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Effect of Staurosporine on the Intracellular Localization of Hepatitis B Virus Core Protein

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Abstract

Core protein of Hepatitis B virus (HBV) play an important role in infection of HBV into the liver cells. Core protein is also including in the HBV genome targeting into the nucleus through modulating carboxyl residues by phosphorylation. Nuclear localication Signal (NLS) in HBV core protein is inside the virion structure and it must be unmasked in order to function, perhaps by phosphorylation. Phosphorylation of of HBV core protein in turn could begin to alter capsid conformation. Staurosporine is a natural product originally isolated from bacterium *Streptomyces staurosporeus*. Staurosporine was discovered to have biological activities ranging from anti-fungal to anti-hypertensive. The interest in these activities resulted in a large investigative effort in chemistry and biology and the discovery of the potential for anti-cancer treatment. The main biological activity of Staurosporine is the inhibition of protein kinases through the prevention of ATP binding to the kinase. In the present study, we have studied the intracellular localization of EGFP-Core fusion protein with triple HBV core and SV-40 nuclear localization signal at its carboxyl terminal in presence and absence of Staurosporine. We also to study the effect of Staurosporine treatment on the intracellular localization of EGFP-Core fusion protein in the hepatocyte cells line of HepG2 cell. Results showed that effect of Staurosporine is prevent the nuclear localization of EGFP-Core fusion protein into nucleus through an inhibition of the phosphorylation of core protein. Stauroporine also prevents cell division so that passive trapping of core protein is inhibited.

Key words: Staurosporine, HBV, core protein, intracellular localization, NLS, HepG2 cell

Introduction

Hepatitis B virus (HBV) causes acute and chronic hepatitis, which is an important cause of cirrhosis of the liver and hepatocellular carcinoma (HCC). In its replication cycle, assembly of HBV virion begins with polymerization of the core protein to enclose a complex of the 3.2-kilobase RNA pregenome and reverse transcriptase (Bartenschlager *et al.*, 1990). Upon completion of capsid assembly, the pregenome is retrotranscribed (Summers and Mason 1982; Bartenschlager and Schaller, 1992). The resulting DNA-containing nucleocapsid then acquires a lipoprotein envelope from the host cell (Gerelsaikhan *et al.*, 1996).

The HBV capsid, also known as core protein or core antigen (HbcAg). This protein consist of 183 residue amino acid with two domains: the assembly domain that forms the contiguous shell, and the protamine domain that is responsible for RNA packaging (Nasal, 1992). Dimeric capsid spontaneously assembles into icosahedral capsids when expressed in

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heterologous systems (Cohen and Richmond, 1982) or from purified protein (Wingfield *et al.*, 1995).

The expression of the HBV core protein into human hepatocyte cell line has been studied by Haryanto and Kann (2006). HBV core protein was found to be localized to the cytoplasm, nucleus and both compartment. It was localized predominantly into the nucleus in HuH-7 cells (Haryanto, 2006). HBcAg was found to be distributed in both the nucleus and the cytoplasm in HBV-producing hepatocytes and transgenic mice (Guidotti *et al.*, 1995). It has also been documented that in HbeAg positive patients, a nucleus-dominant distribution of intrahepatic HBcAg is associated with minor hepatitis activity while a cytoplasmic distribution of HBcAg is associated with chronic active liver disease (Chua *et al.*, 2003).

The molecular basis for the shift from nuclear distribution and minor disease activity to cytoplasmic distribution and disease exacerbation has been unclear. Frequent mutations have been found to accumulate in HBV core protein during natural infections with HBV. It is tempting to hypothesize that naturally occurring HBcAg variants contribute to the change in the subcellular localization of HBV core protein (Chuang *et al.*, 1993; Hosono *et al.*, 1995).

The nuclear localization signal (NLS) of HBV core protein is located within the carboxy-terminal 34 amino acids, in which two sequences (145-ETTVVRRRGRSP-156 and 172-RRRRSQSSRESQC-183) are important for nuclear import (Eckhardt *et al.*, 1991). The same residues contain four poly-arginine clusters, of which three, located between amino acids 157 to 183, have an SPRRR motif. The serine residues, in particular the ones penultimate to praline are phosphorylated in vivo (Roossinck MJ and Siddigqui A, 1987). Mutant protein in which serines in the motif are changed to alanine are nonphosphorylated and localize to the nucleus regardless of the phase of the cell cycle, indicating that phosphorylation influences the protein's nuclear localization (Liao and Ou, 1995).

Staurosporine is a microbial alcaloid antibiotic. It has been reported to have antifungal and strong hypotensive effects (Omura *et al.*, 1977). It also seems that Staurosporine is the most potent inhibitor of the phospholipid / Ca^{2+} -dependent protein kinase C with an IC_{50} of 2.7 nM regarding the enzyme from rat brain (Tamaoki *et al.*, 1986). In compare with other known protein kinase C inhibitors such as trifluoperazine, chloropronaline and polymyxin B, Staurosporine is about 3 orders of magnitude more effective. This potent inhibitory effect makes the compound very valuable in investigating the role of protein phosphorylation by protein kinase C, Ca^{2+} mobilization by inositol phosphates and provides a useful tool for the isolation and purification of protein kinase C. It also inhibits cAMP- and cGMP-dependent protein kinase with Ki -values around 7 nM and the protein tyrosine kinase activity of $p60^{v\text{-}src}$ with an IC_{50} value of 6.4 nM (Nakano *et al.*, 1987). Therefore, Staurosporine is a very potent inhibitor of protein tyrosin kinases as well.

Besides direct blocking of protein kinases, Staurosporine exhibits strong cytotoxic effects on various tumor cell lines (Tamaoki *et al.*, 1986) and inhibits platelet aggregation (Oka *et al.*, 1986; Schächtele *et al.*, 1988) with an IC_{50} of 3.4 µM or 11.6 µM, depending on induction of aggregation induced by either collagen or ADP, respectively. The compound does not interfere with the binding of phosphatidylserine and phorbol esters to protein kinase C suggesting different binding sites. Additionally, Staurosporine does not compete with Ca^{2+}, histones, DAG and ATP (Tamaoki *et al.*, 1986).

In the present study, we have studied the intracellular localization of EGFP-Core fusion protein with triple HBV core and SV-40 nuclear localization signal (NLS) at its carboxyl terminal in presence and absence of Staurosporine. We want to know effect of Staurosporine treatment on the intracellular localization of EGFP-Core fusion protein in the hepatocyte cells line of HepG2.

Materials and Methods

The main materials of this study are HBV core fusion protein encoding DNA plasmid, Staurosporine (SIGMA), transfection agent Tfx-20 (PROMEGA), hepatocyte cell line HepG2 and DMEM medium (GIBCO-BRL) and Fetal Calf Serum or FCS (GIBCO-BRL).

Design of EGFP-Core Fusion Protein Encoding Plasmids

The recombinant plasmid pEGFP-Core with single NLS (design by Haryanto and Kann, 2006) was used as original DNA plasmid. The pEGFP-Core was double restriction digested with Ava I and Apa I enzymes. Then the linier plasmid DNA fragment was ligated with synthetic oligonucleotides, that be ordered from MAGV which encoded additional 3 NLS of HBV Core and 3 SV-40. Sequence of synthetic oligonucleotides for pEGFP-Core 3C NLS are:

Sense (129 nucleotides):
5'-CTC GGG AAT CTC AAC CTA GAA GAA GAA CTC CCT CGC CTC GCA GAC GCA GAT CTC AAT CGC CGC GTC GCC CTA GAA GAA GAA CTC CCT CGC CTC GCA GAC GCA GAT CTC AAT CGC CGC GTC GCT AGG GCC-3'

Antisense (123 nucleotides):
5'-CTA GCG ACG CGG CGA TTG AGA TCT GCG TCT GCG AGGCGA GGG AGT TCT TCT TCT AGG GCG ACG CGG CGA TTG AGA TCT GCG TCT GCG AGG CGA GGG AGT TCT TCT TCT AGG ACA TTG AGA TTC-3'.

Sequence of syntheti oligonucleotides for pEGFP-Core 3 SV-40 NLS are:

Sense (92 nucleotides):
5'-CCG GAA ACT ACT GTT GTT CCT AAG AAG AAG AGA AAG GTG CCT AAG AAG AAG AGA AAG GTG CCT AAG AAG AAG AAG AGA AAG GTG TAG GGG CC-3'

Antisense (81 nucleotides):
5' C CTA CAC CTT TCT CTT CTT CTT AGG CAC CTT TCT CTT CTT CTT AGG CAC CTT TCT CTT CTT CTT AGG AAC AAC AGT AGT TT-3'

Each of a pair synthetic oligonucleotide contains the artificial restriction site of enzyme *Ava I* at 5' and *Apa I* at 3' of the strands. Before ligation, all of the oligonucleotides were adjusted in concentration 1 ìg/ìl. Then between sense and antisense has to be hybridized in order to form double strand DNA. The Hibridisation was done by mixing sense and antisense DNA. After gently mixed the Hibridization tube was incubated on the heat block at 95°C for 2 minutes and then cooled down slowly at room temperature and 4°C in refrigerator.

Preparation of hepatocytec cell line HepG2

In 24 well plate, HepG2 cells was grown onto collagenized cover slips one day before transfection. The cell lines were incubated over night at 37°C in incubator CO_2 until 75-80% confluent.

Transfection and staurosporine treatment

All construction of DNA were transfected into HepG2 cells. The transfection reaction consist of 5 ìl DNA plasmid (200 ng/ml) 5 ml Tfx-20 and 300 ml serum free medium. Then it was mixed gently and incubated at room temperature for 5-10 minutes. While, 24 well plate were took out from CO_2 incubator and changed the 10% FCS containing medium with serum free medium. 24 well plate was returned to the CO_2 incubator and continued the incubation for the appropriate length of time before transfection. Transfection was done, by replacing serum free medium with the

mixture of DNA / Tfx-20 reagent / FCS free medium 310 ml per well. The 24 well plate was incubated in the CO_2 incubator at 37 °C for 1 hour. During incubation the 10% FCS containing medium was warmed at 37°C in the waterbath and the inhibitor protein kinase C of Staurosporine in concentration 40 nM / ml direct added in DMEM medium. After 1 hours FCS free medium was replaced with the 2 ml / ml Staurosporine containing DMEM medium and returned the well plat into the CO_2 incubator until 24-48 hours.

Indirect immunostaining and confocal laser microscopy

The transfected cells was immune stained with mouse monoclonal antibody 414 anti NPC (1:500) as primary antibody and labeled with secondary antibody anti mouse, which marked texas red dye (1:100). Then the intracellular localization of EGFP-Core fusion protein determined under confocal laser microscope as described before by Haryanto (2006).

Quantification of intracellular localization

The HBV core fusion protein which found localized in the compartment of HepG2 cells, was quantified manually using confocal laser scann microscope. The Amount of HBV core protein that distributed in the cytoplasm, nucleus or both of cell compartment were quantified in the absolute and relative values.

Results dan Discussion

Intracellular localization study of HBV core protein as EGFP-Core fusion protein with three HBV core NLS (EGFP-Core 3C) and three SV-40 NLS (EGFP-Core 3 SV-40) without Staurosporine treatment shown in figure 1. It indicated that all fusion proteins were either found predominantly in the cytoplasm (upper row) or in the nucleus (middle row) or in rare cases in both cytoplasm and nucleus (lower row) but with a different frequency (see table). EGFP-Core fusion proteins are indicated by the green fluorescence, nuclear pore complexes are depicted as red rings. Studies of intracellular localization of HBV core protein indicated that different species seem to use different mechanisms to localize core proteins to the nucleus. Nuclear localization of HBV core protein, which is karyophilic, depend on the cell cycle, which enhance during G0/G phase but suppressed during the S phase (Yeh *et al*, 1993)

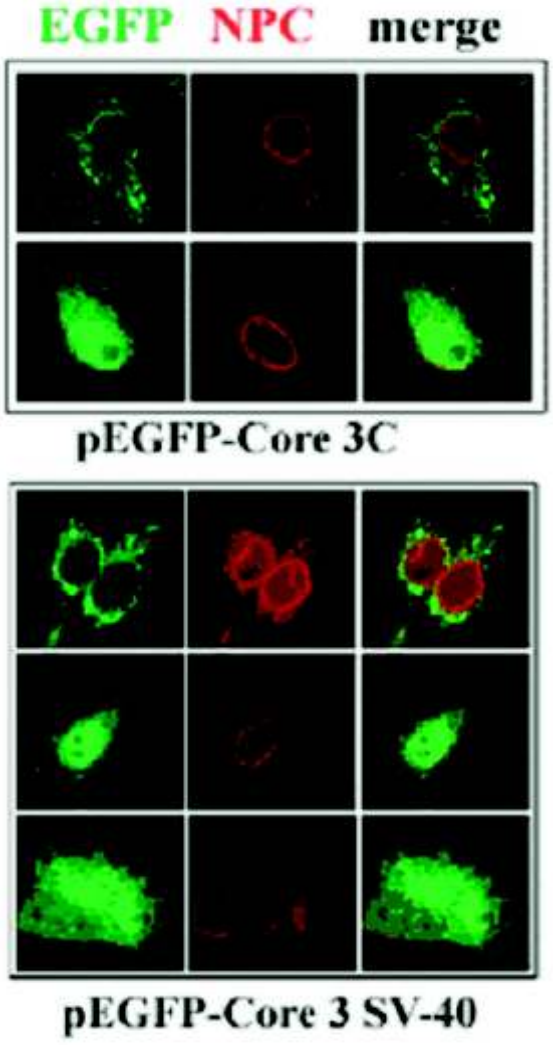

Figure 1. Intracellular Localisation of EGFP-Core 3C (up) and EGFP-Core 3 SV-40 NLS (below) in HepG2 cells without Staurosporine treatment .

Both EGFP-Core 3C and EGFP-Core 3 SV-40 fusion protein found localized predominantly into nucleus because they have strong NLS that responsible on nuclear localization. The macromolecule like EGFP-Core fusion protein up to 28 nm in diameter can enter the nucleus through the central canal of nuclear pore complex (NPC) if they harbor at least one NLS (Feldherr and Akin, 1990). Protein must contain the NLS in order for it to pass through the central transporter channel in signal-mediated protein import. The signal consists of predominantly basic amino acids appearing in either one (monopartite NLS) or two (bipartite NLS) short clusters. It has been shown that HBV nucleocapsids bind to the NPC, following the 'classical' pathway of karyophilic proteins, which is mediated by the cellular proteins importin a and b (karyopherin a and b). The exposure of a corresponding NLS on the surface of the HBV nucleocapsids, which is bound by importin a, appears to be dependent upon genome maturation and/or phosphorylation of the core subunits (Kann et al. 1999).

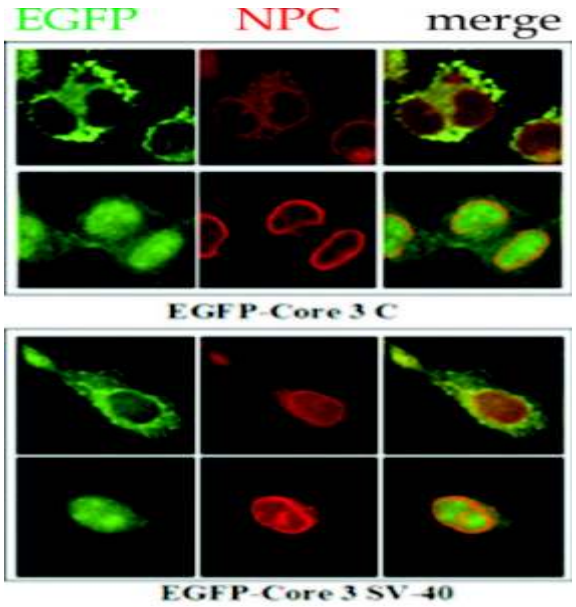

Figure 2. Localization of EGFP-Core 1C in HuH-7 cells treated with Staurosporine

Figure 2 shown that EGFP-Core 3C and 3 SV40 NLS fusion proteins localized in the cytoplasm (upper row) or in nucleus (lower row). The presence of EGFP-Core fusion proteins are indicated by the green fluorescence. The nuclear pore complexes are shown as red rim-like stains.

The mechanism of nuclear localization of EGFP-Core fusion protein is imported to the nucleus is seems to follow natural HBV infection. EGFP-Core fusion protein followed an active nuclear import mediated by cellular nuclear import factors. For interaction with the import factors, the core proteins expose a 'classical' NLS on the surface of fusion protein. The NLS is localized within the carboxy-terminal domain of the core proteins and within the lumen of the EGFP fusion protein capsid. The exposure of the NLS on the capsid surface thus requires a structural change, which is caused by phosphorylation of the carboxy-terminus as reported by Rabe *et al.* (2003). The NLS is bound by the adapter protein importin a and subsequently by the cellular transport receptor importin b. Following the established pathway of nuclear import, importin b mediates the binding to the cytoplasmic fibers of the NPC and the transport through the pore into the nuclear basket (Pante and Kann 2002).

The HBV core protein of the used isolate contains seven phosphorylation sites within the C-terminus comprising amino acids 140-183. One additional phosphorylation site is found upstream of the C-terminus in the flexible linker region at amino acid 139. The degree of phosphorylation, the responsible protein kinase, the used phosphorylation sites and the time point of phosphorylation are not fully understood yet. Phosphorylation at Serine residue 157 is required for pregenome packaging and the residues 164 and 170 are essential for polymerase activity (Gazina *et al.*, 2000; Melegari *et al.*, 2005). Moreover, serine 170

and/or 172 is phosphorylated in capsids from virions (Machida *et al.*, 1991). Some phosphorylation at a non-specified site is essential for nuclear import of the capsids (Kann *et al.*, 1999). However, for entire HBV capsids it is unclear whether the phosphate(s) within the C-terminus is/are required to allow NLS-importin a-interaction or whether phosphorylation induces a structural change that leads to the exposure of the NLS on the capsid surface. Unpublished studies using FITC-BSA-HBV capsid NLS conjugates revealed that phosphorylation within the NLS inhibits the transport capacity of the conjugate. However, for the SV-40 tag it was shown that a phosphate directly adjacent to the NLS has a negative effect on nuclear import while phosphorylation at sites further upstream enhances NLS function (Jans, 1995; Hübner *et al.*, 1997; Jans and Jans, 1994).

In order to analyse the effect of phosphorylation on nuclear transport of the EGFP-Core fusion proteins, cells were treated with a broad-range protein kinase inhibitor after transfection. EGFP-core 3C was used since this protein shows the strongest nuclear localisation thus allowing the best differentiation in case that inhibition of phosphorylation prevents nuclear import. As a control EGFP-Core 3 SV40 NLS was used lacking the C-terminus and the phosphorylation sites downstream of amino acid 143.

Transient expression revealed that the cytoplasmic and the nuclear appearance of both fusion proteins looked identical (figure. 2) to their expression in untreated cells (figure.1). In all compartments the fluorescences looked condensed in some areas being more pronounced in the cytoplasm. Despite of low transfection efficiency, quantification of nuclear and cytoplasmic localisation in different cells however showed a dramatically changed localization (see tabel) in that the vast majority of EGFP-Core fusion proteins were found within the cytoplasm. In the table clearly showed that without Staurosporine treatment EGFP-Core 3C and 3 SV40 NLS predominantly localized within the nucleus. Staurosporine treatment changed the distribution pattern in that the cells exclusively show a cytoplasmic localization of the EGFP-Core fusion proteins. It indicated that only HBV core protein phosphorylation site present in both fusion proteins is located at amino acid 141. In the SV40 Tag NLS no phosphorylation site is present. Assuming that the changes in the distribution pattern is caused by an inhibited phosphorylation of the core protein, one must conclude that this site has an essential function in the viral life cycle in that its phosphorylation is important for allowing the NLS function. However, Staurosporine may have had additional effects. It prevents cell division so that passive trapping of proteins is inhibited. The main biological activity of Staurosporine is the inhibition of protein kinases through the prevention of ATP binding to the kinase. This is achieved through the stronger affinity of staurosporine to the ATP-binding site on the kinase.

Table. Distribution of EGFP, EGFP-Core 3C and pEGFP-Core 3 SV40 NLS in Staurosporine treated HuH-7 cells.

No	Sample	Treatment	Cytoplasm	Nucleus	Both	Total
1.	EGFP-Core 3 C	No	11%	89%	0%	100%
		Staurosporine	91%	9%	0%	100%
2.	EGFP-Core 3 SV40	No	6%	94%	0%	100%
		Staurosporine	93%	7%	0%	100%

The mechanism of the inhibition of protein kinases by Staurosporine is unclear so far. Nakano *et al.*, (1987) state that Staurosporine may bind to the catalytic domain of serine/threorine- as well as tyrosine-kinases. In the presence of ATP, 100 µM even low Staurosporine concentrations (1-8 nM) were capable of inhibiting $p60^{v\text{-}src}$ phosphorylation ruling out a competition of both compounds. As a consequence

Staurosporine should also inhibit phosphorylation in vivo in the presence of physiological ATP concentrations. Herbert *et al.* (1990) showed ATP compete with 3H-Staurosporine binding to protein kinase C with an IC_{50} of ATP, 500 nM in the presence of tritiated inhibitor, 2 nM. He also described that isoquinolinesulfonamides and naphtalenesulfonamides, classes of non-selective protein kinase inhibitors, failed to antagonize 3H-Staurosporine binding to different protein kinases therefore suggesting distinct binding sites.

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References

Bartenschlager R, Junker-Niepmann M, and Schaller H. 1990. The P gene product of hepatitis B virus is required as a structural component for genomic RNA encapsidation. *J Virol.*,64, 5324-5332.

Bartenschlager, R. and Schaller, H.1992. Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome.*EMBO J.*, 11, 3413–3420.

Chua, P. K., Y. M. Wen, and C. Shih. 2003. Coexistence of two distinct secretion mutations (P5T and I97L) in hepatitis B virus core produces a wild-type pattern of secretion. *J. Virol.*, 77, 7673–7676

Chuang, W. L., M. Omata, T. Ehata, O. Yokosuka, Y. Ito, F. Imwki, S. N. Lu, W. Y. Chang, and M. Ohto. 1993. Precore mutations and core clustering mutations in chronic hepatitis B virus infection. Gastroenterology, 104, 263–271.

Cohen, B. J. and Richmond, J. E. (1982). Electron microscopy of hepatitis B core antigen synthesized in E. coli. *Nature (London)*, 296, 677–679.

Eckhardt SG, Milich DR and McLachlan A. 1991. Hepatitis B virus core antigen has two nuclear localization sequences in the arginine-rich carboxyl terminus. *J. Virol.*, *65*, 575-582.

Feldherr CM. And Akin D.1990. The permeability of the nuclear envelope in dividing and nondividing cell culture. *J. Cell Biol.*, *111*,1-8.

Gazina EV, Fielding JE, Lin B and Anderson DA. 2000. Core protein phosphorylation modulates pregenomic RNA encapsidation to different extent in human and duck hepatitis B virus. *J. Virol*., *74*, 4721-4728

Gerelsaikhan T, Tavis JE, and Bruss V. 1996. Hepatitis B virus nucleocapsid envelopment does not occur without genomic DNA synthesis. *J Virol* 70., 4269-4274.

Guidotti, L. G., B. Matzke, H. Schaller, and F. V. Chisari. 1995. High-level hepatitis B virus replication in transgenic mice. *J. Virol.*, 69, 6158–6169.

Haryanto, A. 2006. Expression and intracellular localization study of wild type HBV core protein and its mutants which block nucleocapsid envelopment in HuH-7 cells. *Indonesian Journal of Biotechnology*, 11 (1), 862-869

Haryanto, A. and Kann, M. 2006. Intracellular localization of HBV capsid in hepatocyte cell line after

transfected by the entire HBV genome. *Journal of Veterinary Science,* 24, 93-101.

Herbert, J. M.; Seban, E. and Maffrand JP. 1990. Characterization of specific binding sites for [3H]-staurosporine on various protein kinases. *Biochem. Biophys. Res. Comm*., 171, 189–195.

Hosono, S., P. C. Tai, W. Wang, M. Ambrose, D. Hwang, T. T. Yuan, B. H. Peng, C. S. Yang, C. S. Lee, and C. Shih. 1995. Core antigen mutations of human hepatitis B virus in hepatomas accumulate in MHC class II-restricted T cell epitopes. Virology. 212,151–162.

Hübner S, Xiao CY and Jans DA. 1997. The protein kinase CK2 site ($Ser^{111/112}$) enhances recognition of the Simian Virus 40 large T-antigen nuclear localizationsequence by importin. *J. Biol Chem., 272*,17191-17195.

Liao W and Ou JH. 1995. Phosphorylation and nuclear localization of the hepatitis B virus core protein: significance of serine in the three repead SPRRR motif. *J. Virol., 69,* 1025-1029.

Jans DA and Jans P. 1994. Negative charge at the casein kinase II site flanking the nuclear localization signal of the SV40 large T-antigen is mechanistically important for enhanced nuclear import. *Oncogene.* 9,2961-2968

Jans DA. 1995. The regulation of protein transport to the nucleus by phosphorylation. *Biochem J.,* 311(3), 705-716.

Kann M, Sodeik B, Vlachou A, Gerlich WH, and Helenius A. 1999. Phosphorylation-dependent binding of hepatitis B virus core particles to the nuclear pore complex. *J Cell Biol.,* 145, 45-55

Melegari M, Wolf SK, and Schneider RJ. Hepatitis B virus DNA replication is coordinated by core protein serine phosphorylation and HBx expression. *J Virol.,* 79,9810-9820

Nakano, H.; Murakawa S. and Endo A. 1987. Inhibitory effect of mutastein on the synthesis of artificial, dental plaque by Streptococcus mutans. *J. Antibiot*., 40, 706–708.

Nassal M. 1992. The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. *J Virol.,* 66, 4107-4116.

Oka, S. Kodama, M., Takeda, H. Tomizuka, N. and Suzuki, H. 1986. Staurosporine, a potent platelet aggregation inhibitor from a Streptomyces species. Agric. *Biol. Chem*., 50 (11), 2723–2727.

Omura, S, Iwai Y, Hirano A, Nakagawa A, Awaya J. Tsucha H, Takahashi Y and Masuma R. 1977. A new alkaloid AM-2282 OF Streptomyces origin. Taxonomy, fermentation, isolation and preliminary characterization. *J. Antibiot*., 30, 275–281.

Pante, N. and Kann, M. 2002. Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm. *Mol Biol Cell.,* 13, 425–34.

Rabe B, Vlachou A, Pante N, Helenius A, and Kann M. Nuclear import of hepatitis B virus capsids and release of the viral genome. *Proc Natl Acad Sci U S A.*100, 9849-9854

Roossinck, MJ and Siddigqui A. 1987. In vitro phosphorylation and protein analysis of hepatitis B virus core antigen. *J. Virol., 61*, 955-961.

Schächtele, C. Seifert R and Osswald, H. 1988. Stimulus-dependent inhibition of platelet aggregation by the protein kinase C inhibitors polymyxin B, H-7 and staurosporine. *Biochem. Biophys.*

Res. Comm., 151, 542–547.

Summers, J. and Mason, W. S. 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell.* 29, 403–415.

Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M and Tomita F. 1986. Staurosporine, a potent inhibitor of phospholipid / Ca^{++} dependent protein kinase. *Biochem Biophys Res Commun.*, 135, 397-402.

Wingfield, P. T., Stahl, S. J., Williams, R. W. and Steven, A. C. 1995. Hepatitis core antigen produced in Escherichia coli: subunit composition, conformational analysis, and in vitro capsid assembly. *Biochemistry*.34, 4919–4932.

Yeh CT, Wong SW, Fung YK and Ou JH.1993. Cell cycle regulation of nuclear localization of hepatitis B virus core protein. *Proc. Natl. Acad. Sci.* USA. 90, 6459-6463.