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Functional availability of γ-herpesvirus K-cyclin is regulated by cellular CDK6 and p16INK4a

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ABSTRACT

Viral K-cyclin derived from Kaposi's sarcoma-associated herpesvirus is homologous with mammalian D-type cyclins. Here, we demonstrated the regulatory mechanisms for K-cyclin function and degradation in human embryonic kidney HEK293 and primary effusion lymphoma JSC-1 cell lines. Proteasome inhibitor MG132 treatment induced an accumulation of ubiquitinated K-cyclin in these cells, and co-expression of CDK6 prevented K-cyclin ubiquitination. Also K-cyclin mutants incompetent for CDK6-binding were destabilized by proteasome pathway. Furthermore, silencing of p16INK4a promoted K-cyclin–CDK6 complex formation and hence induced K-cyclin-associated kinase activity in HEK293 cells. These observations indicate that CDK6-bound K-cyclin is functionally stable but monomeric K-cyclin is targeted to ubiquitin-dependent degradation pathway in these cells. Our data suggest that the balance between CDK6 and p16INK4a regulates the availability of functional K-cyclin in human cells.

1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) and Epstein-Barr virus (EBV/HHV-4) are both classified as γ-herpesviruses associated with malignancy. These viruses can manipulate host cell processes and deregulate cellular signaling to promote cell growth and survival [1].

KSHV-associated cell transformation involves the viruses' latent cycle; the main latent genes of KSHV are LANA (ORF73), K-cyclin (ORF72) and vFLIP (ORF71). These genes have been shown to manipulate host cell processes [1–3]. LANA can inactivate RB and p53, similar to other oncogenic viral proteins, and enhance E2F activity to promote S phase entry [4,5]. LANA also manipulates GSK-3β to regulate β-catenin activity and Wnt signaling [6,7]. vFLIP is structurally related to cellular FLIP, and is primarily associated with activation of the NF-κB pathway and anti-apoptotic signaling [8,9].

K-cyclin is thought to be involved in the cell cycle transition [10,11]. K-cyclin is a homolog of mammalian D-type cyclins, particularly cyclin D2 (32% identity and 54% similarity). Similar to cellular D-type cyclins, K-cyclin can form complexes predominantly with CDK6 [12], and the K-cyclin–CDK6 complex can phosphorylate RB protein [10]. The K-cyclin–CDK6 complex have broader substrate specificity than cellular cyclin D–CDK6 and can phosphorylate CDK2 substrates such as ORC1, CDC6, p27Kip1, histone H1, Bcl-2 and p53 [13–15]. In the presence of wild-type p53, K-cyclin expression sensitizes primary cells to various apoptotic stimuli [16], whereas K-cyclin transgenic mice showed lymphomagenesis in the absence of p53 [17], suggesting the malignant potential of K-cyclin *in vivo*.

The cellular cyclin–CDK complexes are usually regulated by their inhibitors called CDK inhibitor (CKI) [18]. The Cip/Kip family of CKIs binds stoichiometrically to the cyclin and CDK subunit [19]. Interestingly, *in vitro* studies showed that the K-cyclin–CDK6 complex is resistant to p27Kip1 and p21Cip1 [11] and can inactivate p27Kip1 and p21Cip1 by phosphorylation [20,21]. p27Kip1 and p21Cip1 regulate cellular cyclin–CDK1/2 members during the cell cycle transition. Therefore, the K-cyclin–CDK6 complex may contribute to cyclin-CDK1/2 activation during cell cycle progression by eliminating the inhibitory activities of p27Kip1 and p21Cip1.

Another CKI, the INK4 family, suppresses cellular D-type cyclin-dependent CDK activity. The INK4 family can bind to the cyclin D–CDK4/6 complex to form a ternary complex [22]. Although initial studies reported that the K-cyclin–CDK6 complex was resistant to p16INK4a [11], and that the K-cyclin–CDK6 complex was constitutively active in KSHV-infected BC3 cells [23], other later studies indicated that the unphosphorylated K-cyclin–CDK6 complex is

Abbreviations: KSHV, Kaposi's sarcoma-associated herpesvirus; HHV-8, human herpes virus-8; EBV, Epstein-Barr virus; HHV-4, human herpes virus-4

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inhibited by the INK4 family member [22,24,25]. These studies suggest that the basal activity of unphosphorylated CDK6 bound to K-cyclin is strong enough to elicit its activities, but the INK4 family can inhibit the unphosphorylated K-cyclin–CDK6 complex.

Currently, it remains unclear whether K-cyclin-associated proteins can regulate intracellular K-cyclin availability. Here, we showed that CDK6-free K-cyclin was targeted to the ubiquitin-dependent destabilization pathway in human cells. We also showed that p16INK4a silencing stimulated intracellular K-cyclin–CDK6 complex formation. These data suggest that the functional availability of K-cyclin is largely dependent on the balance in expression between cellular CDK6 and p16INK4a.

2. Materials and methods

2.1. Plasmids

cDNA for K-cyclin was amplified from the genomic DNA fraction of BCBL cells by standard PCR methods and subcloned into the pIRESpuro plasmid (TAKARA Bio Inc., Shiga, Japan) to construct a C-terminal FLAG-HA-tagged K-cyclin. The murine mutant v-cyclins K104E and E133V were unable to form a complex with cellular CDK4/6 [26]. The mutant K-cyclins K106E and E135V, which have highly conserved amino acid sequences corresponding to v-cyclins K104E and E133V, respectively, were generated using a PCR-based QuikChange™ site-directed mutagenesis kit (Agilent Technologies, Stratagene, La Jolla, CA, USA). Synthetic primers for E135V (5′-GAACTTATAGACCAGGTGAAAGAACTCCTTGAGAAG-3′ and 5′-CTTCTCAAGGAGTTCTTTCACCTGGTCTATAAGTTC-3′) and K106E (5′-CTGTTAGTGGCCAGTGAGCTCAGAAGCCTCACGCC-3′ and 5′-GGCGTGAGGCTTCTGAGCTCACTGGCCACTAACAG-3′) were used with the template pIRESpuro/K-cyclin–FH plasmid. cDNA for CDK6 was amplified by PCR using a HeLa cDNA library and subcloned into the pD3HA plasmid (pcDNA3-modified expression plasmid) to construct a C-terminal myc-tagged CDK6. The LANA-expressing pcDNA3/LANA and histidine-tagged ubiquitin expressing pcDNA3/His-Ub were generated as previously described [27,28].

2.2. Cell culture and transfection

HEK293, BC3 and JSC-1 (from ATCC) cells were cultured in Dulbecco's modified Eagle's or RPMI1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 7% fetal bovine serum and kanamycin (50 μg/mL) at 37 °C in 5% CO_2. To establish the K-cyclin transfectants, HEK293 cells were transfected with the expression plasmid using FuGENE® HD transfection reagents (Roche Diagnostics GmbH, Mannheim, Germany). The mixed populations selected by puromycin (0.5 μg/mL) were designated as 293/K-cyclin, 293/E135V and 293/K106E. 293/LANA was established after screening of G418-resistant pcDNA3/LANA-transfected clones. Full-length LANA expression was confirmed by Western blotting. LANA and K-cyclin co-expressing cells were established by transfection of the K-cyclin plasmid into 293/LANA cells, and the G418- and puromycin-resistant mixed population was designated as 293/LANA + K-cyclin cells.

For siRNA experiments, cells were transfected with siRNA using Lipofectamine™ 2000 (Life Technologies Corp., Invitrogen, Carlsbad, CA, USA). Control scramble (AllStars negative control) and p16INK4a-targeted (Hs_CDKN2A_15) siRNAs were purchased from Qiagen (Hilden, Germany).

2.3. Western blotting

Cells were lysed in SDS buffer (50 mM Tris–HCl [pH 8.0], 2% SDS and 10% glycerol) and sonicated. Other soluble cell extracts were prepared using NEB100 lysis buffer, and SDS–PAGE and Western blotting were performed as previously described [29]. Western blotting signals were detected using an ECL-Plus chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ, USA) or a Super Signal West Dura Extended Duration Substrate (Thermo Fisher Scientific Inc., Pierce, Rockford, IL, USA). Anti-HA (3F10) and anti-c-myc (9E10) monoclonal antibodies were purchased from Roche Diagnostics (Indianapolis, IN, USA). Anti-CDK6 and anti-p16INK4a antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and anti-GAPDH antibody was from Millipore Corp., Chemicon (Billerica, MA, USA). Anti-LANA antibody was from Advanced Biotechnologies Inc. (Columbia, MD, USA).

2.4. Nickel–NTA–agarose purification

Transfected cells were lysed in 1 mL of buffer A (8 M urea, 0.1 M Na_2HPO_4–NaH_2PO_4 [pH 8.0], 0.3 M NaCl and 10 mM imidazole) per sample at 48 h after transfection. The lysate was sonicated for 1 min to reduce viscosity and then mixed on a rotator with 10 μL of nickel–NTA–agarose (Qiagen) for 2 h at room temperature. The beads were washed three times with 250 μL of buffer A, twice with 250 μL of buffer A diluted 1:4 with buffer B (25 mM Tris–HCl [pH 6.8] and 20 mM imidazole), and twice with 250 μL of buffer B. Purified proteins were eluted with buffer C (0.1 M EDTA and 250 mM imidazole). The proteins were analyzed by Western blotting.

2.5. Immunoprecipitation and in vitro immunocomplex kinase assay

Transfected cells were lysed on ice for 5 min in NEB100 lysis buffer. The cleared cell lysates were subjected to immunoprecipitation with anti-FLAG M2 affinity gel (Sigma–Aldrich). The immunocomplexes were eluted with 0.2 mg/mL of FLAG peptide (Sigma–Aldrich). The phosphorylation reaction was performed in a buffer containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM ATP and 10 μCi [γ-^{32}P]ATP with 5 μL of the eluted proteins. The reactions were stopped by the addition of 4× Laemmli SDS sample buffer, resolved by SDS–PAGE and analyzed with a FLA 7000 fluoro image analyzer (Fujifilm, Tokyo, Japan).

3. Results

3.1. Ubiquitination of K-cyclin

Previous studies suggested that K-cyclin is not a substrate for ubiquitination [23,30]. We also found that the viral K-cyclin protein level in BC3 cells was unaffected by the proteasome inhibitor MG132 treatment (data not shown). However, ubiquitination of K-cyclin itself remains unclear. To confirm it in other cells, we expressed exogenous K-cyclin tagged with FLAG and HA at the C-terminus (K-cyclin–FH) in another line of KSHV-infected cells because JSC-1 cells did not express a detectable level of K-cyclin. To our surprise, the K-cyclin–FH protein level was increased in JSC-1 cells after MG132 treatment, but not in BC3 cells (Fig. 1A). In addition, MG132 treatment induced the expression of the high-molecular weight K-cyclin–FH protein in JSC-1 and HEK293 cells (Fig. 1B). Since MG132 inhibits ubiquitin-dependent proteasomal degradation, the high-molecular weight K-cyclin–FH was considered to be a polyubiquitinated protein. To test this hypothesis, K-cyclin–FH and histidine-tagged ubiquitin (His-Ub) were co-expressed in HEK293 cells to isolate the His-Ub-conjugated protein. MG132 treatment induced the accumulation of the high-molecular weight K-cyclin–FH protein (Fig. 1C, left panel, lanes 6 and 7). Importantly, K-cyclin–FH protein was detected in the His-Ub-conjugated sample collected from MG132 treated cells using Ni–NTA beads (Fig. 1C, right panel, lane 7). Thus, these data indicate that K-cyclin was subjected to ubiquitination at least in JSC-1 and HEK293 cells, but probably not in BC3 cells.