A novel role for RAX, the cellular activator of PKR, in synergistically stimulating SV40 Large T Antigen-dependent gene expression

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Running Title: RAX, a regulator of SV40 gene expression

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SUMMARY

The double-stranded (ds) RNA-binding protein RAX was discovered as a stress-induced cellular activator of the dsRNA-dependent protein kinase (PKR), a key regulator of protein synthesis in response to viral infection and cellular stress. We now report a novel function of RAX, independent of PKR, to enhance SV40 promoter (origin)/enhancer-dependent gene expression. Several mammalian cell lines including COS-7, CV-1 and HeLa cells were tested. Results reveal that the SV40 large T antigen is required for RAX-mediated, synergistic enhancement of gene expression. RAX augments SV40 regulatory element-dependent DNA replication and transcription. The mechanism requires the SV40 enhancer, a viral transcriptional element that is necessary for efficient SV40 DNA replication in vivo. Mutational analysis reveals that the dsRNA-binding domains of RAX are required for the gene-expression enhancing function. Thus, in addition to stimulating PKR activity, RAX can positively regulate both SV40 large T antigen-dependent DNA replication and transcription in a mechanism that may alter the interaction of the cellular factor(s) with the SV40 enhancer via RAX’s dsRNA-binding domains. This novel function of RAX may have implications for regulation of mammalian DNA replication and transcription due to the many similarities between the viral and cellular processes.
INTRODUCTION

SV40 is a small DNA virus which has served as a powerful model system for dissecting fundamental biological processes including, DNA replication, transcription and neoplastic transformation (1-4). The SV40 genome consists of a 5.2 kb circular duplex DNA molecule with a 300 bp regulatory region which contains SV40 promoter (including the viral origin of DNA replication) and enhancer elements (2, 5). SV40 DNA replication and transcription occurs in the nucleus of the host cell where the SV40 genome is complexed with histones to form a nucleosomal structure (i.e. minichromosome) which is indistinguishable from cellular chromatin, indicating the similarities between viral and cellular DNA replication and transcription (2, 6, 7). The SV40 large T antigen (T-Ag) is a multifunctional viral protein with a DNA helicase activity and a transcriptional activity and plays an essential role in SV40 DNA replication and transcription (1-4). The T-Ag has been found to interact with a number of cellular proteins that regulate DNA replication and transcription (e.g. DNA polymerase α, human TFIIB-related factor (hBRF), pRb and p53) and to modify cellular regulatory processes that may efficiently promote viral gene expression and replication in permissive cells (1-4). However, the mechanisms by which SV40 T-Ag cooperates with the cellular proteins in vivo to facilitate viral DNA replication and transcription have not been fully elucidated.

The interferon-inducible double-stranded (ds) RNA dependent protein kinase (PKR) is a major regulator of host antiviral defense and normal cellular protein synthesis (8-11). For
example, viral infection leads to PKR activation with phosphorylation of its physiologic substrate, the α subunit of eukaryotic initiation factor-2 (eIF2α), that leads to inhibition of translation of mRNA (12). PKR also shuts down protein synthesis in uninfected cells following cellular stress responses that lead to inhibition of cell growth and apoptosis, including interleukin-3 (IL-3) growth factor withdrawal, serum deprivation, and treatment of cells with tumor necrosis factor-α and lipopolysaccharide (11, 13, 14). Recently, RAX and its human homologue, PACT, were independently discovered as the first cellular activators of PKR (15, 16). RAX/PACT is also a dsRNA-binding protein that directly binds and activates PKR following cellular stresses (such as IL-3 withdrawal, sodium arsenite, hydrogen peroxide, serum deprivation or ceramide treatment) that lead to cell death (15-18). In addition, it is now clear that PKR is a key regulator of protein synthesis but may also regulate other fundamental cellular processes including cell proliferation, apoptosis, differentiation and signal transduction (19-22). Findings reported here indicate that RAX has a novel, unexpected function to augment SV40 T-Ag-dependent DNA replication and transcription that is dependent on its dsRNA-binding domains but independent of PKR.

**MATERIALS AND METHODS**

*Cell culture and transfection*

COS-7, CV-1 and HeLa cells were obtained from American Type Culture Collection
and were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). All transient transfection or co-transfection in these cells was performed using LipofectAMINE (Life Technologies, Inc.).

**Luciferase reporter constructs and expression constructs**

pGL3-luciferase reporter vectors (including pGL3-promoter vector and pGL3-basic vector) and pRL-*Renilla* luciferase reporter vectors (including pRL-SV40 vector and pRL-CMV vector) were obtained from Promega. pGL3-promoter vector contains the SV40 early promoter element while pGL3-basic vector lacks promoter or enhancer elements. pRL-SV40 vector and pRL-CMV vector contain the SV40 early promoter/enhancer elements and the human CMV (cytomegalovirus) early promoter/enhancer elements, respectively. pGL3-SV40, pGL3-CMV and pGL3-RSV luciferase reporter vectors were also created by subcloning the promoter/enhancers of SV40, CMV and RSV (Rous sarcoma virus) into the pGL3-basic vector, respectively. The SV40 promoter/enhancer sequence in the pGL3-SV40 and pRL-SV40 vectors contains the SV40 core origin of DNA replication and auxiliary elements including the 21bp repeats and the 72bp enhancer elements (23). pSV40 E-TAL luciferase reporter vector was created by subcloning the SV40 enhancer sequence from pRL-SV40 vector into pTAL-luciferase reporter vector (CLONTECH) that contains a TATA-like promoter (P_TAL) region from the Herpes simplex virus thymidine kinase (HSV-TK) promoter.

The hemagglutin (HA) epitope-tagged RAX cDNA (15) was subcloned into expression
vectors pcDNA3 (Invitrogen) and pGC1, respectively. The pGC1 vector was derived from the pGL3-CMV vector by deleting the luciferase reporter gene cDNA. Expression plasmids of Flag-tagged RAX and its mutants (such as K84A, K177A, ΔII and ΔI/II) were also created using expression vectors pIRESneo (CLONTECH) and pcDEF3 (15, 24), respectively. Site-directed mutagenesis experiments were performed using the Transformer Kit (CLONTECH). In K84A and K177A mutants the lysine codon was mutated to an alanine codon. ΔII and ΔI/II mutants were created by deleting the cDNA fragments coding amino acid residues 125-194 or residues 63-219 using the restriction enzymes followed by blunt-end ligation.

An expression construct of SV40 large T antigen was created by subcloning the T-Ag cDNA fragment of SV40 genome/pRB322 into the pIRESneo vector. A Flag-tagged eIF2α cDNA was also subcloned into the pcDNA3 vector.

The expression vectors pcDNA3 and pcDEF3 contain the SV40 promoter/enhancer elements that functions as the neomycin promoter while pGC1 and pIRESneo do not contain such SV40 regulatory elements.

**Co-expression of RAX and eIF2α**

Co-transfection of 0.5 μg of Flag-RAX/pIRESneo (empty vector as a control) with 0.5 μg of Flag-eIF2α/pcDNA3 into COS-7 cells was performed in 6-well plates. After 48 h cells were lysed in buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail (Sigma)). 50 μg of the lysate was loaded into a 10% SDS-PAGE gel followed
by western blot analysis using an anti-Flag antibody (Roche Molecular Biochemicals) and the same blot was also stained by Fast-Green dye.

**Luciferase assays**

Co-transfection of 1 µg of HA-RAX/pGC1 (empty vector as a control) with 0.1 µg of pGL3-SV40 vector, pGL3-RSV vector or pGL3-CMV vector into COS-7 cells was performed in 6-well plates. After 48 h cells were lysed and assayed for luciferase activity using the Luciferase assay system (Promega). 0.1 µg of pGL3-SV40 luciferase vector was also co-transfected into CV-1 cells with total 1 µg of plasmids containing empty vector, RAX alone, T-Ag alone or both RAX and T-Ag followed by luciferase assays. The plasmid combinations were 0.5 µg of pGC1 plus 0.5 µg of pIRESneo for the empty vector, 0.5 µg of HA-RAX/pGC1 plus 0.5 µg of pIRESneo for the RAX alone, 0.5 µg of pGC1 plus 0.5 µg of T-Ag/pIRESneo for the T-Ag alone, and 0.5 µg of RAX/pGC1 plus 0.5 µg of T-Ag/pIRESneo for the both RAX and T-Ag, respectively. Similar experiments were also carried out in HeLa cells. The total protein was measured for normalizing luciferase activity readings.

To rule out the possibility that the observed effect of RAX on luciferase activity might be an artifact of a particular RAX expression vector or luciferase reporter vector used, other RAX expression plasmids (including HA-RAX/pcDNA3 and Flag-RAX/pIRESneo) and other luciferase reporter vectors (including pRL-SV40 and pRL-CMV Renilla luciferase vectors as well as pGL3-control vector (Promega), which contains both SV40 promoter and enhancer
elements and was alternatively used as a pGL3-SV40 vector) were also tested in the studies. Similar results were obtained and some examples were shown in the Results section.

Treatment of the PKR inhibitor 2-aminopurine (2-AP) was performed (25). After the HA-RAX/pGC1 plasmid (empty vector as a control) was co-transfected with the pGL3-SV40 luciferase vector into COS-7 cells using the LipofectAMINE method, 2-AP was immediately added into the DMEM media to a final concentration of 10 mM. After 48 h cells were lysed and assayed luciferase activity as described above.

Southern blot analysis

Co-transfection in COS-7 and CV-1 cells was carried out as described before. For plasmid isolation, cells were harvested and lysed in the plasmid lysis buffer (0.6% SDS, 10mM EDTA). The lysate was added with NaCl to a final concentration of ~1 M and incubated on ice overnight. The lysate was then centrifuged for 15 min at 13 g to pellet chromosome DNA. The plasmids in the supernatant were extracted by phenol/chloroform and then precipitated by ethanol. The purified plamids were linearized by the restriction enzymes and then digested by Dpn I (26) followed by Southern blot analysis using the luciferase cDNA insert or the Flag-eIF2α cDNA insert as probe that was radiolabeled using RadPrime DNA labeling system (Life Technologies, Inc.).

Northern blot analysis

Co-transfection was performed as described above. Total RNAs were isolated using the
Trizol method (Life Technologies, Inc.) and probed with the radiolabeled luciferase cDNA insert.

35S-methionine labeling and immunoblotting

Co-transfection was performed as described above. After 48 h, proteins in COS-7 cells that transiently express HA-RAX (or empty vector as a control) were pulse-labeled with 35S-methionine for 60 min to assess protein synthesis (27). The cells were lysed in buffer B (10 mM HEPES, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 20 mM sodium fluoride, 20 mM sodium pyrophosphate, 20 mM b-glycerophosphate, 20 mM sodium molybdate, 20 mM microcystin-LR and protease inhibitor cocktail (Sigma)) followed by SDS-PAGE, Western transfer and autoradiography. The same blot was also immunoblotted using antibodies against phospho-eIF2α (Cell Signaling Technology), eIF2α (15), HA (12CA5, Roche Molecular Biochemicals) and SV40 T-Ag (BD PharMingen).

Poly(I)•poly(C)-agarose beads binding assays

Expression plasmids of Flag-tagged wild-type and mutant RAX as described earlier were transfected into COS-7 cells, respectively. After 48 h cells were lysed in buffer A. Aliquots (50 µg) of lysates were incubated with 20 µl of poly(I)•poly(C) or poly(C)-agarose beads (Amersham Pharmacia Biotech) for 1.5 h at 4°C. These beads were washed three times with buffer A before boiling in SDS-PAGE sample buffer. The eluted samples and 50 µg of the whole cell lysate (as input) were then loaded onto a 10% SDS-PAGE gel followed by
immunoblotting using an anti-Flag antibody.

RESULTS

RAX selectively stimulates SV40 promoter/enhancer-dependent gene expression

To investigate the mechanism(s) by which RAX may regulate PKR, COS-7 cells were co-transfected with plasmids containing Flag-tagged RAX and Flag-tagged eIF2α. Surprisingly, Western blot analysis revealed that Flag-RAX could significantly enhance the expression of the Flag-eIF2α plasmid that was created in a pcDNA3 vector (Invitrogen, Fig. 1A). Fast-Green staining confirmed that expression of total cellular proteins was not affected (Fig. 1B). RAX-mediated augmentation of exogenous gene expression was unexpected because RAX was initially identified and characterized as a cellular activator of PKR that inhibits protein synthesis (15, 16). Since viral promoter/enhancers (such as those of SV40, CMV and RSV) are commonly used to drive exogenous gene expression, we tested whether RAX could affect the transcriptional activity of these promoter/enhancer elements. A commercial pGL3-luciferase reporter vector (Promega) containing these viral promoter/enhancer elements was constructed and co-transfected into COS-7 cells along with a plasmid containing either an HA-tagged RAX or an empty vector. Luciferase expression results indicated that HA-RAX could significantly enhance SV40 promoter/enhancer-dependent gene expression (i.e. ~10-fold increase) but had
only a minimal effect on gene expression driven by the CMV or RSV promoter/enhancers (i.e. ~1-fold increase, Fig. 2A). Western analysis demonstrates equal levels of HA-RAX or T-Ag expression (Fig. 2B).

**SV40 large T antigen is required for RAX-mediated enhancement of luciferase reporter gene expression**

COS-7 cells were derived from CV-1 monkey kidney cells following transformation with a replication origin-defective SV40 mutant that encodes the wild type T-Ag (28). Abundant expression of T-Ag occurs in COS-7 but not CV-1 cells (Fig. 2B and 3B). To test whether T-Ag expression was required for the RAX-mediated enhancement of luciferase gene expression, CV-1 cells were also co-transfected with a pGL3 SV40-luciferase vector and the plasmids containing HA-tagged RAX and/or T-Ag. Results indicate that while expression of HA-RAX alone had essentially no effect on luciferase gene expression, co-expression with the T-Ag can synergistically enhance the activity by over 45 fold (Fig. 3A). Since both HA-RAX and T-Ag were expressed at similar levels in CV-1 cells when transfected individually or together, variable expression levels could not account for HA-RAX-mediated enhancement of luciferase expression (Fig. 3B). Furthermore, in addition to monkey COS-7 and CV-1 cells that are naturally SV40 permissive (28), RAX was also demonstrated to synergize with the T-Ag to stimulate SV40 promoter/enhancer-dependent luciferase gene expression in human HeLa cells.
These findings clearly demonstrate that T-Ag expression is necessary for the synergistic enhancing effect of RAX on luciferase gene expression.

To rule out a nonspecific effect from the pGL3-Firefly luciferase reporter gene vector that might account for any differential reporter gene expression observed, a second luciferase reporter gene from another species was also tested. The pRL Renilla luciferase reporter vector (Promega) that contains either SV40 or CMV promoter/enhancer elements was expressed in COS-7 cells (Fig. 4B). Results confirm that HA-RAX exerts a selective and specific effect that was not dependent on the luciferase gene since expression of the two different luciferase enzymes were both synergistically enhanced. These data rule out a nonspecific, species-specific enhancement of gene expression. Therefore, it is concluded that RAX-mediated enhancement of luciferase gene expression is specific for the SV40 promoter/enhancer elements and independent of the plasmid or reporter genes employed.

**RAX-mediated enhancement of gene expression occurs at the level of both DNA replication and mRNA expression**

The wild type SV40 origin of DNA replication is contained in both the pGL3- and pRL-luciferase reporter vectors. Plasmids containing this origin can replicate in cells expressing the T-Ag, such as COS-7 cells (28). Therefore, we tested whether HA-RAX could enhance SV40 origin-dependent plasmid DNA replication. To distinguish a newly replicated pGL3-luciferase
plasmid in mammalian cells from the plasmid that was transfected into the cells, Dpn I endonuclease was used since it would only cleave the transfected plasmid containing Dpn I sites that were methylated during plasmid preparation in growing bacteria (26). Southern blot analysis using a radiolabeled luciferase cDNA probe revealed that pGL3-plasmid DNA replication occurred following transfection into mammalian cells and was significantly enhanced by HA-RAX (Fig. 5A). These findings indicated that RAX can stimulate SV40 promoter (origin)/enhancer-dependent DNA replication. This novel property of RAX was verified when it was shown that HA-RAX could also increase the replication of an unrelated Flag-eIF2α/pcDNA3 plasmid in COS-7 cells (Fig. 5B). The pcDNA3 vector contains both CMV promoter/enhancer elements (for expressing the gene of interest) and the SV40 promoter (origin)/enhancer elements to allow replication of the Flag-eIF2α/pcDNA3 in COS-7 cells. These findings help to explain how RAX can enhance expression of Flag-eIF2α/pcDNA3 (Fig. 1A). Since RAX only slightly or moderately increased CMV promoter/enhancer activity (Fig. 2A and 4B) while significantly enhancing the Flag-eIF2α expression, the mechanism may result primarily from RAX-enhancement of SV40 promoter (origin)/enhancer-dependent plasmid replication. Furthermore, since both pGL3-luciferase and the Flag-eIF2α/pcDNA3 plasmids are different plasmid constructs, these results strongly suggest that RAX-mediated augmentation of DNA replication is not an artifact of a particular plasmid construct but rather is specific for the SV40 promoter (origin)/enhancer elements.
To test whether the T-Ag was required for RAX-mediated enhancement of DNA replication, plasmid replication studies were carried out in CV-1 cells that lack T-Ag expression. Results indicated that expression of the T-Ag is required for RAX-mediated enhancement of SV40 promoter (origin)/enhancer-dependent DNA replication (Fig. 5C).

Northern analysis confirmed that HA-RAX synergistically increased SV40 promoter/enhancer-dependent luciferase mRNA expression in COS-7 and CV-1 cells (Fig. 6A and B). Results in CV-1 cells indicated that although the basal level of luciferase mRNA expression was undetectable, probably due to the small amount of luciferase vector used in the cotransfection, HA-RAX synergized with the co-expressed T-Ag to stimulate mRNA expression (Fig. 6B, comparing lanes 1 and 2 with lanes 3 and 4). Since (luciferase) gene transcription primarily occurs downstream of plasmid replication, HA-RAX-enhancement of mRNA expression resulted, at least in part, from enhanced plasmid replication. As shown in Fig. 5 and 6, the magnitude of HA-RAX-mediated enhancement of mRNA expression appeared to be greater than that of plasmid DNA synthesis, especially in CV-1 cells, indicating that RAX likely stimulated SV40 regulatory element-dependent DNA transcription as well. Since the T-Ag can function in both SV40 DNA synthesis and transcriptional activation that are tightly coupled processes, RAX may cooperatively stimulate these two processes.

*RAX’s dsRNA-binding domains are required to enhance luciferase gene expression*
RAX/PACT contains three putative dsRNA-binding motifs that are required for PKR activation (15, 16, 29). The first two dsRBMs display high homology with the first dsRBM of PKR (15, 16, 30). K84 and K177 are conserved lysine residues located within the first and second dsRBMs that are necessary for RAX’s dsRNA-binding (15, 16, 29). Constructs containing site-directed mutation (i.e. K84A and K177A) or deletion of one or both of these domains (i.e. ∆II and ∆I/II) were created to examine a role for RAXs dsRNA-binding property (Fig. 7A). Binding to poly(I)•poly(C)-agarose beads indicated that the first two dsRBMs were essential for dsRNA-binding while the third dsRBM alone failed to bind (Fig. 7B). Since only one of the first two dsRBMs of RAX is sufficient for poly(I)•poly(C)-binding in vitro under physiological salt concentrations (150 mM NaCl), a potential difference in the binding affinity between wild-type RAX and mutants in only one of the dsRNA-binding domains could not be detected (Fig. 7B). However, when a more stringent binding condition (500 mM NaCl) is used, the RAX mutants display lower poly(I)•poly(C)-binding than wild type RAX in vitro (data not shown). However, whether differential dsRNA-binding of the wild type and mutant RAX proteins occurs in vivo is not yet clear. Now, using these RAX constructs, we tested whether the dsRNA-binding property can affect RAX-augmented SV40 T-Ag-dependent gene expression. pGL3-SV40 luciferase assays were performed in COS-7 cells co-transfected with the wild-type or a mutant Flag-tagged RAX as described above. Results reveal that deletion of the first two dsRBMs completely abolishes RAX-mediated augmentation of luciferase gene expression.
while mutating or deleting only a single such domain demonstrates reduced stimulation of expression (Fig. 7C). These findings indicate that the first-two dsRNA-binding domains of RAX may be necessary for RAX-mediated enhancement of gene expression.

**PKR is not required for RAX-mediated enhancement of gene expression**

Since RAX is a cellular activator of PKR, we tested whether PKR was involved in RAX-enhancement of SV40 T-Ag-dependent gene expression. A potential effect of RAX on eIF2α phosphorylation and protein synthesis was examined. COS-7 cells were transfected with either an HA-tagged RAX plasmid or an empty vector and metabolically labeled with 35S-methionine to measure steady-state levels of protein synthesis. Western blot analysis was performed using a phospho-eIF2α specific antibody to assess eIF2α phosphorylation since eIF2α is the physiologic substrate of activated PKR (31). Results clearly demonstrate that neither global cellular protein synthesis nor PKR activity was affected by the HA-RAX expressed in COS-7 cells (Fig. 8A and B). In addition, expression levels of the T-Ag and eIF2α in these cells were also not affected by HA-RAX expression (Fig. 8B).

In addition to regulating protein synthesis, PKR has been reported to participate in other fundamental cell processes including signal transduction and activation of transcription factors (22). To assess whether PKR activity might play a role in RAX-mediated enhancement of reporter gene expression, 2-aminopurine (2-AP), a potent inhibitor of PKR, was tested (25).
HA-RAX was co-transfected with the pGL3-SV40 luciferase reporter vector in the presence or absence of a high concentration of 2-AP (10mM) that could inhibit PKR activity (25). Results indicated that 2-AP addition had essentially no effect on HA-RAX enhancement of luciferase gene expression (Fig. 8C). Therefore, the enhancement of reporter gene expression likely resulted from augmentation of T-Ag-dependent DNA replication and transcription rather than affecting the rate of protein synthesis. These data also indicated that RAX-enhancement of DNA replication and gene expression is independent of PKR activity.

*RAX augments T-Ag-dependent gene expression in a mechanism that involves the SV40 enhancer*

Whether RAX can co-operate directly with the T-Ag was tested to determine if a stable association between HA-RAX and T-Ag could be detected. Results indicate this was not the case (data not shown). Alternatively, a potential role of the SV40 enhancer, which has no T-Ag binding site but contains binding sites for several cellular transcription factors (32-34), was tested. HA-RAX was co-transfected with a pGL3-luciferase vector containing either the SV40 promoter (origin) element alone or the SV40 promoter (origin) and enhancer elements together. Results reveal that expression of HA-RAX augments SV40 promoter (origin)/enhancer-dependent luciferase gene expression significantly more than the expression observed from the plasmid containing the SV40 promoter (origin) element alone (Fig. 9A). Furthermore, HA-RAX
also stimulates the SV40 enhancer transcriptional activity independent of the SV40 origin of replication (Fig. 9B). These data suggest that the SV40 enhancer may play a critical role in RAX-stimulated SV40 regulatory element-dependent DNA replication and transcription.

**DISCUSSION**

Results reported here support a novel, unexpected function for RAX, the cellular activator of PKR, to synergize with the T-Ag to stimulate SV40 promoter (origin)/enhancer-dependent luciferase reporter gene expression (Fig. 2-4). RAX-mediated augmentation of luciferase gene expression occurs at the level of both plasmid DNA replication (Fig. 5) and mRNA expression (Fig. 6) but independent of PKR (Fig. 8). Furthermore, using different promoter/enhancer elements (SV40, CMV and RSV) or reporter genes (Firefly luciferase, *Renilla* luciferase and Flag-eIF2α), results confirm that RAX-mediated augmentation is specific for the SV40 promoter (origin)/enhancer elements. Therefore, in addition to its regulatory role in activating PKR, we can conclude that RAX can also function as a positive regulator of SV40 regulatory element-dependent DNA replication and gene expression.

Results were obtained in several mammalian cell lines (COS-7, CV-1 and HeLa) indicating that the T-Ag is required for RAX-mediated stimulation of SV40 promoter (origin)/enhancer-dependent DNA replication and gene expression (Fig. 2-6). Although a significant synergy can be observed between the RAX and T-Ag, attempts to detect a stable
direct association between these two proteins was not successful. These data suggest that RAX may indirectly co-operate with the T-Ag to stimulate DNA replication and gene expression. Interestingly, ectopic expression of RAX in COS7 cells enhances the association between the T-Ag and an as yet undetermined ~75 kDa cellular protein (data not shown). Efforts are ongoing in the lab to identify this ~75 kDa protein.

It is now well recognized that a plasmid containing the SV40 promoter (origin)/enhancer elements can be assembled into chromatin structure following the transfection of CV-1 or COS cells (35, 36). Such nucleosomal assembly indicates that plasmid DNA replication and transcription closely resembles SV40 DNA replication and transcription in vivo. Thus, RAX-mediated enhancement of gene expression may result from its stimulatory role as a cellular regulator of SV40 DNA replication and transcription in vivo. The SV40 promoter (origin)/enhancer sequence contained in the reporter vectors employed consists of an intact viral origin of DNA replication and auxiliary elements including the 21 bp repeats and the 72bp enhancer elements (23). The T-Ag directly interacts with the viral origin region and weakly binds to the 21 bp repeats, while the 72bp enhancer elements do not contain a T-Ag binding site but contain other cellular transcription factor-binding sites (e.g. a binding site for AP-1) (32, 34). While the auxiliary transcriptional elements (i.e. the 21 bp repeats and the SV40 enhancer) are not essential for viral DNA replication, they can enhance replication activity and are necessary for the efficient SV40 DNA replication in vivo (33, 37-39). Interestingly, the SV40
enhancer was found to play a critical role in RAX-mediated enhancement of gene expression (Fig. 9). Thus, RAX may enhance the T-Ag function indirectly to stimulate DNA replication and transcription by altering the interaction of cellular factors (such as AP-1) with the SV40 enhancer. Recently, the SV40 enhancer (including the AP-1 element) was reported to direct chromatin remodeling in SV40 chromosomes during activation of early transcription (40). Since binding of transcriptional factor-mediated chromatin remodeling can play a role not only in transcriptional activation but also viral DNA replication, RAX may regulate (or cooperate with) cellular factors such as AP-1 that bind to the SV40 enhancer. While speculative, this could modify the nucleosomal structure and stimulate SV40 DNA replication and transcription. Further studies will be required to elucidate this mechanism.

Recently, an ~70-amino acid dsRNA-binding motif (dsRBM) that specifically binds dsRNA has been identified in a growing family of dsRNA-binding proteins, including those expressed in viruses, bacteria, and lower and higher eukaryotes (41, 42). RAX/PACT contains three such dsRNA-binding motifs (dsRBMs) that possess differential dsRNA-binding properties (Figure 7B, 29). The first two dsRBMs of RAX/PACT are essential for dsRNA-binding. It is known that dsRNA-binding domains are not only necessary for dsRNA-binding but also can mediate protein-protein interactions in either an RNA-dependent or an RNA-independent manner (15, 16, 29, 43, 44). These domains have been found to be critical for the function and regulation of dsRNA-binding proteins (42). For example, the dsRNA-binding domain of PKR
plays an essential role in its dimerization and enzymatic activity, interactions with regulators of PKR, and targeting to the ribosome (43, 44). While our results indicate that the dsRNA-binding domains of RAX are essential for its enhancement of SV40 promoter (origin)/enhancer-dependent DNA replication and gene expression (Fig. 7C), the mechanism is not yet clear.

RAX/PACT was initially discovered as a dsRNA-binding protein that interacts with and activates PKR during stress signaling (15, 16). Given this, it is surprising that PKR activity is apparently not involved in RAX-mediated enhancement of T-Ag-dependent DNA replication and gene expression (Fig. 8). This PKR-independent, “rogue” property of RAX may occur by T-Ag co-opting of RAX in the absence of an applied stress that would otherwise mediate RAX activation of PKR. Alternatively, T-Ag may somehow prevent PKR activation by potentially inactivating the PKR pathway. This latter possibility seems likely since it has been recently shown that expression of the SV40 T-Ag is able to inhibit a PKR-mediated translational block in virus-infected cells at a step downstream of eIF2α phosphorylation (45). Interestingly, dsRNA-binding proteins are reported to effect various biological functions (42). For example, PKRs function is not limited to regulating protein synthesis but is also involved in regulating cell proliferation, signal transduction and other cellular processes (22, 46). Other dsRNA-binding proteins are also reported to be involved in diverse biological processes including rRNA processing (e.g. RNase III), pre-mRNA editing (e.g. ADAR1), mRNA localization (e.g. Staufen) and transcriptional activation (e.g. RHA, 42, 47-50). Thus, a novel function for RAX in
regulating SV40 DNA replication and transcription is not inconsistent with the “multifunctional”
nature of dsRNA-binding proteins.

In summary, we have discovered a novel regulatory function for RAX, the cellular
activator of PKR, in synergizing with the T-Ag to stimulate SV40 promoter (origin)/enhancer-
dependent DNA replication and gene expression. RAX requires its dsRNA-binding domains and
the SV40 enhancer to positively regulate SV40 DNA replication and transcription. Thus, RAX
may play a PKR-independent role in regulating mammalian DNA replication and transcription
due to the many similarities between the viral and cellular processes.

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REFERENCES


FOOTNOTES

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Abbreviations: 2-AP, 2-aminopurine; eIF2α, the α subunit of eukaryotic initiation factor-2; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PKR, double-stranded RNA-dependent protein kinase; RAX, PKR-associated protein X; T-Ag, SV40 large T antigen.

FIGURE LEGENDS

FIG. 1. RAX-mediated enhancement of Flag-eIF2α expression in COS-7 cells. Flag-RAX/pIRESneo (empty vector as a control) was co-transfected with Flag-eIF2α/pcDNA3 into
COS-7 cells. At 48 h post-transfection, cells were lysed and 50 µg of the lysate was loaded into a 10% SDS-PAGE gel as described under “Materials and methods” (A) Western blot analysis using an anti-Flag antibody. (B) Fast-Green staining. V – empty vector pIRESneo; RAX – Flag-RAX/pIRESneo. Lanes 1, 2 and 3 are triplicates for the vector while lanes 4, 5 and 6 represent triplicates for Flag-RAX.

**FIG. 2. Effects of RAX on luciferase reporter gene expression in COS-7 cells.** 1 µg of HA-RAX/pGC1 plasmid (empty vector as a control) was co-transfected into COS-7 cells with 0.1 µg of pGL3-luciferase reporter vector containing the promoter/enhancer of SV40, RSV or CMV (pGL3-SV40, pGL3-RSV or pGL3-CMV). After 48 h cells were lysed and assayed for luciferase activity as described under “Materials and methods”. (A) Relative luciferase activity. Luciferase expression is displayed relative to that of the vector. V – empty vector pGC1; RAX – HA-RAX/pGC1. (B) Western blot analysis using antibodies of anti-HA and anti-T-Ag. Lanes 1 and 4 – pGL3-SV40; Lanes 2 and 5 – pGL3-RSV; Lanes 3 and 6 – pGL3-CMV.

**FIG. 3. Role of T-Ag in RAX-mediated enhancement of gene expression.** 0.1 µg of the pGL3-SV40 luciferase reporter vector was co-transfected into CV-1 cells with 1 µg of plasmids containing empty vector (V), HA-RAX alone, T-Ag alone or both HA-RAX and T-Ag. After 48 h cells were lysed and assayed for luciferase activity as described under “Materials and
methods”. (A) Relative luciferase activity. (B) Western blot analysis using antibodies of anti-HA and anti-T-Ag. Lane 1 Control – the lysate from COS-7 cells that was transfected with HA-RAX.

**FIG. 4.** (A) **RAX-mediated enhancement of luciferase reporter gene expression in HeLa cells.** pGL3-SV40 luciferase reporter vector was co-transfected into HeLa cells with plasmids containing RAX and/or T-Ag followed by luciferase assays similarly as described in FIG. 3. (B) **Effects of RAX on Renilla luciferase reporter gene expression in COS-7 cells.** pRL-SV40 and pRL-CMV Renilla luciferase reporter vectors were used to repeat luciferase assays as described in FIG. 2.

**FIG. 5.** **RAX-mediated enhancement of DNA replication.** Co-transfection in COS-7 and CV-1 cells was performed as described in FIG. 1-3. The plasmids were isolated, linearized and Dpn I-digested and then probed with a radiolabeled luciferase cDNA insert or Flag-eIF2α cDNA insert, as described under Materials and methods. Southern blot analysis of plasmid replication of (A) pGL3-SV40 luciferase vector in COS-7 cells, (B) Flag-eIF2α/pcDNA3 in COS-7 cells and (C) pGL3-SV40 luciferase vector in CV-1 cells. Lane 1 control the Dpn I digested pGL3-SV40 vector or Flag-eIF2α/pcDNA3 plasmid that was isolated from bacteria preparation and used as reference for the transfected plasmid.
FIG. 6. RAX-mediated enhancement of mRNA expression. Co-transfection in COS-7 and CV-1 cells was performed as described in FIG. 2 and 3. Total RNAs were isolated and probed with the radiolabeled luciferase cDNA insert as described under “Materials and methods”. Northern blot analysis in (A) COS-7 and (B) CV-1 cells. Luc – luciferase mRNA. Ethidium bromide staining of 18 S rRNA was used as a loading control (bottom panels).

FIG. 7. Mutational analysis of the role of the dsRNA-binding domains in RAX-enhancement of gene expression. (A) Schematic representation of wild-type (wt) and mutant RAX. (B) Poly(I)•poly(C)-agarose beads-binding assays. Expression plasmids of Flag-tagged wt and mutant RAX in the pcDEF3 vector were transfected into COS-7 cells, respectively. After 48 h cells were lysed and aliquots (50 µg) of lysates were incubated with poly(I)•poly(C)-agarose beads which were then washed with the lysis buffer followed by SDS-PAGE and immunoblotting using an anti-Flag antibody, as described under “Materials and methods”. Poly(C)-agarose beads-binding of wt RAX was used as a negative control. I – input; B – poly(I)•poly(C) or poly(C)-beads bound protein. pIC – poly(I)•poly(C)-beads-binding; pC – poly(C)-beads-binding. (C) Luciferase assays. The plasmids of Flag-tagged wt type and mutant RAX were co-transfected with the pGL3-SV40 luciferase reporter vector into COS-7 cells followed by luciferase assays as described in FIG. 2.
FIG. 8. RAX-mediated enhancement is PKR-independent. (A) \(^{35}S\)-metabolic labeling. The HA-RAX/pGC1 plasmid (empty vector as a control) was co-transfected with the pGL3-SV40 luciferase vector into COS-7 cells. After 48 h, proteins in COS-7 cells that transiently express HA-tagged RAX were pulse-labeled with \(^{35}S\)-methionine for 60 min to assess protein synthesis. The cells were lysed followed by SDS-PAGE, Western transfer and autoradiography.

(B) Western blot analysis. The same blot was also immunoblotted using antibodies against phospho-eIF2\(\alpha\), eIF2\(\alpha\), HA and SV40 T-Ag. (C) The PKR inhibitor 2-AP treatment. The co-transfection was performed as described in FIG. 2 in the presence or absence of 10 mM 2-AP followed by Luciferase assays.

FIG. 9. Role of the SV40 enhancer in RAX-mediated enhancement of gene expression (A) 0.1 \(\mu\)g of pGL3-luciferase reporter vectors containing the SV40 promoter (origin) element only (pGL3-SV40-P) and both SV40 promoter (origin) and enhancer elements (pGL3-SV40-P&E) were co-transfected with HA-RAX into COS-7 cells followed by luciferase assays as described in FIG. 2A. Luciferase expression is displayed relative to that of the vector co-transfected with pGL3-SV40-P. Western blot analysis of protein expression using antibodies against HA and SV40 T-Ag. V – empty vector pGC1; RAX – HA-RAX/pGC1. (B) pSV40 E-TAL luciferase reporter vector containing the SV40 enhancer alone (pSV40-E-TAL) and pTAL control reporter vector (pTAL) were co-transfected with HA-RAX into COS-7 cells followed by luciferase
assays and western blot analysis as described in (A). Luciferase expression is displayed relative
to that of the vector co-transfected with pTAL vector.
Fig. 1.
Fig. 2.

A

Relative Luciferase Activity

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<tr>
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B

kDa

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T-Ag

HA-RAX
Fig. 3.

A

![Graph showing Relative Luciferase Activity with different plasmids: V, RAX, T-Ag, RAX+T-Ag, pGL3-SV40.]

B

![Western blot images showing T-Ag and HA-RAX with lanes labeled: Control, V, RAX, T-Ag, RAX+T-Ag.]

Arrows indicate bands for T-Ag and HA-RAX.
Fig. 4.

**A**

![Graph showing relative luciferase activity for HeLa cells with different plasmids: V, RAX, T-Ag, RAX+T-Ag. The Y-axis represents relative luciferase activity, and the X-axis lists the plasmids. The graph shows a significant increase in luciferase activity when using RAX+T-Ag plasmid.](image)

**B**

![Graph showing relative luciferase activity for COS-7 cells with different plasmids: V, RAX. The Y-axis represents relative luciferase activity, and the X-axis lists the plasmids. The graph shows a significant increase in luciferase activity when using RAX plasmid.](image)
Fig. 5.

**A**

COS-7

1 2 3

- Replicated pGL3-SV40
- Dpn I-cleaved pGL3-SV40

**B**

COS-7

1 2 3

- Replicated Flag-eIF2α/ pcDNA3
- Dpn I-cleaved Flag-eIF2α/ pcDNA3

**C**

CV-1

1 2 3 4 5

- Replicated pGL3-SV40
- Dpn I-cleaved pGL3-SV40
Fig. 6

A

COS-7

28S →

18S →

EtBr

V

RAX

B

CV-1

28S →

18S →

Luc

EtBr

V

RAX

T-Ag

RAX+T-Ag

18S
Fig. 7.

A

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C

![Graph showing Relative Luciferase Activity for different plasmids](image)
Fig. 8.

A

B

C

Relative Luciferase Activity

25

20

15

10

5

0

+2ΔP

-ΔP

V

RAX

Plasmids
Fig. 9.

A

![Graph A showing relative luciferase activity for different plasmids: V, RAX, pGL3-SV40-P, and pGL3-SV40-P&E.]

B

![Graph B showing relative luciferase activity for different plasmids: V, RAX, pTAL, and pSV40 E-TAL.]

1 2 3 4

T-Ag

1 2 3 4

T-Ag

V RAX pGL3-SV40-P V RAX pGL3-SV40-P&E

HA-RAX

V RAX pTAL V RAX pSV40 E-TAL

HA-RAX
A novel role for RAX, the cellular activator of PKR, in synergistically stimulating SV40 large T antigen-dependent gene expression
Mingli Yang, Takahiko Ito and W. Stratford May

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