Biochemical Characterization of Suramin as a Selective Inhibitor for the PKA-Mediated Phosphorylation of HBV Core Protein *in Vitro*

Motohito OKABE,^a Masato ENOMOTO,^b Haruki MAEDA,^b Kazuyuki KUROKI,^c and Kenzo OHTSUKI^{*,b}

^a Genetical Biochemistry, School of Allied Health Sciences, Kitasato University; ^b Genetical Biochemistry and Signal Biology, Graduate School of Medical Sciences, Kitasato University; 1–15–1 Kitasato, Sagami-hara 228–8555, Japan; and

^c Division of Cell Biology, Cancer Research Institute, Kanazawa University; 13–1 Takaramachi, Kanazawa 920–0934, Japan. Received May 2, 2006; accepted June 13, 2006; published online June 20, 2006

The inhibitory effect of suramin on the phosphorylation of GST-HBV core fusion protein (GST-Hcore) and two GST-Hcore fusion polypeptides (Hcore157B and Hcore164B) by two α -type cAMP-dependent protein kinases (PKAI α and PKAII α) was biochemically investigated *in vitro*. It was found that (i) this phosphorylation was inhibited by suramin at a low concentration (IC_{50} =approx. 10 nM); (ii) a relative high dose of suramin was required to inhibit an autophosphorylation of PKAII α (IC_{50} =approx. 0.7 μ M) and the PKAII α -mediated phosphorylation of histone H2B (IC_{50} =approx. 0.4 μ M); (iii) the PKAII α -mediated phosphorylation of Hcore157B was more sensitive to suramin than the phosphorylation of Hcore157B by Ca^{2+} -dependent protein kinase (PKC); and (iv) suramin had a high binding affinity for Hcore157B, but not for histone H2B *in vitro*. These results suggest that suramin selectively inhibits the PKA-mediated phosphorylation of HBV-CP through the direct binding *in vitro* of suramin to the Arg-rich C-terminal region (containing three potential phosphorylation sites for PKA) on HBV-CP.

Key words suramin; cAMP-dependent protein kinase; human hepatitis B virus core protein

Earlier studies have revealed that (i) hepatitis B virus (HBV) core protein (HBV-CP) is a 21 kDa phosphoprotein (185 amino acid residues) in intact cells¹; (ii) the arginine (Arg)-rich C-terminal region (positions 157–185) on HBV-CP contains potent phosphorylation sites for several intracellular protein kinases,² such as cAMP-dependent protein kinase (PKA), Ca^{2+} -dependent protein kinase (PKC), cyclin-dependent protein kinase Cdc2 and serine (S)/arginine (R) protein-specific kinase; and (iii) the phosphorylation of HBV-CP by these protein kinases is implicated in promoting DNA replication of HBV and is critical for subsequent pregenomic RNA packaging.^{2–5} These earlier studies suggest that the selective inhibition of HBV-CP phosphorylation by potent protein kinase inhibitors may be involved in the prevention of viral multiplication in HBV-infected cells.

In our preliminary experiments, at least four sulfated compounds [suramin (a compound with six sulfo groups, Fig. 1B); cholesterol-3'-sulfate (CH-3S); fucoidan; and Evans blue] effectively inhibited the PKAII α -mediated phosphory-

lation of glutathione-S-transferase (GST)-Hcore (full length polypeptide of HBV-CP) and two GST-Hcore fusion polypeptides (Hcore157B: positions 157–185 and Hcore164B: positions 164–185, Fig. 1A) *in vitro*. These preliminary results suggested that suramin may interact *in vitro* with the positively charged Arg-rich C-terminal region, containing potent phosphorylation sites for PKA and PKC, on HBV-CP *in vitro*. It has been reported that (i) suramin inhibits PKAII α (IC_{50} =approx. 0.66 mM) much less potently than type I PKC (IC_{50} =approx. 29 μ M) using histone III-S as a phosphate acceptor *in vitro*⁶; and (ii) suramin also inhibits reverse transcriptase of human immunodeficiency virus type 1 (HIV-1) *in vitro*⁷ and *Trypanosoma* malate dehydrogenase *in vivo*.⁸ Furthermore, it has been demonstrated that (i) suramin acts as a potent inhibitor for core protein-associated two enzyme activities (reverse transcriptase and DNA polymerase) of duck hepatitis B virus (DHBV) *in vitro*⁹; and (ii) the drug effectively blocks the early stages of DHBV infection in cultured cells.¹⁰ However, the suramin-induced inhibitory mechanism involved in the viral replication in DHBV- or HBV-infected cells remains to be elucidated, and there are no reports concerning the inhibitory effect of suramin on the PKAII α -mediated phosphorylation of HBV-CP *in vitro*. Therefore, the present study was undertaken to (i) further characterize suramin as a selective inhibitor for the phosphorylation of HBV-CP by two PKAs (PKAI α and PKAII α) using three GST-Hcore fusion polypeptides (GST-Hcore, Hcore157B and Hcore164B) *in vitro*; (ii) detect the direct binding of suramin to the Arg-rich C-terminal region between 157 and 185 of HBV-CP; and (iii) determine the inhibitory kinetics of suramin on the PKAII α -mediated phosphorylation of Hcore157B *in vitro*.

MATERIALS AND METHODS

Chemicals and Reagents [γ -³²P]ATP (3000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. (Arling-

Fig. 1. (A) The Primary Sequences of Two GST-HBV Core Polypeptides [Hcore157B (Positions 157–185) and Hcore164B (Positions 164–185)] Containing Three Potent Phosphorylation Sites (□, R-R-X-S/T) for PKA and Potent Phosphorylation Sites (—, S/T-X-R) for PKC and (B) Chemical Structure of Suramin

* To whom correspondence should be addressed. e-mail: ken@kitasato-u.ac.jp

ton Heights, U.S.A.); cyclic adenosine monophosphate (cAMP), dithiothreitol (DTT), phosphatidyl-L-serine (PS), suramin (polysulfonated naphthylurea), histone H2B (calf thymus), protamine sulfate, protamine 1B (rainbow trout sperm) and two PKAs (PKAI α and PKAII α , bovine heart muscle) from Sigma Chemical (St. Louis, U.S.A.); PKC (specific activity: 1–2 μ mol phosphate/min/mg protein transferred to histone) from Molecular Probes, Inc. (Oregon, U.S.A.); NF449 (a derivative of suramin) from Calbiochem (Darmstadt, Germany); and diacylglycerol (D-2-Diolein) from Funakoshi Co., Ltd. (Tokyo, Japan). Galloyl pedunculagin (GP, a potent PKA inhibitor¹¹) was a generous gift from Dr. Tanaka (Nagasaki University, Japan).

Preparation of GST-Hcore and Two GST-Hcore Fusion Polypeptides The HBV core sequences were amplified from HBV DNA and their nucleotide sequences were identified by polymerase chain reaction (PCR) using 5' and 3' primers bearing BamHI and XhoI sites, respectively.¹² PCR products were cleaved and ligated into the BamHI–XhoI site between glutathione-S-transferase (GST) and the chitin-binding domain (CBD) of pGST-CBD plasmid DNA, which expresses a GST fusion protein in *Escherichia coli* under the control of isopropyl- β -D-thiogalacto-pyranoside (IPTG)-inducible promoter. Expression and purification of these fusion proteins (GST-Hcore, Hcore157B and Hcore164B) were carried out as described by Kuroki *et al.*¹³ Plasmids were used to transform competent Rosetta cells, and the IPTG-induced cultured cells were lysed by sonication. These extracts were applied onto a glutathione-sepharose column and the bound GST fusion proteins on the affinity-column were eluted separately with 50 mM Tris–HCl (pH 8.0) containing 20 mM reduced glutathione.

Detection of ³²P-Phosphorylated Polypeptides by SDS-PAGE Followed by Autoradiography To detect GST-Hcore, Hcore157B, Hcore164B, protamine 1B, protamine sulfate or histone H2B (approx. 5 μ g each) phosphorylated by PKAII α or PKAI α (approx. 50 ng) *in vitro*, reaction mixtures (total 50 μ l) comprising 40 mM Tris–HCl (pH 7.6), 2 mM DTT, 1 mM Mn²⁺, 1 μ M cAMP and 5 μ M [γ -³²P]ATP (500 cpm/pmol) were incubated for 30 min at 30 °C. In the case of PKC, reaction mixtures comprising 40 mM Tris–HCl (pH 7.6), 2 mM DTT, 1 mM Mn²⁺, 1 mM Ca²⁺, 1 μ M PS, type I PKC (approx. 50 ng) and 5 μ M [γ -³²P]ATP (500 cpm/pmol) were incubated for 30 min at 30 °C. Protein phosphorylation by two PKAs or PKC was arrested by the addition of an equal volume of sample buffer. ³²P-Labeled polypeptides in the reaction mixtures were detected by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography, as described in our previous paper.¹⁴

Assay for the Phosphorylation of Hcore157B by Two PKAs (PKAI α and PKAII α) *in Vitro* The phosphorylation of Hcore157B by two PKAs (PKAI α and PKAII α) was determined *in vitro*. After incubation for 30 min at 30 °C, the phosphorylation of Hcore157B by two PKAs was arrested by the addition of 0.5 ml 20% trichloroacetic acid (TCA) and 0.25 ml 0.1 M sodium pyrophosphate containing bovine serum albumin (approx. 1 mg/ml) and 10 mM EDTA. The TCA-insoluble precipitates were trapped on a glass membrane filter (Advantec GF/75, Tokyo, Japan), and then washed successively with 2% TCA and 100% ethanol. After

drying, the ³²P-radioactivity on the membrane filter was measured with a liquid scintillation spectrometer, as stated in our previous report.^{11,14}

Measurement of the Binding-Affinity of Suramin *in Vitro* by Using a QCM The binding affinities of suramin and CH-3S with GST-Hcore, Hcore157B, protamine 1B, histone H2B or GST were measured by using a quartz crystal microbalance (QCM, Initium, Tokyo, Japan), as described originally by Sun *et al.*¹⁵ GST-HBV-CP, Hcore157B, protamine 1B, histone H2B or GST (approx. 2 μ g each) was directly immobilized on a QCM plate in 8 ml of 40 mM Tris–HCl (pH 7.6) at 25 °C until an equilibrium was attained. The indicated concentrations of suramin or CH-3S were added to the equilibrated solution. The resonance frequency of the QCM was defined as the 0 position after equilibrium. The frequency change in the QCM responding to GST-Hcore, Hcore157B, protamine 1B, histone H2B or GST was recorded for specific time points. The stability of the 27-MHz QCM frequency in the solution was 5 Hz for 12 h at 25 °C.

RESULTS

Phosphorylation of GST-Hcore Fusion Polypeptides by Two PKAs *in Vitro* To detect the phosphorylation of GST-Hcore and two GST-Hcore fusion polypeptides (Hcore157B and Hcore164B) by two PKAs (PKAI α and PKAII α) *in vitro*, these three polypeptides (approx. 5 μ g each) were separately incubated for 30 min at 30 °C with PKAII α and 5 μ M [γ -³²P]ATP (500 cpm/pmol) in the presence or absence of 1 μ M cAMP. Figure 2A shows that (i) PKAII α phosphorylates GST-Hcore *in vitro* (lane 2); and (ii) this phosphorylation is significantly stimulated by 1 μ M cAMP (lane 3), but completely inhibited by 1 μ M GP (lane 4, Fig. 2A). Under the same experimental conditions, both Hcore157B (lane 2) and Hcore164B (lane 3, Fig. 2B) were highly phosphorylated by PKAII α in the presence of 1 μ M cAMP *in vitro*. No difference in the PKAII α -mediated phosphorylation kinetics between Hcore157B and Hcore164B in the presence of 1 μ M cAMP *in vitro* was observed (Fig. 2C). Similar phosphorylation of Hcore157B as well as Hcore164B by PKAI α instead of PKAII α was observed *in vitro* (data not shown). However, no phosphorylation of recombinant GST by these two PKAs (PKAI α and PKAII α) *in vitro* was detected. Only phosphoserine was detected in Hcore157B, containing Ser-170 and Ser-178, and GST-Hcore phosphorylated by two PKAs in the presence of 1 μ M cAMP *in vitro* (data not shown).

The Inhibitory Effects of Five Sulfated Compounds on the PKAII α -Mediated Phosphorylation of Hcore157B *in Vitro* The inhibitory effects of five sulfated compounds [suramin, NF449 (a derivative of suramin), Evans blue, CH-3S and fucoidan] on the phosphorylation of Hcore157B by PKAII α were compared *in vitro*. Figure 3A shows that (i) suramin is the most effective inhibitor of the PKAII α -mediated phosphorylation of Hcore157B among the five tested compounds; and (ii) the IC₅₀ of suramin (approx. 10 nM) is approx. 5-fold lower than that of Evans blue (IC₅₀=approx. 50 nM), approx. 7-fold lower than that of NF449 (IC₅₀=approx. 70 nM), approx. 10-fold lower than that of CH-3S (IC₅₀=approx. 0.1 μ M) and approx. 20-fold lower than that of fucoidan (IC₅₀=approx. 0.2 μ M).

Fig. 2. Phosphorylation of GST-Hcore and Two GST-Hcore Fusion Polypeptides (Hcore157B and Hcore164B) by PKAIIα *in Vitro*

(A) To determine the phosphorylation of GST-Hcore by PKAIIα *in vitro*, reaction mixtures (total 50 μl) comprising 40 mM Tris-HCl (pH 7.6), PKAIIα (approx. 50 ng), 3 mM Mn²⁺, 2 mM DTT, GST-Hcore (approx. 5 μg) and 5 μM [γ -³²P]ATP (500 cpm/pmol) were incubated for 30 min at 30 °C in the presence or absence of 1 μM cAMP. ³²P-labeled GST-Hcore in the reaction mixtures was detected by autoradiography after SDS-PAGE. Lane 1, PKAIIα alone; lane 2, PKAIIα incubated with GST-Hcore; lane 3, lane 2 + 1 μM cAMP; and lane 4, lane 3 + 1 μM GP. (B) Either Hcore157B or Hcore164B (approx. 5 μg each) was incubated with PKAIIα (approx. 50 ng) in reaction mixtures as described in the legend of Fig. 2A. After incubation for 30 min at 30 °C, ³²P-labeled Hcore157B or Hcore164B in reaction mixtures containing 1 μM cAMP were detected by autoradiography after SDS-PAGE. Lane 1, GST (control); lane 2, Hcore157B; and lane 3, Hcore164B. (C) Either Hcore157B or Hcore164B was incubated with PKAIIα (approx. 50 ng) in the presence of 1 μM cAMP in reaction mixtures, as described in the legend of Fig. 2A. After incubation for the indicated periods (0–150 min) at 30 °C, ³²P-labeled Hcore157B (—○—) or Hcore164B (—●—) in reaction mixtures was detected by autoradiography after SDS-PAGE. 100% represents maximum phosphorylation of Hcore157B or Hcore164B by PKAIIα after incubation for 150 min at 30 °C.

Fig. 3. (A) The Inhibitory Effects of Suramin, NF449, Evans Blue, CH-3S and Fucoidan on the Phosphorylation of Hcore157B by PKAIIα *in Vitro*

After incubation (30 min at 30 °C) of Hcore157B (approx. 5 μg) with PKAIIα (approx. 50 ng) and 5 μM [γ -³²P]ATP (500 cpm/pmol) in the presence of the indicated concentrations of suramin (—■—), NF449 (—○—), Evans blue (—◇—), CH-3S (—●—) or fucoidan (—△—), ³²P-phosphorylated Hcore157B in the reaction mixtures was detected by autoradiography after SDS-PAGE. 100% represents the phosphorylation of Hcore157B by PKAIIα in the absence of these five inhibitors. Mean values from three different experiments.

(B) The Inhibitory Effect of Suramin on the PKAIIα-Mediated Phosphorylation of GST-Hcore, Hcore157B, Protamine 1B or Histone H2B *in Vitro*

After incubation (30 min at 30 °C) of PKAIIα with either GST-Hcore, Hcore157B, protamine 1B, protamine sulfate or histone H2B (approx. 5 μg each) and 5 μM [γ -³²P]ATP (500 cpm/pmol) in the presence of the indicated concentrations of suramin, ³²P-phosphorylated GST-Hcore (—○—), Hcore157B (—●—), protamine 1B (—◆—), protamine sulfate (—◇—) or histone H2B (—△—) in the reaction mixtures was detected by autoradiography after SDS-PAGE. 100% represents the phosphorylation of these proteins by PKAIIα in the absence of suramin.

(C) The Inhibitory Effect of Suramin on the PKAIIα-Mediated Phosphorylation of Hcore157B and on Autophosphorylation of PKAIIα *in Vitro*

After incubation (30 min at 30 °C) of PKAIIα with Hcore157B (approx. 5 μg) and 5 μM [γ -³²P]ATP (500 cpm/pmol) with the indicated concentrations of suramin, ³²P-phosphorylated Hcore157B (—○—) or autophosphorylation of PKAIIα (—●—) in the reaction mixtures was detected by autoradiography after SDS-PAGE. 100% represents the PKAIIα-mediated phosphorylation of Hcore157B and autophosphorylation of PKAIIα in the absence of suramin.

The inhibitory effects of suramin on the PKAIIα-mediated phosphorylation of five distinct substrates (GST-Hcore, Hcore157B, protamine 1B, protamine sulfate and histone H2B) were determined *in vitro*. The PKAIIα-mediated phosphorylation of GST-Hcore and Hcore157B was inhibited by suramin in a dose-dependent manner *in vitro* (Fig. 3B). There were similar suramin-induced inhibitory kinetics in the PKAIIα-mediated phosphorylation between GST-Hcore and Hcore157B (Fig. 3B). In contrast, higher concentrations of suramin were required to inhibit the PKAIIα-mediated phosphorylation of protamine 1B (IC₅₀ = approx. 0.2 μM), protamine sulfate (IC₅₀ = approx. 1.0 μM), and histone H2B (IC₅₀ = approx. 0.4 μM, Fig. 3B). There was approx. 40-fold dose-

discrepancy in the suramin-induced inhibition of the PKAIIα-mediated phosphorylation between Hcore157B and histone H2B *in vitro*. The IC₅₀ of suramin on the autophosphorylation of PKAIIα was approx. 0.7 μM, which were approx. 70-fold higher than that observed with the PKAIIα-mediated phosphorylation of Hcore157B *in vitro* (Fig. 3C). The IC₅₀ of suramin on the PKC-mediated phosphorylation of Hcore157B was found to be approx. 50 nM, which was approx. 5-fold higher than its effect on the PKAIIα-mediated phosphorylation of Hcore157B *in vitro* (Fig. 4). These results suggest that suramin (i) may inhibit the PKA-mediated phosphorylation of GST-Hcore as well as Hcore157B through its direct binding to their Arg-rich domains (positions 157—

Fig. 4. The Inhibitory Effect of Suramin on the Phosphorylation of Hcore157B by PKAII α or PKC *in Vitro*

After incubation (30 min at 30 °C) of PKAII α (—●—) or PKC (—○—) with Hcore157B (approx. 5 μ g) and 5 μ M [γ - 32 P]ATP (500 cpm/pmol) in the presence of the indicated concentrations of suramin, 32 P-phosphorylated Hcore157B in the reaction mixtures was detected by autoradiography after SDS-PAGE. 100% represents the PKAII α -mediated phosphorylation of Hcore157B and the phosphorylation of Hcore157B by PKC in the absence of suramin. Mean values from three different experiments.

Fig. 5. The Inhibitory Kinetics of Suramin on the PKAII α -Mediated Phosphorylation of Hcore157B *in Vitro*

The phosphorylation kinetics of Hcore157B by PKAII α were determined by incubation for 30 min at 30 °C with 5 μ M [γ - 32 P]ATP (500 cpm/pmol) in the presence (—○—) or absence (—●—) of suramin (final concentration: 30 nM) *in vitro*. Lineweaver-Burk plots. Mean values from three different experiments.

185) containing three potential phosphorylation sites for PKA; and (ii) more potently affects the phosphorylation of Hcore157B by two PKAs rather than that by PKC *in vitro*.

Inhibitory Kinetics of Suramin on the PKAII α -Mediated Phosphorylation of Hcore157B *in Vitro* The inhibitory kinetics of suramin on the PKAII α -mediated phosphorylation of Hcore157B was determined *in vitro*. An apparent K_m of PKAII α for Hcore157B shifted from 0.08 to 0.18 μ g/ml when incubated with 30 nM suramin, whereas the V_{max} of Hcore157B shifted from 0.57 to 0.56 nmol/ μ g/min in the presence of 30 nM suramin (Fig. 5). Under the same experimental conditions, similar results were obtained with PKAI α instead of PKAII α *in vitro* (data not shown). These results show that direct binding of suramin to Hcore157B reduces its affinity for two PKAs (PKAI α and PKAII α) *in vitro*.

The High Binding Affinity of Suramin for Hcore157B *in Vitro* The binding affinities of suramin with GST-Hcore, Hcore157B, protamine 1B, histone H2B and GST were determined *in vitro* by using a QCM. As expected, suramin directly bound to GST-Hcore, Hcore157B and protamine 1B with different affinities, but did not bind to GST *in vitro* (Fig. 6A). The higher binding affinity of suramin with Hcore157B was observed, but not significantly with histone H2B (Fig.

Fig. 6. (A) The Binding Affinities of Suramin with Either GST, GST-Hcore, Hcore157B, Protamine 1B or Histone H2B *in Vitro*

Using a QCM (Initium, Tokyo), the binding affinities of suramin with these polypeptides (approx. 2 μ g each) were examined in 8 ml of 40 mM Tris-HCl (pH 7.6) at 25 °C. Suramin (final concentration: 1 μ M) was added to equilibrated solutions containing GST (a), GST-Hcore (b), Hcore157B (c), protamine 1B (d) or Histone H2B (e). Mean values from three different experiments.

(B) The Binding Affinities of CH-3S with GST, GST-Hcore, Hcore157B, Protamine 1B or Histone H2B *in Vitro*

The binding affinities of CH-3S with these polypeptides (approx. 2 μ g each) were examined, under the same experimental conditions as in (A). CH-3S (final concentration: 1 μ M) was added to equilibrated solutions containing either GST (a), GST-Hcore (b), Hcore157B (c), protamine 1B (d) or histone H2B (e). Mean values from three different experiments.

6A). In contrast, the binding affinity of CH-3S with these basic proteins was compared *in vitro*. The binding affinity of CH-3S to these three basic proteins (GST-Hcore, Hcore157B and protamine 1B) was approx. 2-fold higher than that observed with suramin *in vitro* (Fig. 6B). The significant binding affinity of CH-3S with histone H2B was observed (Fig. 6B). These results show that (i) suramin has a higher binding affinity for Hcore157B in comparison with that observed for protamine 1B *in vitro*; and (ii) both suramin and CH-3S may prevent the phosphorylation of Hcore157B by two PKAs through their direct binding to the near phosphorylation sites for two PKAs on these substrate proteins *in vitro*.

DISCUSSION

In the present study, the inhibitory effect of suramin on the phosphorylation of GST-Hcore and two GST-Hcore fusion polypeptides (Hcore157B and Hcore164B) by two PKAs (PKAI α and PKAII α) was biochemically investigated *in vitro*. It was found that (i) PKAII α highly phosphorylated GST-Hcore, Hcore157B as well as Hcore164B in the presence of 1 μ M cAMP *in vitro* (Figs. 2A and 2B); (ii), in the presence of 1 μ M cAMP, both Hcore157B and Hcore164B, containing Ser-170 and Ser-178, were phosphorylated time-dependently by PKAII α in a similar kinetic *in vitro* (Fig. 2C); (iii) this phosphorylation was inhibited by suramin in a dose-dependent manner (Fig. 3A); and (iv) the IC₅₀ (approx. 10 nM) of suramin on the PKAII α -mediated phosphorylation of Hcore157B was significantly lower than those determined with other substrates, such as protamine 1B, protamine sulfate and histone H2B *in vitro* (Fig. 3B).

The inhibitory effect of suramin on the PKAII α -mediated phosphorylation of Hcore157B did not correspond to the sulfate number of the sulfated inhibitors, because (as shown in

Fig. 3A) the PKAII α -mediated phosphorylation of Hcore157B was significantly more sensitive to suramin than NF449, which contains eight sulfates. The IC₅₀ (approx. 0.7 μ M) of suramin on the autophosphorylation of PKAII α was about 70-fold higher than that observed for the PKAII α -mediated phosphorylation of Hcore157B *in vitro* (Fig. 3C). In contrast, the IC₅₀ (approx. 10 nM) of suramin on the PKAII α -mediated phosphorylation of Hcore157B was approx. 5-fold lower than that (approx. 50 nM) determined with the phosphorylation of Hcore157B by PKC *in vitro* (Fig. 4). In addition, inhibitory kinetic experiments (Fig. 5) revealed that direct binding of suramin to Hcore157B significantly reduced the binding affinity of the protein substrate for PKAII α *in vitro*.

Suramin may directly bind to the near two potential phosphorylation sites (Ser-170 and Ser-178) for PKA on the Arg-rich C-terminal region at positions between 164 and 185 (protamine-like sequence) of HBV-CP, thus selectively preventing the phosphorylation of HBV-CP by two PKAs (PKAI α and PKAII α) *in vitro*. This speculation is supported by the *in vitro* experimental results that (i) two PKAs (PKAI α and PKAII α) phosphorylate only Ser-residues on Hcore157B as well as Hcore164B (Fig. 2B); (ii) the PKA-mediated phosphorylation of Hcore157B is more sensitive to suramin than the phosphorylation of Hcore157B by PKC (Fig. 4); and (iii) suramin has a high binding affinity for Hcore157B, but not for histone H2B (Fig. 6A). The direct binding of suramin to the Arg-rich C-terminal region on HBV-CP is similar to the observations reported for the binding sites of suramin on human neutrophil elastase, ryanodine receptor (RYR1) and cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel.^{16–18} Mely *et al.* reported that suramin strongly binds to six Arg-residues on human neutrophil elastase, which is a 30 kDa glycoprotein belonging to the class of serine proteinases.¹⁶ Furthermore, it has been reported that suramin interacts with the calmodulin binding sites on the ryanodine receptor (RYR1)¹⁷ as well as CFTR for chloride channel.¹⁸ There is a structural similarity in the Arg-rich domains, containing glutamine (Glu)-residues beside two potential phosphorylation sites for PKA among these three suramin-binding proteins (neutrophil elastase, RYR1 and CFTR) and Hcore164B (positions 164–185, Fig. 1A). However, there are no Glu-residues in the Arg-rich amino acid domains on protamine 1B. These structural differences of the suramin-binding domains between HBV-CP and protamine 1B may be one reason why they exhibit different substrate activities for two PKAs (PKAI α and PKAII α) *in vitro*. Therefore, it seems likely that the Arg-rich regions (positions 164–185), containing Glu-169 and Glu-179, on

HBV-CP may include the potent binding sites for suramin *in vitro*.

To clearly understand the physiological effect of suramin on the PKA-mediated phosphorylation of HBV-CP, further analytical studies will be required to: (i) detect the selective inhibition of suramin on the growth of HBV through its direct physiological interaction with HBV-CP and the suramin sensitivity to the PKA-mediated phosphorylation of HBV-CP in virus-infected cells; and (ii) determine the critical suramin-binding sites on HBV-CP.

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