Modulation of the Nuclear Transcription Factor of Activated T Cells by Duck Hepatitis B Virus

By

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Running title: NFAT activation by DHBV

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24   Abstract:

25   During infection with hepadnaviruses besides the infectious agent a high number of

26   subviral particles without nucleocapsids are produced, which are able to change the

27   infection dramatically. In addition, it was observed that the activation of the nuclear

28   factor of activated T cells, regulated usually in cells of the immune system, was strongly

29   influenced after infection. When primary duck liver cells were infected with purified

30   virions of duck hepatitis B virus the activation of this factor was reduced in a similar

31   way as it was achieved by inhibition of calcineurin, a cellular phosphatase necessary to

32   control the factor, whereas the addition of subviral particles inhibited this reduction. It

33   was found that the large surface protein of the virus was responsible for the reduced

34   activity. Although this protein was embedded in similar amounts into the envelopes of

35   both particles, only virions were able to inhibit the activity of the nuclear factor. An

36   explanation of the different performances of the particles in primary duck liver cells

37   apparently depends on the individual mode of insertion of the large surface proteins into

38   the viral membrane. Furthermore, the nuclear factor of activated T cells could only be

39   detected in liver sinusoidal endothelial cells, which was shown being attracted by virions

40   but not by subviral particles.

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INTRODUCTION

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50   Transcription factors of the family of nuclear factors of activated T cells (NFAT)

51 are expressed in most cells of the immune system and play a crucial role at the

52 transcription of cytokines and other gene products important for an immune answer (13,

53 27). In this context, the activation of NFAT proteins caused by the T cell receptor-CD3

54 complex of stimulated T cells as a presumption for the induction of cytokines was

55 intensively studied (44). Soon, the close relationship of the regulation of NFAT by the

56 calcium/calmodulin-dependent serine/threonine protein phosphatase 2B (now specified

57 as phospho-protein phosphatase 3, also called calcineurin) was evident (29). De-

58 phosphorylation of NFAT was shown to be a necessary requirement for its translocation

59 from the cytoplasm into the nucleus. For this reason, the NFAT signaling pathway was

60 the target of immunosuppressive drugs like Cyclosporin A (CsA), which, after binding

61 to the cellular chaperone-like protein cyclophylin A, inhibits the phosphatase-activity of

62 calcineurin (54).

63   Naturally, the members of the family of hepadnaviruses with the human hepatitis B

64 virus (HBV) as prototype have found a way to avoid a strong immune attack of the host

65 as a prerequisite for the establishment of a persistent virus infection (50). Therefore, the

66 events during the first hours of an infection are certainly critical, which especially

67 depends on the efficiency to avoid an activation of early immune factors like

68 interleukin-2 as a key element for the stimulation of cytotoxic and helper T cells. In this

69 connection, the demonstration was essential that the X protein of HBV was able to

70 activate the responding NFAT molecule (38) as well as to regulate the intracellular

71 calcium level (2). On the basis of these observations, activation of NFAT could have

72 vital consequences for the initiation of the immune answer against viral infections. An *in*

73 *vitro* model was established where liver cells from duck embryos containing all other

74 immunological active components like liver sinusoidal endothelial and Kupffer cells

75 were present. The aim was to prove, whether the application of the virions of duck HBV

76 (DHBV) or the so-called subviral particles (SVPs), usually existing in an about 1,000

77 fold excess, differently influence the modulation of NFAT.

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98   **Cells and purification of virus.** The preparation of embryonic primary duck liver

99   cells (PDLCs) by digestion with collagenase and culturing conditions were the same as

100   already described (6, 55). In addition, the purification of DHBV as well as SVPs from

101   sera of duck carriers were performed as reported (6, 14), where the different viral

102   entities were separated in a steep 0-40 % Urografin (3,5-diacetamido-2,4,6-tri-

103   iodobenzoic acid, Schering AG, Berlin, Germany) gradient diluted in GNTE buffer (0.2

104   M glycine, 0.2 M NaCl, 0.02 M Tris, and 0.002 M EDTA, pH 7.5) followed by a flat self-

105   forming Urografin gradient with an initial concentration of 26.5 %. Alternatively, virus

106   particles were obtained from the supernatants of LMH-D2 cells, originating from a

107   chicken hepatoma cell line after the stable transfection with a DHBV-overlength

108   genome (16, 32), which were maintained in D-MEM-F12 medium (GIBCO, Eggenstein,

109   Germany) supplemented with 10 % FCS, 100 U/ml penicillin, 100 μg/ml streptomycin

110   und 2mM glutamine at 37°C and 5 % CO_2. If not stated otherwise, PDLCs were infected

111   with a multiplicity of infection (MOI) of 0.01. In some cases, cells were treated with

112   1μM of CsA or alternatively with a mixture of 40nM phorbol myristyl acetate and 4μM

113   ionomycin.

114   **Transfection of hepatoma cells and PDLCs.** The human hepatoma cell line HuH-

115   7 was grown with 10 % fetal calf serum in D-MEM (GIBCO). Usually, transfection

116   experiments were performed by the $Ca_3(PO_4)_2$-method as described (8) using the

117   mutants 1285C (synthesizing DHBV without the small surface protein [S]) and 1165A

118   (synthesizing DHBV without the large surface protein [L]); both mutants were kindly

119   provided by J. Summers (58). Further transfection experiments were carried out using

either the vector pSH107c, a kind gift of G.R. Crabtree, where the full length human NFAT cDNA (52) was subcloned into the eukaryotic expression vector pBJ5 (26) or the eukaryotic expression vector pRK5, where downstream of the CMV promoter the DNA fragments for the core (C) protein (pRK5-C) or L of DHBV3 (pRK5-preS) were introduced within the restriction sites NruI and NotI after PCR amplification. Cells were harvested 96 hours after plating for the analysis of protein expression. Transfection efficiencies were controlled after staining the cell nuclei using Hoechst 33342 (Invitrogen, Karlsruhe, Germany) by a green fluorescence protein-expressing vector. The use of this combination revealed efficiencies of about 30 % in HuH-7 cells, whereas in pRK5-C-transfected cells only an efficiency of about 10 % was obtained. Transfections in PDLCs were only satisfactorily achieved by use of Fugene (Roche, Indianapolis, USA) instead of the $Ca_3(PO_4)_2$-method.

Quantification of DHBV and SVPs. The number of viral genome molecules as determined by dot blot hybridization was considered equivalent to the number of virions since viremic duck sera seem to contain very few defective genomes (31). Viral genomic equivalents and the number of SVPs were determined as described elsewhere (6).

Detection of viral proteins. Viral proteins were examined in disrupted cells separated on 5 to 20 % polyacrylamide gradient gels (37) as described (6) or alternatively analyzed by indirect immune fluorescence using rabbit anti-C or anti-preS antisera followed by incubation with Alexa Fluor488-conjugated secondary antibody (46). Cell nuclei were counterstained using Hoechst 33342.

Measurement of NFAT activity. The analysis of NFAT activation was carried out with 5 μg of the cis reporter vector pNFAT-Luc (Stratagene, LaJolla, USA), in which a

144 response element for NFAT is located upstream of a TATA box and a luciferase reporter

145 gene (Fig. 1a, top). Control transfections performed with the pCIS-CK and pAP1-Luc

146 plasmids (Stratagene) didn't disclose any increase or decrease of luciferase activities

147 over background levels and were for reason of clarity not shown. In general, liver cells

148 were infected with DHBV for 4 h and directly thereafter transfected with pNFAT-Luc

149 for 24 h using the FuGene method (Fig. 1a, bottom). Supplementary analyses, where

150 NFAT-transfections were performed one day earlier or later, were less successful

151 (Bruns, unpublished data). In accordance with the experimental flow chart (Fig. 1b) one

152 day after infection/transfection the supernatant was replaced by fresh medium and 2

153 days later cells were harvested, washed with phosphate-buffered saline, and disrupted

154 according to the manufacturer's protocol. After binding of the de-phosphorylated NFAT

155 to its response element the luciferase gene could be determined in a Microluminat

156 luminometer (Berthold, Wildbad, Germany). Usually, the luciferase activities are shown

157 as columns representing the means of three calculations and expressed as relative light

158 units (RLU) or as percentage of 100 % unstimulated cells with vertical bars as standard

159 errors.

160 **Analysis of PDLC sub-populations.** For the detection of phagocytic activities of

161 liver-specific macrophages (Kupffer cells) PDLCs were incubated one day after plating

162 with a suspension of colloidal Indian ink in PBS for 10 min and analyzed by con-focal

163 microscopy. In order to check the presence of liver sinusoidal endothelial cells (LSECs)

164 fluorescein-conjugated acetylated low density lipoproteins (acLDL: Biodpy FL-acLDL

165 or Alexa Fluor594-acLDL; Molecular Probes, Leiden, The Netherlands) were added to

166 PDLC cultures one day after plating for 2 h, whereupon cells were examined by con-

167 focal microscopy (3, 5).

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RESULTS

170   **DHBV and SVPs generate distinct NFAT activations in liver cells.** The first

171   experiments were performed to find out, whether the addition of virions of DHBV,

172   SVPs, or both have an effect on the calcium/calmodulin-dependent pathway of NFAT.

173   For this reason, virus particles were separated first over a 0-40 % Urografin gradient,

174   which, equally like sucrose gradients, regularly leads only to a partial separation of virus

175   particles because of similar densities and the high surplus of SVPs. Therefore, the

176   isolated DHBV or SVP particles of the first gradient were divided further by use of two

177   flat gradients of 26.5 % leading to the enrichment of DHBV at the bottom of one and of

178   SVPs at the top of the other gradient (6, 14). The initial idea for designing this

179   experiment was to find out, whether the enhancement of virus replication usually

180   obtained after the application of SVPs (6) was caused by an increased release of calcium

181   ions. Because of the difficulties of the exact measurement of calcium ions, we planned

182   to determine it indirectly by calculation of NFAT, which will only be activated by

183   calcineurin in the presence of calcium ions. According to protocol, PDLCs were mock-

184   infected, infected with DHBV with or without SVPs, or with SVPs alone and co-

185   transfected with pNFAT-Luc (Fig. 1). The measurements represented a certain basal

186   level of constitutively active NFAT, which was regularly detected in uninfected liver

187   cells (Fig. 2a and b, CO). In contrast, after infection with virions at MOI 0.01 a drastic

188   reduction of the NFAT activity could be observed (Fig. 2a and b, DHBV). When in

189   addition to the low amount of virions a high surplus of SVPs was added, the lower

190   NFAT activity was moderately restored using the preparation of partially purified

191   particles (Fig. 2a, DHBV+SVPs), but nearly fully re-established using the preparation of

highly purified virus particles (Fig. 2b, DHBV+SVPs). On the other side, the application of the empty particles alone didn't reveal a strong difference in comparison to the luciferase activity of uninfected cells (Fig. 2a and b, SVPs).

NFAT inactivation by DHBV resembles the decrease caused by the inhibition of calcineurin with CsA. A further control was performed to certify the specific nature of NFAT modulation caused by the virus particles. Because of the known inhibiting activity of CsA for calcineurin, which is necessary for the nuclear translocation of dephosphrylated NFAT, we proved whether the addition of CsA was able to inhibit the activation of NFAT (Fig. 2c). The influence of CsA on NFAT was investigated in uninfected cells (CO), in cells infected with virions (DHBV), or empty particles (SVPs). The results demonstrated that an addition of CsA reduced the NFAT activity in uninfected PDLCs (CO) in a comparable manner (CO- versus CO+ = 58 %), as was achieved by virions without further treatment (CO- versus DHBV- = 64 %). The NFAT activation in PDLCs was even more reduced, when virions were supplied together with CsA (DHBV- versus DHBV+ = 43 %), whereas the inhibition of CsA on NFAT activation was weak after the application of SVPs (SVPs- versus SVPs+ = 20 %).

NFAT inactivation is largely independent of the infectious dose. A strong inhibition of NFAT activation was already measured in PDLCs infected with low doses of virions. This outcome was unexpected and could only be explained, when NFAT modulation was induced not only by the viral input alone but also by the release of some additional cellular or viral factors after infection. The relatively short incubation time of four days before measuring the activation of NFAT also speaks in this favor, because at that time point there is only a few progeny of virions, which could only be detected by PCR analysis (Chang and Bruns, unpublished data). On the other side, an activation of

216 input virus could not be ruled out. For clarification, whether viral input or output was

217 able to influence the NFAT activation, an experiment was performed, where in PDLCs

218 the infection was continuously increased (Fig. 3). Again, a strong reduction of the

219 luciferase activity was observed, when every one hundredth cell obtained an infectious

220 particle (MOI 0.01). In general, the measurements remained low, even when 10 fold and

221 100 fold higher viral inputs per cell were applied (MOI 0.1 and 1), whereas a further

222 increase (MOI 10) resulted in reduced inhibitions of NFAT activation. An explanation

223 for the latter observation could be that after the addition of 10 purified virions per cell a

224 contamination with SVPs could not be avoided anymore.

225 **NFAT is inactivated by the large surface protein of DHBV.** Originally NFAT

226 was identified in T cells as a key factor for the expression of cytokines, but there were

227 expanding reports of NFAT activation in a wide range of other cells (28). We wanted to

228 know whether hepatocytes are also competent to activate this factor. I was found that the

229 application of pNFAT-Luc to different hepatoma cell lines disclosed, although less

230 pronounced as in PDLCs, again a basic level of NFAT activation (Bruns, unpublished

231 data). In the next experiment, HuH-7 cells were transfected with vectors responsible for

232 the production of two DHBV mutants (58). The first one (1165A) with a stop codon

233 inside the preS part of L (alteration of $TG^{112}G$ into $TA^{112}G$) lead to the production of

234 DHBV containing S only, whereas the second one (1285C) with a point mutation at the

235 S start codon (alteration of $AT^{162}G$ into $AC^{162}G$) resulted in the production of DHBV

236 containing L only (Fig. 4a). Cultures of HuH-7 cells were transfected with increasing

237 amounts (0 to 6 µg) of 1285C and decreasing amounts (6 to 0 µg) of 1165A in parallel

238 together with 3 µg pNFAT-Luc. Thus, each cell culture was treated in total with 6 µg of

239 the viral plasmids in various combinations and 3 µg of pNFAT-Luc for the measurement

240 of NFAT activation. The results revealed a gradual decrease of NFAT activition with the

241 continuous increase of 1285C, but not with 1165A (Fig. 4b). This tendency could be

242 confirmed after the application of 1285C or 1165A alone, where 1285C exhibited a

243 maximal inhibition of the NFAT activity, whereas 1165A exhibited luciferase activities

244 similar to control levels. Western blots performed in parallel demonstrated in cells the

245 continuous increase or decrease of the proteins in correlation to the amounts of viral

246 plasmid DNAs applied (Fig. 4c, left). Transfection with 1165A alone resulted in a

247 secretion of particles (most probably SVPs), whereas this was not the case, when 1285C

248 was used (Fig. 4c, right). In summary, the transfection experiments using DHBV

249 without S or DHBV without L, gave a first clue that L could be responsible for the

250 inhibition of NFAT activition.

251 As both vectors, 1285C and 1165A, were able to synthesize C, but only NFAT-

252 inactivating virions contain a nucleocapsid and not SVPs, the next experiments were

253 performed with vectors responsible for the expression of either C or L alone. The study

254 was carried out again in HuH-7 cells, but now with the protein expression vector pRK5

255 encoding either L or C of DHBV together with pNFAT-Luc. Because of the relative low

256 measurements of luciferase activities acquired with these vectors all three parallel

257 experiments are displayed. Identical amounts of cells were used for transfections, which

258 were verified at the end of the experiment by amido black staining of the PVDF

259 membrane (Fig. 5a). The cells were transfected alternatively with pRK5-C or pRK5-

260 preS for the expression of C (lanes 3) or L (lanes 4), respectively. As controls cells

261 transfected with pRK5 (lanes 2), untreated cells (lanes 5), cells treated with CsA (lanes

262 6) or transfected with the GFP-containing vector (lanes 1) were introduced; the latter

263 was used to calculate the transfection efficiencies, which were about 30 % in this

264　　experiment. The Western blots with anti-C (Fig. 5b) or anti-S antisera (Fig. 5c) revealed

265　　similar viral protein expression in the experiments I and II, whereas experiment III

266　　expressed slightly more L and less C. In all three experiments, it was apparent that the

267　　application of pRK5-preS, responsible for the expression of L, lead to an inhibition of

268　　NFAT activation, which were similar to measurements observed in CsA-treated cells

269　　(Fig. 5d). Instead cells, which were transfected with pRK5 or the C-expressing vector

270　　resulted in some stimulation of NFAT activation, similarly as was demonstrated for the

271　　GFP-containing plasmid. A possible explanation for the latter effects could be the

272　　presence of some internal sequences similar to the NFAT response element.

273　　**NFAT initiates migrations of core and large surface proteins towards the**

274　　**nucleus.** Transfection experiments were carried out in HuH-7 cells with pRK-C and

275　　pRK-preS to analyze the intracellular locations of the translated products by indirect

276　　immune fluorescence. Naturally, both DHBV proteins appear broadly distributed as dot-

277　　like structures in the cytoplasm (Fig. 6a and b); the decision whether the small amounts

278　　of C, detectable in association with the stained nuclei, was localized within or above the

279　　nucleus could not be made unmistakably (Fig. 6a). On the other side, though only in

280　　about 1 % of positive cells, these small nuclear dots were strongly enlarged and nearly

281　　exclusively enriched within the nucleus, when the cells were co-transfected with

282　　pSH107c for the parallel production of NFAT proteins (Fig. 6c). In some cases C was

283　　concentrated near the nuclear pores (Fig. 6c, arrows). Another form of convergence of

284　　the antigen via NFAT was observed with an anti-preS antibody in cells co-transfected

285　　with pRK-preS and pSH107c; in this case, the preS proteins were strongly focused

286　　within small areas around the nucleus in about 10 % of stained cells, but never inside the

287　　nucleus (Fig. 6d); in other cells various stages of enrichment around the nucleus could

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288 also be detected (not shown). These actions were reversible, when at the same time CsA

289 was added (Fig. 6e and f). At present, we interpret these findings to mean that NFAT

290 molecules were able to transport bound viral antigens towards the nucleus; but only C,

291 probably because of its minor size and/or the presence of a nuclear localization signal

292 (42, 43), was able to pass the nuclear membrane.

293   **Detection of NFAT in LSECs of a duck liver.** As our protocol allowed the

294 preparation of total PDLCs, we wanted to know which other liver cells could be detected

295 associated with hepatocytes. We found that, similarly as described for the human liver

296 (17), parenchymal (hepatocytes) as well as non-parenchymal cells were obtained in a

297 ratio of about 60 to 40 % (Fig. 7b), where the latter could be furthermore subdivided

298 into about 70 % Kupffer cells, 30 % LSECs, and 3 % fat storing (Ito) cells (17). By use

299 of colloidal Indian ink and acLDL the biological activities against foreign material,

300 phagocytosis of ink particles by Kupffer cells (Fig. 7a) and uptake of Fluorescein-

301 stained acLDL by LSECs (Fig. 7c), could be demonstrated. Further co-immune

302 fluorescence studies with anti NFATc and acLDL uptake revealed an association of

303 NFAT proteins with LSECs *in vitro* and *in vivo* (3, 5, Maenz and Bruns, unpublished

304 data).

305   **Co-localization of NFAT and viral proteins was discovered in LSECs.** Our first

306 hypothesis was to identify NFAT molecules in hepatocytes, which represent the final

307 targets for hepadnaviruses. The additional observation of NFAT activation in hepatoma

308 cell lines increased our suspicion that NFAT molecules could be detected in the same

309 cells, which were also infected by DHBV. Unexpectedly this was not the case.

310 Moreover, NFAT was found in LSECs and the question came up again, similarly as

311 already proposed (3), which role these cells play during infection.

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312 Earlier experiments have shown that LMH-D2 cell cultures lead to production and

313 release of viral progeny consisting of virions, nucleocapsids (NCs), and SVPs in a ratio

314 of about 1 : 1 : 10-100, i.e. the high excess of SVPs usually found after infections *in vivo*

315 (ducks) or *in vitro* (PDLCs) was reduced about 10 to 100 fold in such cultures; this

316 tendency strongly facilitated the efficiency of virus purification (Bruns, unpublished

317 data). Virus particles were obtained from the supernatants of LMH-D2 cells and

318 thereafter separated, contrary to the usual protocol, first by a flat self-forming Urografin

319 gradient with the initial concentration of 26.5 % and thereafter by the conventional 0-40

320 % Urografin gradient. After the first ultracentrifugation step DHBV particles (virions

321 and NCs) were collected from the bottom and SVPs from the top. The efficiency of

322 separation was proved by the analysis of the surface proteins of virions and SVPs in a

323 Western blot (Fig. 8a). The high density of NCs enabled the successive separation from

324 virions using a 0 to 40 % gradient; both subunits could easily be discriminated by DNA

325 dot blots of the fractions collected after ultracentrifugation being positive for NCs and

326 virions (Fig. 8b, left), but negative for SVPs (Fig. 8b, right). Thereafter, purified virions,

327 NCs, and SVPs were added to PDLC cultures. In order to visualize also the uptake of

328 virus particles into non-parenchymal cells the virus dose was increased to 500 particles

329 per cell. After an adsorption time of 2 h acLDL was added and the cells were incubated

330 for further 2 h before fixation and treatment of cells with anti-viral antibodies (anti-

331 DHBc for the detection of virions and NCs, anti-DHBpreS for the detection of virions

332 and SVPs). Only in cells incubated together with acLDL and virions a co-staining could

333 be identified using anti-DHBc and (possibly due to the presence of contaminating SVPs)

334 to a lesser extent using anti-DHBpreS antisera (Fig. 8e, middle); no co-staining could be

335 visualized in cells, where NCs (Fig. 8e, left) or SVPs (Fig. 8e, right) were added. These

336   findings let us suggest that virions are associated, at least in the beginning of an

337   infection, with LSECs, whereas no SVPs or NCs could be detected.

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361   DISCUSSION

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363   One key event of the regulated transcription response to immune stimuli is the

364 calcium/calmodulin-dependent signal cascade leading to the activation of NFAT (54).

365 The NFAT signaling pathway is also influenced by a number of viruses including

366 human immunodeficiency virus (33, 45), African swine fever virus (18, 48, 49), Rhesus

367 monkey rhadinovirus (10), hepatitis C virus (1), and human herpesvirus 8 (53), which

368 either induce a cellular state permissive for viral infection and replication or suppress

369 immune detection and clearance of the virus. For herpes simplex virus it was shown that

370 the nuclear translocation and activation of NFAT was blocked (56). In this case, it could

371 be demonstrated that virus binding was not sufficient to inhibit the nuclear translocation

372 of NFAT, but that entry of the virion and early gene transcription were required.

373 Moreover, it was detected that a consequence of blocking the NFAT import was the

374 reduction in NFAT-dependent transcriptional activation from the interleukin-2 promoter.

375 All in all, these examples include both stimulatory and inhibitory interventions acting at

376 various levels of the cascade.

377   Besides the immune modulating role of T cell activation, the range of NFAT target

378 genes is expanding to differentiation of non-immune cells (9, 28). Hence, it could be

379 explained for the HBV-transfected hepatocytes that the viral X protein was able to

380 activate NFAT by a CsA-sensitive pathway (38) and regulate intracellular calcium level

381 (2). In addition, it was shown that NFAT1-C repressed the transcriptional activity of the

382 enhancer II and the pregenomic promoter (EnII/Cp) of HBV. As the NFAT1-C

383 responsive site GGAGA was located at the position of the nucleotides 1603 to 1618, the

384   HBx-driven transcriptional activation of the EnII/Cp was cancelled in a dose dependent

385   manner (40). So far, all the experiments with hepadnaviruses were performed by

386   transfection experiments of hepatoma cell lines. Here, we show for the first time that

387   activation of NFAT is diminished by the application of virions during an acute infection

388   of PDLCs, similarly, as can be seen after the addition of the specific calcineurin

389   inhibitor CsA. The specificity was be proved by different experiments using purified

390   DHBV with or without purified SVPs for infection, where a high surplus of SVPs over

391   virions neutralized the inactivation of NFAT. On the first sight this observation seemed

392   to be contradictory to findings exhibiting an enhancement, when PDLCs were infected

393   with low MOI and contained at the same time high amounts of SVPs (6). However,

394   these studies performed *in vitro* do certainly not reflect the local situation present in the

395   liver of a host with an effective immune system at the early phase of an infection.

396   However, the phenomenon of enhancement could play a role later-on starting with the

397   second round of infection.

398   Whether activation or inhibition of NFAT is the result of a regulation at the level of

399   the intracellular calcium balance in infected cells, similarly as described for the spike

400   protein of mouse hepatitis virus (35), the envelope protein gp120 of human

401   immunodeficiency virus (11), or the X protein of HBV (2, 38), still remains an open

402   question. Our investigation demonstrated that the activation of NFAT could again be

403   recovered from its suppressed state, when SVPs were added imitating an event, where

404   the calcium influx into the cytoplasm was elevated by treatment of cells with ionophores

405   (Bruns, unpublished data).

406   So far it is not clear, whether the activation of other transcription factors like NFκB,

407   similarly as was shown e.g. for human immunodeficiency virus and herpesvirus 8 (53),

408   plays a role during the infection with DHBV, but the strong reduction of NFAT

409   activation at a low infection dose has undoubtedly an implication during the acute

410   infection *in vivo* in as far as to avoid a recruitment of immune cells like T lymphocytes

411   or macrophages because of the lack of cytokine stimulation. This would imply that at the

412   beginning of an infection and at a moment, when only a few SVPs are present, more

413   infectious agents would escape the destruction by immunological active cells and could

414   explain, why it is possible to infect ducklings with DHBV using extreme low virus doses

415   (31). Later, with the presence of higher amount of virus particles, especially SVPs, the

416   immune system will then be mobilized as usual, but now with the possible consequence

417   of enhancement or inhibition depending on virus burden (6, 34). Interestingly, it seems

418   that the hepadnaviral L, responsible for the first interaction with the host-cell receptor

419   and for many other biological activities during the viral life cycle (4, 6, 7, 12, 15, 20, 22-

420   25, 30, 36, 39, 41, 51, 57, 59, 60) is also the most important candidate of NFAT

421   inactivation and for some time our main concern was how L-containing virions were

422   able to reduce NFAT activity in one case, whereas L-containing SVPs did not. Our

423   observations of the diverse fashion of activation could be possibly explained with the

424   different orientation of the molecules within the envelope of both particles, as our data

425   indicated that this protein is usually located on the outside of virions, whereas in SVPs

426   most of the proteins are hidden in the interior obviously as a consequence of

427   phosphorylation, although it was demonstrated that phosphorylation of L does not

428   influence the infectivity (19, 21). Alternatively, the variable presentation of L in virions

429   and SVPs could also result in different pathways for both particles within parenchymal

430   and/or non-parenchymal cells of the liver (3). At least, some possible interactions

431   between NFAT and viral products could be demonstrated by co-transfection

432   experiments, where a strong intracellular migration was initiated for C and L of DHBV

433   in the presence of NFAT, which could again be blocked by CsA.

434   One detail needs further consideration, as it became obvious that reduction of

435   NFAT inactivation was similarly strong, when infections were performed with low as

436   well as higher virus doses. Certainly, by the nature of whole liver cell preparation with

437   its difficulties for total segregation into single cells the prediction of virus dose per cell

438   can only be a rough estimation, which also ignores the detail that nearly half of the cell

439   number is not of the hepatocellular type. Moreover, the low transfection efficiency of

440   about 10 % of total liver cells will also minimize the probability that one cell in a culture

441   got both virus by infection and pNFAT via transfection. Under these circumstances, a

442   direct influence of virions on NFAT activity is rather unlikely. Therefore, we believe

443   that other factors released from infected cells could be involved. Infection studies

444   revealed that besides SVPs also the post-translationally modified core protein, the e-

445   antigen of DHBV, is secreted in high amounts within the first days of infection (Bruns,

446   unpublished data). Here, we could demonstrate that an inhibition of NFAT activation by

447   purified SVPs can be excluded. For this reason, the objective of our next studies will

448   concentrate on purified e-antigen, which is now available (47).

449   Taken together, these data indicate that inactivation of NFAT was caused by the

450   virions but not by the SVPs of DHBV, which could have substantial consequences

451   during the early state of an infection of a host, where only a few number of virions is

452   available and the number of SVPs not high enough to influence the infection (6, 34).

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481   FIGURE LEGENDS

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483   Fig. 1. Protocol for the measurement of NFAT activation in PDLCs. (a) Scheme,

484   modified from the manufacturer's instruction manual, of the plasmid pNFAT-Luc

485   containing the response element for NFAT, the TATA box, and the Luciferase gene

486   (top). Binding of the de-phosphorylated trans-activator TA (NFAT) to the NFAT

487   response element after infection of PDLCs with DHBV and co-transfection with

488   pNFAT-Luc (bottom). (b) Experimental flow chart displaying the time points of various

489   treatments of PDLCs between plating and harvest.

490   Fig. 2. Modification of the NFAT activity in PDLCs by virions or SVPs of DHBV.

491   (a) PDLCs were either mock-infected (CO), infected with partly purified virions at MOI

492   0.01 (DHBV) or infected together with partly purified 10 SVPs/cell (DHBV+SVPs), or

493   treated with 10 SVPs/cell alone (SVPs). (b) Repetition of the experiment described

494   above, but this time highly purified virions and SVPs were used. (c) PDLCs, either

495   uninfected (CO), infected with highly purified virions at MOI 0.01 (DHBV), or treated

496   with highly purified 10 SVPs/cell (SVPs) were alternatively mock-treated (-) or treated

497   with 1 μM of the calcineurin-inhibitor CsA (+).

498   Fig. 3. Independence of NFAT activation by the titer of infectious virus. PDLCs

499   were transfected and either mock-infected (MOI 0) or infected with different amounts of

500   purified virions (MOI 0.01 to MOI 10).

501   Fig. 4. Analysis of NFAT activation after transfections with DHBV-S- and DHBV-L-

502   mutants. (a) Scheme according to Summers and co-workers (58) demonstrating the point

503   mutations within the viral DNA to obtain either the DHBV mutant 1165A by insertion

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504   of a stop codon at the carboxy terminus of the preS part of L (left) or to obtain the

505   DHBV mutant 1285C by destruction of the start codon for S (right). (b) HuH-7 cells

506   were transfected with pNFAT-Luc and the plasmids 1285C and 1165A in the

507   concentrations shown. Quantification of NFAT activation showed a continuous

508   reduction of luciferase activities with increasing amounts of 1285C and at the same time

509   decreasing amounts of 1165A. (c) Examinations by Western blot with an anti-S

510   antiserum, performed in parallel, revealed a gradual increase of S equivalent to the

511   added amounts of 1165A, whereas only a minor difference of L was noticed after an

512   increased application of 1285C (left). A lower release of virus particles into the

513   supernatants was observed with increased accumulation of 1285C (right). The first line

514   represents DHBV-positive duck serum (DS) as control; the positions of the viral

515   envelope proteins P18 (S) as well as P36 (L) and its cleavage product P28 (L) are shown

516   on the right.

517   Fig. 5. Effect of the proteins C and L of DHBV on NFAT activation. HuH-7 cells

518   were transfected three times in parallel (Experiments I to III) using an expression vector

519   without any insert (pRK5) or a vector containing alternatively the viral C (pRK5-C) or L

520   (pRK5-preS) as inserts. A green fluorescent protein (GFP)-containing plasmid was used

521   for the calculation of the transfection efficiencies. Mock-treated cells (CO) and cells

522   treated with CsA provided further controls. Cells were harvested after 4 days and

523   divided into two portions: one half was used for measuring the luciferase activities (d),

524   the other half was analyzed by Western blot using first antibodies against the core (b)

525   and then against the surface proteins of DHBV (c); finally, the blot was stained with

526   Amido Black to compare the total amount of cellular proteins (a). The marker proteins

527   for the estimation of the molecular weights are shown on the left side, whereas a

DHBV-positive duck serum on the right side was applied to demonstrate the positions of the viral surface proteins. Note that only CsA-treatment or transfection with the L-expression vector lead to the inhibition of NFAT activation (d).

Fig. 6. Indirect immune fluorescence of HuH-7 cells for the demonstration of the localizations of DHBV proteins after NFAT production. Cells were transfected with pRK5-C or pRK5-preS for the expressions of viral C (a, c, e) and L proteins (b, d, f) alone (a, b) or in combination with NFAT proteins expressed by the vector pSH107c (c-f). As controls, CsA was added to the co-transfected cell cultures (e, f). Arrowheads show possible concentrations of C around nuclear pores.

Fig. 7. Analysis of parenchymal and non-parenchymal subsets of duck liver cells. A total PDLC preparation was examined three days after cultivation by confocal microscopy demonstrating the partition into hepatocytes (rich in vacuoles) and non-parenchymal cells (b). Addition of colloidal ink was used to visualize the phagocytic activities of Kupffer cells distributed over the whole culture (a), whereas the specific uptake of Fluorescein-labeled acLDL served for the recognition of LSECs mainly found within the area of non-parenchymal cells (c).

Fig. 8. Investigation of the association of LSECs with NCs, virions, or SVPs of DHBV. (a) Virus particles were separated first in a self-forming Urografin gradient of 26.5 % and collected either from the bottom-fractions, where virions and NCs were concentrated, or the top-fractions containing the SVPs; the amount of particles were calculated and checked in a Western blot before further separation (note that here an anti-DHBpreS antiserum was used, which could not recognize NCs). (b) A steep Urografin gradient was then used to separate the collected DHBV sample into virions and NCs, which were both detected by measuring the DNA content per fraction in a dot

552 blot (left), or to prove the purity of the SVPs containing no DNA (right). (c) The isolated

553 particles (NCs, virions, SVPs) were then applied to PDLC cultures and checked after an

554 adsorption time of 4 h with an anti-DHBc antiserum (red) for the discovery of NCs and

555 virions (upper row) or with an anti-DHBpres antiserum (red) for the recognition of

556 virions and SVPs (lower row); note that co-immune fluorescence with acLDL (green),

557 which was added 2 h before harvest, was only detectable with purified virions (middle

558 pictures).

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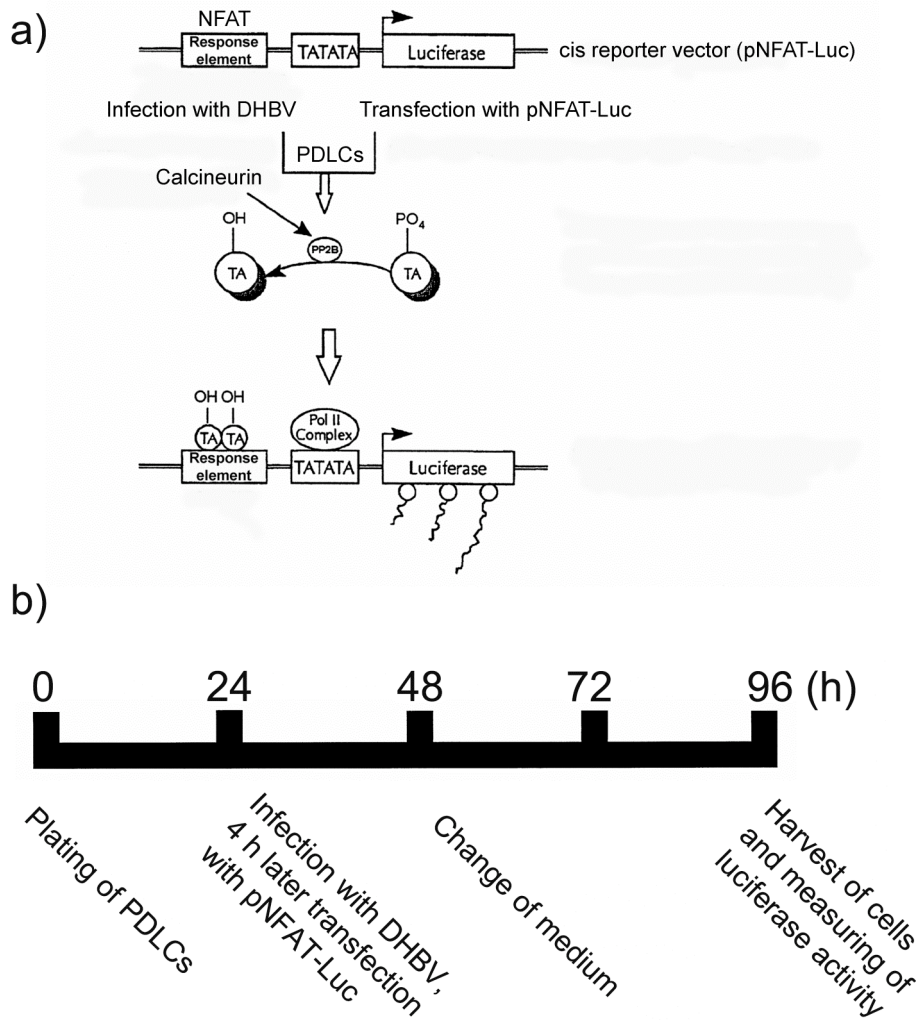
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Fig. 1

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a) Partially purified virus particles of the 1st (0-40%) Urografin gradient

b) Highly purified virus particles of the 2nd (26.5%) Urografin gradient

c) Highly purified virus particles of the 2nd (26.5%) Urografin gradient
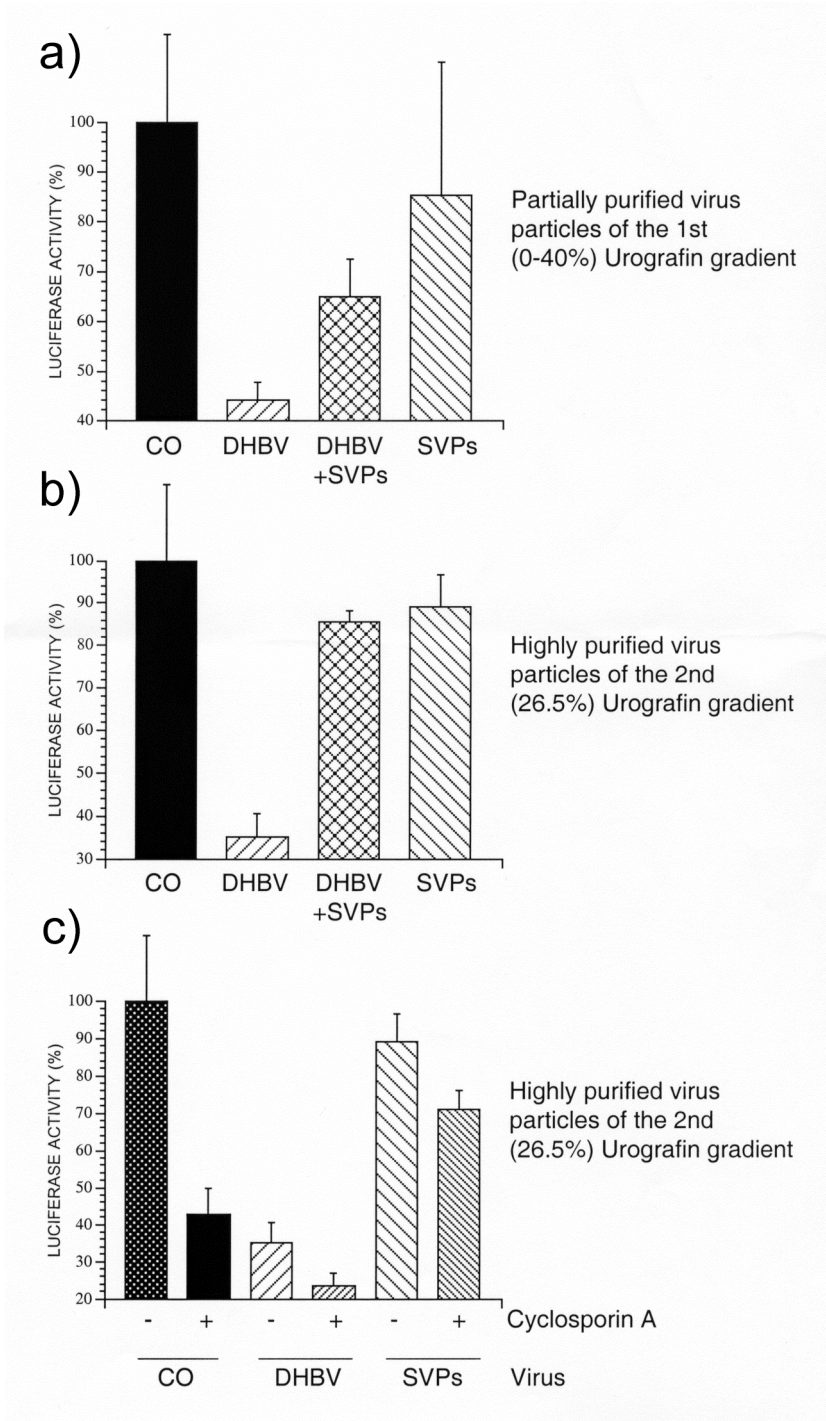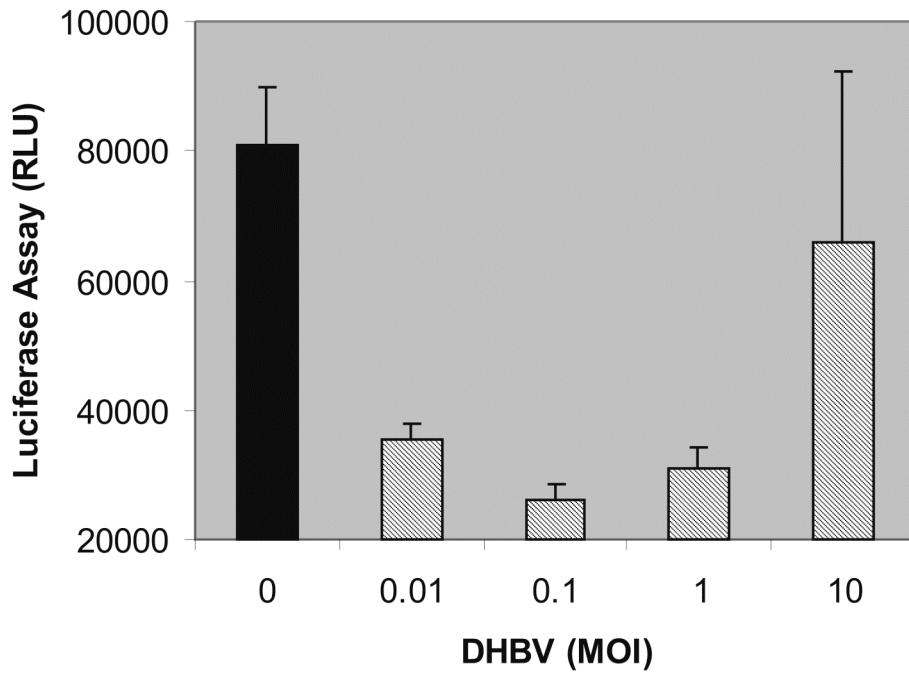
Fig. 2

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Fig. 3

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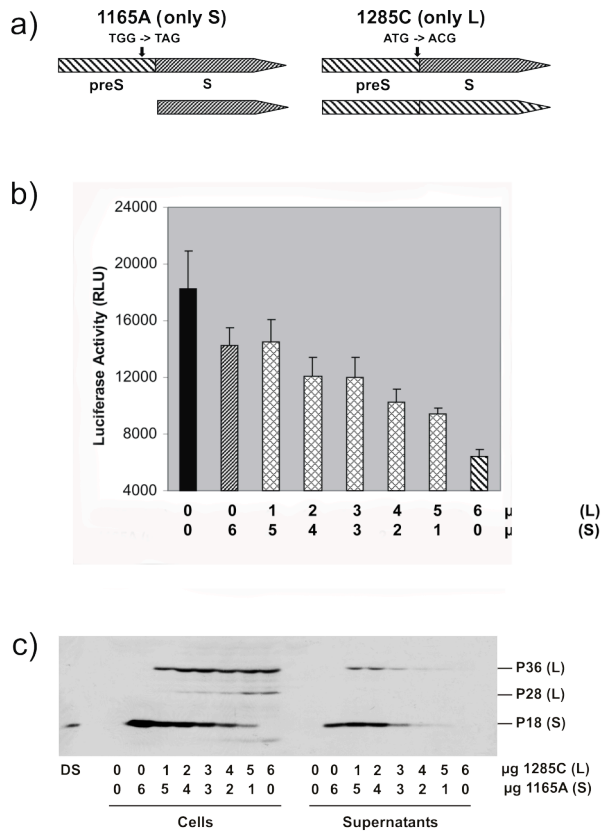Fig. 4

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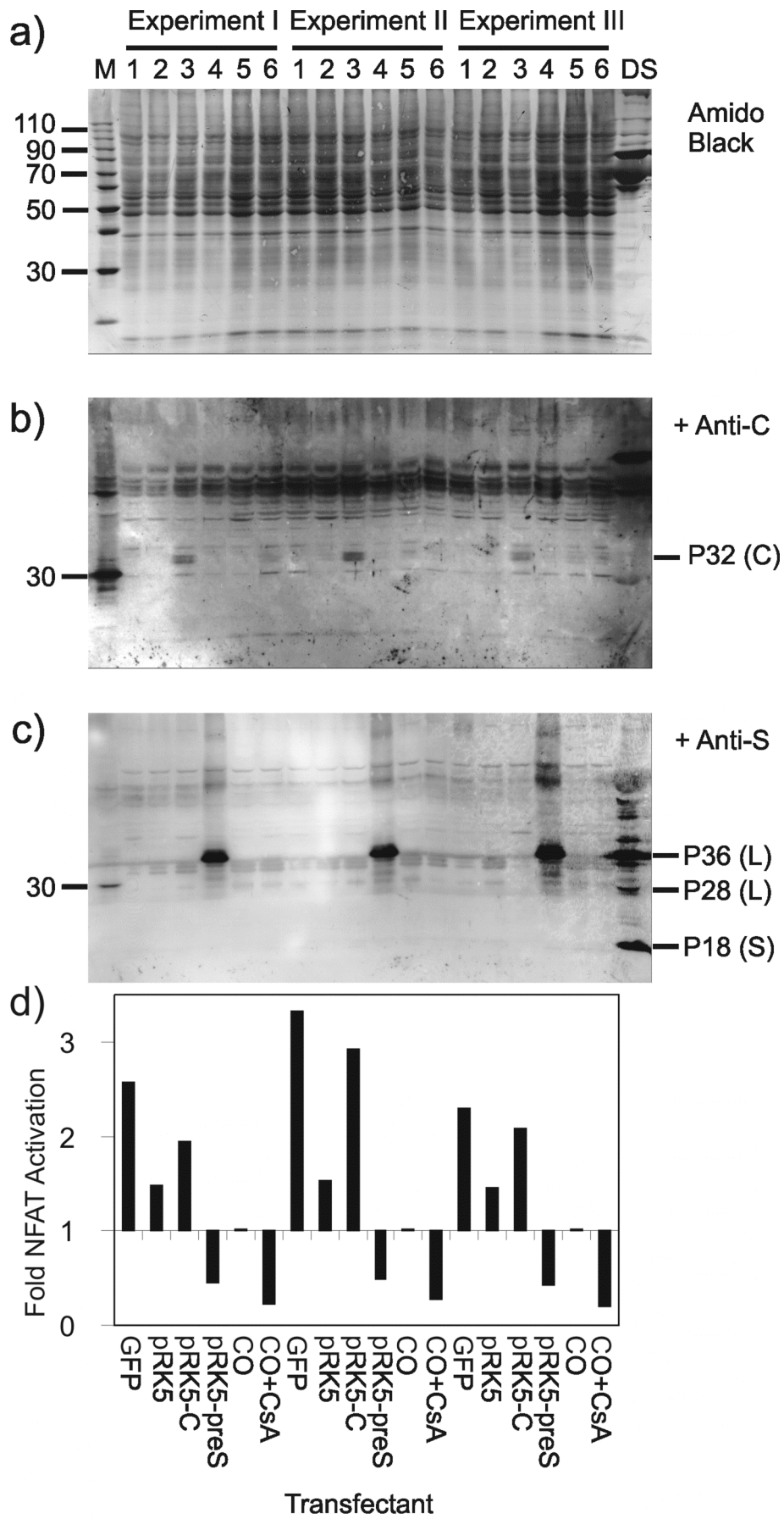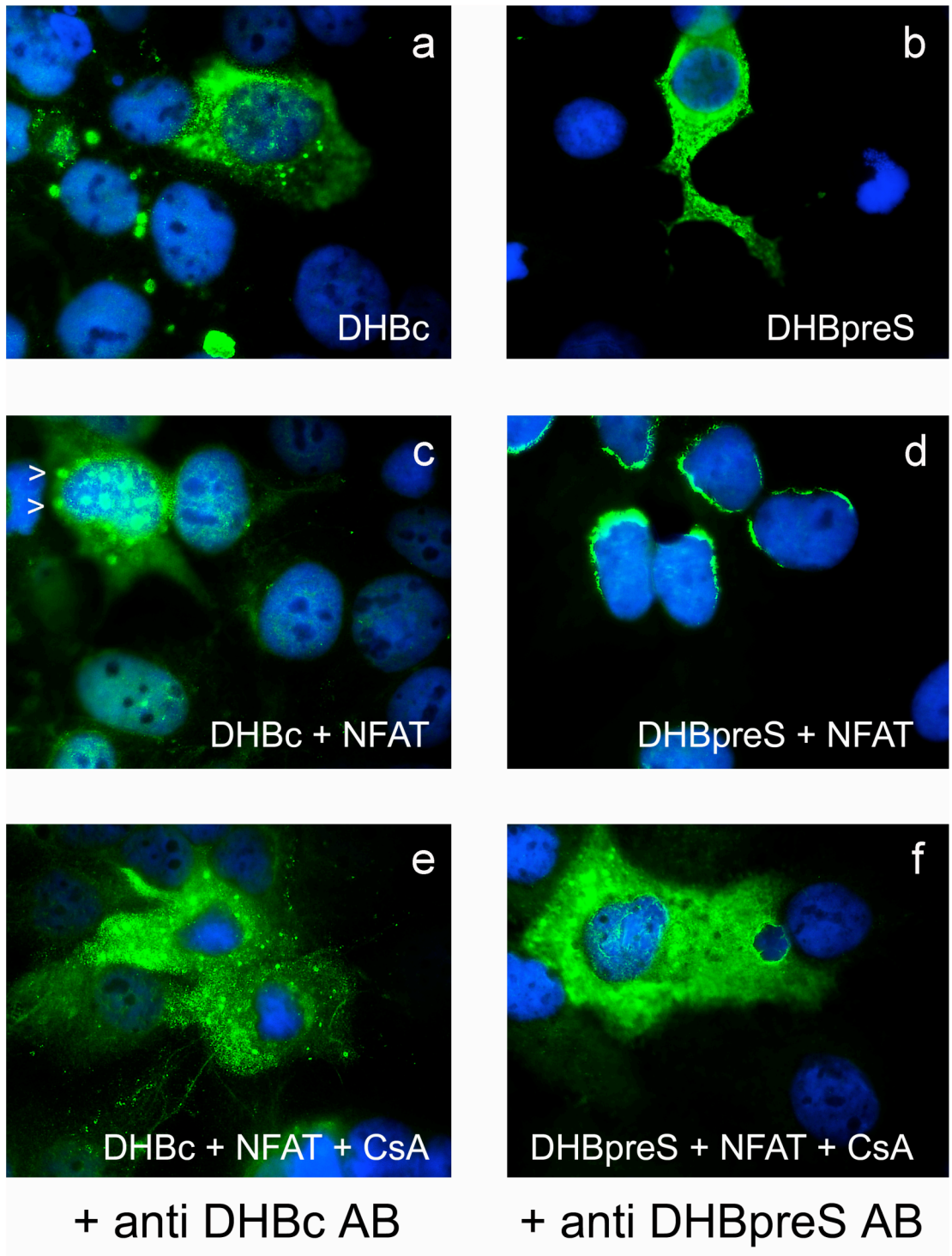775
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a)

Experiment I Experiment II Experiment III

M 1 2 3 4 5 6   1 2 3 4 5 6   1 2 3 4 5 6   DS

Amido Black

110 —
90 —
70 —
50 —

30 —

b)

+ Anti-C

P32 (C)

30 —

c)

+ Anti-S

P36 (L)
P28 (L)

30 —

P18 (S)

d)

Fold NFAT Activation

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0

Transfectant

GFP
pRK5
pRK5-C
pRK5-preS
CO
CO+CsA
GFP
pRK5
pRK5-C
pRK5-preS
CO
CO+CsA
GFP
pRK5
pRK5-C
pRK5-preS
CO
CO+CsA

Fig. 5

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DHBc

DHBpreS

DHBc + NFAT

DHBpreS + NFAT

DHBc + NFAT + CsA

DHBpreS + NFAT + CsA
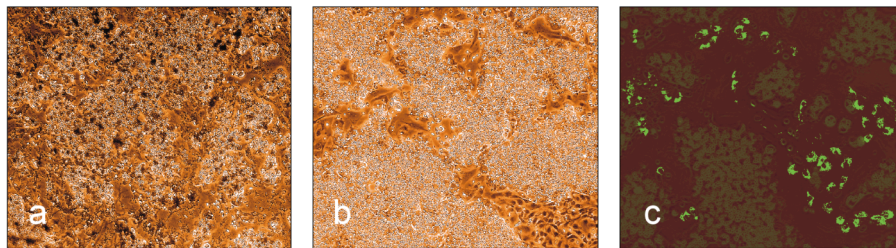
+ anti DHBc AB

+ anti DHBpreS AB

Fig. 6

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10 ×

Fig. 7

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a)

preS
P36
P28

DHBV   SVPs   DHBV   SVPs

u.d.   1/10

b)

DHBV

NCs   virions

SVPs

c)

acLDL

+ NCs   + virions   + SVPs

+ anti-DHBc

+anti-DHBpreS

Fig. 8

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