VRK1 phosphorylates CREB and mediates *CCND1* expression

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Accepted 25 June 2008
Journal of Cell Science 121, 3035-3041 Published by The Company of Biologists 2008
doi:10.1242/jcs.026757

Summary

Vaccinia virus B1 kinase plays a key role in viral DNA replication. The homologous mammalian vaccinia-related kinases (VRKs) are also implicated in the regulation of DNA replication, although direct evidence remains elusive. Here we show that VRK1 regulates cell cycle progression in the DNA replication period by inducing cyclin D1 (CCND1) expression. Furthermore, depletion of VRK1 in human cancer cells reduces the fraction of cells in S phase at a given time. VRK1 specifically enhances activity of the cAMP-response element (CRE) in the *CCND1* promoter by facilitating the recruitment of phospho-CREB to this locus. VRK1 phosphorylates CREB at Ser133 in vitro and the expression of a kinase-dead mutant of VRK1 or knockdown of VRK1 using siRNA fails to activate CREB and subsequently activate CRE. Finally, we show that VRK1 is a critical link in the *CCND1* gene expression pathway stimulated by Myc overexpression. Our results indicate that VRK1 is a novel regulator of *CCND1* expression.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/18/3035/DC1

Key words: VRK, CRE, CREB, Myc, CCND1

Introduction

DNA replication is an essential process occurring in all lifeforms that requires tight regulation to preserve genetic integrity. Vaccinia virus B1 (vvB1) kinase plays a critical role in viral DNA replication (Rempel et al., 1990) by regulating intermediate viral gene expression (Kovacs et al., 2001). Human vaccinia-related kinase 1 (VRK1) can functionally rescue the DNA replication deficiency of vaccinia virus harboring a temperature-sensitive mutation of vvB1 under nonpermissive temperature conditions (Boyle and Traktman, 2004). This capacity requires the kinase activity of VRK1 because a catalytically inactive allele of VRK1 fails to rescue viral replication and suggests that VRK1 mimics the function of vvB1 kinase (Boyle and Traktman, 2004).

Recent studies suggest several possible mechanisms by which VRK1 controls cell cycle progression: (1) phosphorylation of histone H3 at Thr3 and Ser10, which is essential in chromatin condensation during mitosis (Kang et al., 2007); (2) phosphorylation of the barrier-to-autointegration factor (BAF), which is important in the formation of proper chromatin and nuclear envelope structure (Nichols et al., 2006); and (3) transcriptional activation of genes involved in cell proliferation, such as retinoblastoma, cyclin-dependent kinase-2 (CDK2), and survivin (Santos et al., 2006). Furthermore, the high level of expression of VRK1 in proliferating tissue and in numerous cancer cell lines, implies a role for VRK1 in cell cycle regulation (Nezu et al., 1997). To date, however, there is no direct evidence for a role of VRK1 in DNA replication.

It is believed that cyclin D1 (CCND1), cyclin D2 (CCND2) and cyclin D3 (CCND3), which act as regulatory factors of the CDK partners CDK4 or CDK6, are the rate-limiting factors controlling the G1 to S phase transition (Tashiro et al., 2007). The finding that cyclin-D-null embryos survive to day 13.5, in a similar manner to normal control embryos, suggests that D-type cyclins are dispensable during cell cycle progression in embryonic development (Kozar et al., 2004). Moreover, although hematopoiesis is slightly impaired, mice lacking both CDK4 and CDK6 are viable and develop normally before dying during the late stages of embryonic development as a result of severe anemia (Malumbres et al., 2004). Nevertheless, it is clear that CCND1-CDK4 complexes are critical for the initiation of malignant cancer cell growth (Hulit et al., 2004).

The D-type cyclins are not tightly regulated during the cell cycle and are strongly mitogen dependent (Sherr, 1993). As a result, the CDKs formed by the association of D-type cyclins with CDK4 or CDK6 act as mitogen sensors during G1 phase to link signals from the extracellular environment to other CDKs comprising the core cell-cycle machinery (Sherr and Roberts, 2004). This link is activated by the mitogen-stimulated Ras- and Myc-signaling pathways (Yu et al., 2005). Once induced, CCND1 associates with CDK4 or CDK6 leading to an activated complex. The expression of *CCND1* is mainly regulated at the transcriptional level (Laurance et al., 2001). The cAMP-response element (CRE) in the *CCND1* promoter is critical for the transcriptional induction of the gene (Moriuchi et al., 2003; Pradeep et al., 2004).

In the present study, we investigate the mechanism underlying the regulation of DNA replication in human cancer cells by VRK1. Our results demonstrate that VRK1 is critical in the regulation of the *CCND1* gene expression pathway once activated by Myc.

Results

Involvement of VRK in DNA synthesis

To evaluate the role of the VRK family in DNA replication of mammalian cells, the fraction of cells in S phase at a given time in asynchronously growing HeLa cells was determined. Cells were labeled with bromodeoxyuridine (BrdU) for 1 hour, after transient expression of each VRK isotype of 24 hours, to identify cells undergoing DNA synthesis. The relative number of cells expressing VRK and showing BrdU labeling differed according to the VRK isotype. Approximately 50% of cells expressing VRK1 were positive for BrdU labeling, whereas the population of BrdU-

positive cells expressing VRK2 and VRK3 was significantly lower at 20%, a level consistent with cells transfected with the control vector (Fig. 1A). The effect of VRK1 on increased number of cells in S phase was more distinct if cells were allowed to have BrdU incorporation for only a short time (1 and 3 hours, see supplementary material Fig. S1) compared with a longer time of incorporation (12 and 24 hours, supplementary material Fig. S1). The requirement for VRK1 in DNA synthesis was further investigated by depleting VRK1 using siRNA. For this experiment, HeLa cells were pretreated with BrdU for 6 hours to allow cells to replicate and incorporate BrdU. The level of VRK1-depleted cells (siVRK1) labeled with BrdU was approximately 20%, whereas the level of BrdU-labeled cells treated with control siRNA was approximately 80% (Fig. 1C). These results suggest that VRK1 plays an essential role in DNA replication.

Regulation of CCND1 expression by VRK1

To investigate the mechanism underlying the regulation of DNA replication by VRK1, we examined the effect of VRK1 on the expression of cyclins. Transient overexpression of VRK1 specifically elevated the level of cyclins with a role in the G1-S transition, including CCND and cyclin E1 (CCNE1). The level of CCND was markedly increased whereas the level of CCNE was only slightly increased and located in both the cytoplasm and nucleus. By comparison, there was no significant change in the levels of cyclins involved in the G2-M transition, including cyclin A1 (CCNA1) and cyclin B1 (CCNB1) (Fig. 2A). To further investigate the VRK1-induced elevation of CCND expression, we utilized specific antibodies against each of the three isotypes of D-type cyclins (CCND1, CCND2 and CCND3) found in mammalian cells. CCND1 was the only isotype elevated by VRK1 (Fig. 2A). Consistent with these results, the siRNA-mediated knockdown of VRK1 in HeLa cells led to a specific decrease in the level of CCND1 and no change in the level of CCND2 or CCND3 (Fig. 2B). Control siRNA and the siRNA-mediated knockdown of VRK3 had no effect on the level of CCND1 (Fig. 2B). VRK1 modulates the expression of CCND1 in a time-dependent manner. As the siVRK1 incubation time was increased, the endogenous level of VRK1 gradually reduced as did the level of CCND1 (Fig. 2C). When proteosomal degradation is blocked with the proteasome inhibitor MG132, the protein level of CCND1 slightly increased whereas its mRNA level remained unchanged; inhibition of transcription with actinomycin D (ActD) significantly reduced both protein and mRNA level of CCND1 (Fig. 2D). Interestingly, overexpression of VRK1 resulted in elevation of mRNA level of CCND1 (Fig. 2D). These results suggest that the VRK1-mediated regulation of *CCND1* expression occurs via induction of transcription rather than by an increase in protein stability. To verify the requirement of VRK1 kinase activity in this process, we used a kinase-dead mutant (K179E) generated by site-directed mutagenesis of the active site (Kang et al., 2007; Vega et al., 2004). This mutant VRK1 failed to allow either cytoplasmic or nuclear accumulation of CCND1 (Fig. 3A,B), indicating that VRK1 kinase activity is required for the expression of *CCND1*.

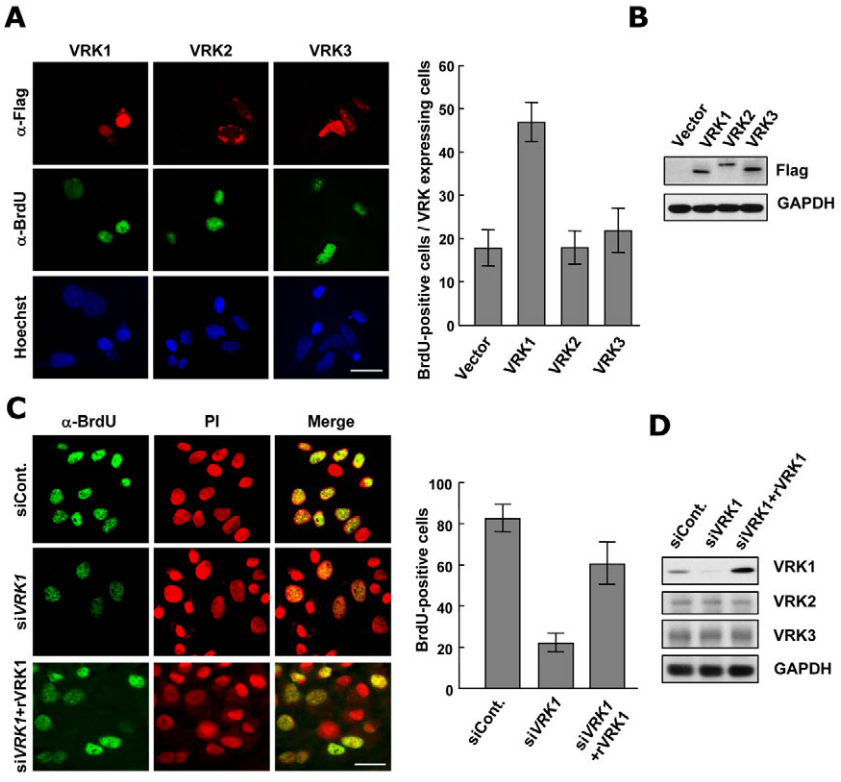

Fig. 1. Requirement of VRK1 for DNA replication. (A) Immunofluorescence of VRK1, VRK2 and VRK3 (red), BrdU (green) and DNA (Hoechst 33342, blue) from cells expressing pFlag-VRK. (B) The expression levels of Flag-labeled VRK1, VRK2 and VRK3 were determined by immunoblotting with the indicated antibodies. (C) HeLa cells transfected with control siRNA, siVRK1(CAAGGAACCTGGTGTTGAA; sequence effective only on human VRK1 not rat VRK1), or siVRK1 plus Flag-ratVRK1 (rVRK1) were immunostained for BrdU (green) and DNA (red, propidium iodide, PI). (D) The level of endogenous VRKs was measured by immunoblotting with the indicated antibodies. The error bars represent the mean ± s.d. from $n>300$ transfected cells. Scale bars: 20 μm.

VRK1 enhances *CCND1* expression through the CRE element

The mechanism underlying transcriptional induction of CCND1 was investigated using luciferase reporter assays to examine activation of the CRE and the activating protein 1 (AP1) element in the human *CCND1* promoter. VRK1 activates the CRE element but has no effect on the AP1 element in the *CCND1* promoter (Fig. 4A). Furthermore, the kinase-dead mutant of VRK1 did not activate CRE (Fig. 4A). These results suggest that VRK1 induces the activation of the CRE. CRE and AP1 activity were further investigated using a chromatin immunoprecipitation (ChIP) assay. Transient overexpression of VRK1 resulted in more robust binding of phospho-CREB (CREB-*P*) to CRE (Fig. 4B), the most critical element in the *CCND1* promoter (Laurance et al., 2001). The binding of ATF2 to CRE also increases with VRK1 overexpression (Fig. 4C). Based on these results, we conclude that VRK1 induces *CCND1* expression through activation of the CRE in the *CCND1* promoter. This is consistent with previous reports on the critical role of CRE in the *CCND1* promoter (Laurance et al., 2001; Moriuchi et al., 2003; Pradeep et al., 2004).

Physical interaction of VRK1 and CREB

VRK1 is known to bind several transcription factors, including p53 (Vega et al., 2004), c-Jun

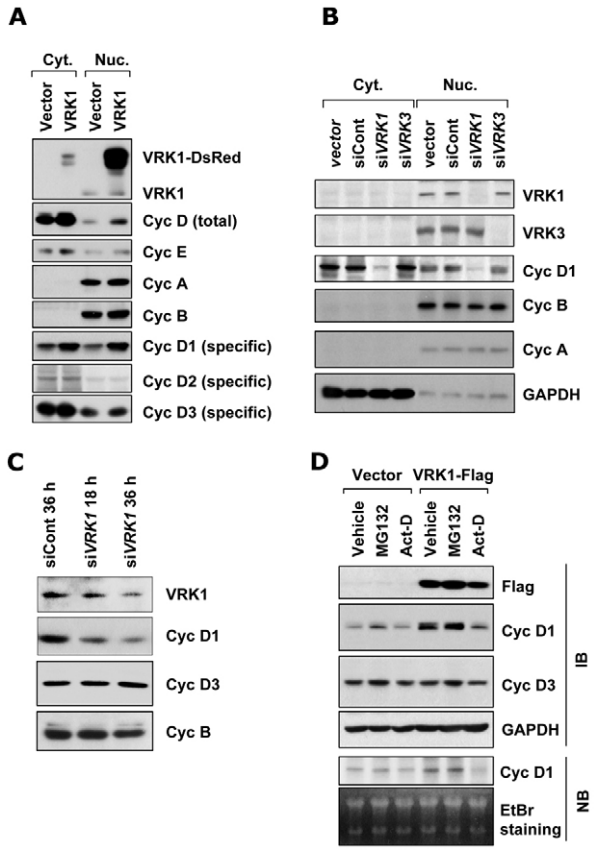

Fig. 2. Induction of CCND1 by VRK1. (A) Cytoplasmic (Cyt.) and nuclear (Nuc.) lysates from HeLa cells transfected with control vector (vector, pDsRed1-C1) or pDsRed1-C1-VRK1 were immunoblotted with the indicated antibodies. (B) HeLa cells transfected with mock vector (pFlag-CMV2), control siRNA (siCont), siVRK1 or siVRK3 were fractionated into cytoplasmic and nuclear lysates and then immunoblotted with the indicated antibodies. (C) Total protein lysates from HeLa cells transfected with control siRNA (siCont) or siVRK1 for the indicated periods were immunoblotted with the indicated antibodies. (D) HeLa cells transfected with control vector (vector, pFlag) or pFlag-VRK1 were treated with DMSO (vehicle), MG132 or actinomycin D (ActD) for 5 hours, followed by immunoblotting (IB) with the indicated antibodies and northern blotting (NB) with an antisense *CCND1* probe. Ethidium bromide (EtBr) stained 18S and 28S ribosomal RNA (rRNA) was used as a loading control.

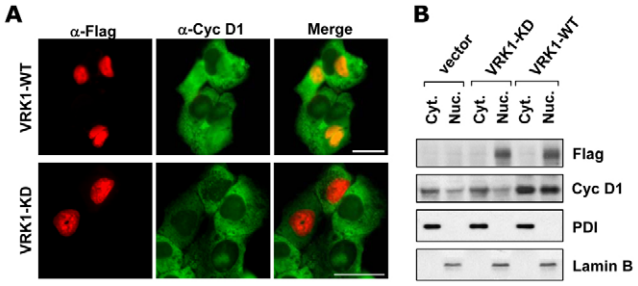

Fig. 3. Kinase activity of VRK1 is required for the nuclear accumulation of CCND1. (A) Immunofluorescence of CCND1 (green) and VRK1 (red) from cells expressing wild-type (WT) or the kinase-dead (KD) mutant of VRK1. (B) Cytoplasmic (Cyt.) and nuclear (Nuc.) lysates of HeLa cells transfected with mock vector (pFlag-CMV2), kinase-dead (VRK1-KD) or wild-type (VRK1-WT) VRK1 were immunoblotted with the indicated antibodies. Protein disulfide isomerase (PDI) and Lamin B were used as markers for the cytoplasm and nucleus, respectively. Scale bars: 20 μm.

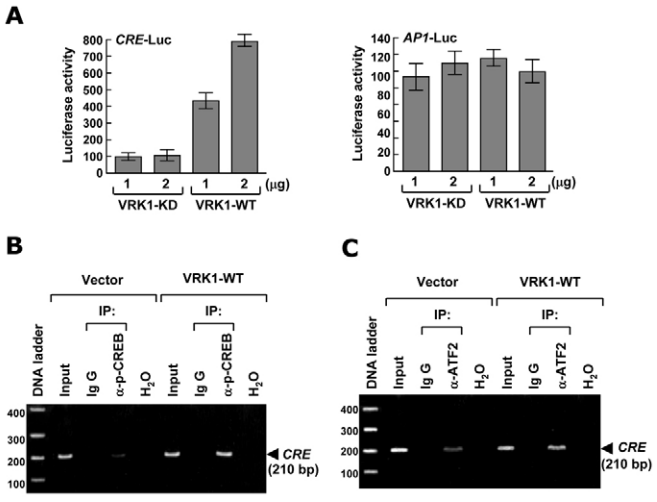

Fig. 4. Activation of cAMP-response element (CRE) in the *CCND1* promoter by VRK1. (A) The luciferase activity of cells transfected with kinase-dead (KD) or wild-type (WT) VRK1 was measured. The results represent mean ± s.d. from experiments performed triplicate. (B,C) ChIP assay measuring the CREB-*P* (B) and ATF2 (C) binding to CRE in the *CCND1* promoter of HeLa cells transfected with either KD-VRK1 or WT-VRK1.

(Sevilla et al., 2004a) and the cAMP-dependent transcription factor ATF2 (Sevilla et al., 2004b). Immunoprecipitation confirmed the presence of the CRE-binding protein (CREB) in the VRK1 immune complex (Fig. 5A). Moreover, using a GST-pulldown assay we also confirmed that CREB was associated with VRK1, and not VRK2 or VRK3, the other VRK isotypes (Fig. 5B). The functional motifs within the CREB protein are well characterized (De Cesare et al., 1999) and are depicted in Fig. 5C. To identify the motif associated with VRK1, we prepared a series of plasmid constructs containing CREB domain fragments and used the purified recombinant proteins in binding assays with VRK1. The results show that VRK1 forms a stable complex with full-length CREB but not with the CREB domain fragments (Fig. 5C). The mRNA level, protein level and kinase activity of VRK1 increases gradually from the G1 to the G2-M phase of the cell cycle, peaking at prometaphase (Kang et al., 2007). Consistent with this report, we showed that the protein level of VRK1 peaked in G2-M phase, decreased dramatically at the end of mitosis, then increased slightly in G1-S phase (Fig. 5D,E). By comparison, the expression profile of CREB was inversely proportional to that of VRK1 (Fig. 5D,E). Despite the difference in protein expression between CREB and VRK1 during the cell cycle, the association between these proteins occurs in a cell-cycle-dependent manner, is proportional to the level of CREB, and peaks during late G1 phase to early S phase (Fig. 5D,E).

Phosphorylation of CREB at Ser133 by VRK1

When the CREB protein is phosphorylated at its kinase-inducible motif, it folds into an active form then binds to the CREB-binding protein to activate target gene transcription (Dyson and Wright, 2005). The phosphorylation of CREB occurs in a cell-cycle-dependent manner, showing the highest level in the S-phase-arrested state followed by a gradual reduction (Saeki et al., 1999). Given the association of VRK1 with CREB, the possibility of VRK1 phosphorylating CREB was investigated. In vitro, we showed that

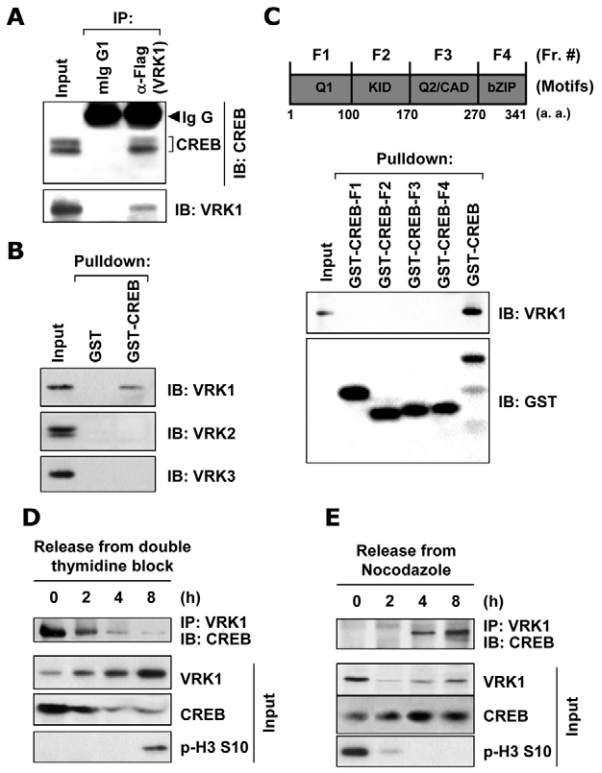

Fig. 5. Physical interaction of VRK1 with CREB in a cell-cycle-regulated manner. (A) Protein extracts from HeLa cells transfected with pFlag-VRK1 were analyzed by immunoprecipitation with an anti-Flag antibody. Coprecipitated CREB proteins were detected by immunoblotting using the anti-CREB antibody. Mouse immunoglobulin-G1 (mIg-G1) was used as an immunoprecipitation control. (B) Total cell lysates from HeLa cells were incubated with the GST-CREB protein and pulled down with glutathione-conjugated beads. Bound proteins were evaluated by immunoblotting using anti-VRK isoform-specific antibodies. (C) Recombinant full-length or fragments of CREB (F1-F4) were used for binding analyses with VRK1. Bound VRK1 protein was detected by immunoblotting using the anti-VRK1 antibody. Recombinant proteins were detected using the anti-GST antibody. bZIP, basic leucine zipper; CAD, constitutive activation domain; KID, kinase-inducible domain; Q, glutamine-rich domain. (D,E) The cell cycle was synchronized in S phase using a double thymidine block (D) or in M phase by treatment with nocodazole (E), and then released at the indicated time by removing the cell cycle inhibitors. Expression of VRK1 and CREB was assessed by immunoblotting and interactions were investigated by immunoprecipitation. P-H3 S10 (phosphorylated histone 3) was used as a mitotic marker.

VRK1 phosphorylates CREB at Ser133, a known phosphorylation site for CREB activation (Johannessen et al., 2004), but not at Ser142, a known phosphorylation site for CREB inactivation (Sun and Maurer, 1995) (Fig. 6A). CREB phosphorylation was detected only in full-length CREB and not in CREB domain fragments, consistent with the inability of CREB fragments to associate with VRK1 (Fig. 6A). Although the level of phosphorylation was significantly reduced for the S133A mutant CREB, VRK1-induced phosphorylation was not completely abolished. However, the kinase-dead VRK1 failed to phosphorylate CREB (Fig. 6A). This result suggests that, in addition to Ser133, VRK1 phosphorylates other residues in CREB. Consistent with in vitro kinase assay data, the CREB-*P* Ser133 level was enhanced in HeLa cells when wild-type VRK1 (WT-VRK1) was transiently expressed. The level did not change when kinase-dead VRK1 (KD-VRK1) was introduced (Fig. 6B,D). Moreover, the level of CREB-*P* Ser133 was markedly reduced when endogenous VRK1 was depleted with targeted siRNA (Fig. 6C). However, the total CREB level was not altered by WT-VRK1 expression (Fig. 6C,E). Together, these results show that endogenous VRK1 is involved in the phosphorylation of CREB at Ser133.

VRK1 mediates Myc-induced *CCND1* expression

Myc plays a pivotal role in cell growth and proliferation via the transcriptional activation of numerous genes. Oligonucleotide microarray analysis identified potential Myc target genes in human B cells in which proliferation depends on the expression of Myc (Schuhmacher et al., 2001). VRK1 was identified as one of around 90 Myc target genes transcriptionally activated 4-8 hours after induction of Myc. To confirm VRK1 as an authentic target of Myc, we investigated the involvement of VRK1 in the *CCND1* expression signaling pathway in which Myc is a potent upstream activator (Lee and Sicinski, 2006). Transient expression of Myc increased the level of expression of VRK1 and CCND1 (Fig. 7A,B, siCont) compared with the level observed in untransfected control cells (Fig. 7B, arrowheads). Despite overexpression of Myc, VRK1-depleted cells

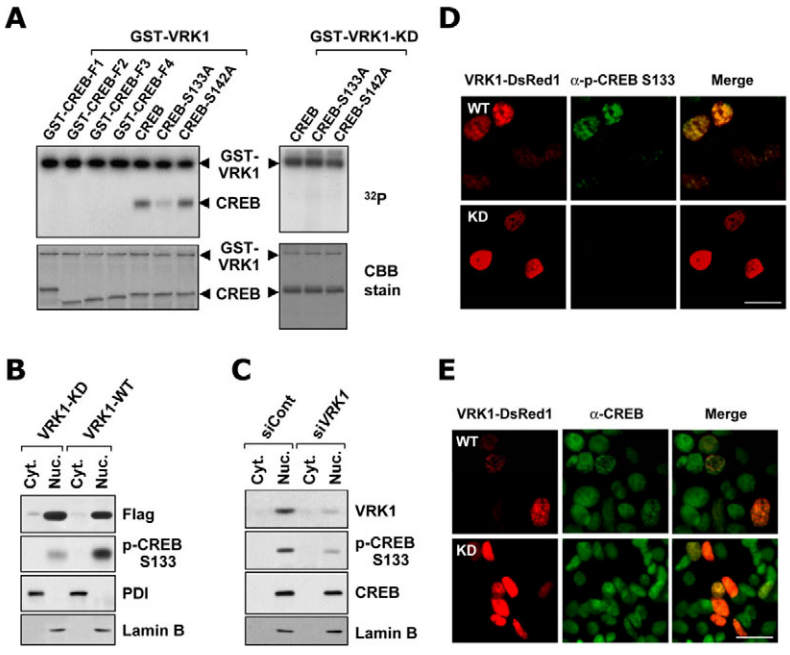

Fig. 6. VRK1 phosphorylates CREB at Ser133. (A) Phosphorylation of CREB by wild-type or kinase-dead VRK1 was assessed by in vitro kinase assays. GST-CREB-fragments (F1-F4), wild-type CREB or site-directed mutated CREB (S133A and S142A) proteins were used as substrates. (B,C) Cytoplasmic and nuclear lysates from HeLa cells transfected with kinase-dead (KD) or wild-type (WT) VRK1 (B), or with control siRNA (siCont) or siVRK1 (C) were immunoblotted with the indicated antibodies. Protein disulfide isomerase (PDI) and Lamin B were used as fraction markers for the cytoplasm and nucleus, respectively. (D,E) HeLa cells transfected with WT-VRK1 or KD-VRK1 were immunostained for CREB-*P* S133 (green) (D) or CREB (E). Scale bars: 20 μm.

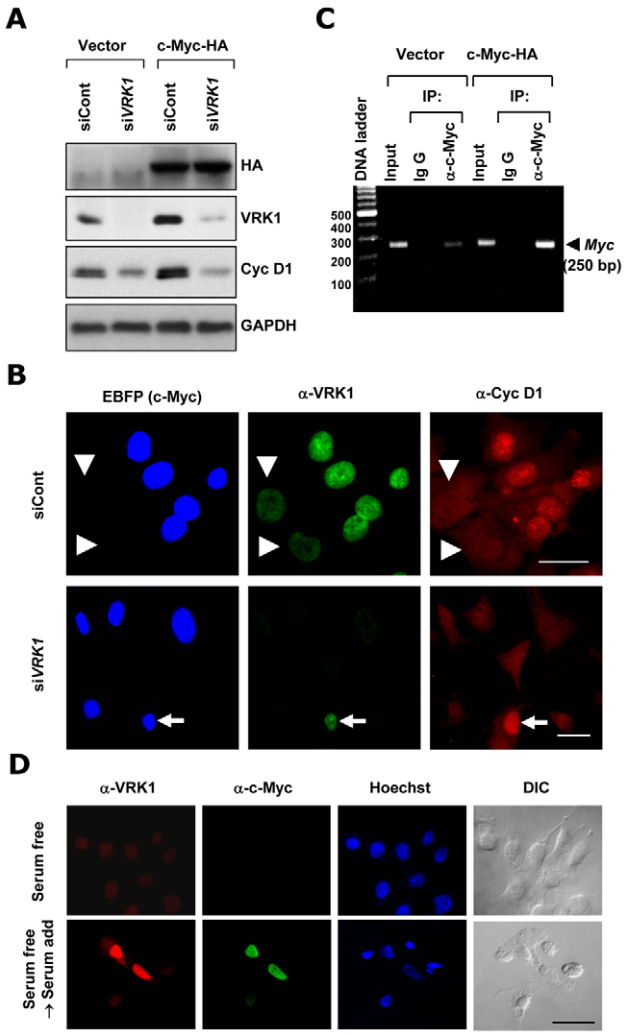

Fig. 7. VRK1 mediates Myc-induced *CCND1* expression. (A) HeLa cells were cotransfected with pHA empty vector (Vector) or pHA-Myc and control siRNA (siCont) or siVRK1. Cells were immunoblotted with the indicated antibodies. (B) Immunofluorescence of Myc-EBFP (blue), VRK1 (green) and CCND1 (red) from cells transfected with siCont or siVRK1. Arrowheads and arrow denote untransfected cells and cells that do not show knockdown, respectively. Scale bars: 20 μm. (C) ChIP assay measuring Myc binding to conserved Myc-binding sites in the *VRK1* promoter of HeLa cells transfected with pHA empty vector or pHA-Myc. (D) Cells were maintained without serum for 24 hours then fetal bovine serum was added to 5% and cells incubated for a further 3 hours. Cells were then immunostained for VRK1 (red), Myc (green) and DNA (Hoechst 33342, blue). DIC, digital interference contrast image. Scale bar: 30 μm.

failed to induce the expression of *CCND1* (Fig. 7A,B). A higher level of CCND1 expression was observed in cells in which VRK1 was not completely knocked down (Fig. 7B, arrow). The Myc consensus binding site (CACGTG) is located in the *VRK1* promoter 2130 bp upstream of the transcription start site. ChIP analysis demonstrated that binding of Myc to the *VRK1* promoter is enhanced in cells overexpressing Myc (Fig. 7C). However, there was no difference in the binding of Myc to the *VRK1* promoter in the presence or absence of siVRK1 (supplementary material Fig. S2). We also found that both Myc and VRK1 are induced by the addition of serum to serum-depleted cells (Fig. 7D). On the basis of these results, we conclude that VRK1 is involved in the Myc-induced *CCND1* expression pathway. In summary, Myc stimulates VRK1 expression via direct binding to the *VRK1* promoter. Subsequently, VRK1 phosphorylates CREB, and facilitates CREB binding to the CRE of the *CCND1* promoter, which then leads to an accumulation of CCND1, which eventually promotes the G1-S transition in the cell cycle. However, this model still needs to be tested.

Discussion

The association of VRK1 and CREB occurs specifically during the G1 to S phase transition in the cell cycle. The level of VRK1 is at its lowest level during this period whereas the level of CREB is maintained relatively high. The VRK1 level increases gradually from G1 to the early mitotic phase. There is a significant reduction in the level of VRK1 during progression of the mitotic phase from anaphase to barely detectable levels during telophase and early G1 phase through proteasome-dependent degradation (Kang et al., 2007). The level of VRK1 is highest at the G2-M phase transition, with most of the protein being associated with the chromatin, which then condenses into chromosomes through phosphorylation of histone H3 at Thr3 and Ser10 residues (Kang et al., 2007). The intracellular environments between G1-S and G2-M are quite different. The phosphorylation of Thr3 and Ser10 of histone H3 in the global region of the chromatin, which occurs during G2-M for chromatin condensation, does not occur during G1-S. However, several kinases, including ribosomal S6 kinase 2 (RSK2) and mitogen- and stress-response kinases MSK1 and MSK2, are known to phosphorylate histone H3 Ser10 in the specific promoter region of target genes for expression during interphase (Nowak and Corces, 2004). Although the mechanism underlying the specific interaction between VRK1 and CREB in G1-S is unclear, it is possible that the intracellular environment during the G1-S transition period provides optimal conditions for binding of the two proteins. Our results emphasize the integral role of VRK1 during cell cycle progression.

Protein kinases such as protein kinase A, mitogen-activated protein kinases (MAPKs), and Ca^{2+}/calmodulin-dependent protein kinases phosphorylate CREB at Ser133, which is required for the transcriptional activation of specific stimuli-induced genes (De Cesare et al., 1999). Signaling pathways may also target additional sites on CREB, or proteins that associate with CREB, thereby allowing the regulation of distinct programs of gene expression under different conditions (Ravnskjaer et al., 2007). The expression profile of phosphorylated CREB during cell cycle progression shows that phosphorylation peaks during the G1-S transition and then gradually decreases from S phase to M phase (Saeki et al., 1999). Consistent with previous data, our results demonstrate that association of CREB with VRK1 occurs in a cell-cycle-dependent manner from late G1 to early S phase. The association of ATF2 with the CRE of the *CCND1* promoter is also facilitated by VRK1. Thus the transcriptional activation of *CCND1* via CRE is enforced by VRK1. The kinase activity of VRK1 also appears to influence the nuclear accumulation of CCND1. Although the induction of *CCND1* itself is not crucial, the nuclear accumulation of CCND1 is a key feature of many cancer types (Gladden and Diehl, 2005). The aberrant *CCND1* expression common to many human cancers highlights the potential of CCND1 as a therapeutic target. Further investigation of the precise molecular signaling mechanisms underlying VRK1-mediated *CCND1* induction will provide valuable information for the treatment of cell-cycle-related diseases.

CCND1 expression can be induced by a variety of serum-response factors, including Myc, although the consensus Myc-binding site on the *CCND1* promoter does not appear to be functional. In the present study, depletion of VRK1 resulted in the failure of Myc-induced *CCND1* expression. We clearly demonstrate that Myc activates the *VRK1* promoter, which has a Myc-binding element. Since *VRK1* shows temporal expression during cell cycle progression, it is possible that some transcription factors other than Myc play a role in regulating *VRK1* expression at different cell cycle stages.

Accumulation of VRK1 stimulates the expression of *CCND1* by phosphorylating CREB, which subsequently activates the CRE on the *CCND1* promoter. Although several substrates of VRK1 have been characterized, including BAF, p53, c-Jun, ATF2 and CREB, the upstream signaling pathway leading to the transcriptional induction of VRK1 is not known. The present study identifies Myc as the first known regulator that can activate the signaling pathway linked to activation of *VRK1* expression.

Materials and Methods

Plasmids and antibodies

pFlag-CMV2-VRKs and pGEX4T-1-VRK1 have been described previously (Kang and Kim, 2006). The kinase-dead form of VRK1 was generated by site-directed mutagenesis of lysine 179 to glutamic acid as described previously (Kang et al., 2007; Vega et al., 2004). Triple AP1 or CREs from human CCND1 were subcloned into the pGL3-basic luciferase reporter vector (Promega, Madison, WI) as was the Myc-binding element from human VRK1 as described previously (Watanabe et al., 1996). Myc was subcloned into pHA (a gift from S. K. Jang, POSTECH, Korea) or pEBFP-C1 vector (Clontech, Palo Alto, CA). As depicted in Fig. 5C, full-length CREB, CREB fragments, and site-directed mutations (S133A or S1442) of CREB, were generated by subcloning each construct into the pGEX4T-3-TEV vector (gift from K. J. Kim, POSTECH, Korea). When necessary, the GST moiety was removed from CREB by treatment with TEV protease (gift from K. J. Kim, POSTECH, Korea) as described previously (Kwon et al., 2005). Antibodies against VRK isotypes were prepared as described previously (Kang and Kim, 2006). Antibodies were purchased as follows: anti-Flag epitope (M2) from Sigma (St Louis, MO); anti-HA epitope (clone 12CA5) from Roche Applied Science (Mannheim, Germany); anti-ATF2 from Upstate Biotechnology (Lake Placid, NY); anti-GAPDH and anti-BrdU from Calbiochem (San Diego, CA); anti-p38 MAPK, anti-c-Jun, anti-CREB and anti-phospho-CREB Ser133 from Cell Signaling Technology (Danvers, MA); and anti-cyclins, anti-GST, anti-Lamin B and anti-PDI were from Santa Cruz Biotechnology (Santa Cruz, CA)

Immunocytochemistry

For immunocytochemical analysis, HeLa cells were cultured to 50-60% confluency on 15 mm glass chips coated with poly-D-lysine. Transfected cells were maintained for an additional 24 hours and visualized by microscopy. When necessary, cells were treated with 10 μM MG132, 5 μg/ml actinomycin D or vehicle (DMSO) for 5 hours. Standard procedures were then followed as previously described (Kang and Kim, 2006). For bromodeoxyuridine (BrdU) staining, cells were treated with 10 μM BrdU for 1 hour or for 6 hours and standard procedures were followed as described previously (O'Keefe et al., 1992). After washing, cells were counterstained with 10 ng/ml propidium iodide or 20 ng/ml Hoechst 33342 stain to visualize the nuclei. An Axioplan2 fluorescence imaging microscope (Carl Zeiss, Jena, Germany) equipped with an ApoTome (Carl Zeiss) was used to capture fluorescence images. The siRNA duplex targeting VRK1 (siVRK1) was mixture of the two effective siRNA duplexes (GCTAAGCTTAAGAATTCTG and CAAGGAACCTGGTGTTGAA). In addition to siVRK1, the VRK3-specific siRNA (GAGUUCAUUAGCAUGGACC) and the control scrambled siRNA (siCont) were obtained from Dharmacon (Lafayette, CO).

Northern blot analysis

Total RNA from cells transfected with plasmids indicated in Fig. 2D was isolated using TRI reagent (Molecular Research Center), according to the manufacturer's instructions. A probe for detecting the mRNA of *CCND1* was generated by PCR amplification, and radioisotope-labeled by random primer extension (Boehringer Mannheim). Probes were purified using a push column beta shield device and NucTrap probe purification columns (Stratagene). An aliquot of total RNA (5 μg) was subjected to northern blot analysis. The blot stained with ethidium bromide was photographed to check the quantity and quality of the nucleic acids.

Luciferase assays

Cells were grown in six-well plates and transfected with 0.5 μg luciferase reporter containing CRE or AP1 sequences of human *CCND1*, 0.5 μg pcDNA3-β-galactosidase and 1 μg wild-type or kinase-dead pFlag-VRK1 expression vectors. Metafectine reagent (Biontex, Munich, Germany) was used for transfection throughout the experiments according to the manufacturer's instructions. Total cell extracts were prepared 24 hours post transfection and assayed for luciferase activity using MicroLumat Plus LB 96V (Berthold Technologies, Bad Wildbad, Germany). Transfection efficiencies were normalized by cotransfection with a β-galactosidase expression plasmid.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously (Elsby et al., 2006). Input and immunoprecipitated DNA were purified and used to amplify promoter elements of human *CCND1* for CRE and AP1 or *VRK1* for the Myc element. The following PCR primers were used: CRE F, 5′-ATGGCTTTTGGGCTCTGCCC-3′; CRE R, 5′-CTCGCTGCTACTGCGCCGAC-3′; AP1 F, 5′-ACCAATTAGGAACTTCGGTG-3′; AP1 R, 5′-CCTTGACCAGTCGGTCCTTG-3′; Myc F, 5′-GTTGCTGATTTA-ATTGTGCT; Myc R, 3′-GATTCACACATCTCATGGGT. The PCR products for CRE and AP1 were 210 bp and the product for Myc was 250 bp.

Protein interaction assay, preparation of cytoplasmic and nuclear extracts

GST-pulldown assay, immunoprecipitation and preparation of cytoplasmic and nuclear extracts were carried out as described previously (Kang and Kim, 2006).

Protein kinase assay

In vitro kinase assay was performed with 100 ng of either recombinant GST-VRK1 or kinase-dead of GST-VRK1 protein and 300 ng of substrate CREB proteins. The standard procedure for in vitro kinase assay of VRK1 activity was carried out as described previously (Kang et al., 2007).

This study was supported by grants from the National R&D Program for Cancer Control (0520250-2), the National Cancer Center, the Ministry of Health & Welfare and the Frontier Program for Human Functional Genome (FG06-2-19) of the Ministry of Science and Technology. This work was also supported by the Biotech grant from POSCO and the Brain Korea 21 program of the Ministry of Education.

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