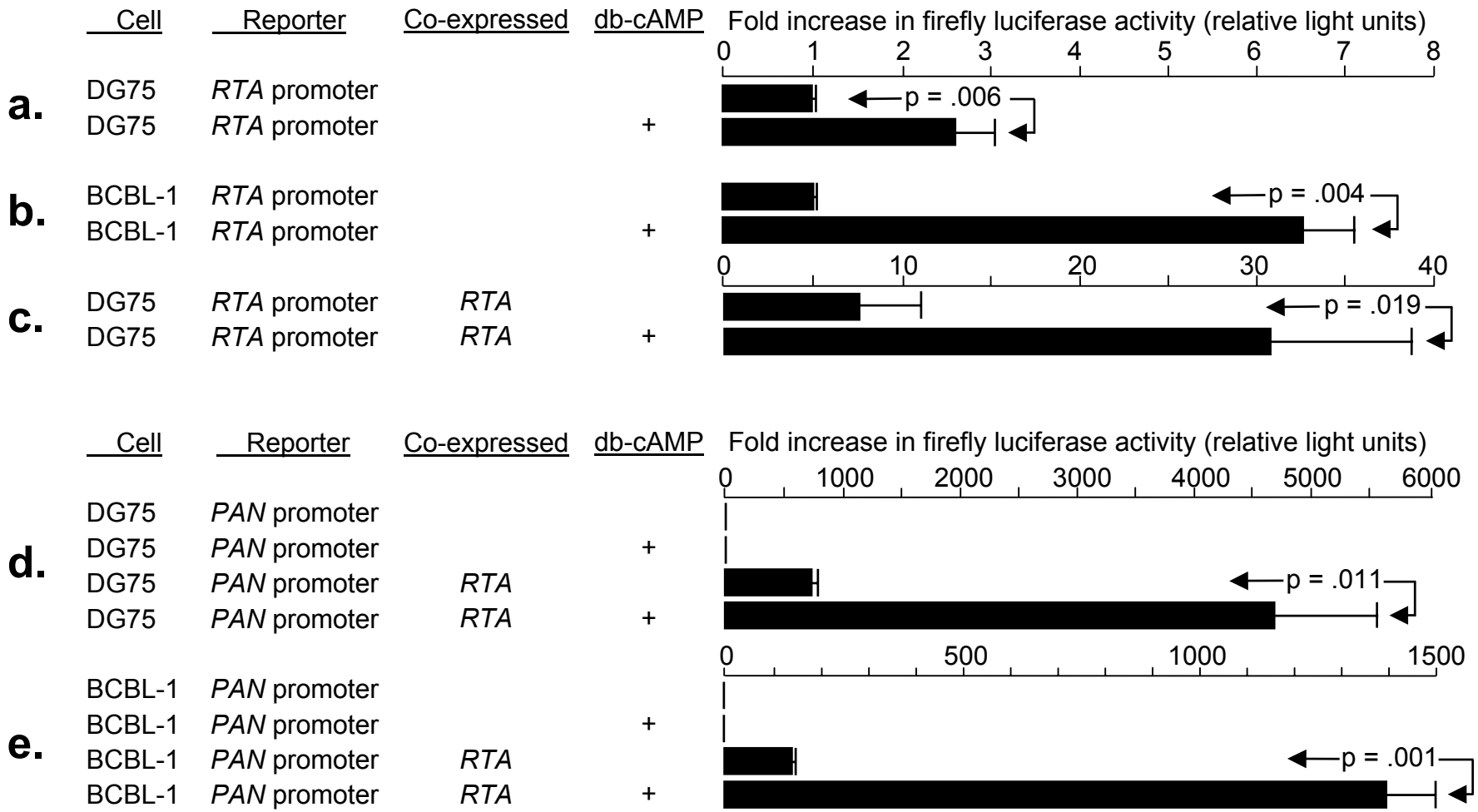
Figure 1

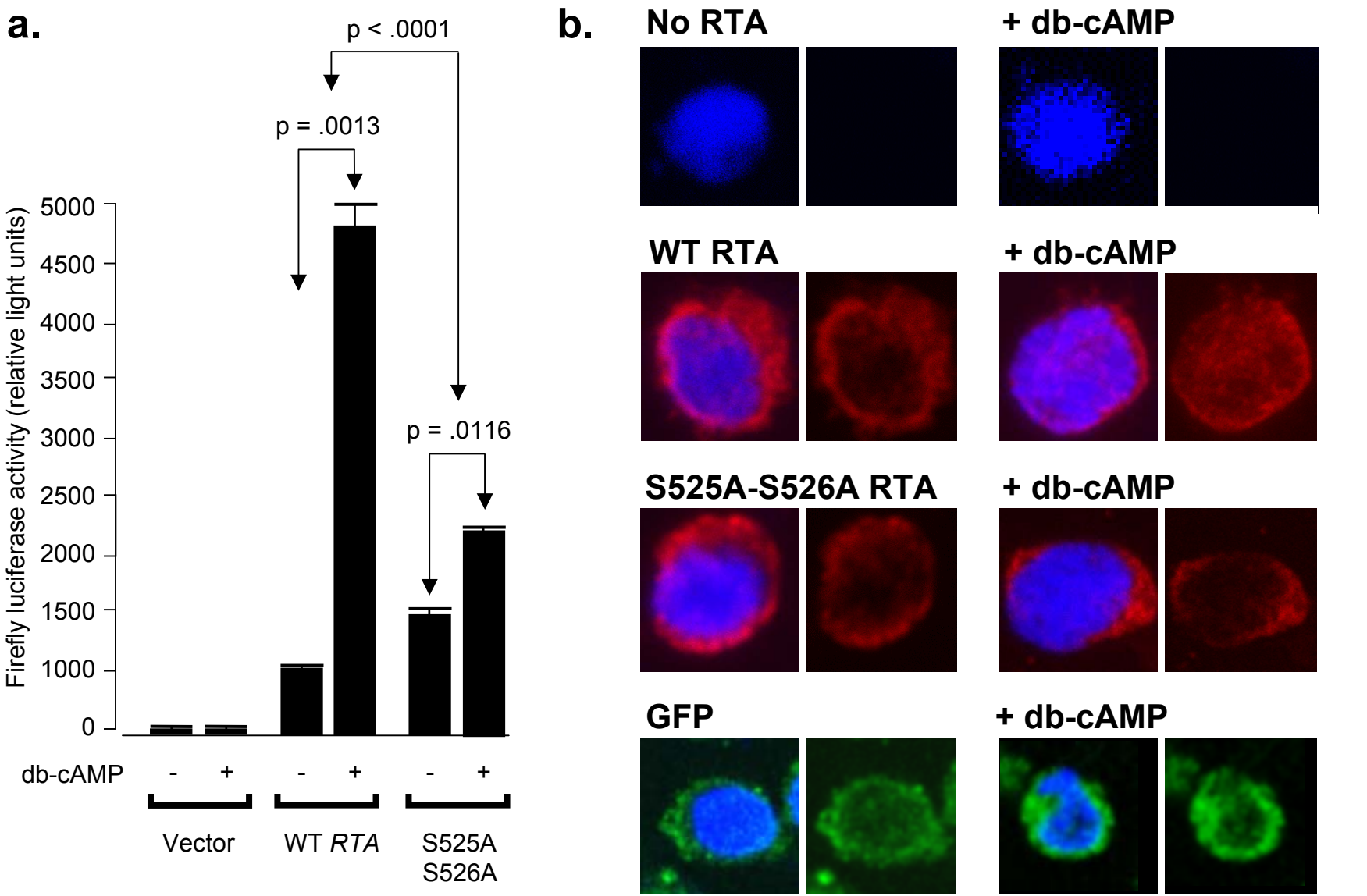

a.
KS-1:
Experiment 1
Experiment 2
Unstim.
PMA
10 μM NE
Unstim.
PMA
10 μM NE
KS patient serum
MW 49,800
β-actin
b.
BCBL-1:
Norepinephrine
PMA
0
100 pM
1 nM
10 nM
100 nM
1 μM
10 μM
100 μM
KS patient serum
MW 49,800
β-actin
c.
Immediate-early: ORF50
Fold-increase over untreated
400
300
200
100
60
40
30
20
10
6
4
3
2
1
0
12
24
36
48
Hours post-treatment
PMA
NE 100 μM
NE 10 μM
Untreated
d.
Late: ORF29
3000
2000
1,000
500
300
200
100
50
30
20
10
5
3
2
1
0
12
24
36
48
Hours post-treatment
PMA
NE 100 μM
NE 10 μM
Untreated


a.
10 μM
10 μM
10 μM
10 μM
10 μM
NE
PMA
Unstim.
100 μM
10 μM
1 μM
.1 μM
100 μM
Propranalol
KS patient serum
MW 49,800
β-actin
b.
ORF50
ORF29
Fold increase in copies/GAPDH
64
32
16
8
4
2
1
NE μM 0 10 10 10 0 10 10 10
Phentolamine (-α) μM 10 10
Propranalol (-β) μM 10 10
c.
No RT
+ Control
BCBL-1
KS-1
BCBL-1
KS-1
β1-AR
β2-AR
β3-AR
GAPDH


ORF50
ORF29
Fold increase in copies/GAPDH
64
32
16
8
4
2
1
NE μM
KT-5720 (-PKA) μM
PKC 19-27 (-PKC) μM
db-cAMP(+PKA) μM
0 10 10 10 10
1
1 10
100 300 1000
0 10 10 10 10
1
1 10
100 300 1000


GFP
Empty vector
GFP
GFP + PKA$_c$
0.2%
75%
56%
Counts
FL1-H
ORF50 ORF29
p = .007
p = .002
Fold increase in copies/GAPDH
Empty vector
+ GFP
+ PKAc
Empty vector
+ GFP
+ PKAc


Cell
Reporter
Co-expressed
db-cAMP
Fold increase in firefly luciferase activity (relative light units)
a.
DG75 RTA promoter
DG75 RTA promoter +
0 1 2 3 4 5 6 7 8
p = .006
b.
BCBL-1 RTA promoter
BCBL-1 RTA promoter +
p = .004
0 10 20 30 40
c.
DG75 RTA promoter RTA
DG75 RTA promoter RTA +
p = .019
Cell
Reporter
Co-expressed
db-cAMP
Fold increase in firefly luciferase activity (relative light units)
0 1000 2000 3000 4000 5000 6000
d.
DG75 PAN promoter
DG75 PAN promoter +
DG75 PAN promoter RTA
DG75 PAN promoter RTA +
p = .011
0 500 1000 1500
e.
BCBL-1 PAN promoter
BCBL-1 PAN promoter +
BCBL-1 PAN promoter RTA
BCBL-1 PAN promoter RTA +
p = .001


a.
p < .0001
p = .0013
p = .0116
Firefly luciferase activity (relative light units)
5000
4500
4000
3500
3000
2500
2000
1500
1000
0
db-cAMP
-
+
-
+
-
+
Vector
WT RTA
S525A
S526A
b.
No RTA
+ db-cAMP
WT RTA
+ db-cAMP
S525A-S526A RTA
+ db-cAMP
GFP
+ db-cAMP