Biochemical characterization of HIV-1 Rev as a potent activator of casein kinase II in vitro

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Abstract The stimulatory effects of several DNA-binding basic proteins (histone and protamine) and HIV-1 Rev with arginine (Arg)-rich clusters on the activity of casein kinase II (CK-II) were investigated in vitro. It was found that recombinant Rev (rRev) and the synthetic oligo-fragments corresponding to the amino acid sequences of its Arg-rich cluster stimulate CK-II activity in a dose-dependent manner. The activated CK-II phosphorylates several cellular and viral proteins in HIV-1 infected human MOLT-4 cells, and also phosphorylates HIV-1 structural proteins, including recombinant reverse transcriptase (rRT). These phosphorylations are selectively inhibited by CK-II inhibitors, such as quercetin, oGA (a glycyrrhetinic acid derivative) and NCS-chrom (an enediyne containing antibiotic). The data presented here suggest that HIV-1 Rev acts as an effective potent activator of CK-II, which may be a cellular mediator promoting HIV-1 replication in virus-infected cells.

Key words: Casein kinase II; Casein kinase-II activator; Casein kinase-II inhibitor; Human immunodeficiency virus-1; Human immunodeficiency virus-1 regulator of expression of virion proteins; Human MOLT-4 cell

1. Introduction

Recent reports have demonstrated that casein kinase II (CK-II), a second messenger-independent nuclear serine/threonine protein kinase [1], plays an important role in positive and negative transcriptional regulation by specific phosphorylation of several transcriptional factors (Sp1 [2], Ap1 [3], serum response factor [4], upstream binding factor [5] and steroid hormone receptor [6]). Recently, we reported that certain DNA-binding sperm proteins (protamines in fishes and histones H2B2 and H2B3 in sea urchin) with oligo-Arg clusters function as potent CK-II activators in vitro [7]. A computer based protein sequence search for similar proteins containing an Arg-rich cluster revealed: two DNA-binding proteins (DNA terminal protein at positions 361–366 of human adenovirus type 5 [8] and nuclear antigen 3C at positions 74–80 of the Epstein-Barr virus [9]); and four retroviral gene products (Rev at positions 35–50 and Tat at positions 49–57 of HIV-1 [10]; Rev at positions 41–49 of simian immunodeficiency virus (SIV) [11]; and Rex (regulator in *pX* gene) at positions 3–15 of human T-cell leukemia virus type 1 [12].

The stimulatory effects of two recombinant HIV-1 proteins (rRev and rTat) on CK-II activity was quantitatively determined using in vitro CK-II assay systems. Also, the inhibitory effects of some anti-viral substances on the CK-II-catalyzed phosphorylation of viral proteins were examined. CK-II can easily be partially purified from sea urchin eggs [7] and mouse cells [13] as a hetero-complex with p99 (ERp99, a protein from the Hsp-90 family) and p56 (calreticulin) using Mono Q or glycyrrhizin-affinity column chromatography (HPLC). This hetero-complex provides convenient material for an in vitro assay of CK-II activity, since CK-II in the complex effectively phosphorylates p99 and p56 by incubation with ATP or GTP in the presence of a suitable CK-II activator [13].

Our findings reveal a novel function of HIV-1 Rev as an effective potent activator of CK-II, which may therefore be a cellular mediator responsible for promoting HIV-1 replication in virus-infected cells. In addition, some anti-viral substances, such as quercetin, oGA [13] and NCS-chrom [14], are characterized here as potent inhibitors of CK-II, which is in agreement with recent findings that other CK-II inhibitors (flavonoids and benzothiophenes) selectively inhibit HIV-1 transcription [15].

2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham (Arlington Heights, USA); poly-Arg, dephosphorylated α-casein (bovine milk) and quercetin (3,3′,4′,5,7-pentahydroxyflavone) from Sigma Chemical (St. Louis, USA); histones (calf thymus) from Boehringer-Mannheim Biochemicals (Germany); and HIV-1 IIIB lysate (approx. 5.72×10^9 vp/ml (a sucrose density gradient purified virus: 1 mg protein/ml)) from ABI Advanced Bio-technologies Inc. (MD, USA). Rabbit polyclonal antibodies to recombinant HIV-1 proteins, such as RT, gp41 and capsid proteins (p24 and p17), were obtained from Intracel Co. (Cambridge, MD, USA). Neocarzinostatin (NCS, a macromolecular antitumor antibiotic) was obtained from Kayaku Antibiotic Research Laboratories Co., Ltd. (Tokyo). NCS-chrom was extracted from NCS with 10% (v/v) methanol containing 0.1 N HCl, as reported previously [14]. A glycyrrhetinic acid derivative (oGA [13]) was kindly supplied by Minophagen Pharmaceutical Co., Ltd. (Tokyo).

2.2. Purification of rRev

Crude protein was extracted from *E. coli* transfected with pGEX-2T/HIV-1 *rev* and grown overnight in soybean-casein digest (SCD)

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Abbreviations: CK-II, casein kinase II; DTT, dithiothreitol; ERp99, 99-kDa endoplasmic reticulum protein; GST, glutathione-*S*-transferase; HIV-1, human immunodeficiency virus type 1; oGA, glycyrrhetinic acid derivative; NCS, neocarzinostatin; NCS-chrom, NCS-chromophore; NLS, nuclear localization signal; PMSF, phenylmethylsulfonyl fluoride; poly-Arg, poly-L-arginine; Rev, regulator of expression of virion proteins; rRev, recombinant Rev; rRT, recombinant reverse transcriptase; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; Tat, transactivator of transcription

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medium containing ampicillin (100 μg/ml) at 37°C. The fusion protein was induced with 0.1 μM IPTG (isopropyl-β-D-thiogalactopyranoside) for 6 h. The bacterial pellet (about 15 g wet weight) was then sonicated for 60 s and mixed gently for 30 min in buffer (40 mM Tris-HCl (pH 7.2), 2 mM DTT, protease inhibitors (0.1 μM PMSF, 0.5 μM aminobenzamidine and 1 μg/ml leupeptin) and 1% Triton X-100) containing 1.0 M NaCl. After centrifugation (20 000 × *g* for 10 min at 4°C), the supernatant (about 25 mg protein) was applied on a glutathione-affinity column after dialysis against buffer containing 0.1 M NaCl. The partially purified GST-Rev fusion protein was cleaved specifically with thrombin. To obtain rRev, the reaction mixture was directly applied onto a column of Superdex 200pg (HPLC). Elution was carried out with 20 mM phosphate buffer (pH 6.8) containing 0.5 M NaCl. The purified 13-kDa polypeptide was identified as a Rev protein since the N-terminal partial amino acid sequence (MAGRSGDSDE) corresponded to that of HIV-1 Rev.

2.3. Partial purification of CK-II from MOLT-4 cells

CK-II in a 1.5-M NaCl extract from human MOLT-4 cells (approx. 8×10^8 cells) was partially purified by means of phosphocellulose column chromatography. CK-II was eluted with 1.0 M NaCl after washing well with 0.4 M NaCl. Two effective phosphate acceptors (p99 and p56) for CK-II in the 1.0-M NaCl fraction were detected. CK-II and its effective phosphate acceptors in the phosphocellulose fraction were further purified by means of gel filtration on Superdex 200pg (HPLC), as previously reported [13]. By determination of their N-terminal partial amino acid sequences, p99 and p56 were identified as ERp99 (a protein from the Hsp-90 family) and calreticulin (Ca^{2+}-binding protein), respectively, as reported previously [7,13].

2.4. Assay for CK-II activity

CK-II activity was measured in the standard reaction mixture (50 μl), which comprised 40 mM Tris-HCl (pH 7.6), 2 mM DTT, 3 mM Mn^{2+}, 20 μM [γ-^{32}P]ATP (1000 cpm/pmol) and the indicated amount of CK-II fraction containing native phosphate acceptors (p99 and p56) prepared from MOLT-4 cells. After incubation for the indicated periods at 30°C in the presence or absence of the indicated CK-II activators, the ^{32}P-labeled p99 and p56 in the reaction mixtures were detected by autoradiography after SDS-PAGE, as reported previously [7,13].

3. Results

3.1. Characterization of rRev as a potent activator of CK-II in vitro

The biochemical characteristics of protein phosphorylation by CK-II in the fraction prepared from MOLT-4 cells exactly corresponded to those characterized with CK-IIs from sea urchin eggs [7] and mouse cells [13], because CK-II activity (phosphorylation of p99 and p56) was greatly stimulated by basic polypeptides, such as salmine A1, poly-Arg (about 100 Arg residues) and poly-Lys (about 100 Lys residues), but completely inhibited by heparin (a CK-II inhibitor) (data not shown).

Using purified GST (glutathione-*S*-transferase)-Rev fusion protein (GST-Rev) and rRev obtained from GST-Rev after cleavage with thrombin (Fig. 1A), it was found that CK-II activity was significantly stimulated when co-incubated with rRev (Fig. 1B, lane 5), but not with GST (lane 4). rRev was phosphorylated by CK-II in vitro (Fig. 1B, lane 5), as has been reported by others [16]. Under the same experimental conditions, rTat required higher doses (over 3 μg/ml) to induce significant stimulation of CK-II activity, and its stimulatory effect was about one-third of rRev-induced CK-II stimulation (data not shown).

Three synthetic oligo-fragments that act as nuclear localization signals (NLSs) were used to determine the stimulatory kinetics of CK-II activity (see Fig. 2 legend). The Rev oligo-fragment (RQARRNRRRRWRERQR) stimulated CK-II-catalyzed phosphorylation of p99 up to about 7.2-fold at 1 μg/ml, in a manner similar to that observed with rRev (Fig. 2A). In contrast, rTat and p120 oligo-fragments (Arg-rich cluster regions) required higher doses (over 5 μg/ml) to induce comparable CK-II activity (Fig. 2B). The marked stimulation of CK-II activity induced by the HIV-1 Rev oligo-fragment was also observed with SIV Rev oligo-fragment (RRQRRRRWRRR) (data not shown). Slight stimulation of CK-II activity was induced with a minimum hepta-fragment related to the Rev sequence at positions 38–44 (RRNRRRR), but not with a hepta-fragment of HIV-1 Tat at positions 51–57 (KRRQRRR) nor of the NLS domain at positions 126–132 (PKKKRKV) of SV-40 large T antigen. Slight stimulation of CK-II activity was also observed when a Lys-residue of the Tat hepta-fragment was substituted with an Arg-residue. The rRev-induced stimulation of CK-II activity was reproduced when p99 and p56 were substituted with other CK-II phosphate acceptors, such as casein or phosvitin.

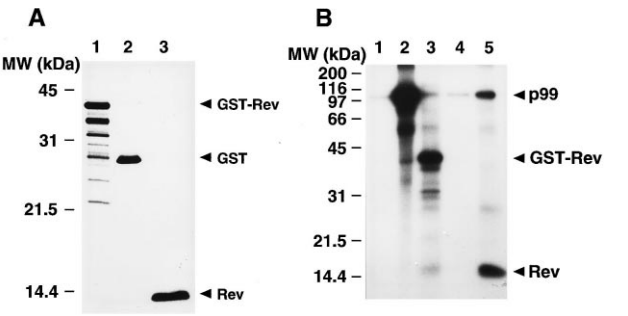

Fig. 1. Selective purification of a GST-rRev fusion protein extracted from *E. coli* transfected with pGEX-2T/HIV-1 *rev* by glutathione-affinity column chromatography. A: After treatment (2 h at 30°C) of GST-rRev (approx. 3 mg) with thrombin (2 units/ml), rRev was further purified by gel filtration on a Superdex 200pg column (HPLC). SDS-PAGE (Coomassie Brilliant Blue R-250 staining) detected GST-rRev (lane 1) in the glutathione-affinity fraction and the purified Superdex fractions S-I (GST, lane 2) and S-II (rRev, lane 3). B: To test the stimulatory effects of GST-rRev, GST and rRev on CK-II activity, the CK-II fraction (Mono Q fraction, approx. 2 μg) prepared from normal MOLT-4 cells was incubated for 20 min at 30°C with 20 μM [γ-^{32}P]ATP (1000 cpm/pmol) and 3 mM Mn^{2+} in the absence (lane 1) or presence (approx. 1 μg each) of CK-II activators, poly-Arg (about 100 residues, lane 2), GST-rRev (lane 3), GST (lane 4), or rRev (lane 5). Note that GST (lane 4), cleaved from GST-rRev fusion protein by thrombin, has no effect by itself.

These results show that, in vitro, (i) rRev is a more effective activator of oligomeric CK-IIs ($\alpha 2\beta 2$, $\alpha\alpha'\beta 2$ or $\alpha' 2\beta 2$) than rTat; and (ii) the Arg-rich cluster (NLS and Rev response element (RRE) binding domains) of HIV-1 Rev is responsible for activation of oligomeric CK-IIs. However, Arg-rich basic polypeptides, such as salmine A1 and poly-Arg, have been shown to have no stimulatory effect on the activity of α-monomeric CK-II (CK-IIα) [17].

3.2. Phosphorylation of viral proteins by CK-II in HIV-1 infected cells and HIV-1 lysate

To detect the viral gene products specifically phosphorylated by CK-II in HIV-1-infected cells, the CK-II fractions (phosphocellulose fractions) were prepared from HIV-1-infected and uninfected MOLT-4 cells. Total phosphorylation of substrate proteins contained in the CK-II fraction from HIV-1-infected cells was about 1.4 times higher than that determined in the kinase fraction from uninfected control

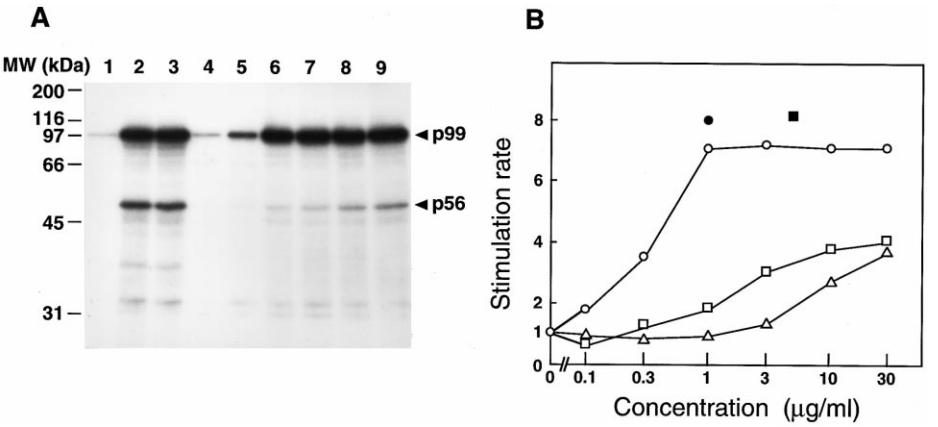

Fig. 2. The stimulatory effects of rRev and three synthetic Arg-rich oligo-fragments corresponding to the amino acid sequences of Rev, Tat and p120 on CK-II activity in vitro. The partially purified CK-II fraction (Mono Q fraction, approx. 5 μg) was incubated for 20 min at 30°C with 20 μM [γ-^{32}P]ATP (1000 cpm/pmol) in the presence or absence of the indicated basic oligo-fragments. The ^{32}P-labeled p99 and p56 in the reaction mixtures were detected by autoradiography after SDS-PAGE. CK-II fraction alone (lane 1 in A) or with rRev (1 μg, lane 2 in A; ● in B); rRev (5 μg, lane 3 in A; ■ in B) or rRev oligo-fragment (lanes 4 through 9, ○–○) at doses of 0.1 μg (lane 4), 0.3 μg (lane 5), 1 μg (lane 6), 3 μg (lane 7), 10 μg (lane 8), or 30 μg (lane 9). The stimulatory effects of synthetic oligo-fragments related to Tat (△–△) and p120 (□–□) on CK-II activity were determined under the same experimental conditions. The Arg-rich oligo-fragments were: positions 35–50 (RQARRNRRRRWRERQR) of HIV-1 Rev; 49–61 (RKKRRQRRRPPQG) of HIV-1 Tat; and 41–55 (KRLSSRARKRAAKRR) of human cell nuclear 120-kDa protein (p120). In B, a stimulation rate of 1 represents the CK-II-mediated phosphorylation of p98 determined in the absence of the CK-II activators.

cells, after incubation for 20 min at 30°C with 20 μM [γ-^{32}P]ATP in the presence of rRev as a CK-II activator. As shown in Fig. 3A, in the absence of CK-II activators (lanes 1 and 3), a 99-kDa cellular polypeptide (p99) was mainly detected as a ^{32}P-labeled polypeptide. In contrast, in the presence of CK-II activators, a number of cellular polypeptides highly phosphorylated by CK-II were detected (Fig. 3A). Interestingly, in HIV-1-infected cells (Fig. 3A, lane 4), at least six phosphorylated proteins, with molecular weights of approx. 120 (a), 51 (b), 41 (c), 33 (d), 28 (e), and 24 kDa (f), corresponded closely to the molecular sizes of HIV-1 gene products: gp120 (external envelope protein, 120 kDa), p51 (reverse transcriptase, 51 kDa), gp41 (transmembrane domain of envelope protein, 41 kDa), p32 (integrase, 32 kDa), p27 (Nef, 27 kDa) and p24 (capsid protein, 24 kDa), respectively.

To further confirm phosphorylation of HIV-1 structural proteins by CK-II in vitro, HIV-1 lysate was directly incubated for 20 min at 30°C with purified CK-II and 20 μM [γ-^{32}P]ATP in the presence of rRev as a CK-II activator. Fig. 3B (lane 3) shows that several polypeptides with molec-

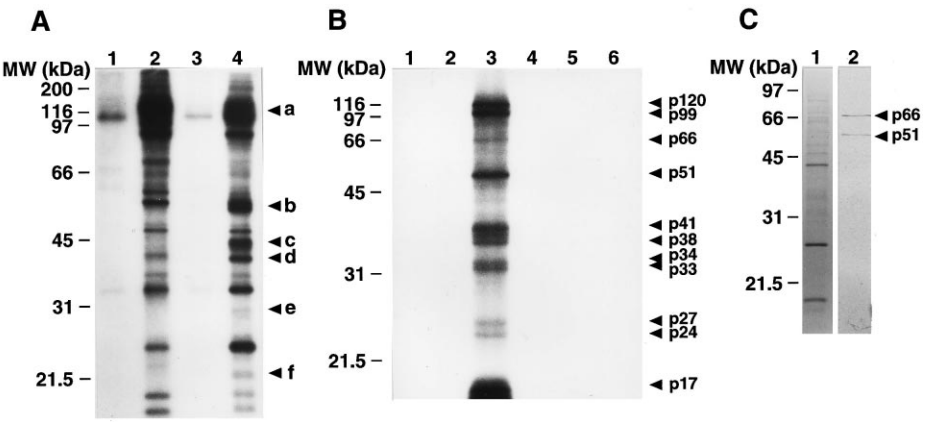

Fig. 3. Detection of polypeptides phosphorylated by CK-II in HIV-1-infected and uninfected MOLT-4 cells in vitro. A: The CK-II fractions were separately prepared from the 1.5-M NaCl extracts of HIV-1-infected and uninfected cells (control) by one-step purification (phosphocellulose column chromatography). The partially purified CK-II fractions (approx. 10 μg) from HIV-1-infected and uninfected MOLT-4 cells were separately incubated for 20 min at 30°C wiht 20 μM [γ-^{32}P]ATP (1000 cpm/pmol) and 3 mM Mn^{2+} in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of rRev (approx. 5 μg). The ^{32}P-labeled polypeptides were then detected by autoradiography after SDS-PAGE: uninfected cells, lanes 1 and 2; HIV-1-infected cells, lanes 3 and 4. B: Detection of ^{32}P-labeled polypeptides after incubation of HIV-1 lysate with purified CK-II in the presence of rRev as a CK-II activator or three CK-II inhibitors in vitro. HIV-1 IIIB lysate (approx. 5 μg) was incubated for 20 min at 30°C with approx. 30 ng purified CK-II fraction (Mono Q fraction [13]), 20 μM [γ-^{32}P]ATP (1000 cpm), 3 mM Mn^{2+} and rRev (approx. 5 μg). Lane 1, HIV-1 lysate alone; lane 2, HIV-1 lysate incubated with purified CK-II; lane 3, lane 2+rRev; lane 4, lane 3+10 μM oGA; lane 5, lane 3+2 μM quercetin; and lane 6, lane 3+0.15 μM NCS-chrom. C: Identification of RT (p66 and p51) in HIV-1 lysate by Western blotting after SDS-PAGE. Lane 1, the structural proteins on the gel were detected by staining with amide black after SDS-PAGE; and lane 2, detection of two polypeptides (p66 and p51) on the gel by Western blotting using rabbit polyclonal antibody to recombinant HIV-1 RT.

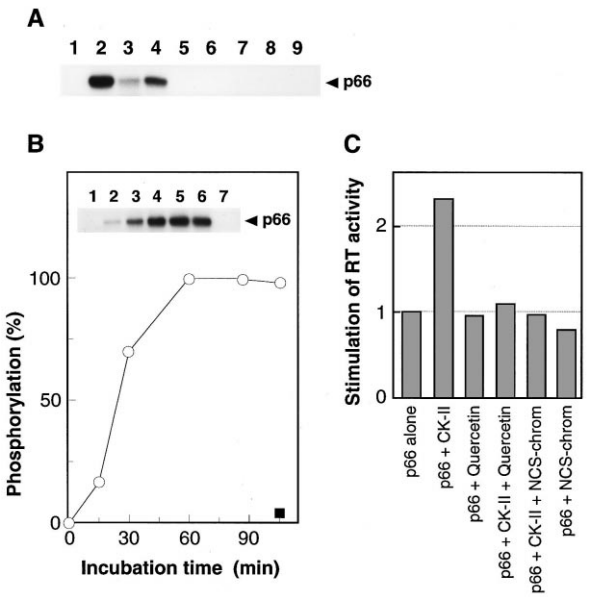

Fig. 4. Characterization of CK-II-mediated phosphorylation of rRT (p66) in vitro. A: rRT was purified to an apparently homogeneous polypeptide (approx. 66 kDa, p66) from the crude protein extract from *E. coli* BL21 transfected with pET21a(+)/HIV-1 rRT by means of DEAE-cellulose column chromatography, gel filtration on a Superdex 200pg column and Mono Q column chromatography (HPLC), successively. To characterize the CK-II-mediated phosphorylation of rRT, the purified rRT was incubated for 20 min at 30°C with CK-II and 20 μM [γ-^{32}P]ATP (1000 cpm/pmol) in the presence of CK-II activators or inhibitors. The ^{32}P-labeled rRT (p66) in the reaction mixtures was detected by autoradiography after SDS-PAGE. Lane 1, rRT alone; lane 2, rRT+CK-II; lane 3, lane 2+Rev Arg-rich oligo-fragment; lane 4, lane 2+Tat Arg-rich oligo-fragment; and rRT incubated with CK-II in the presence of CK-II inhibitors (GTP (20 μM, lane 5), heparin (0.2 μg/ml, lane 6), oGA (10 μM, lane 7), quercetin (2 μM, lane 8) or NCS-chrom (0.15 μM, lane 9)). B: Purified rRT (approx. 1.0 μg) was incubated for the indicated periods at 30°C with purified CK-II (approx. 20 ng) and 20 μM [γ-^{32}P]ATP (2000 Ci/pmol). The autoradiogram spectrophotometer values are means of three repetitions. 100% represents maximum phosphorylation of rRT by CK-II under the given experimental conditions. Insert: Incubation for 0 min (lane 1), 15 min (lane 2), 30 min (lane 3), 60 min (lane 4), 90 min (lane 5), 120 min (lane 6), and for 120 min without CK-II (lane 7, ■). C: To phosphorylate rRT (approx. 1.0 μg) by CK-II, the protein was incubated for 60 min at 30°C with purified CK-II (approx. 30 ng), cold 50 μM ATP and 10 mM Mg^{2+} in the presence or absence of a CK-II inhibitor (2 μM quercetin or 0.15 μM NCS-chrom) at pH 8.0. After full phosphorylation of rRT, RT activity was determined by subsequent incubation for 20 min at 30°C with 10 μM [α-^{32}P]dGTP (2000 cpm/pmol) under the RT assay conditions [20]. The RT activity of phosphorylated rRT (mean of five different experiments) was statistically significant ($P < 0.001$ using Student's *t*-test). The stimulation rate 1 represents the incorporation of approx. 5.2 pmol/μg/min of [α-^{32}P]dGTP into the synthesized DNA fraction under the given experimental conditions.

ular weights of approx. 120, 99, 51, 41, 38, 33 and 17 kDa in HIV-1 lysate are highly phosphorylated by CK-II, but at least four polypeptides (66, 38, p27 and 24 kDa) are poorly phosphorylated. Under the same experimental conditions, similar protein phosphorylation by CK-II in HIV-1 lysate was observed when other CK-II activators were used, such as poly-Arg and Rev Arg-rich oligo-fragments (data not shown). As shown in Fig. 3B, the CK-II-catalyzed phosphorylation of the viral proteins was completely inhibited by co-incubation with potent CK-II inhibitors known to exhibit anti-viral effects: oGA [13,18] (10 μM, lane 4); quercetin [19] (2 μM; lane 5); or NCS-chrom [14] (0.15 μM, lane 6). The comparative experiments confirmed that a 99-kDa phosphorylated polypeptide (p99) detected in Fig. 3B (lane 3) is ERp99 copurified with CK-II from mouse liver [13]. Western blotting identified p66 and p51 in HIV-1 lysate as reverse transcriptase (Fig. 3C), and three major bands (41-, 24- and 17-kDa polypeptides) on the gel (Fig. 3C, lane 1) were also identified as gp41 and capsid proteins (p24 and p17), respectively.

From these results, it is concluded that (i) at least six polypeptides (gp120, RT (p66 and p51), gp41, capsid proteins (p27 and p17)) positively identified in HIV-1 lysate are viral gene products phosphorylated by CK-II; and (ii) three compounds (oGA, quercetin and NCS-chrom) with anti-viral effects selectively inhibit the CK-II-catalyzed phosphorylation of the viral proteins in vitro.

3.3. Biological significance of the CK-II-catalyzed phosphorylation of rRT (p66) in vitro

A computer-based protein sequence search, using GenetyxMac/CD, revealed that HIV-1 RT (p66: RT (p51)-RNase H (p15), 560 amino acids) has at least nine phosphorylation sites (**S/T**-X-X-E/D) for CK-II as well as four sites for Ca^{2+}-dependent protein kinase (C-kinase) and three sites for tyrosine kinase. Indeed, both p66 and p51 in HIV-1-infected cells (Fig. 3A) and HIV-1 lysate (Fig. 3B) were slightly phosphorylated by CK-II. Although recombinant RNase H-free RT (p51) was an effective phosphate acceptor rather than that of recombinant RT (rRT, p66), the RT activity of RNase H-free rRT (p51) was much less than that determined with rRT. Therefore, rRT was used as a substrate for CK-II in the following analytical experiments. As expected, purified rRT functioned as a phosphate acceptor for CK-II in vitro (Fig. 4A, lane 2 and Fig. 4B). In an assay system using purified CK-II and rRT, however, phosphorylation of rRT by CK-II was significantly reduced when incubated with Arg-rich oligo-fragments related to HIV-1 Rev (Fig. 4A, lane 3) or HIV-1 Tat (lane 4). In addition, GTP (10 μM, lane 5) and four CK-II inhibitors (heparin (0.1 μg/ml, lane 6), oGA (10 μM, lane 7), quercetin (2 μM, lane 8) and NCS-chrom (0.15 μM, lane 9)) effectively inhibited CK-II-catalyzed phosphorylation of rRT (Fig. 4A). Phosphorylation of rRT (p66) by CK-II in the absence of the CK-II activators reached a plateau at 60 min under the given experimental conditions (Fig. 4B).

To determine the physiological significance of the CK-II-catalyzed rRT phosphorylation, RT activity was measured after its incubation with CK-II and cold ATP in the presence or absence of the CK-II inhibitors (Fig. 4C). The RT activity of rRT was about 2.3-fold stimulated after its full phosphorylation by CK-II and this stimulation was inhibited when the phosphorylation step included co-incubation with 2 μM quercetin or 0.15 μM NCS-chrom, which alone did not affect rRT activity (Fig. 4C). These results suggest that CK-II mediates the stimulation of RT activity of rRT in vitro.

4. Discussion

The results presented here suggest that CK-II is a cellular mediator for activation of cellular factors involved in HIV-1 transactivation and for stimulation of the physiological activities of viral proteins in virus-infected cells. This possibility is

strongly supported by the following evidence: (i) rRev and its related Arg-rich fragments can effectively stimulate CK-II activity without substrate specificity (Figs. 1–3); (ii) the activated CK-II phosphorylates several cellular and viral proteins in HIV-1-infected cells (Fig. 3A) and several viral proteins in HIV-1 lysate (Fig. 3B); and (iii) CK-II mediates stimulation of rRT activity (Fig. 4). In addition, both CK-II [21] and HIV-1 Rev [22] are abundant in the nucleoplasm.

The finding that three anti-viral substances (oGA, quercetin and NCS-chrom) inhibit CK-II activity in a dose-dependent manner without substrate specificity (Fig. 3), is basically consistent with the recent report by Critchfield et al. [15], who demonstrated that potent HIV-1 inhibitors, such as chrysin (a flavonoid), benzothiophene and 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB), inhibit CK-II-catalyzed phosphorylation of cellular proteins involved in HIV-1 transcription. Therefore, it seems that the selective inhibition of CK-II-catalyzed phosphorylation of cellular and viral proteins may be the mechanism by which CK-II inhibitors inhibit HIV-1 transactivation at the cellular level. Moreover, NCS-chrom will be potentially useful as an effective inhibitor of CK-II for basic studies on its physiological role in the biochemical processing of HIV-1 replication in virus-infected cells, because the enediyne containing antibiotic selectively inhibits the CK-II-mediated stimulation of rRT activity in vitro at low doses (less than 0.15 μM). NCS-chrom also effectively induces the base-catalyzed cleavage of single-stranded DNA (ssDNA) with a bulge structure, such as transcription response element (TAR) DNA transcribed from HIV-1 RNA, at T-residues [23].

It is well known that the NLS domain (RQARRNRRRR-WRERQR) of HIV-1 Rev is involved in specific binding of RRE and nuclear/nucleolar localization [22], and that its activation domain can interact with one or more cellular factors required for Rev-induced transactivation in HIV-1-infected cells [24]. Recently, it has been reported that phosphorylation of HIV-1 Rev by CK-II, but not by CK-I, is implicated in down-regulation of Rev at the cellular level [16]. We also detected phosphorylation of rRev by CK-II (Fig. 2B, lane 5), and have observed that the binding ability of rRev to RRE is significantly reduced through its phosphorylation by CK-II in vitro (unpublished observation). This suggests that (i) formation of the HIV-1 Rev-RRE complex may be regulated by CK-II; and (ii) the protein-protein interaction between Rev and its cellular mediators, such as human Rev-interacting protein (hRIP) [25], involved in the activation of viral transcription may also be regulated by CK-II in HIV-1-infected cells.

In the in vitro system using purified functional factors (CK-II, rRT and rRev or rTat), it was found that an Arg-rich oligo-fragment (NLS domain) of HIV-1 Rev significantly reduced the CK-II-catalyzed phosphorylation of rRT (Fig. 4A, lane 3). Indeed, p66 (HIV-1 RT) was not much phosphorylated when the CK-II fraction from HIV-1-infected cells was incubated with 20 μM [γ-^{32}P]ATP (1000 cpm/pmol) and 3 mM Mn^{2+}, in the presence of rRev as a CK-II activator (Fig. 3A, lane 4). Both p66 and p51 in HIV-1 lysate were also poorly phosphorylated by CK-II after direct incubation of the lysate with purified CK-II and 20 μM [γ-^{32}P]ATP (1000 cpm/pmol) in the presence of rRev (Fig. 3B, lane 3). These results suggest that (i) HIV-1 Rev may be an effective potent activator for the CK-II-catalyzed phosphorylation of several cellular factors and some HIV-1 gene products, such as gp120, gp41 and capsid proteins (p27 and p17), which are synthesized from the late structural mRNAs of HIV-1 [22]; and (ii) the CK-II-mediated stimulation of HIV-1 RT (p66) activity may be unrelated to the cellular level of Rev in HIV-1-infected cells. However, for understanding clearly the biological role of HIV-1 Rev in the CK-II-catalyzed phosphorylation of cellular transcriptional factors and viral gene products during HIV-1 replication, further analytical studies will be required to discover: (i) the protein-protein interaction between CK-II, RT and Rev or Tat in vitro; and (ii) the regulatory mechanisms involved in the physiological activities of cellular transcriptional factors (Sp-1, Ap-1 and NFκB), viral gene products (gp120, gp41, capsid proteins (p27 and p17) and protease (p11)) and Rev-interacting proteins (RIPs) through their specific phosphorylation by the Rev-activated CK-II in HIV-1-infected cells.

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