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Factors involved in the cell density-dependent regulation of nuclear/cytoplasmic distribution of the 11.5-kDa Zn^{2+}-binding protein (parathymosin-α) in rat hepatocytes

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We dedicate this publication to the memory of Dr Ingeborg A. Brand

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SUMMARY

Although the 11.5 kDa Zn^{2+}-binding protein (ZnBP, parathymosin-α) possesses a functional bipartite nuclear localization signal it was found in most tissues in the cytoplasm. The cultivation of freshly isolated rat hepatocytes for 24 hours under standard conditions was associated with an almost complete translocation of ZnBP from the cytoplasm to the nuclei. Here we demonstrate, that this translocation is negatively correlated with cell density. The translocation of ZnBP to the nucleus can be inhibited or abolished by inhibitors of protein synthesis (cycloheximide) or transcription (actinomycin D). Moreover, cycloheximide can induce a relocation of ZnBP to the cytoplasm when applied after the appearance of ZnBP in the nuclei. DMSO, an inhibitor of dedifferentiation of cultured hepatocytes, abolishes also the translocation of ZnBP into the nucleus. Thinly seeded cells keep their ZnBP in the cytoplasm if they are co-cultured with plasma membranes from Morris MH7777 hepatoma cells or antibodies against E-cadherin indicating the involvement of cell adhesion proteins. We have enriched a protein from the cytosol of fresh hepatocytes which inhibits the translocation of ZnBP, but not that of albumin-NLS into the nucleus in a permeabilized cell system. Such an activity could not be found in the cytoplasm of permanent cell lines which harbor ZnBP only in the nucleus. A model for the regulation of the nuclear import of ZnBP is proposed.

Key words: 11.5 kDa-Zn^{2+}-binding protein, Nuclear transport, Cell density, Cycloheximide, Cell adhesion protein

INTRODUCTION

The 11.5 kDa Zn^{2+}-binding protein (ZnBP, parathymosin-α) is a reversible inhibitor of phosphofructokinase-1 (Brand and Söling, 1986; Trompeter et al., 1989). The acidic protein contains 55% acidic amino acids which are mostly concentrated in a central acidic cluster conferring the cation binding and the phosphofructokinase-1 inhibitory activities (Brand et al., 1988; Trompeter et al, 1989) and is able to bind to a large variety of cytosolic proteins, including several glycolytic enzymes when affinity chromatography is performed with cytosol from rat liver or brain (Brand and Heinickel, 1991). Although ZnBP is found almost exclusively in the cytoplasm in liver, brain, adrenal gland, smooth muscle, kidney, lung, spleen, and testis, it is found mostly in the nuclei in the intestinal mucosa cells of the crypts of Lieberkühn and in pancreatic duct cells (Brand et al., 1991). The permanent cell lines NRK (normal rat kidney cells) and H35 Reuber hepatoma contain ZnBP exclusively in the nucleus (Trompeter et al., 1996). As injection of ZnBP into *Xenopus* oocytes led to a nuclear uptake of ZnBP (Watts et al., 1990), the motif -PKRQKT- in ZnBP was proposed to function as a nuclear localization signal (NLS). Immunofluorescence studies with COS-cells overexpressing ZnBP wild type and ZnBP with mutations of the PKRQKT-motif demonstrated an active nuclear import of ZnBP (Trompeter et al., 1996). Furthermore, overexpression of fusion proteins between ZnBP and a truncated form of calreticulin showed that ZnBP contains a functionally active bipartite NLS (Trompeter et al., 1996). This study also demonstrated, that ZnBP can be enriched in the nucleus by passive diffusion, even when only the N-terminal half or the central acidic cluster is expressed.

We have previously demonstrated that in freshly isolated rat hepatocytes ZnBP was located almost exclusively in the cytoplasm, whereas after 24 hours of culture ZnBP in most cells had been translocated to the nuclei (Trompeter et al.,

1996). These changes occured independently of changes in the amount of total ZnBP or of ZnBP m-RNA. We now demonstrate that the nuclear import of ZnBP in rat hepatocytes is negatively correlated with cell density and can be blocked by inhibitors of protein- or mRNA-synthesis.

An effect of cell density on the nuclear import of proteins has been described previously for the hox 1.3 homeodomain protein (Odenwald et al., 1987), the nuclear import of angiogenic factors (Moroianu and Riordan, 1994), the human metallothionein IIA (Kou et al., 1994), and for the von Hippel-Lindau tumour suppressor gene product (Lee et al., 1996), but for none of these proteins data on potential mechanisms involved were presented. Here, we have not only added a new example of a protein whose nuclear/cytoplasmic distribution is affected by cell density, but show for the first time also results which for the case of ZnBP shed some light on possible mechanisms involved. A cytoplasmic protein is described which inhibits nuclear import of ZnBP, but not that of albumin-NLS. Such an activity could not be isolated from the cytosol of permanent cell lines which harbor ZnBP only in the nucleus

MATERIALS AND METHODS

Materials

Polyclonal antibodies against ZnBP were raised in rabbits according to standard procedures. The monoclonal antibody DECMA-1 which recognizes a membrane-proximal part of the extracellular domain of E-cadherin (Ozawa et al., 1991) was a kind gift from R. Kemler (Freiburg, FRG). The monoclonal antibody against LI-cadherin as well as plasma membranes from Morris MH7777 hepatoma cells were kindly provided by R. Tauber (Berlin, FRG). Importins and and Ran/TC4 were kind gifts from D. Görlich (Heidelberg, FRG) and H. Ponstingl (Heidelberg, FRG), respectively.

Isolation and culture of isolated rat hepatocytes

Isolation and culture of primary hepatocytes from male Wistar rats was performed according to the method of Berry et al. (1991a). The isolated cells were washed twice with sterile modified Krebs-Ringer-solution (120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2. mM KH_2PO_4, 20 mM Hepes, 0.4% (w/v) BSA, pH 7.4) and separated from non-parenchymal cells and debris by centrifugation at 50 ***g*** for 5 minutes through a Percoll cushion (43.1 ml Percoll, 24.8 ml M199 Earle in 6.5 ml 1.4 M NaCl, 50 mM KCl, 8 mM $MgCl_2$, 4 mM KH_2PO_4, 16 mM Na_2HPO_4, pH 7.4). The pelleted hepatocytes were resuspended and cultured in M199 Earle supplemented with 100 nM dexamethasone, 1 nM insulin, 415 IE penicillin/ml, and 150 μg/ml streptomycin. Cell viability was verified by exclusion of trypan blue. The cells were diluted to the desired densities, seeded in 24-well (14 mm/well) plates on collagen-coated coverslips (Berry et al., 1991b), and cultured at 37°C. At the indicated times the cells were fixed with 8% paraformaldehyde, permeabilized with 0.3% (v/v) Triton X-100 in PBS, and stained for ZnBP using a 1/1000 diluted polyclonal rabbit anti-rat ZnBP antiserum and a 1/200 diluted FITC-labeled goat anti-rabbit antiserum. The samples were embedded with Mowiol 4-88 and analyzed with a Zeiss Axioplan micoscope with a Zeiss Plan Neofluar 40×/0.75 objective and a Plan neofluar 100×/1.30 oil objective equipped with a digitized CCD-camera. Data aquisition was supported by the KAPPA image base data aquisition software (Kappa, Gleichen, FRG). Data documentation and analysis was performed using Adobe Photoshop 4 software. For each experiment the distribution of ZnBP was evaluated in at least 300 to 400 cells. All experiments were performed at least twice.

Co-cultivation of isolated rat hepatocytes with plasma membranes from Morris MH7777 hepatoma cells

Plasma membranes from Morris MH7777 hepatoma cells (equivalent to 140 μg of protein/well) were seeded together with the hepatocytes (5×10^4 cells/well) and the nuclear/cytoplasmic distribution of ZnBP was assessed by immunofluorescence after 3 or 6 hours of cultivation as given above.

Modification and overexpression of ZnBP

Wild-type ZnBP was modified by PCR-based addition of a N-terminal tag containing 7 cysteine residues (see Fig. 1) used for coupling of the fluorochrome tetramethylrhodamine maleimide. The resulting PCR-product was subloned into *Escherichia coli* expression vector pGEX2T (Pharmacia) using *Eco*RI sites added to the PCR-primers. *E. coli* BL21 cells were transformed with pGEX2T containing the tagged ZnBP-cDNA. Cells were harvested, resuspended in homogenizing buffer (50 mM Tris-base, pH 7.4 (4°C), 250 mM sucrose, 100mM NaCl, 1 mM EDTA) and lysed with lysozyme (1 mg/ml culture volume) for 30 minutes on ice. Lysis was completed by sonication (10 times, 10 seconds at 30% maximal output). The GST-cys-ZnBP fusion protein was purified from the lysate by GSH-affinity chromatography. Expression and purity were controlled by western and immunoblotting.

Fluorochrome labeling of GST-Cys-ZnBP and BSA

GST-cys-ZnBP was labeled with tetramethylrhodamine maleimide (TMR) after dialysis against 20 mM Hepes, pH 7.4. The reaction was stopped by addition of a 100-fold excess of reduced glutathione, and the labeled protein (GST-F-ZnBP) was dialyzed against transport buffer TB (20 mM Hepes, pH 7.4, 100 mM K-acetate, 5 mM Mg-acetate, 2 mM DTT, 1 mM EGTA). BSA was labeled with tetramethylrhodamine isothiocyanate (TRITC). The reaction was stopped with 1 M NH_4Cl, and unbound TRITC removed by gel filtration over Sephadex G-25 equilibrated with TB. Labeled BSA was coupled to a peptide (-CYTPPKKKRK) containing the wild-type NLS of the SV40 large T-antigen by using the bifunctional crosslinker sulfo-SMCC as given by Moore and Blobel (1992). The fluorescent protein was designated F-BSA-NLS.

Permeabilized cell assay

NRK-cells (8×10^4 cells) were seeded on coverslips and cultured for 48 hours at 37°C in MEM/5% FCS. Upon subconfluency, cells were washed in ice-cold TB and permeabilized for 5 minutes with 80 mg digitonin/2 ml TB per well as described by Adam et al. (1990). Cells were washed three times with TB. Endogenous cytosol was removed by 5 additional washings with TB. The import mix contained in 40 μl of TB (final concentrations) BSA 10 mM, ATP 1 mM, GTP 0.2 mM, creatine phosphate 5 mM, creatine kinase 20 U/ml, importin-α 25 μg/ml, importin-β 15 μg/ml, RanTC4 5 μg/ml. Unless otherwise mentioned, the cells were incubated with the import mix and 1 μg of F-BSA-NLS or 1 μg GST-F-ZnBP in the dark for 30 minutes at 37°C. Cells were then fixed for 30 minutes in 9% paraformaldehyde at room temperature, washed 3 times in TB and embedded with Mowiol 4-88.

Preparation of rat liver cytosol

Freshly isolated rat liver tissue (12 g) was cut into small pieces and homogenized with 2.5 volumes (v/w) of 20 mM Hepes/KOH, pH 7.4, 250 mM sucrose. The homogenate was centrifuged at 1000 ***g*** and the supernatant filtered through cheese-cloth and spun for 10 minutes at 10,000 ***g***. The supernatant was filtered again and spun for 90 minutes at 100,000 ***g***. The resulting supernatant ('cytosol') was dialyzed against TB, fractionated and stored at –80°C until used.

Enrichment of a ZnBP-import inhibiting activity

Rat liver cytosol (6 ml) prepared as given above was concentrated by Centricon-30 centrifugation to 500 μl and loaded onto a Superdex 200 HiLoad 16/60 column equilibrated with TB. The column was run at

0.25 ml/min and 2 ml fractions were collected. Fractions inhibiting the nuclear import of ZnBP (see Results) were combined and loaded onto a MonoQ HiLoad 16/10 anion exchange column equilibrated in buffer A (20 mM Hepes/KOH, pH 7.4). Following washing, bound proteins were eluted with a linear NaCl gradient from 0 to 1 M in buffer A at 1 ml/minute. Fractions (2 ml) were pooled as given in Fig. 7B. The pooled fractions were tested for inhibiting the nuclear import of ZnBP and analyzed by SDS-gel electrophoresis and by non-denaturing PAA-electrophoresis (Safer, 1998).

RESULTS

Effects of cell density

Hepatocytes seeded at densities of 5×10^4 and 1×10^5 cells/well showed a nuclear localization of ZnBP in most of the cells already after 2 hours of cultivation (Fig. 2a and b), whereas in cells seeded at a density of 1.5×10^5 cells/well ZnBP remained in the cytoplasm after 2 hours of cultivation (Fig. 2c). Even after 24 hours of cultivation, ZnBP remained excluded from the nucleus in cells seeded at a density of 1.5×10^5 cells/well (Fig. 2i), whereas at this time point practically all cells grown at the two lower densities showed a nuclear localization of ZnBP (Fig. 2g and h). At concentrations below 5×10^4 cells/well ZnBP became translocated to the nucleus within 30 to 60 minutes (results not shown here). This makes it unlikely that ZnBP at higher cell concentrations remained in the cytoplasm as a consequence of high levels of other proteins competing with ZnBP for nuclear transport as in this case these competing proteins would have to be removed or degraded within this short time interval.

As many cells exhibited ring-like structures associated with the nuclei, an accumulation of ZnBP at the nuclear envelope rather than a nuclear import had to be excluded. However examination of cells by confocal laser microscopy (Fig. 2j,k,l) demonstrated that ZnBP was indeed localized within the nuclei of most cells and not at the cytoplasmic side of the nuclear envelope. In some cells, ring-like structures were observed even inside the nuclei (Fig. 2l), demonstrating that imported ZnBP tends to locate at the inner nuclear membrane. ZnBP did not seem to be associated with nucleoli (see e.g. Fig. 2k).

Isolated plasma membranes inhibit nuclear import of ZnBP

The effect of cell density on the retention of ZnBP in the cytoplasm pointed to the possibility that cell-cell interaction might play a role. Therefore, we co-incubated rat hepatocytes seeded at 5×10^4 cells/well with isolated plasma membranes (140 μg protein) from Morris MH7777 hepatoma cells. Morris MH7777 express several unspecific surface proteins (Loch et al., 1992; Vedel et al., 1983) and high concentrations of cadherin E (Vestweber and Kemler, 1985) and cadherin LI (Berndorff et al., 1994). Co-incubation with the plasma membranes from MH7777 cells for 3 hours resulted in a significant inhibition of nuclear import of ZnBP from 76% in the control cultures to 31% in the cultures co-incubated with the MH7777 membranes. After 6 hours, only 34% of the cells co-incubated with MH7777 membranes contained ZnBP in their nuclei as compared to 86% in control cells (Fig. 3).

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5'-GGA ATT CAT TGT GGA GCA TGC TCG CTA TGT GGC TCA
          His Cys Gly Ala Cys Ser Leu Cys Gly Ser

TGC GCT GGT TGT CTA GAG TGT GCA GCA TGT TCG GAG
Cys Ala Gly Cys Leu Glu Cys Ala Ala Cys Ser Glu

AAG AGC GTG GAA GCA-3'
Lys Ser Val Glu Ala
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Fig. 1. PCR-primer used to generate the N-terminal cys-tag of GST-cys-ZnBP. A tag of 20 amino acids with 7 cysteine residues was added to the N terminus of ZnBP by PCR. Cysteine residues and their codons are printed in bold, the N terminus of wt-ZnBP is underlined. The *Eco*RI-site used to clone the PCR-product into pGEX2T is given in italics.

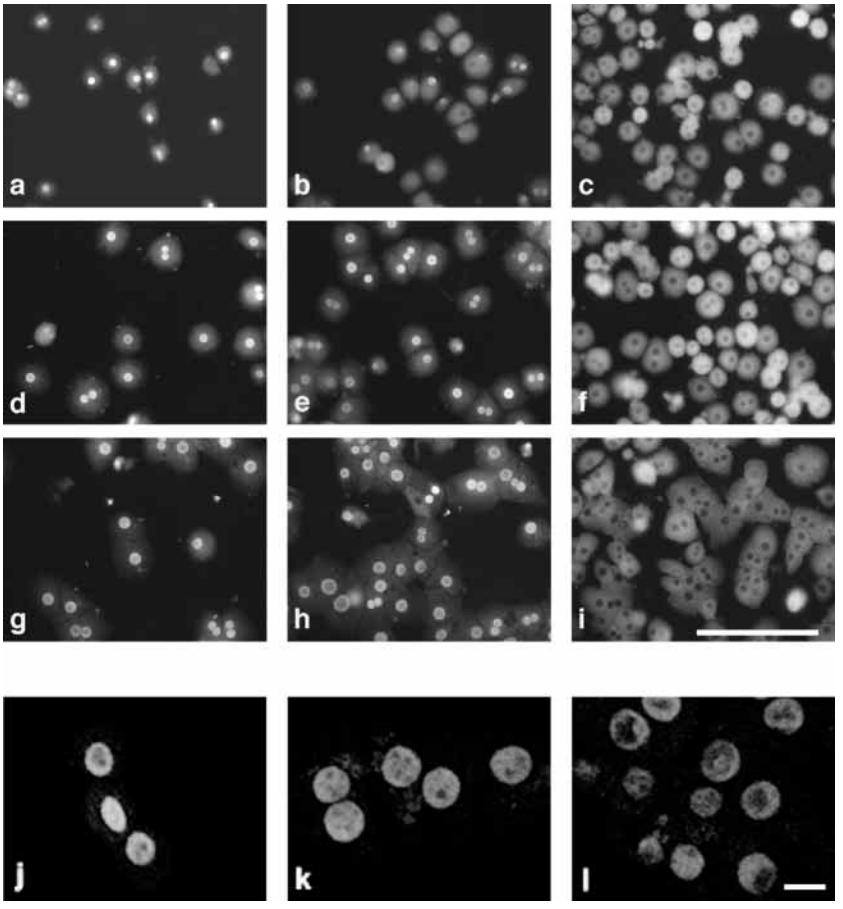

Fig. 2. Cell density-dependent nuclear import of ZnBP. Rat hepatocytes cultured at densities of 5×10^4 cells/well (a,d,g), 1×10^5 cells/well (b,e,h) and 1.5×10^5 cells/well (c,f,i) were analyzed by indirect immunofluorescence for the intracellular distribution of ZnBP 2 hours (a,b,c), 6 hours (d,e,f), or 24 hours (g,h,i) after seeding. Bar, 100 μm. (j,k,l) Intranuclear ZnBP as revealed by confocal laser microscopy in cells cultured at 2.5×10^4 cells/well for 3 hours (j), at 5×10^4 cells/well (k) or 1×10^5 cells/well (l) for 24 hours. Bar, 25 μm.

Table 1. Cycloheximide (CHX) initiates transport of ZnBP from the nucleus to the cytosol

	Localization of ZnBP	
	Cytoplasmic	Nuclear
	(% observed cells)	
Control, 6 hours	14	86
Control, 9 hours	11	89
CHX, 9 hours	71	29
Control, 24 hours	1	99
CHX, 24 hours	99	1

Hepatocytes were seeded at a density of 5×10^4 cells/well and cultured for 6 hours. Then cycloheximide at a final concentration of 1 mM was added and the cultivation continued for another 3 hours (CHX, 9 hours) or 18 hours (CHX, 24 hours). Control cells were incubated for the same times without cycloheximide. At the given time points the cells were fixed and analyzed for the distribution of ZnBP as given in Materials and Methods. For each condition 300 to 400 cells were analyzed.

Antibodies against a cytoplasmic domain of E-cadherin inhibit nuclear import of ZnBP

In order to inhibit a potential homophilic interaction between E-cadherins from neighbouring cells we incubated rat hepatocytes seeded at 1×10^5 cells/well with the rat monoclonal antibody DECMA-1 against E-cadherin (Ozawa et al., 1991). To our suprise the antibody inhibited nuclear import of ZnBP under these conditions. Only 15% of the cells showed nuclear localization of ZnBP as compared to 60% in control cells incubated without the antibody. Antibodies against LI-cadherin had no effect on ZnBP localization (results not shown here). Our results can be explained by antibody induced crosslinking of E-cadherin and are in line with a recent report of Ozawa and Kemler (1998) that crosslinking of E-cadherin activates E-cadherin-dependent signalling.

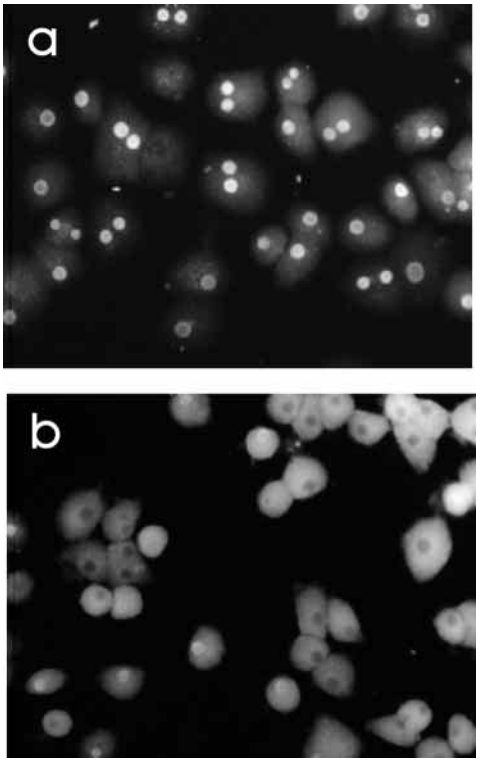

Fig. 3. Plasma membranes from Morris 7777 hepatoma cells inhibit the translocation of ZnBP to the nucleus in rat hepatozytes. Freshly prepared rat liver hepatocytes were seeded and cultured at a density of 5×10^4 cells/well for 6 hours in the absence (a) or presence (b) of purified plasma membranes (140 μg/well) from Morris 7777 hepatoma cells.

Effects of cycloheximide and actinomycin D

We tested the possibility, that the effect of low cell density is associated with the increased synthesis of a specific protein (proteins). If this were so, one would expect an inhibition of translocation of ZnBP to the nucleus under the condition of inhibited protein synthesis. To test this hypothesis, rat hepatocytes were cultured at a density of 5×10^4 cells/well for 3 hours in the absence or presence of 1 mM cycloheximide and at a density of 1×10^5 cells/well for 6 hours in the absence or presence of 10 μM actinomycin D as given in the methods section. Under both conditions, 100% of the control cells contained ZnBP in the nucleus, whereas the translocation of ZnBP into the nucleus was abolished in almost 100% of the cells by both compounds (Fig. 4a-d).

We investigated further, whether the inhibition of protein synthesis at a time point when ZnBP had already been translocated to the nucleus, could induce a relocation of ZnBP to the cytoplasm. To this end, rat hepatocytes were cultured at a density of 1×10^5 cells/well for 6 hours, by which time 86% of the cells contained ZnBP in the nuclei. The cultivation was then continued for another 3 hours or 18 hours in the absence or presence of 1 mM cycloheximide. As given in Table 1, already after 3 hours, an increased number of cycloheximide treated cells showed a relocation of ZnBP to the cytoplasm, and after 18 hours of cycloheximide practically all cells had their ZnBP translocated back to the cytoplasm whereas control cells kept ZnBP exclusively in the nuclei.

DMSO abolishes the translocation of ZnBP to the nucleus in thinly seeded cells

It is well established that cultured rat hepatocytes start to de-differentiate relatively soon after start of culture (Padgham et al., 1993; Villa et al., 1991). We, therefore, considered the possibility that the state of de-differentiation might affect the localization of ZnBP in cultured hepatocytes. As DMSO is known to induce differentiation in various cell lines (Isom et al., 1985; Kost and Michalopoulos, 1991) and to inhibit de-differentiation of cultured hepatocytes (Arterburn et al., 1995) we cultured freshly isolated rat hepatocytes at a density of 1×10^5 cells/well in the presence and absence of 2% (v/v) DMSO. After 6 hours all control cells had translocated ZnBP to the nucleus (Fig. 4e), whereas practically all cells cultured in the presence of DMSO retained ZnBP in the cytoplasm (Fig. 4f). Even after 24 hours all cells cultured in the presence of DMSO excluded ZnBP from their nuclei (results not shown here).

Nuclear import of ZnBP can be specifically inhibited by a cytoplasmic protein fraction

Next, we tested whether rat liver cytosol contains an activity able to specifically retain ZnBP in the cytoplasm. From affinity chromatography experiments, ZnBP is well known to bind to a large variety of cytosolic proteins, including several glycolytic enzymes, in vitro (Brand and Heinickel, 1991) and since several different mechanisms like binding to and modifying of ZnBP by a cytosolic component might mediate the cytosolic retention of ZnBP, we directly approached inhibition of nuclear import of ZnBP mediated by rat liver cytosol by using the permeabilized cell assay (Adam et al., 1990). To this end, we analyzed the import of fluorescently labeled ZnBP in this assay using NRK cells and an import mix consisting of importin α, importin β, the small GTPase

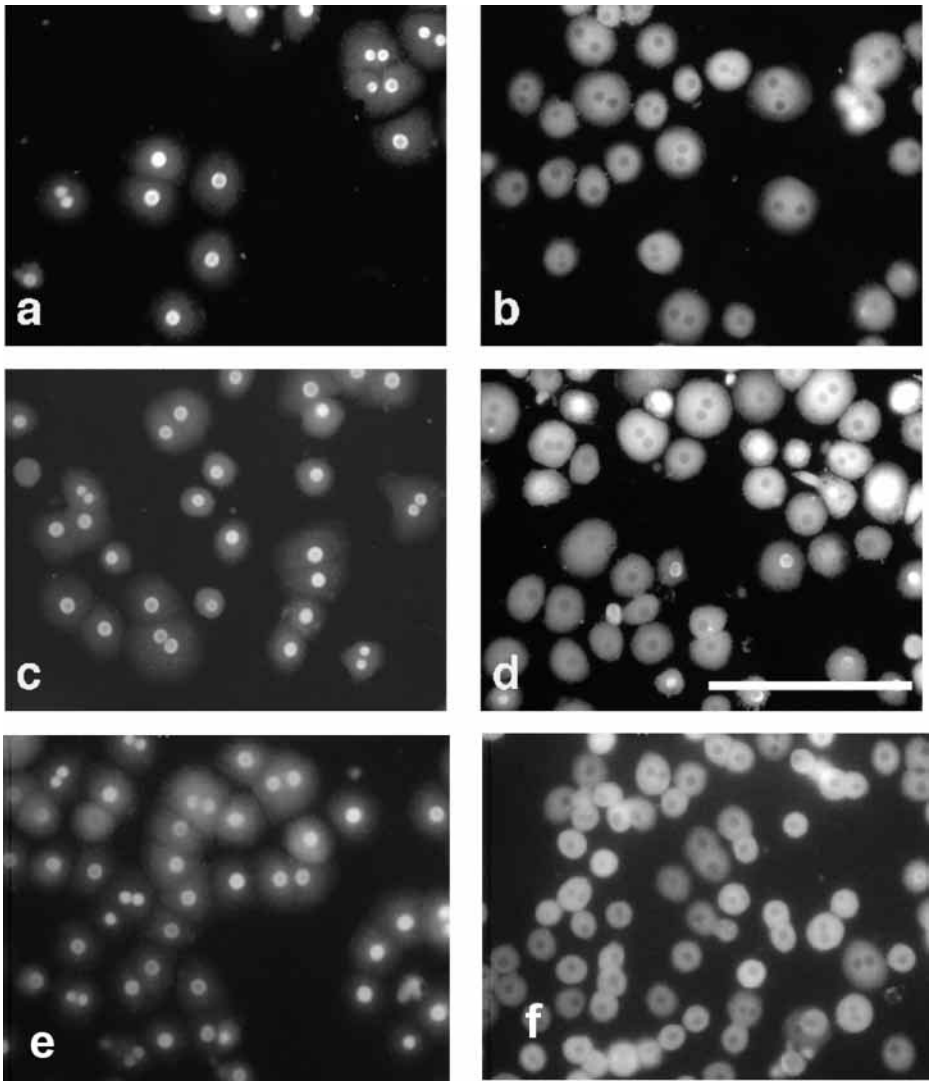

Fig. 4. Effect of inhibition of protein synthesis and of DMSO on the nuclear/cytoplasmic distribution of ZnBP. (a-d) Freshly prepared rat hepatocytes were cultured for 3 hours (a and b) or 6 hours (c and d) in the absence (a and c) or presence of 1 mM cycloheximide (5×10^4 cells/well, b) or of 10 μM actinomycin D (1×10^5 cells/well, (d) and analyzed for the localization of ZnBP. (e and f) Freshly prepared rat hepatocytes (5×10^4 cells/well) were cultured for 6 hours in the absence (e) or presence (f) of 2% (v/v) DMSO and analyzed for the localization of ZnBP. Bar, 100 μm.

Ran/TC4, and an ATP regenerating system. The fluorochrome tetramethylrhodamine maleimide was linked to a Cys-tag located N-terminally of the ZnBP in a recombinant GST-ZnBP fusion protein (GST-F-ZnBP). Tetramethylrhodamine isothiocyanate labeled BSA linked to the SV40 large T-antigen NLS (F-BSA-NLS) served as control protein. Both substrates, GST-F-ZnBP as well as F-BSA-NLS were imported into the nucleus of permeabilized NRK-cells only in the presence of the afore mentioned import factors (Fig. 5). This demonstrates that nuclear import of both substrates involves the recognition of their NLS sequences. Since rat liver cells in situ contain ZnBP almost exclusively in the cytoplasm (Brand et al., 1991), the putative protein inhibiting the nuclear import of ZnBP should exist at least in small amounts in rat liver cytosol. To test this we analyzed whether increasing amounts of GST-F-ZnBP were able to titrate out such a protein. In these experiments, dialyzed rat liver cytosol (20 μg protein/assay) was added to the import assay. As shown in Fig. 6 the uptake of F-BSA-NLS occured in 100% of the cells independently of the concentration of F-BSA-NLS. In the case of GST-F-ZnBP, however, 25% and 15% of the cells excluded the fusion protein from their nuclei at a concentration of 100 ng/assay and 300 ng/assay, respectively. At 500 ng/assay almost 100% of the cells showed a nuclear uptake of GST-F-ZnBP, but considerable amounts of the fusion protein remained in the cytoplasm. At 1000 ng/assay, all cells exhibited a clear nuclear uptake, although some fusion protein remained in the cytoplasm.

In contrast, when using cytosols from HeLa-, NRK-, or Reuber H35 hepatoma cells no inhibition of the nuclear import of GST-F-ZnBP was detected at any substrate concentration (data not shown).

These findings demonstrate that small amounts of a free import inhibiting protein are present in the cytosol of fresh hepatocytes despite the endogenous cytosolic ZnBP, whereas no such activity seems to exist in the cytosol of permanent cell lines which is in line with our observation that these cells contain ZnBP almost exclusively in the nucleus.

To substantiate the existence of an inhibitory protein further, we fractionated rat liver cytosol on a Superdex 200 HiLoad 16/60 gel filtration column and tested the fractions in the protein import assay. Proteins (5 μg/assay) from fractions 2 and 3 of the elution profile (Fig. 7A), corresponding to a molecular mass >250 kDa led to a cytoplasmic retention of GST-F-ZnBP in 38% to 51% of the analyzed cells (see example in Fig. 8), while 5 μg protein from pool 4 was still able to retain GST-F-ZnBP in the cytoplasm of 5-8% of the inspected cells. Proteins from fractions 5-16 had no effect on the nuclear import of GST-F-ZnBP. None of the tested fractions was able to inhibit the nuclear import of F-BSA-NLS (Fig. 8).

In another Superdex 200 gel filtration experiment, in which 5 μg protein of the peak fraction inhibited nuclear import of GST-F-ZnBP in 51% of the cells, the active fraction was purified further by MonoQ HiLoad 16/60 anion exchange chromatography. The most active fraction eluted at 400-415 mM NaCl (Fraction 9 in Fig. 7B). It inhibited at 5 μg protein/assay the nuclear import of GST-F-ZnBP in 100% of the inspected cells (Fig. 9) without inhibiting the nuclear import of the F-BSA-NLS (Fig. 9). Proteins from the neighboring fractions 8 and 10 did not have an inhibitory effect on the nuclear import of GST-F-ZnBP (Fig. 9). Fraction 9 showed one main protein band during native gel electrophoresis (Fig. 10) but spread up into several bands when

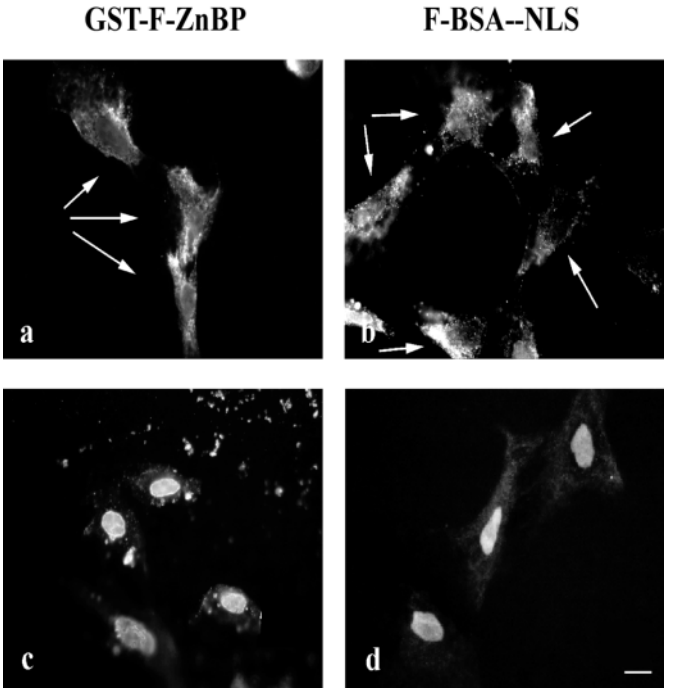

Fig. 5. Nuclear import of fluorochrome-labeled GST-ZnBP fusion protein in permeabilized NRK cells depends on the presence of the nuclear import factors importin α, importin β and RanGTP. Nuclear import of GST-F-ZnBP (a and c) and F-BSA-NLS (b and d) was tested in the absence (a and b) and presence (c and d) of the import factors. Arrows indicate GST-F-ZnBP or F-BSA-NLS accumulated in the cytoplasm.

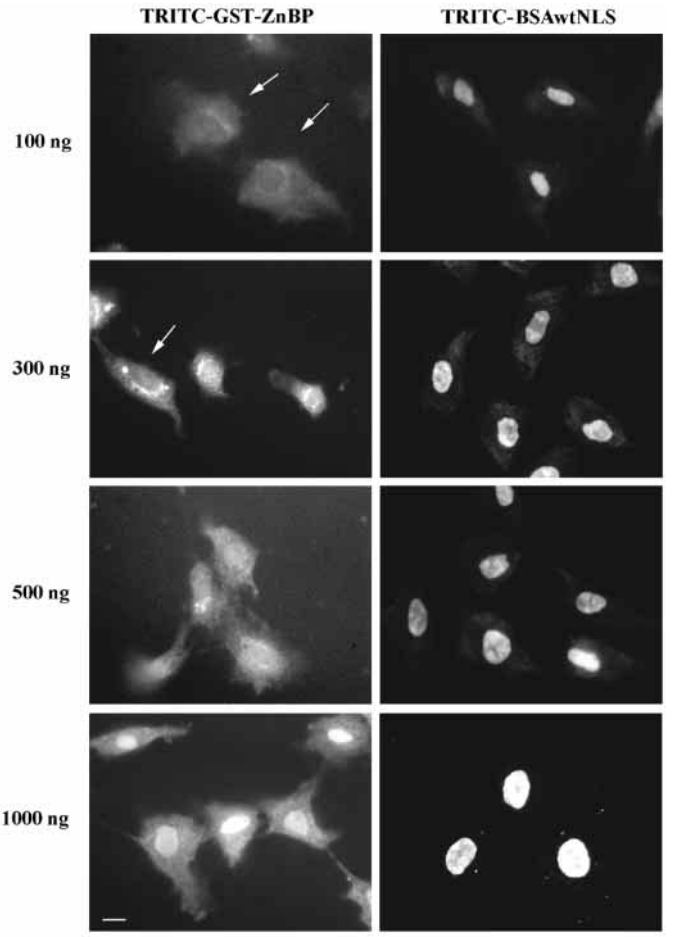

Fig. 6. Inhibition of nuclear import of GST-F-ZnBP by rat liver cytosol is overcome by increasing concentrations of GST-F-ZnBP. Increasing amounts (100-1000 ng) of GST-F-ZnBP (left panels) or F-BSA-NLS (right panels) were added to the permeabilized cell assay in the presence of rat liver cytosol (corresponding to 20 μg protein/assay) and nuclear import factors and checked for nuclear import. At 100 ng GST-F-ZnBP nuclear import could not be observed in 25% of the cells (see arrows), whereas all cells showed nuclear import of F-BSA-NLS at the same concentration. At 300 ng GST-F-ZnBP still 15% of the cells showed little or no nuclear uptake, whereas at 500 and 1000 ng/ assay of GST-F-ZnBP all cells showed nuclear uptake, but even then GST-F-ZnBP could still be observed in the cytoplasm.

separated by SDS-PAGE (data not shown here). Proteins from fraction 9 lost their inhibitory action on nuclear import of GST-F-ZnBP after heat inactivation, indicating the proteinaceous nature of this activity (data not shown). The nuclear import of fluorescently labeled cys-ZnBP generated from GST-cys-ZnBP by thrombin cleavage was also inhibited by proteins from fraction 9 (results not shown here), indicating that protein B does not inhibit the nuclear import of ZnBP by an unspecific interaction with GST.

DISCUSSION

Our previous studies had revealed a cytoplasmic localization of ZnBP in most mammalian tissues including hepatocytes (Brand et al., 1991). Isolated hepatocytes preserved this distribution only when they were cultured at high density as shown by conventional immunofluorescence and by confocal laser microscopy. Confocal laser miscroscopy showed clearly that 'nuclear' ZnBP which sometimes appeared in ring-like structures during conventional immunofluorescence microscopy was localized within the nuclei. In addition, confocal laser microscopy revealed occasionally ring-like structures inside the nuclei (Fig. 2j-l), which may possibly reflect preferential binding of ZnBP to structures located near the nucleoplasmic side of the nuclear envelope. Binding of ZnBP to intranuclear structures has been proposed earlier (Trompeter et al., 1996). The cytoplasmic distribution of ZnBP in most tissues and in densely seeded hepatocytes occurs inspite of the fact that the protein contains a functionally active bipartite NLS (Trompeter et al., 1996). This raises the following questions: (1) how is the NLS of ZnBP inactivated so that ZnBP is retained in the cytoplasm? (2) which regulatory mechanisms determine the cytoplasmic/nuclear distribution of ZnBP? (3) how can cell density interfere with these regulatory mechanisms? The fact that the cytoplasmic retention of ZnBP in isolated cultured rat hepatocytes depends on cell density indicates that cell-cell contact may play an important role.

One possibility would be a dramatic increase in the amount of other proteins with an NLS which could compete for binding to the importins or other steps of the importin-dependent nuclear import. The latter possibility seems unlikely for the case of ZnBP for the following reasons: (1) at low cell density translocation of ZnBP into the nucleus can occur within 30-60

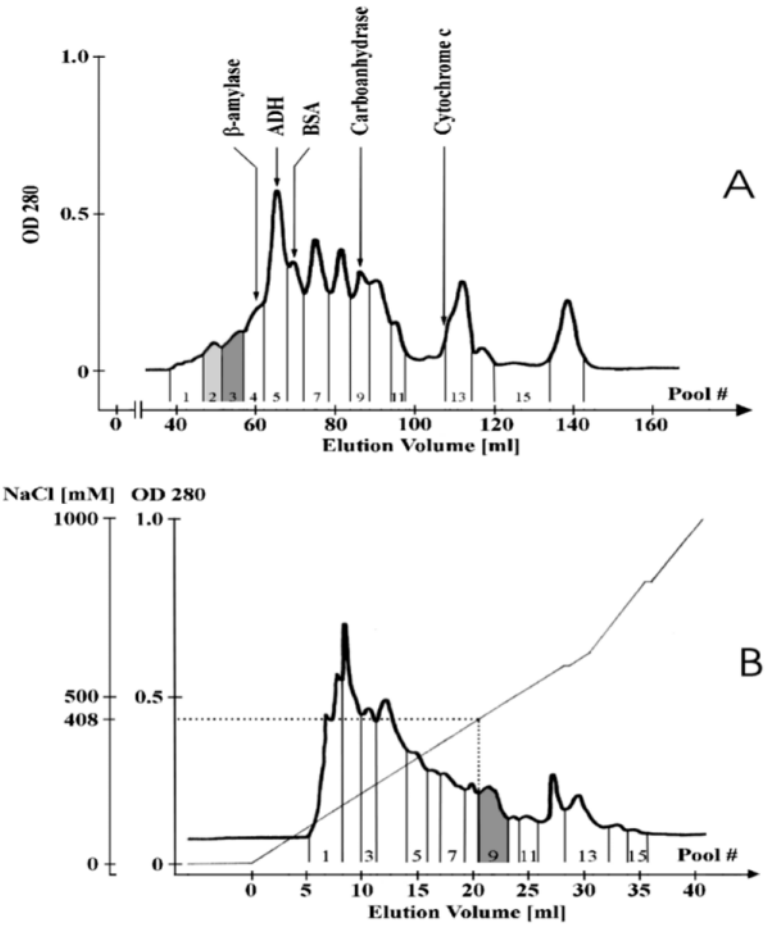

Fig. 7. Elution profiles of the inhibitory protein activity during gel filtration of rat liver cytosol over Superdex 200 HiLoad (a) and subsequent anion exchange chromatography on MonoQ HiLoad (b). Shaded fractions (fractions 2 and 3 in a and fraction 9 in b) showed the highest inhibition of nuclear import of GST-F-ZnBP (see Figs 8 and 9). The activity elutes from the Superdex 200 with an app. molecular mass of >250 kDa.

minutes which could only be explained by assuming that the competing proteins were removed and/or degraded during this short interval; (2) we have shown recently (Trompeter et al., 1996) that due to its small size ZnBP can be translocated to the nucleus also by diffusion i.e. in an NLS-independent way, although less effectively. Hence, the existence of competing NLS-proteins should not block completely the appearance of ZnBP in the nucleus. In addition, putative postranslational modifications of ZnBP changing its secondary structure and thereby masking the NLS would also not inhibit nuclear import by passive diffusion.

A protein with a functional NLS could also be retained in the cytoplasm by binding to a cytoplasmic protein(s) which itself is unable to reach the nucleus. We used the permeabilized cell assay (Adam et al., 1990) to determine whether cytosol from rat liver contains an activity able to inhibit the active nuclear import of ZnBP. Freshly prepared rat liver cytosol was indeed able to inhibit the nuclear import in a small percentage of cells at small but not at higher concentrations of GST-F-ZnBP without having any effect on the nuclear import of F-BSA-NLS at similar concentrations. By gel filtration and anion exchange chromatography this activity could be enriched from fresh rat liver cytosol. It had an apparent molecular mass >250 kDa and was heat-labile. It did not inhibit nuclear import of F-BSA-NLS under identical experimental conditions, pointing to its specificity for ZnBP. The most enriched protein fraction blocked nuclear import of GST-F-ZnBP in 100% of the cells at a concentration of 5 μg/assay and appeared as one main band with a molecular mass >250 kDa during native electrophoresis, but as several protein bands with molecular masses >50 kDa during SDS-PAGE. Thus this import inhibiting protein may be a hetero-oligomer. Since the import of GST-F-ZnBP and F-BSA-NLS required only importins, the small GTPase Ran TC4, and an energy-regenerating system it seems likely that import inhibiting protein inhibits the nuclear import of ZnBP by interfering with the recognition of the NLS of ZnBP by importins. No protein inhibiting nuclear import of ZnBP could be isolated from the cytosol of permanent cell lines (HeLa, NRK, Reuber hepatoma cells) in accordance with the fact that in these cells ZnBP was always observed in the nucleus independently of cell density. This supports our conclusion, that the retention of ZnBP in the cytosol of freshly isolated or densely seeded rat hepatocytes is accomplished by a cytoplasmic proteinacous factor (factors).

How might this fraction achieve the cytosolic retention of ZnBP? Theoretically, the cytosolic factor could possess a proteolytic activity specific for ZnBP thus diminishing nuclear uptake of ZnBP just by destroying ZnBP in the cytosol. This seems unlikely for the following reasons: (a) the inhibitory factor was enriched from cytosol freshly prepared from the livers of fed rats which contain all their endogenous ZnBP in the cytoplasm; (b) the shift in the nuclear/cytoplasmic distribution of ZnBP in isolated hepatocytes is not accompanied by a loss of ZnBP (Trompeter et al., 1996); (c) inhibition of translocation of ZnBP by cycloheximide, actinomycin or DMSO in thinly seeded hepatocytes is not accompanied by a loss of ZnBP in the cytoplasm but rather by an accumulation (Fig. 4).

We propose that we have enriched a protein (proteins) which binds to ZnBP and thereby (1) masks and inactivates the NLS of ZnBP and (2) increases the molecular weight of the complex to prevent it from passive diffusion into the cytoplasm. It appears that the inhibition of nuclear ZnBP-uptake is specific as the translocation of F-BSA-NLS was not inhibited. F-BSA-NLS contains in contrast to GST-F-ZnBP more than one NLS. Therefore, a cytoplasmic factor interacting directly with the NLS could be less effective in the case of F-BSA-NLS. However, the following observations make this less likely: (a) under conditions were the enriched cytoplasmic fraction blocked nuclear uptake of GST-F-ZnBP in 100% of the cells, in none of the cells nuclear uptake of F-BSA-NLS was inhibited; (b) if F-BSA-NLS would bind via its NLS to the cytoplasmic factor, it should compete with GST-F-ZnBP for this factor at low concentrations of cytosol or of the enriched factor, i.e. F-BSA-NLS should under these conditions enhance nuclear uptake of GST-F-ZnBP. This was not the case (results not shown here). It seems unlikely that the enriched protein is phosphofructokinase-1, as this should appear during in SDS-

PAGE as a uniform protein band of about 80 kDa. In this context one should also realize that the binding of ZnBP to PFK-1 is not very specific (Brand and Heinickel, 1991) and may just indicate the ability of ZnBP to react relatively easily with other proteins. An elucidation of the precise mechanism of ZnBP-retention will only be possible after final purification of the cytoplasmic factor(s) and functional assays with the recombinant protein(s).

An inhibition of recognition of the NLS of ZnBP by a cytosolic protein resembles the masking of the NLS of the transcription factor NF-κB (Baeuerle, 1991) by I-κB in the cytoplasm. This concept requires that in sparsely seeded hepatocytes, where ZnBP is translocated to the nuclei, the NLS of ZnBP is no longer masked. This could result either from a lack of the cytosolic protein or from its temporary inactivation. As inhibition of protein synthesis by cycloheximide after translocation of ZnBP into the nucleus in thinly seeded cells leads to a reappearence of ZnBP in the cytoplasm, it seems unlikely that a lack of the import-inhibiting protein due to degradation is responsible for the nuclear import of ZnBP into the nucleus of thinly seeded cells. It is much more likely that in thinly seeded cells, this protein has only lost its (putative) capability to block the interaction between ZnBP and the importins. The cycloheximide experiments indicate (1) that the inactivation of the import-inhibiting protein is reversible, and (2) that its conversion from an active to an inactive state (e.g. by phosphorylation/dephosphorylation) might be catalyzed by

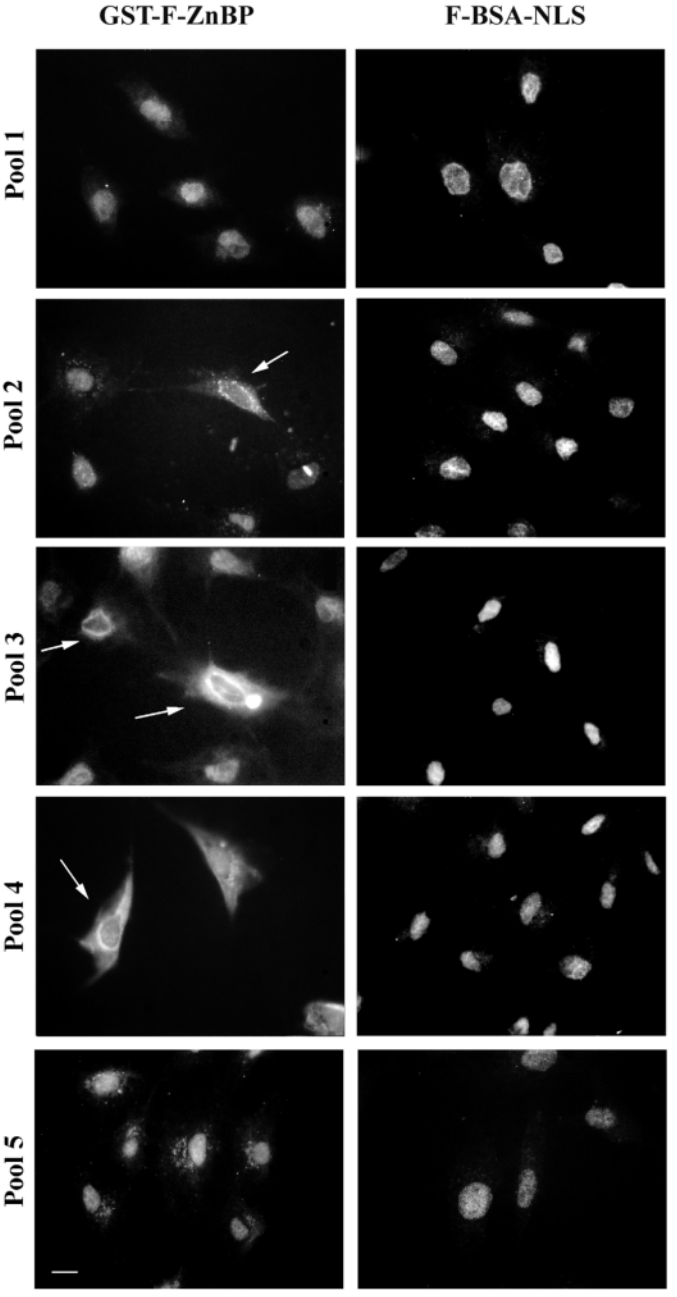

Fig. 8. Inhibition of nuclear import of GST-F-ZnBP, but not of F-BSA-NLS by fractions from Superdex 200 gel filtration of rat liver cytosol. Aliquots corresponding to 5 μg of protein from the fractions of the Superdex 200 gel filtration of rat liver cytosol (see Fig. 7a) were added to permeabilized NRK-cells and nuclear import of GST-F-ZnBP and F-BSA-NLS was examined in the presence of import factors as given in the methods section. None of the fractions inhibited nuclear import of F-BSA-NLS, whereas proteins from fraction 1 abolished or inhibited nuclear import of GST-F-ZnBP in 5%, from fraction 2 in 34%, from fraction 3 in 38%, and from fraction 4 in 8% of the cells. No inhibition was obtained with proteins from fraction 5 or later fractions. Arrows mark cells with cytoplasmic GST-F-ZnBP.

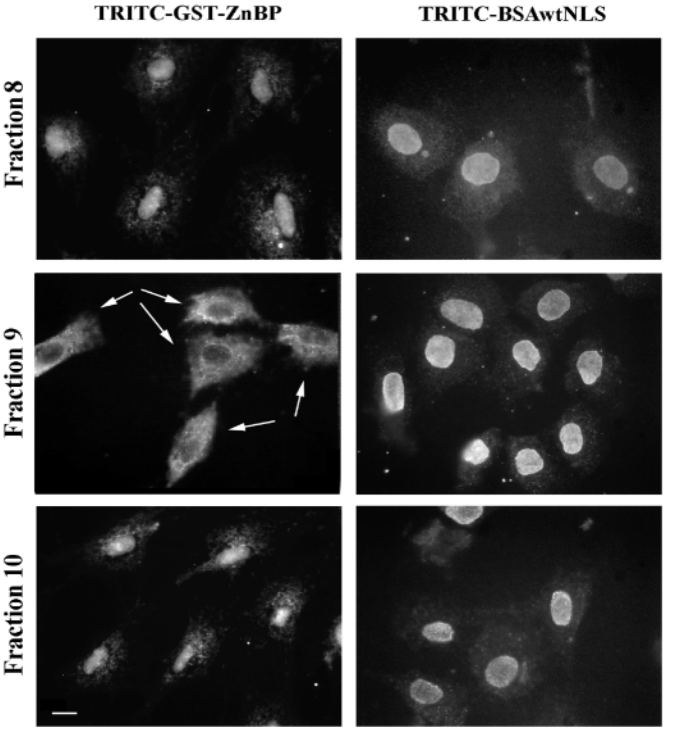

Fig. 9. Inhibition of nuclear import of GST-F-ZnBP by a fraction from rat liver cytosol enriched by MonoQ HiLoad anion exchange chromatography. Fractions 2 and 3 obtained by Superdex 200 gel chromatography were pooled, loaded onto a MonoQ Hiload 16/10 anion exchange column, and eluted with a NaCl-gradient as indicated in Fig. 7B. Aliquots from the collected fractions corresponding to 5 μg of protein were added to the import assay as in Fig. 8. None of the fractions inhibited the nuclear translocation of F-BSA-NLS, whereas fraction 9 abolished nuclear uptake of GST-F-ZnBP in 100% of the cells. Aliquots from fractions 8 or 10 were almost without effect. The arrows indicate GST-F-ZnBP accumulating in the cytoplasm.

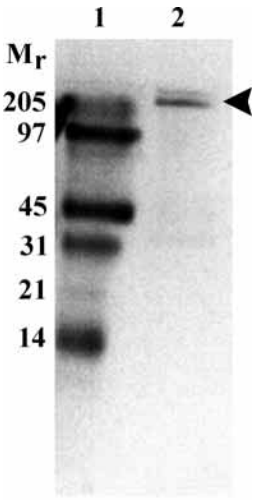

Fig. 10. Analysis of the enriched inhibitory protein(s) by native PAA-gel electrophoresis. Rat liver cytosol was fractionated by Q-Sepharose anion exchange chromatography and tested for inhibitory activity. Fractions containing inhibitory activity were combined and further fractionated by anion exchange chromatography via Mono-Q-HiLoad. Fractions with inhibitory activity were subsequently separated further by size exclusion chromatography via Superdex S-200. The fraction containing proteins of >250 kDa was concentrated by Centricon 30 centrifugation. Protein (5 μg) from this fraction inhibited nuclear uptake of ZnBP in 100% of analyzed cells (not shown here). The enriched protein (5 μg) was processed by native PAA-electrophoresis and stained with Coomassie Blue. Lane 1: molecular mass markers; lane 2: inhibitory protein.

a regulatory protein R. Protein R must have a high turnover and, therefore, its amount has to be strongly sensitive to inhibition of protein synthesis. In thinly seeded cells the activity of protein R would be relatively high, in densely seeded cells low. This may be an oversimplification as protein R may not act directly on the import inhibiting protein but via additional factors, but this would not change the basic concept.

Under condition of inhibited protein synthesis, ZnBP returns to the cytoplasm, which demonstrates that the import inhibiting protein must have regained its activity. The proposed high-turnover protein R can under these conditions not be responsible for this reactivation and other mechanism have to be taken into account. If the import inhibiting protein turns out to be a complex of several polypeptides, it might contain an endogenous activity keeping it in its active state. Also, an additional protein might ensure the active state of the import inhibiting protein. In both cases, the intracellular localization of ZnBP would be dependent on the relative activities of R and its counterpart. If the activity of R dominates, the import inhibiting protein would become inactive and allow ZnBP to be imported. If R is not present or inactive, the import inhibiting protein would become active and keep ZnBP in the cytoplasm.

How can this hypothetical type of regulation be linked to the recognition of cell density? We have shown here, that thinly seeded hepatocytes retain ZnBP in the cytoplasm when cultured in the presence of plasma membranes from Morris MH7777 hepatoma cells, which are known to be rich in cadherins. This indicates that cell/cell interaction can affect the nuclear/cytoplasmic distribution of ZnBP. The import inhibiting protein is a cytoplasmic protein and, therefore, the hypothetical protein R supposed to catalyze directly or indirectly its reversible inactivation, most likely exists also in the cytoplasm. It could also be that protein R is reversibly associated with the plasma membrane. Such an association could then affect its turnover. However, more likely additional factors are involved which transmit cell density signals from the plasma membrane to components regulating the turnover of protein R.

Surprisingly, the monoclonal antibody DECMA-1 directed against the extracellular domain of E-cadherin inhibited the nuclear translocation of ZnBP in rat hepatocytes. As cadherin E-mediated signalling requires dimerized E-cadherins (Ozawa and Kemler, 1998), it seems possible, that the antibodies induced a cross-linking of E-cadherins. This may have led to similar intracellular responses as homophilic interactions of E-cadherins from neighbouring cells. Nevertheless, this experiment shows the participation of E-cadherin in regulating the intracellular distribution of ZnBP.

Isolated rat hepatocytes in culture show all signs of rapid de-differentiation together with the translocation of ZnBP into the nucleus. But this relationship is more than circumstantial as indicated by the fact that DMSO not only inhibits de-differentiation of hepatocytes (Ai et al., 1995; Kuliczkowski et al., 1995; Otsuka-Murakami and Nishimoto, 1995), but also abolishes completely over a long time the translocation of ZnBP from the cytoplasm to the nucleus. It should also be noted, that the amount of ZnBP in human breast cancer is dramatically increased (Tsitsiloni et al., 1998), an observation we could confirm for hepatocarcinoma cells (unpublished results). In contrast, benign breast tumors did not show increased ZnBP expression (Tsitsiloni et al., 1998). Although these observations point to a putative role of ZnBP in the regulation of de-differentiation and/or proliferation, its precise function still remains to be elucidated as well as the detailed mechanism that regulates its nuclear import in a cell-density dependent manner. It still remains unclear, whether ZnBP fullfills its function in the cytoplasm, in the nucleus or in both compartments and whether the in-vitro binding to several cytosolic proteins (Brand and Heinickel, 1991) is of physiological relevance. It has been shown recently (Kondili et al., 1996) that ZnBP can bind to histone H1. In view of the fact that ZnBP is an extremely acidic protein and that histone H1 under identical conditions binds also to poly (glutamate) the specificity of this interaction and its physiological occurrence and function need to be proved. In speculating about a physiological role of ZnBP in the nucleus one should keep in mind that ZnBP shares similarities with prothymosin-α, a nuclear protein of similar size and acidity also possessing an active NLS. The expression of prothymosin-α is under the control of c-myc (Lutz et al., 1996; Ben-Yosef at al., 1998). It modulates the interaction of histone H1 with chromatin (Karetsou et al., 1998; Gomez-Marquez and Rodriguez, 1998), and overexpression of the protein accelerates proliferation and retards differentiation in HL-60 cells (Rodriguez et al., 1998). It has also been reported that prothymosin-α stimulates the phosphorylation of EF-2 and thus could also affect reactions in the cytosol (Vega et al., 1998). Unfortunately, a systematic analysis of the cytoplasmic/nuclear distribution of prothymosin-α in normal mammalian tissue cells is lacking. Iguchi et al. (1998) have observed that high concentrations of Zn^{2+} induce necrosis in LNCaP- and in PC-3 cells. This effect was accompanied by an increased expression of ZnBP but also of other small proteins including metallothionein. As we could not observe an increased level of ZnBP and ZnBP-m-RNA in hepatocytes under conditions where ZnBP became translocated to the nucleus (Trompeter et al., 1996), we consider it unlikely that the Zn^{2+}-induced effects described by Iguchi et al. (1998) can be related to the cell density-induced changes in the nuclear/cytoplamic distribution of ZnBP.

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