Variable Nuclear Cytoplasmic Distribution of the 11.5-kDa Zinc-binding Protein (Parathymosin-α) and Identification of a Bipartite Nuclear Localization Signal*

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The 11.5-kDa zinc-binding protein (ZnBP, parathymosin-α), a potent inactivator of 1-phosphofructokinase, is found only in the cytoplasm of most tissues despite the presence of the putative nuclear localization signal PKRQKT. Recent reports on nuclear uptake of ZnBP could not exclude the participation of unspecific diffusion. We show here that wild-type ZnBP overexpressed in COS cells accumulates exclusively in the nucleus but that ZnBP with a mutated or deleted PKRQKT motif appears both in the nucleus and in the cytoplasm. In contrast, fusion proteins between ZnBP and parts of the endoplasmic reticulum protein calreticulin required the intact PKRQKT motif for nuclear import. The motif RKR, located nine amino acids upstream of the PKRQKT motif, is also involved in the active nuclear import of ZnBP. In contrast to rat hepatocytes and kidney cells in situ, which have ZnBP almost exclusively in the cytosol, we find ZnBP in Reuber H35 hepatoma cells and normal rat kidney cells only in the nuclei. Freshly isolated rat hepatocytes translocate their ZnBP to the nucleus in <24 h during standard cell culture conditions.

The 11.5-kDa zinc-binding protein (ZnBP) was first described as a factor capable of inactivating 1-phosphofructokinase in a Zn²⁺-dependent and reversible manner (1). ZnBP is found in the cytoplasm of liver, brain, adrenal gland, smooth muscle, kidney, lung, spleen, and testis, whereas it is only weakly detectable in skeletal muscle and adipose tissue (2). Interestingly, in epithelial cells of the intestinal mucosa and in duct cells of the exocrine pancreas, it has preferentially been observed in the nuclei (2).

As shown by affinity chromatography, ZnBP is able to bind in a Zn²⁺-dependent manner not only to 1-phosphofructokinase but also to other glycolytic and gluconeogenic enzymes, e.g. aldolase, hexokinase/glucokinase, and others (3).

The 1-phosphofructokinase-inactivating property has been located on a tryptic 43-amino acid peptide containing four acidic clusters of glutamic acid and aspartic acid representing two specific and two unspecific zinc binding sites (4). The sequence of this peptide was used to isolate and sequence both a full-length cDNA clone from a rat liver library (5) and the corresponding genomic DNA (6). The cDNA sequence revealed the identity of ZnBP with rat parathymosin-α (7) and showed the presence of the motif PKRQKT, which resembles the nuclear localization signal (NLS) TKKQKT of prothymosin (8) as well as the known "prototype" signal PKKKRKV of SV40 large T-antigen (9).

The injection of parathymosin-α (ZnBP) and prothymosin into Xenopus oocytes resulted in the accumulation of the injected proteins in the nucleus (10). However, it was not excluded in these experiments, whether the relatively small ZnBP (M, = 11,471) may have entered the nucleus simply by diffusion through the nuclear pore and thereafter been retained in this compartment. Therefore, it remained an open question whether the sequence PKRQKT could function as a NLS and actively translocate ZnBP into the nucleus. Fusion of human growth hormone lacking the signal sequence to the N termini of prothymosin and parathymosin-α (ZnBP) resulted in HeLa S3 cells in a nuclear localization of both fusion proteins, supporting the idea that prothymosin and ZnBP are imported into the nucleus in an active manner (11).

To examine the structure of the putative NLS in ZnBP and its influence on the intracellular localization, we have now overexpressed in COS cells wild-type and point-mutated cDNAs with modified or deleted NLS as well as fusion constructs with a segment of calreticulin (12) lacking its ER import sequence. Since the fusion constructs code for a 48-kDa protein, intracellular distribution of the ZnBP fusion protein should no longer be determined by simple diffusion. Using these constructs, we can show that the import of ZnBP into the nucleus requires an active bipartite nuclear targeting sequence. As ZnBP was found in most rat organs (including liver and kidney) to be restricted to the cytosol (2), we have also analyzed by immunofluorescence the localization of endogenous ZnBP in NRK and H35 Reuber hepatoma cells and studied the effect of standard cell culture conditions on the distribution of ZnBP in isolated rat hepatocytes. Surprisingly, ZnBP was found almost exclusively in the nucleus not only in the two permanent cell lines, but also in hepatocytes cultured for 12–24 h, indicating a potential role of ZnBP during proliferation or differentiation.

EXPERIMENTAL PROCEDURES

Materials

All restriction and DNA-modifying enzymes came from Life Technologies, Inc. Taq polymerase was from Cetus Corp., Weiterstadt, Germany. Pfu polymerase was from Stratagene. The expression vector pCMV2 (13) was a kind gift of Dr. Gerald Thiel (Institute for Genetics, University of Cologne, Germany). Rat calreticulin cDNA was a gift of Dr. Birte Sönichsen (this institute). Dihydrofolate reductase DNA was
Bipartite Nuclear Localization Signal in ZnBP

Methods

In vitro Mutagenesis—All mutagenesis reactions were performed using the T7- Geli (Pharmacia LKB) and the M13K07 (Stratagene) vectors. The 936-bp full-length EcoRI cDNA-fragment coding for ZnBP-WT (wild-type) (5) was subcloned into M13mp18, and single-stranded DNA was used as a template to hybridize phosphorylated 24-mer mutagenic oligonucleotides. Polymerization/ligation reactions, MspI/HhaI cuts, and exonuclease III digestion were performed according to the supplier's instructions. The resulting DNA was then transformed into competent Escherichia coli DH5α cells (Stratagene). Mutant plasmids were picked and single-stranded as well as replicative form DNA were prepared. The primary structures of the mutants were verified by sequencing using the Pharmacia 7 Sequencing kit. The mutation ZnBP-D1 was introduced via two-step PCR. The first amplification was performed to introduce the amino acid exchange, and the second was performed to add a C-terminal antigenic c-myc tag. EcoRI inserts of the replicative form DNA as well as the ZnBP-WT EcoRI fragment were subcloned into expression vector pCMV2 (13) and used for transient transfection of COS cells. This vector offers a human cytomegalovirus promoter as well as a SV40 origin of replication. All subcloning and DNA analyzing procedures followed established methods (14).

Fusion Gene Construction—Calcitulin-ZnBP fusion genes were constructed as follows. The 1544-bp EcoRI calcitulin cDNA fragment used in our experiments contains 12 bp of the 5′-nontranslated region, a 51-bp region coding for the ER import sequence (including a BglI site), as well as an acidic cluster and a KDEL coding sequence near the 3′-end. A 1239-bp BglI fragment was used as a template for PCR reactions. Primers were synthesized on an Applied Biosystems 381A synthesizer and designed to delete the BglI import sequence but to reconstitute the 5′-nontranslated region at the N terminus together with an ATG. The N-terminal primer carried in addition SstI and EcoRI sites at its 5′-end. The C-terminal primer excluded the acidic cluster, and contained 5′- and 3′-termini of calcitulin and contained a MluI site, allowing in-frame fusion of the calcitulin PCR product to the N terminus of the ZnBP variants.

The PCR-generated translated regions of the ZnBP variants contain a 5′-MluI site and a 3′-HindIII site for vector ligation. PCR reactions were performed in a Biomed 60 thermocycler using either 2.5 units of Taq polymerase (calcitulin) or 2.5 units of Pfu polymerase (ZnBP) and 0.75 μM concentrations of each primer. Primers were annealed at 60 °C for 1 min, polymerization time was 1.5 min (1 s extend/cycle) at 72 °C, and denaturation occurred at 92 °C for 2 min. 35 cycles were performed in each reaction. Resulting PCR products of 1076 bp (calcitulin) and 324 bp (ZnBPs) in length were analyzed and purified on 0.7%-agarose gels. DNAs were digested with SstI/MluI (calcitulin) or EcoRI/HindIII (ZnBPs).

ZnBP fragments were subcloned into the SstI/HindIII precut pGEM-4Z vector (Promega, Heidelberg, Germany). Recombinant colonies were identified by blue-white color phenotype, and plasmid DNA was restricted analyzed. Plasmid DNA of pGEM-ZnBP was then linearized with SstI and MluI, and calcitulin PCR fragments were subcloned into the vector-ZnBP constructs, resulting in the complete fusion genes. Fusion gene clones were analyzed by Southern hybridization of various restriction fragments, and EcoRI/HindIII fragments were subcloned into pCMV2. To generate the fusion construct CR-ZnBP-D1, the ZnBP fragment of CR-ZnBP-WT was replaced with MluI/HindIII by the two-step PCR product, ZnBP-D1, mentioned above.

Calcitulin dihydrofolate reductase fusion genes were based on three different dihydrofolate reductase PCR products, which all coded for the same part of dihydrofolate reductase (amino acids 2-186). The first contained an EcoRI site at its 5′-end followed by a Kozak sequence (15), an ATG, and restriction sites HindIII and MluI for later introduction of ZnBP variants. Its 3′-end contained the motif PKRKKT, a c-myc tag, and a XbaI site. The second PCR product contained the same 5′-structure as the first one, but it possessed only a BglI site (and no stop codon) at its 3′-end used for fusion with the third PCR product, which contained a 5′-BglI site and shared its 3′-end with the first PCR fragment. This first dihydrofolate reductase PCR product was cloned into pCMV2 directly, resulting in vector pD. The other two were subsequently cloned into pGEM-4Z and thereby fused by the BglI sites. The resulting EcoRI/XbaI fragment (containing a dimeric dihydrofolate reductase fragment) was subcloned into pCMV2, resulting in vector pDD. The HindIII/MluI sites present in the 5′-region of the dihydrofolate reductase inserts in pD and pDD were used to introduce PCR-generated fragments of ZnBP spanning an N-terminal portion (amino acids 1-34, ZnBP-N) or the central acidic cluster (amino acids 35-72, ZnBP-C). In additional constructs, the entire coding region of ZnBP in its variants ZnBP-WT and ZnBP-Thr91 was subcloned into pD and pDD.

All overexpression constructs were sequenced to verify the fidelity of PCR reactions and fusion borders as well; pCMV2 constructs were used for overexpression studies.

Cell Culture and Blotting Techniques—COS cells as well as H35 Reuber hepatoma cells were cultivated in Dulbecco's modified Eagle's medium (Seromed, Berlin, Germany) including 10% FCS. NRK cells were grown in minimum Eagle's medium α (Life Technologies, Inc.) containing 5% FCS (heat-inactivated).

Perfusion of rat livers was performed as described by Kleinecke et al. (16). Fresh hepatocytes were cultivated on collagen-plated coverslips and maintained in medium M199 (Life Technologies, Inc.) containing 15% (w/v) of both penicillin and streptomycin, 10 −5 M dexamethasone, and 10 −6 M porcine insulin. Cells were grown at 37 °C for 45 min, 4 h, or 24 h. Subsequent immunofluorescence studies of all cell lines were performed as described below.

For Northern blot studies, the 936-bp ZnBP cDNA (5) was labeled with 32P using the random priming method (17). Total RNA from fresh hepatocytes as well as from hepatocytes cultivated for 4 and 24 h was isolated as described in Ref. 18. Northern blotting onto GeneScreen plus membranes (Dupont NEN) and hybridization were performed at 42 °C as described in Ref. 14. Hybridized membranes were washed twice in 2 × SSC (5 min), twice in 2 × SSC, 1% SDS at 60 °C (30 min), and twice in 0.1 × SSC at 20 °C (30 min) and exposed to x-ray film for 3-24 h. For Western blotting, transfected cells were analyzed using purified anti-ZnBP. Immunoblots of ZnBP-transfected cells, as well as of rat hepatocyte cell lysates were performed and stained as given in Ref. 2, fusion protein blotting followed standard procedures (14), with additional renaturation at 42 °C in Tris-buffered saline for at least 6 h (2).}

Transient Transfection of COS Cells and Immunofluorescence—Rabbit ZnBP-antibody p42 (2) was affinity-purified on a ZnBP-coupled Sepharose 4B column. DNAs used for transfections were purified on QIAGEN columns (Qiagen, Hilden, Germany) according to the supplier's instructions. About 7 × 105 COS cells were transfected with 1 μg of DNA using the calcium phosphate method (14). 48 h after transfection, cells were analyzed by immunofluorescence.

COS, NRK, and H35 Reuber hepatoma cells as well as rat hepatocytes were fixed in 3% paraformaldehyde and permeabilized with 0.3% Triton X-100. Double immunofluorescence was performed using affinity-purified rabbit anti-ZnBP (diluted 1:50 in phosphate-buffered saline, 10% FCS) or monoclonal mouse anti-SV40 large T-antibody (Dianova, 1:100 in phosphate-buffered saline, 10% FCS) following standard procedures. Goat anti-rabbit fluorescein isothiocyanate conjugate and goat anti-mouse tetramethylrhodamine isothiocyanate conjugate were obtained from Dianova, Hamburg, Germany. A 1:200 dilution in phosphate-buffered saline, 10% FCS. Samples were embedded in Moviol and visualized at emission wavelengths of 519 nm (fluorescein isothiocyanate) and 572 nm (tetramethylrhodamine isothiocyanate), respectively. To visualize ZnBP from rat hepatocytes with peroxidase-coupled goat anti-rabbit antibodies, endogenous peroxidase of permeabilized hepatocytes was inhibited by incubation of cells with 0.5% H2O2 in Tris-buffered saline for 15 min at room temperature. Incubation with the ZnBP-antibody was performed as described above followed by incubation with goat anti-rabbit peroxidase conjugate (diluted 1:2000 in Tris-buffered saline, 10% FCS). The staining reaction was performed with 5 ml of 100 mg/ml citrate (pH 6), 100 μl of N,N-diethyl-p-phenylenediamine (Eastman Kodak Co.) solution (12 mg/ml in acetonitrile/HeO; 4:1), and 500 μl 0.3% (w/v) 4-chloro-1-naphthol (in CH3OH and 2% 30% H2O2) for 30 min at room temperature. The staining was stopped by washing in Tris-buffered saline.

RESULTS

Construction and Overexpression of Mutant cDNAs—Three point mutations (ZnBP-Stop91, ZnBP-Scr92, and ZnBP-Thr91) were introduced into the 936-bp cDNA of ZnBP (see Fig. 1). Mutation ZnBP-Stop91 converts the codon AAG for Lys91 in the PKR92 QKT motif into the stop codon TAG, yielding a protein lacking the last 11 amino acids including the putative NLS.

ZnBP-Scr92 and ZnBP-Thr91 exchange Ser and Thr for the original Arg92 and Lys91, respectively, thereby expressing the
mutated nuclear localization signals PKS92QKT and PT91RQKT.

By Western blotting, it was assured that the affinity-purified anti-ZnBP antibody reacted only with the overexpressed wild-type or mutated ZnBPs (Fig. 2, upper panel), but not with endogenous COS cell proteins (Fig. 2, lower panel, lane E). Using this antibody, ZnBP-WT transiently overexpressed in COS cells was found almost exclusively in the nucleus (Fig. 3A). In contrast, protein expressed from ZnBP-Stop91 was observed not only in the nucleus but also in the cytoplasm (Fig. 3C). Expression products from ZnBP-Ser92 and ZnBP-Thr91 revealed the same intracellular distribution as ZnBP-Stop91, i.e., they gave a prominent nuclear staining with additional fluorescence from the cytoplasm (Figs. 3, E and G). A significant difference between the mutants could not be observed. Intracellular localization of SV40 large T-antigen in the same cells was used as control for intact nuclei. These results indicate that the putative NLS (PKRQKT) had influenced the nuclear localization of ZnBP in COS cells, as mutated and deleted forms of this motif were accompanied by an additional cytoplasmic localization.

Construction and Overexpression of Fusion cDNAs—Since a 11.5-kDa protein is able to diffuse passively into the nucleus (19), we examined the localization of a larger fusion protein containing ZnBP to distinguish between passive diffusion and NLS-dependent active nuclear import. To this end, we overexpressed fusion constructs between a segment of calreticulin (12) and ZnBP, resulting in a fusion protein of approximately 48 kDa (see Fig. 2, lower panel, and Fig. 4). A 1239-bp BglI fragment of a full-length calreticulin cDNA, which lacks part of the ER import sequence, served as template for PCR. The N-terminal primer was designed to reconstitute the 5'-nontranslated region, possibly important for translation efficiency (15), but to delete the complete ER import sequence, thus resembling the N terminus of the mature calreticulin. The C terminus excluded the acidic clusters and the C-terminal ER

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**Fig. 2.** Western blot of wild-type ZnBP and ZnBP mutants (upper panel) and of fusion proteins consisting of parts of calreticulin and wild-type ZnBP or mutated ZnBP (lower panel) in COS cells. Upper panel, lane A, purified ZnBP (0.5 μg); lane B, expressed ZnBP-WT; lane C, expressed ZnBP-Ser92; lane D, expressed ZnBP-Thr91; lane E, ZnBP-Stop91. Lower panel, lane M, M, markers; lane A, expressed CR-ZnBP-WT; lane B, CR-ZnBP-Stop91; lane C, CR-ZnBP-Thr91; lane D, CR-ZnBP-Ser92; lane E, lysis from nontransfected cells. Cells were homogenized as given under "Experimental Procedures," and 50 μg of total protein was separated by SDS-PAGE and immunoblotted. Note that ZnBP displays an apparent molecular mass of 19 kDa in SDS gels (1). Although CR-ZnBP-WT is an expression product from clonal DNA, it appears occasionally as a double band in these blots (lane A).
Fig. 3. Immunofluorescence of COS cells overexpressing constructs ZnBP-WT (A and B), ZnBP-Stop91 (C and D), ZnBP-Ser92 (E and F), and ZnBP-Thr91 (G and H). Double-immunofluorescence was performed using anti ZnBP antiserum (panels A, C, E, and G) and anti-SV40 large T-antigen antiserum (panels B, D, F, and H). All COS cells display SV40 large T-antigen immunoreactivity in their nuclei. Wild-type ZnBP (A) appears almost exclusively in the nucleus, whereas mutant variants (C, E, and G) are detected also in the cytosol.

Fig. 4. Structure and size of calreticulin-ZnBP fusion genes overexpressed in COS cells. Fusion genes with the location of their functional parts are shown in shaded boxes. Functional protein motifs are printed in italics. ZnBP parts of the constructs are printed in boldface italics. The ATG marks the start and an asterisk the stop codon of each open reading frame. The calreticulin lacks the signal sequence as well as the C-terminal acidic clusters and the KDEL motif.

retention signal KDEL and was fused in frame to PCR products of the translated regions of the various ZnBP-DNAs. Fig. 4 illustrates the structure of these fusion DNAs. The resulting four fusion DNAs (designated CR-ZnBP-WT, CR-ZnBP-Stop91, CR-ZnBP-Ser92, and CR-ZnBP-Thr91) were subcloned into pCMV2 and overexpressed in COS cells as described above. Western blot examination of the fusion proteins revealed that the purified anti-ZnBP antibodies were also able to recognize the fusion proteins, which displayed a molecular mass of about 48 kDa (Fig. 2, lower panel). The results of the overexpression of these constructs in COS cells are shown in Fig. 5. Like ZnBP-WT, the CR-ZnBP-WT expression product was found exclusively in the nucleus (Fig. 5A), displaying the same distribution as SV40 large T-antigen (Fig. 5B). In contrast, protein expressed from CR-ZnBP-Stop91 was only detected in the cytoplasm (Fig. 5C). A significant number of the CR-ZnBP-Stop91 transfected cells displayed a weak signal over their nuclei, but this result was obtained only from an overlay of cytoplasm as indicated by laser scan microscopy, which showed that the nuclei were essentially free from immunoreactive antigen (results not shown here). Fusion proteins from CR-ZnBP-Ser92 and CR-ZnBP-Thr91 again showed the same distributions as the CR-ZnBP-Stop91 expression product (Figs. 5, E and G).

Nuclear Retention of ZnBP—Although the results described so far strongly support an active, NLS-mediated import of ZnBP into the nucleus, these experiments do not allow any conclusions to be drawn concerning an interaction of ZnBP with nuclear components. Therefore, we constructed two pCMV2-based vectors (pD and pDD), which overexpressed dihydrofolate reductase (pD) and a dimeric form of dihydrofolate reductase (pDD). The first (pD) should still be able to diffuse into the nucleus, whereas the other (pDD) should be excluded from the nucleus due to its larger molecular mass. The structure of these constructs is given in Fig. 6. The motif PKRQKT as well as a c-myc tag were added to the C terminus to allow for nuclear import and detection of the expressed proteins. The N-terminal restriction sites HindIII and MluI allowed the in-frame subcloning of any PCR-generated ZnBP fragment into pD and pDD. In addition to the complete ZnBP-DNAs (ZnBP-WT and ZnBP-Thr91), resulting in pD-ZnBP-WT, pDD-ZnBP-WT, pD-ZnBP-Thr91, and pDD-ZnBP-Thr91, we introduced PCR products coding only for the central acidic cluster spanning amino acids 35–72 of ZnBP into the nucleus, these experiments do not allow any conclusions to be drawn concerning an interaction of ZnBP with nuclear components. Therefore, we constructed two pCMV2-based vectors (pD and pDD), which overexpressed dihydrofolate reductase (pD) and a dimeric form of dihydrofolate reductase (pDD). The first (pD) should still be able to diffuse into the nucleus, whereas the other (pDD) should be excluded from the nucleus due to its larger molecular mass. The structure of these constructs is given in Fig. 6. The motif PKRQKT as well as a c-myc tag were added to the C terminus to allow for nuclear import and detection of the expressed proteins. The N-terminal restriction sites HindIII and MluI allowed the in-frame subcloning of any PCR-generated ZnBP fragment into pD and pDD. In addition to the complete ZnBP-DNAs (ZnBP-WT and ZnBP-Thr91), resulting in pD-ZnBP-WT, pDD-ZnBP-WT, pD-ZnBP-Thr91, and pDD-ZnBP-Thr91, we introduced PCR products coding only for the N-terminal fragment spanning amino acids 1–34 (pD-ZnBP-N and pDD-ZnBP-N) or for the central acidic cluster spanning amino acids 35–72 of ZnBP (pD-ZnBP-C and pDD-ZnBP-C). Proteins expressed from pD (pD without ZnBP-insert, pD-ZnBP-WT, pD-ZnBP-Thr91, pD-ZnBP-N, and pD-ZnBP-C) had molecular masses of 26.5–38 kDa, enabling them to passively diffuse into the nucleus, whereas proteins expressed from pDD (pDD-ZnBP, pDD-ZnBP-Thr91, pDD-ZnBP-N, and pDD-ZnBP-C) had molecular masses >60 kDa, excluding them from passive diffusion into the nucleus. This concept allows the direct comparison of the nuclear/
cytoplasmic distribution of ZnBP-containing dihydrofolate reductase-ZnBP fusion chimeras with that of control constructs expressing only the dihydrofolate reductase fusion partner. Moreover, it permits the distinction between distribution effects based on active import and effects resulting from nuclear interaction of the expressed proteins. All constructs were overexpressed in COS cells. Expressed proteins were detected by immunofluorescence with an anti-c-myc antibody. As a control, pD without ZnBP insert was also expressed. Results are given in Figs. 7 and 8.

Surprisingly, all proteins expressed from pDD vector (except for pDD-ZnBP-WT) remained in the cytoplasm, despite their C-terminal motif PKRQKT (Fig. 8, B–D). In contrast pDD-ZnBP-WT, expressing wild-type ZnBP in the dihydrofolate reductase environment was found almost exclusively in the nucleus (Fig. 8A), pDD-ZnBP-Thr91, as well as the calreticulin fusion protein of this variant (CR-ZnBP-Thr91, Fig. 5G), remained in the cytoplasm (Fig. 8B). This demonstrates that the motif PKRQKT alone is necessary but not sufficient to function as an active NLS.

The overexpression of the pD constructs gave a clear hint for a nuclear retention of ZnBP (Fig. 7). Protein expressed from pD alone (without ZnBP-sequences but containing the PKRQKT-motif) appeared equally distributed between the cytoplasm and the nucleus (Fig. 7A). Insertion of wild-type ZnBP (pD-ZnBP-WT) resulted in a nearly exclusive nuclear localization due to its active import (Fig. 7B). In contrast to the distribution of products expressed from pD without ZnBP-insert, all other ZnBP inserts (pD-ZnBP-Thr91, pD-ZnBP-N, and pD-ZnBP-C) led to a clear shift of the expressed proteins toward the nucleus (Fig. 7, C–E), although the products of the corresponding pDD constructs had remained in the cytoplasm, indicating the absence of NLS-mediated transport. Therefore, the nuclear import of pD chimeras must have resulted from passive diffusion into the nucleus. The increased nuclear retention of the products of pD-ZnBP-Thr91, pD-ZnBP-N, and pD-ZnBP-C as compared with that of pD (Fig. 7A) points to an interaction of ZnBP-sequences with nuclear components.

ZnBP Contains a Bipartite NLS—The finding that constructs expressed from pDD remain in the cytoplasm despite their motif PKRQKT suggests that this motif alone is not sufficient to direct a protein into the nucleus, although mutations of this motif inhibit active import. ZnBP contains the sequence RKR nine amino acids upstream of this motif. This raised the possibility that this sequence might contribute to a bipartite NLS as it had been found in nucleoplasmin (9) and other nuclear proteins. To analyze the influence of the RKR motif on the intracellular distribution of ZnBP, we constructed an additional mutation of ZnBP, namely ZnBP-Δ1, in which the lysine in the RKR motif is exchanged for an alanine, and a C-terminal c-myc tag was added. The ZnBP-fusion protein of CR-ZnBP-WT was replaced by this mutant. Otherwise, this fusion gene displayed the same structure as the other calreticulin fusion genes (see Fig. 4) and was overexpressed in COS cells. Expressed proteins were detected by the anti-c-myc antibody. CR-ZnBP-Δ1 was restricted to the cytoplasm and did not appear in the nucleus (Fig. 7E). This result was similar to that obtained with CR-ZnBP-Stop91, CR-ZnBP-Thr91, and CR-ZnBP-Ser92, which possess a wild-type RKR-motif but a deleted or mutated PKRQKT-sequence (see Fig. 5) and demonstrates the contribution of the lysine residue in the RKR motif to the function of the NLS of ZnBP. Therefore, the functionally active nuclear import motif of ZnBP is a bipartite NLS.

Localization of Endogenous ZnBP—We have described previously (2) that in most rat organs ZnBP is located mainly in the cytoplasm. However, in the crypts of Lieberkühn of the intestinal mucosa and in the pancreatic duct cells, ZnBP exists mainly in the nuclei (2). This raised the possibility that the intracellular distribution of ZnBP might be somehow related to

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**Fig. 6. Structure and size of dihydrofolate reductase-ZnBP fusion genes overexpressed in COS cells.** Fusion genes with the location of their functional parts are shown in boxes. Functional protein motifs are printed in italics. ZnBP-parts of the constructs are printed in boldface italics. The localization of the motif PKRQKT is indicated directly. The ATG marks the start and an asterisk marks the stop codon of each open reading frame.

**Fig. 7. Immunofluorescence of COS cells overexpressing constructs pD (A), pD-ZnBP-WT (B), pD-ZnBP-Thr91 (C), pD-ZnBP-N (D), and pD-ZnBP-C (E).** In contrast to experiments described in Figs. 3 and 5, expressed proteins were detected using the c-myc antibody. Whereas protein expressed from construct pD, which lacks any ZnBP-sequences, was distributed almost evenly throughout the entire cell (A), addition of parts of ZnBP resulted in a shift in the localization toward the nucleus (B–E). pD-ZnBP-WT appeared almost exclusively in the nucleus (B).
In HeLa S3 cells a nuclear accumulation of ZnBP and prothymosin a was observed (10). Clinton and co-workers (11) demonstrated that ZnBP is located mainly in the cytosol in most cell types. Only after several hours of cultivation under conditions where the hepatocytes tend to dedifferentiate (20), ZnBP remained in the cytoplasm, indicating the necessity of the short basic cluster RKR nine amino acids upstream the PKRQKT motif.

The appearance of ZnBP in the nuclei of cultured hepatocytes was independent of a possible increase in synthesis of ZnBP. As shown in Fig. 8, levels of ZnBP transcripts and of ZnBP slightly decreased after 24 h of cultivation. The authors did not analyze whether the proposed nuclear targeting sequence PKRQKT was active, although the fusion protein theoretically was able to enter the nucleus also with a mutated PKRQKT motif but also fusion proteins containing ZnBP linked to dihydrofolate reductase, but lacking an intact NLS, diffuse into the nucleus inspite of their molecular mass of about 34 kDa. Only by constructing even larger fusion proteins with intact or mutated NLS, we could unequivocally show that the NLS is operative. Our results clearly show that ZnBP represents a small protein, capable of diffusion into the nucleus, but possessing an operative NLS. This is different from histone H1. Histone H1 can potentially diffuse into the nucleus and requires an active uptake mechanism sensitive to chilling and energy depletion (21), but for histone H1 an NLS was shown to be dispensable.

**DISCUSSION**

Although previous studies from this laboratory have revealed that ZnBP is located mainly in the cytosol in most tissues (2), we show here that ZnBP contains a functionally active bipartite NLS. Previous experiments, in which ZnBP had been injected into Xenopus oocytes, had shown the uptake of ZnBP into the nucleus (10). Clinton et al. (11) demonstrated in HeLa S3 cells a nuclear accumulation of ZnBP and prothymosin fused to a truncated form of human growth hormone. These fusion proteins had molecular masses of about 34 kDa. However, the authors did not analyze whether the proposed nuclear targeting sequence PKRQKT was active, although the fusion protein theoretically was able to enter the nucleus also by diffusion. Indeed, we have shown here that not only ZnBP with a mutated PKRQKT motif but also fusion proteins containing ZnBP linked to dihydrofolate reductase, but lacking an intact NLS, diffuse into the nucleus inspite of their molecular mass of about 34 kDa. Only by constructing even larger fusion proteins with intact or mutated NLS, we could unequivocally show that the NLS is operative. Our results clearly show that ZnBP represents a small protein, capable of diffusion into the nucleus, but possessing an operative NLS.
has not been identified up to now. The participation of a yet unknown protein with an NLS carrying histone H1 by “piggyback” transport cannot be excluded.

While the dihydrofolate reductase expressed from pDH is largely found in the cytoplasm and only to a limited extent in the nucleus, the pDH expression products containing additional sequences from ZnBP accumulate much more in the nucleus in spite of the fact that none of these constructs have an active (bipartite) NLS. This indicates that the nuclear/cytoplasmic distribution of ZnBP may be determined not only by passive diffusion or NLS-dependent import but also by nuclear retention, probably resulting from interaction of ZnBP or fragments of ZnBP with nuclear components.

Our results show further that the motif KRK situated nine amino acids upstream from the PKRQKT motif is also involved in nuclear targeting of rat ZnBP. The exchange of the central lysine against an alanine abolished the nuclear uptake of the ZnBP-calreticulin fusion protein in spite of an intact PKRQKT motif. In human ZnBP, one finds the motif LKR instead of KRK (22), which could indicate that either the KR motif or even the lysine alone is sufficient. Bovine ZnBP, on the other hand, contains the sequence LVR (23). If this motif were operative as part of a bipartite NLS, it would be in conflict with our observation that the lysine is required for nuclear import. As a cDNA-derived sequence of bovine ZnBP has not been published up to now, a sequencing error cannot be completely excluded. In any case, the sequences in ZnBP from rat and human would be in line with the “consensus” sequence for a bipartite NLS (24), where the upstream situated part of the NLS is composed of 2–3 basic amino acids (e.g., KR in Xenopus nucleoplasmin or KRK in poly(ADP-ribose)polymerase). The motif TTKQKT interpreted as the NLS of prothymosin (8) may also be only part of a bipartite NLS, as 11 amino acids upstream of this motif a KR motif exists in rat as well as in human prothymosin. ZnBP-calreticulin fusion proteins contained still the calreticulin sequence PPKKIKDPD. This sequence had been interpreted as a potential NLS by Michalak et al. (12). However, fusion proteins containing this sequence did not enter the nucleus unless their ZnBP portion contained the intact NLS of ZnBP. This finding could be of importance considering the recent reports of a transcriptional control exerted by intranuclear calreticulin (25, 26).

We have shown here clearly that the NLS of ZnBP is operational. Why then does ZnBP exist in most tissues mainly in the cytosol? One explanation could be that in differentiated cells, ZnBP normally is bound to other cytoplasmic proteins, which would block the interaction of the nuclear targeting signal of ZnBP with importins (27). The binding of ZnBP to this blocking protein would have to be overcome to allow for the formation of free ZnBP, which then could interact with importins and enter the nucleus. An interaction with such blocking proteins might be abolished by decreasing the affinity of these proteins for the nucleus. An interaction with such blocking proteins might free ZnBP, which then could interact with importins and enter the nucleus. The binding protein(s) or show conditions of decreased binding affinities of such binding protein(s) for ZnBP. The fact that ZnBP has been shown to interact not only with 1-phosphofructokinase but also with several other cytoplasmic enzymes (3) would support the idea that under physiological conditions ZnBP can interact also with other cytoplasmic proteins. This property of ZnBP also makes it necessary to examine the possibility that ZnBP transports proteins lacking an NLS into the nucleus by piggy-back transport.

That the cytoplasmic/nuclear distribution of ZnBP undergoes regulation under in vivo conditions is underlined by our following observations. (a) Rat kidney-derived NRK cells and rat hepatocyte-derived Reuber H 35 hepatoma cells contain their ZnBP almost exclusively in the nucleus, whereas ZnBP in the corresponding differentiated cells exist in the cytoplasm (Red. 2 and see Fig. 9). (b) As in intact liver (2), freshly seeded isolated rat hepatocytes had almost no ZnBP in their nuclei as seen in immunofluorescence experiments. But after 8–24 h of cultivation under standard conditions, almost all ZnBP appeared in the nucleus. These results complete our earlier observation (2), that in contrast to most other tissues, intestinal mucosa cells in the zona germinativa of the crypts of Lieberkühn contain ZnBP mainly in the nucleus.

Under standard culturing conditions, hepatocytes start relatively soon to dedifferentiate (20). Furthermore, it has been reported recently, that in human mammary tumor tissue, the levels of prothymosin as well as those of ZnBP had increased dramatically (29). In view of these observations, we have now begun to examine the question whether and how ZnBP might be involved in processes of cell differentiation and/or proliferation and to identify factors responsible for retention of ZnBP in the cytoplasm of differentiated, nonproliferating cells.

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