Vigilin contains a functional nuclear localisation sequence and is present in both the cytoplasm and the nucleus

S. Kügler[a], A. Grünweller[a], C. Probst[a], M. Klinger[b], P.K. Müller[a], C. Kruse[a,*]

[a]*Institut für Medizinische Molekularbiologie, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany*
[b]*Institut für Anatomie, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany*

Received 14 February 1996

Abstract Vigilin is a member of the KH protein family and contains 14 tandemly arranged potential RNA-binding domains. Between KH domains 2 and 3 we have identified a nuclear localization sequence by cloning this sequence into the NH_2-terminal region of phage T7 RNA polymerase as a reporter protein and by showing its transfer into the nucleus. Furthermore we provide experimental evidence that Vigilin is present both in the nucleus and in the cytoplasm in similar concentrations. These observations support the notion that Vigilin may shuttle between nucleus and cytoplasm presumably in contact with RNA molecules.

Key words: Vigilin; KH domain; Nucleocytoplasmic shuttle; Subcellular localization

1. Introduction

A great number of nucleic acid binding proteins are imported into the nucleus following their synthesis in the cytoplasm. This process is mediated by cytoplasmic proteins which recognize a nuclear localization sequence (NLS) in the transported protein and mediate its translocation through the nuclear pores [1–4]. It has also been observed that the NLS is frequently located adjacent to or overlapping with the nucleic acid binding site [5]. A characteristic RNA-binding motif is the KH domain [6–8], which is generally present in one to a few copies in most of the known members of this protein family, which have been detected either in the nucleus [9–11] or in the cytoplasm [12–15]. Vigilin is a unique member of this group in that it contains 14 KH domains in series, which account for the major part of the 150 kDA protein. Vigilin is an ubiquitous protein and is highly conserved between avian and mammalian species [16–18]. There is also circumstantial evidence that Vigilin is part of a tRNA-binding complex associated with the rough endoplasmic reticulum (rER) [19].

In the present study we have analyzed whether the nuclear localization signal located on a predicted loop region between KH domains 2 and 3 of Vigilin is able to direct a reporter protein (phage T7 RNA polymerase) to the nucleus of eukaryotic cells. The subcellular deposition of this chimeric protein was traced by immunocytochemistry. In concert with immunogold labeling for electronmicrographs and immunoblot analysis of subcellular extracts we show that Vigilin is present in similar amounts in both the nuclear and the cytoplasmic protein pool of HEp-2 cells, indicating a functional involvement in the two compartments.

*Corresponding author. Fax: (49) (451) 500 3637.

2. Material and methods

2.1. Anti-Vigilin antibody

A chicken Vigilin cDNA fragment coding for KH domains 3–7 was expressed as a β-galactosidase fusion protein in *E. coli*. The fusion protein was purified by affinity chromatography and used to immunize rabbits according to standard protocols. The antiserum obtained (FP3) is monoreactive on immunoblots and precipitates human Vigilin in a cell free translation system.

2.2. Cloning of the Vigilin NLS into the NH_2-terminus of phage T7 RNA polymerase

Two 60mer oligonucleotides comprising the sense (5′-aattcgctcgcatcaagaagatttatgaggagaagaaaaagaagactacaaccattgcag-3′) and antisense (5′-aattctgcaatggttgtagtcttcttttttcttctcctcataaatcttcttgatgcgagcg-3′) strand coding for 20 amino acids comprising the Vigilin NLS were annealed to generate *Eco*RI sites on both ends of the resulting double stranded DNA fragment. The pSVNUG-G1-A expression vector [20], coding for 5 vector specific amino acids, the SV40 NLS and amino acids 11–883 of the T7 RNA polymerase was cut with *Eco*RI to remove the SV40 NLS. The gel-purified vector was ligated with the double stranded oligonucleotide, generating either a chimeric T7 RNA polymerase with the Vigilin NLS (clone 'VIG-NLS-T7') or a hydrophobic sequence at its NH_2-terminus (clone 'VIG-REV-T7'), depending on the orientation of the insertion. Identity of the clones was determined by DNA sequencing on both strands.

2.3. Transfection and immunostaining of COS-7 cells (ATCC CRL 1651)

1 μg of each vector was LipofectAmine transfected (3×10^5 cells/transfection) using a commercially available kit (Life Technologies, Eggenstein, FRG) according to the manufacturer's instructions. After 48 h, cells were fixed wih 30% methanol/1.75% formaldehyde for 15 min, washed twice with phosphate buffered saline (PBS) and permeabilized with acetone/H_2O (1:1) for 5 min, with acetone for 5 min and with acetone/H_2O at −20°C for 5 min. Cells were washed again with PBS, and endogenous peroxidase was blocked by a 15 min incubation in 0.5% H_2O_2 in PBS. The anti-T7 RNA polymerase antibody was allowed to bind overnight at room temperature in PBS/0.1% BSA. For staining of cells with the immunoperoxidase staining technique the Vectastain Elite ABC kit (Camon, Wiesbaden, Germany) was used according to the manufacturer's instructions.

2.4. Immunostaining of HEp-2 cells (ATCC CCL 23)

For permeabilization of the cell membrane cells were incubated in methanol for 10 min and subsequently placed in acetone for 1 min at −20°C (fix A). For additional permeabilization of the nuclear membrane we used acetone/H_2O (1:1) for 5 min, acetone for 5 min and acetone/H_2O at −20°C for 5 min (fix B). Cells were stained as described above.

2.5. Preparation of protein fractions from HEp-2 cells

In order to avoid disruption of nuclear complexes, we used a modification of the method originally described by Dignam et al. [21]. 5×10^8 subconfluent HEp-2 cells were washed twice with ice-cold PBS, scraped from their dishes with a cell lifter and pelleted by centrifugation at 4°C, $600\times g$, 5 min. After an additional washing step with PBS the compact cell volume was roughly estimated and 5 vol. ice-cold hypotonic buffer (10 mM HEPES/KOH, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 1 mM DTT, 10 mM benzamidine, 0.5 mM PMSF) were added and incubated for 10 min on ice. Cells were then lysed

SSDI S0014-5793(96)00204-9

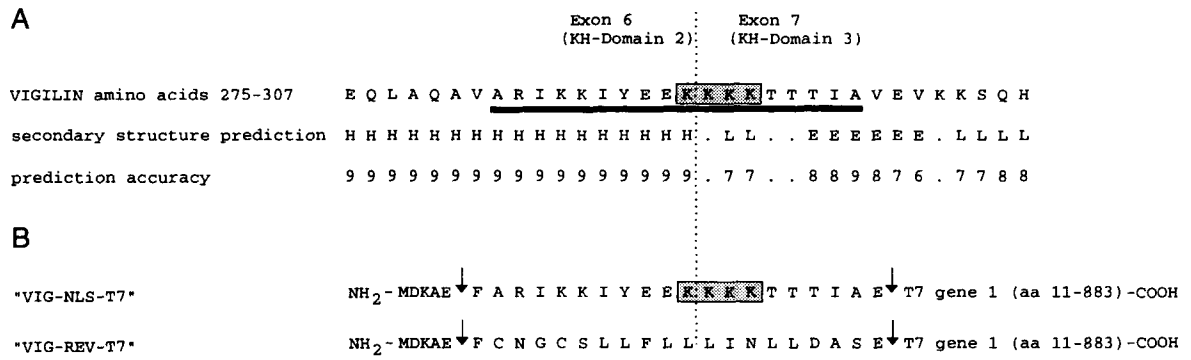

Fig. 1. Location of the NLS in human Vigilin. (A) Amino acids 275–307 of human Vigilin. The NLS is boxed. The secondary structure prediction and the respective prediction accuracies are shown below the sequence (H = α-helical; L = loop; E = extended (β-sheet); · = no prediction with reasonable accuracy). The vertical stippled line demarcates the position of the intron which separates exon 6 (coding for KH domain 2) and exon 7 (coding for KH domain 3). (B) The residues underlined in (A) were inserted into the pSVNU-G1-A expression vector [20], replacing the SV40-NLS of this construct. The amino acid sequence encoded by the two resulting expression constructs is partially shown. Insertion of the double stranded oligonucleotide into the *Eco*RI site (arrows) generated VIG-NLS-T7 (insertion in the correct orientation) and VIG-REV-T7 (insertion in reversed orientation as negative control). The amino terminal residues MDKAE are vector-encoded, the residues F and E at the N- and C-termini of the inserts are generated by the cloning procedure.

by 10 strokes in a pestle-B dounce homogenizer. 0.1 vol. of sucrose buffer (consisting of 9 vol. of 75% sucrose and 1 vol. 10×salt solution (0.5 M HEPES/KOH pH 7.9, 0.1 M KCl, 2 mM EDTA, 10 mM DTT, 7.5 mM spermidine, 1.5 mM spermine)) was added to the suspension and nuclei were pelleted by centrifugation (10 min, 1500×*g*, 4°C). The supernatant was dialysed against dialysis buffer (20 mM HEPES/KOH pH 7.9, 20% glycerine, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) and used as the cytoplasmic protein fraction. The nuclei were resuspended in 1 ml nuclear resuspension buffer (NRB, 20 mM HEPES/KOH pH 7.9, 0.2 mM EDTA, 2 mM EGTA, 25% glycerine, 0.75 mM spermidine, 0.15 mM spermine, 10 mM benzamidine, 0.5 mM PMSF). Nuclei were lysed by stirring 30 min on ice and debris was pelleted by ultracentrifugation (30 min, 117 000×*g*, 4°C). The supernatant was dialyzed as above, precipitated and referred to as the first soluble nuclear protein fraction. The pellet was resuspended in NRB containing 0.4 M $(NH_4)_2SO_4$, stirred and centrifuged as above to precipitate the insoluble nuclear proteins. The supernatant was dialyzed and precipitated. This pool consists of soluble nuclear proteins free of any contamination by membranes of the endoplasmic reticulum. The protein concentration of each fraction was determined according to the method of Bradford [22].

2.6. Electron microscopical studies

Samples of rat pancreas were fixed with glutaraldehyde and reduced osmium [23] and embedded in LR White [24]. Ultrathin sections were mounted on 250-mesh nickel grids, blocked by 0.5% BSA in TBS and incubated with affinity purified FP3 (on Vigilin bound to nitrocellulose membranes), diluted 1:500 in TBS for 18 h at 22°C. For electron optical detection, FP3 was visualized with donkey anti-rabbit IgG, coupled to 12 nM gold (Dianova, Hamburg, Germany). For negative controls, incubations were carried out with FP3 serum which had been preadsorbed on Vigilin.

3. Results and discussion

3.1. Vigilin contains a functional nuclear localization signal

A SV40-type NLS consensus sequence consisting of four consecutive lysyl residues is located between KH domains 2 and 3 of chicken and human Vigilin. Secondary structure prediction using the 'predict protein' EMBL server [25] suggests that the highly charged Vigilin NLS is located on a loop

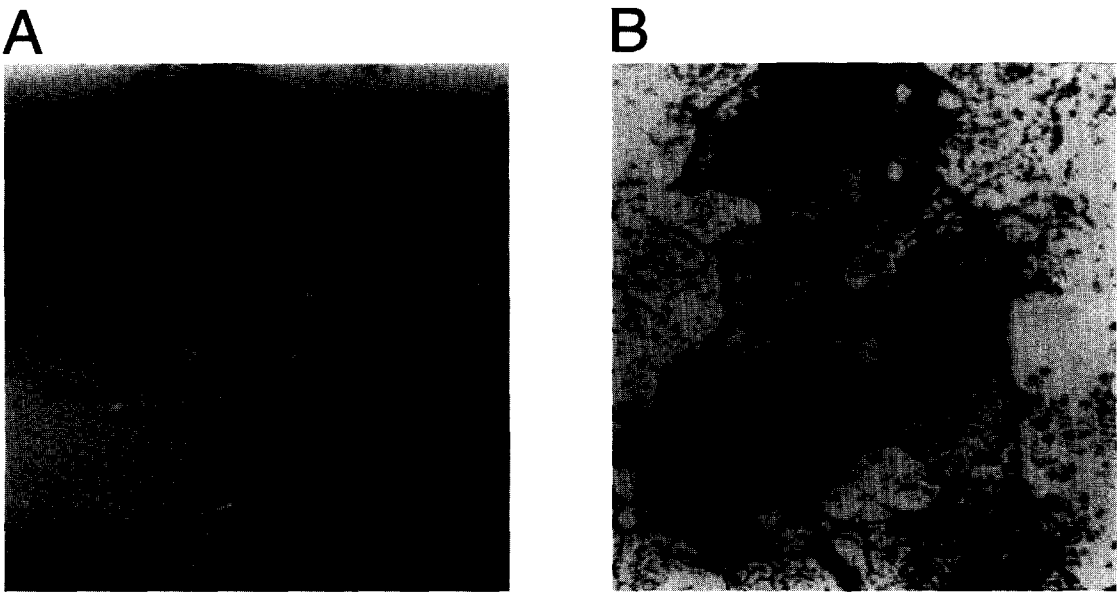

Fig. 2. The Vigilin-NLS as part of the chimeric T7 RNA polymerase. T7 RNA polymerase containing the Vigilin-NLS is directed to the nucleus (A), while the control T7 RNA polymerase containing a hydrophobic stretch of residues in place of the NLS stays in the cytoplasm (B).

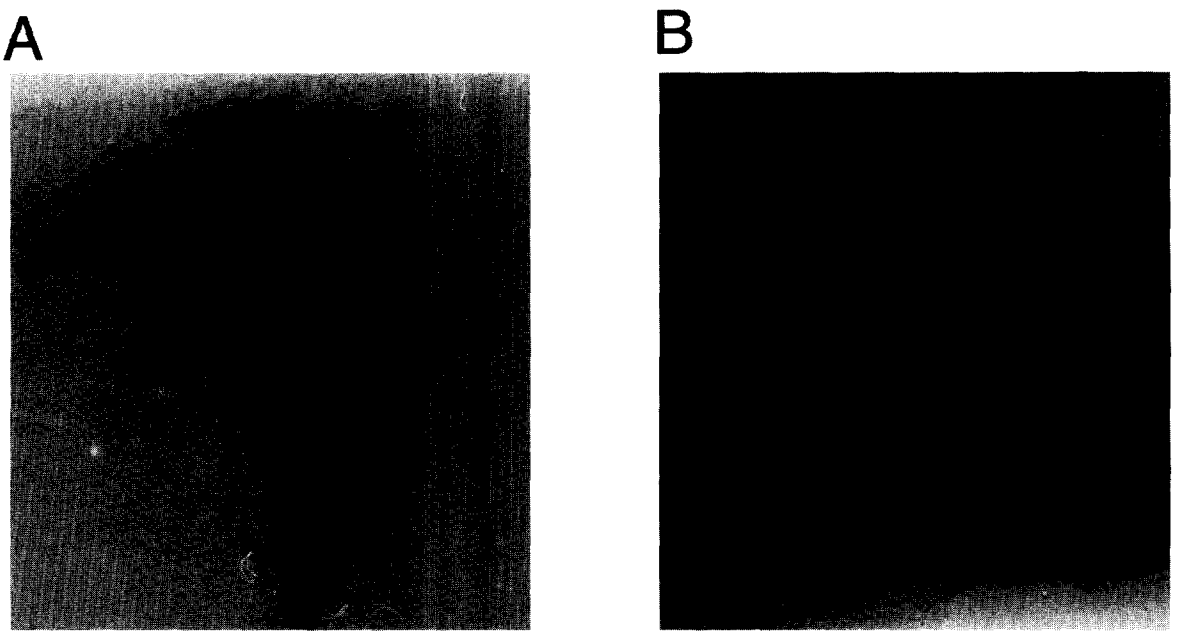

Fig. 3. Vigilin is present both in the cytoplasm and in the nucleus. HEp-2 cells stained with the affinity purified anti-Vigilin antibody (FP3). Permeabilization of plasma membrane only (A) and of plasma and nuclear membranes (B).

between two secondary structures and will thus be accessible to the importins [1,2] (Fig. 1A). This structure prediction (an α-helix at the C-terminal end of domain 2 and a β-strand at the NH_2-terminus of domain 3) is in excellent accordance with NMR data obtained for KH domain 5 of human Vigilin [26]. A functional assay for the putative NLS was performed by inserting a short stretch of amino acids comprising the four lysyl residues into the NH_2-terminus of phage T7 RNA polymerase (Fig. 1B). This reporter protein was chosen because it has previously been shown that it is transported into the nucleus by insertion of the SV40-NLS into its NH_2-terminal region [20]. Tests for compartimental deposition of the chimeric protein were performed using COS-7 cells transfected with expression plasmids encoding either T7 RNA polymerase with the Vigilin-NLS (VIG-NLS-T7) or with a hydrophobic stretch generated by inverse insertion of the same oligonucleotide (VIG-REV-T7) into the expression vector. Fig. 2 clearly shows that the VIG-NLS-T7 construct is directed to the nucleus while the control construct VIG-REV-T7 remains in the cytoplasm. No staining with the anti-T7 RNA polymerase antiserum was observed in mock transfected cells, while a result identical to that shown in Fig. 2A was obtained with

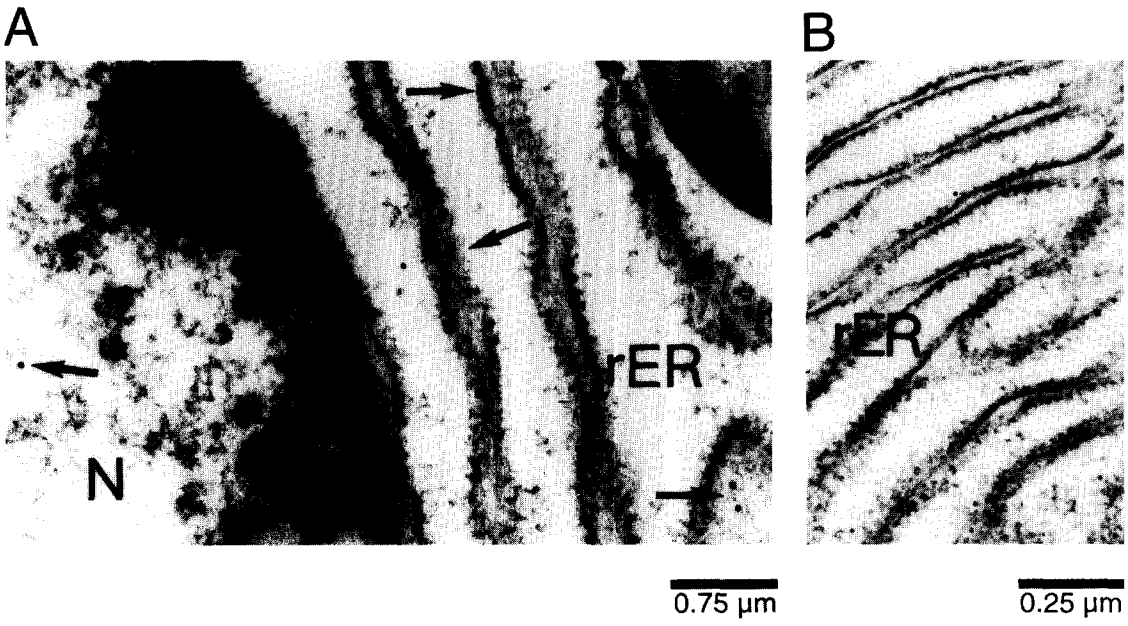

Fig. 4. Subcellular localization of Vigilin by immuno-electron micrography of ultrathin sections of rat liver. (A) Arrows point to gold particles in a region showing parts of the nucleus (N), the rough endoplasmic reticulum (rER) and a mitochondrium (M). (B) Extensive staining of the outer membrane of the rER.

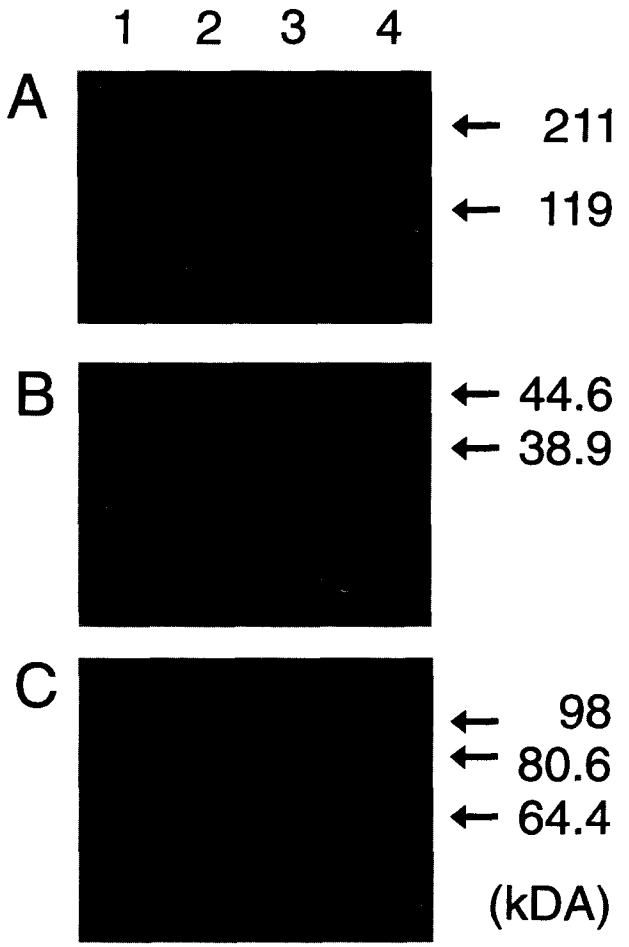

Fig. 5. Immunoblot analysis of protein fractions from HEp-2 cells. Identical amounts of protein from each fraction were loaded on the gels and stained with affinity purified anti-Vigilin antibody FP3 (A); anti-α-SSR antibody (B); and anti-Lamine (type A) antibody (C). Molecular weight markers are shown on the right. Lanes 1: cytoplasmic proteins (20 μg of 21.2 mg total protein from this fraction was loaded on the gels). Lanes 2: first soluble (= low salt) nuclear fraction (20 μg of 5.5 mg). Lanes 3: insoluble nuclear proteins (20 μg of 5 mg). Lanes 4: nuclear proteins soluble in 0.4 M (53%) $(NH_4)_2SO_4$ (20 μg of 9.7 mg).

an expression vector coding for T7 RNA polymerase preceded by the original SV40 NLS (data not shown).

A screening of nuclear KH proteins revealed NLS consensus sequences in Merl [9], in human and *Xenopus* hnRNP K [10] and in the eukaryotic ribosomal S3 proteins, which are imported into the nucleus for ribosomal assembly, but not in Nova [11]. However, Vigilin is the only KH protein with the NLS located adjacent to a putative RNA binding site. As Vigilin consists mainly of 14 putative RNA-binding domains it is possible that Vigilin functions as a transporter protein for RNA from the nucleus to the cytoplasm and one may hypothesize that the binding of RNA adjacent to the NLS might mask the NLS in order to prevent re-import into the nucleus [5]. (The ability of in vitro translated human Vigilin to bind to single stranded ribo-oligonucleotides has been demonstrated; D. Willkomm and S.K., unpublished data.)

3.2. *Vigilin is present both in the cytoplasm and in the nucleus*

When cells are treated so as to allow the anti-Vigilin antibody to penetrate both the cell membrane and the nuclear envelope an even staining of the entire cell is observed (Fig. 3B). In contrast, the nucleus is spared from immunostaining when the plasma membrane only is permeabilized (Fig. 3A). Using immuno-electron microscopy on tissue sections with affinity purified anti-Vigilin antibody FP3, the gold label was found inside the nucleus and at the outer surface of the rER (Fig. 4). Vigilin does not show any subnuclear accumulation, e.g. in the nucleoli. No signals above background were obtained in other cytoplasmic compartments (e.g. mitochondria, lysosomes) in good accordance with organelle fractionation experiments which demonstrated the presence of Vigilin in the microsomal fraction (data not shown) and with the pattern of immunostaining showing a network-like structure in the cytoplasm (Fig. 3).

To explain the cellular distribution pattern of Vigilin one may assume that (i) only a portion of the newly synthesized Vigilin is imported into the nucleus, while another portion is captured by cytoplasmic factors and directed to the rER (note that Vigilin does not contain a signal sequence for insertion into the ER); (ii) all of the newly synthesized Vigilin is imported into the nucleus and the rER fraction represents protein exported from the nucleus. According to present knowledge the latter pathway seems to be more likely since a partial capture would require an unknown factor competing with the importins for Vigilin. Although specific nuclear export signals have been recently identified in a few proteins [27–29], there is also evidence arguing for nuclear export by a default pathway [30,31]. At present experimental evidence is lacking which could show whether Vigilin shuttles continuously across the nuclear envelope like some nucleolar proteins [32], the progesterone receptor [33] or some of the hnRNPs [27], or is stably anchored at the ER after it had passed by the nucleus.

3.3. *Vigilin does not accumulate either in the nucleus or in the cytoplasm during culture of HEp-2 cells*

To confirm the immunocytochemical data which show Vigilin in the nucleus and the cytoplasm (Fig. 3) subfractions of HEp-2 cells were prepared and probed with the anti-Vigilin antibody FP3. Concomitant contaminations of nuclear preparations with endoplasmic membranes were monitored with an antibody against the α-subunit of the signal sequence receptor (α-SSR [34]). Type A lamins were used as nuclear marker proteins. Fig. 5 shows a strong signal for Vigilin in the cytoplasm (Fig. 5A, lane 1) as well as in the first fraction of soluble nuclear proteins, obtained as the supernatant from lysed and pelleted nuclei (Fig. 5A, lane 2). Since both α-SSR and Vigilin are detected in the latter fraction with the same relative signal strength as in the cytoplasm (Fig. 5A,B, lanes 1 and 2), the presence of Vigilin in the first nuclear extract is due to membrane associated protein. A Vigilin containing though membrane free preparation of soluble nuclear proteins was obtained by resuspending the pelleted nuclei in a buffer containing 0.4 M ammonium sulfate (Fig. 5A,B, lane 4), whereas virtually no Vigilin could be detected in the insoluble nuclear protein fraction (Fig. 5A, lane 3). Identical results were obtained with the chicken embryonal fibroblast cell line CEC-32 (data not shown). Apparently, Vigilin can be solubilized from human and chicken nuclei and its nuclear concentration compares well with that in the cytoplasm.

This observation renders Vigilin different from other proteins shuttling between the cytoplasm and the nucleus, which are predominantly nuclear at equilibrium conditions [27,32].

The heat shock related proteins B3 and B4, which are equally concentrated in cytoplasm and nucleus in *Xenopus* oocytes [35], accumulate in the nucleus following heat stress. The mechanisms which govern the subcellular distribution of Vigilin are not known at present.

3.4. Conclusion

In the present study we have demonstrated, for the first time, a functional NLS in a KH protein, Vigilin. The nearness of this NLS to a putative RNA binding domain suggests a functional relationship between the two motifs. Vigilin is also the only KH protein as yet with an unambiguous occurrence in both the cytoplasm and the nucleus, which suggests that it may shuttle between the two compartments. Together with the previous findings that Vigilin is a ubiquitous and highly conserved protein and contains 14 putative RNA binding domains, the presented data suggest a role in RNA transport.

Acknowledgements: We thank Dr. Enno Hartman for providing us with the antibody against α-SSR, Dr. Norbert Radomsky for the anti-Lamine antibody and John J. Dunn and F. William Studier for the T7 RNA polymerase expression vector and the anti-T7 RNA polymerase antibody. We are also grateful to Dr. Roland Hartmann for critical reading of the manuscript and useful comments. The excellent technical assistance of Ms S. Vandersee is gratefully acknowledged. This study was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG Kr-1512/1-1).

References

[1] Görlich, D., Vogel, F., Mills, A.D., Hartmann, E. and Laskey, R.A. (1995) Nature 377, 246–248.
[2] Simos, G. and Hurt, E.L. (1995) FEBS Lett. 369, 107–112.
[3] Melchior, F., Paschal, B., Evans, J. and Gerace, L. (1993) J. Cell. Biol. 123, 1649–1659.
[4] Paschal, B. and Gerace, L. (1995) J. Cell. Biol. 129, 925–937.
[5] LaCasse, E.C. and Lefebvre, Y.A. (1995) Nucleic Acids Res. 23, 1647–1656.
[6] Gibson, T.J., Thompson, J.D. and Heringa, J. (1993) FEBS Lett. 324, 361–366.
[7] Burd, C.G. and Dreyfuss, G. (1994) Science 265, 615–621.
[8] Siomi, H., Choi, M., Siomi, M.C., Nussbaum, R.L. and Dreyfuss, G. (1994) Cell 77, 33–39.
[9] Engebrecht, J. and Roeder, G.S. (1990) Mol. Cell. Biol. 10, 2379–2389.
[10] Siomi, H., Matunis, M.J., Michael, W.M. and Dreyfuss, G. (1993) Nucleic Acids Res. 21, 1193–1198.
[11] Buckanovich, R.J., Posner, J.B. and Darnell, R.B. (1993) Neuron 11, 657–672.
[12] Verheij, C., Bakker, C.E., de Graaff, E., Keulemans, J., Willemsen, R., Verkerk, A.J.M.H., Reuser, A.J.J., Hoogeveen, A.T. and Oostra, B. (1993) Nature 363, 722–724.
[13] Kiledjian, M., Wang, X. and Liebhaber, S.A. (1995) EMBO J. 14, 4357–4364.
[14] Siomi, M.C., Siomi, H., Sauer, W.H., Srinivasan, S., Nussbaum, R.L. and Dreyfuss, G. (1995) EMBO J. 14, 2401–2408.
[15] Wintersberger, U., Kühne, C. and Karwan, A. (1995) Yeast 11, 929–944.
[16] Neu-Yilik, G., Zorbas, H., Gloe, T.R., Raabe, H.M., Hopp-Christensen, T.A. and Müller, P.K. (1993) Eur. J. Biochem. 213, 727–736.
[17] Schmidt, C., Henkel, B., Pöschl, E., Zorbas, H., Purschke, W.G., Gloe, T.R. and Müller, P.K. (1992) Eur. J. Biochem. 206, 625–634.
[18] McKnight, G.L., Reasoner, J., Gilbert, T., Sundquist, K.O., Hokland, B., McKernan, P.A., Champagnes, J., Johnson, C.J., Bailey, M.C., Holly, R., O'Hara, P.J. and Oram, J.F. (1992) J. Biol. Chem. 264, 15936–15942.
[19] Kruse C., Grünweller, A., Notbohm, H., Kügler, S., Willkomm, D.K., Purschke, W. G. and Müller, P.K. (1995) Biol. Chem. Hoppe-Seyler 376, Suppl., 122.
[20] Dunn, J.J., Krippl, B., Bernstein, K.E., Westphal, H. and Studier, F.W. (1988) Gene 68, 259–266.
[21] Dignam, J.D., Lebowitz, R.M. and Roeder, R.G. (1983) Nucleic Acids Res. 11, 1475–1489.
[22] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[23] Tamaki, H. and Yamashina, S. (1994) J. Histochem. Cytochem. 42, 1285–1293.
[24] Klinger, M. (1994) Ann. Anat. 176, 67–73.
[25] Rost, B. and Sander, C. (1994) Proteins 19, 55–72.
[26] Morelli, M.A.G., Stier, G., Gibson, T., Joseph, C., Musco, G., Pastore, A. and Trave, G. (1995) FEBS Lett. 358, 193–189.
[27] Michael, W.M., Choi, M. and Dreyfuss, G. (1995) Cell 83, 415–422.
[28] Wen, W., Meinkoth, J.L., Tsien, R.Y. and Tylor, S.S. (1995) Cell 82, 463–473.
[29] Fischer, U., Huber, J., Boelens, W.C., Mattaj, I.W. and Lührmann, R. (1995) Cell, 475–483.
[30] Schmidt-Zachmann, M.S., Dargemont, C., Kühn, L.C. and Nigg, E.A. (1993) Cell 74, 493–504.
[31] Laskey, R.A. and Dingwall, C. (1993) Cell 74, 585–586.
[32] Borer, R.A., Lehner, C.F., Eppenberger, H.M. and Nigg, E.A. (1989) Cell 56, 379–390.
[33] Guichon-Mantel, A., Lescop, P., Christin-Maitre, S., Loosfelt, H., Perrot-Applanat, M. and Milgrom, E. (1991) EMBO J. 10, 3851–3859.
[34] Görlich, D., Prehn, S., Hartmann, E., Herz, J., Otto, A., Kraft, R., Wiedmann, M., Knespel, S., Dobberstein, B. and Rappoport, T.A. (1990) J. Cell. Biol. 111, 2283–2294.
[35] Mandell, R.B. and Feldherr, L.M. (1990) J. Cell Biol. 111, 1775–1783.