Construction, transfection and production of recombinant vigilin in mammalian expression system

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Abstract

Vigilin is an abundant, highly conserved, ubiquitous protein containing 15 related, but non-identical, K-homolous nucleic acid binding domains. The construction, transfection and production of recombinant vigilin in mammalian expression system were investigated. The whole length of vigilin was amplified by polymerase chain reaction (PCR) and ligated to pCEP-PU vector. The recombinant construct pCEP-PU with vigilin was produced and transfected into Human embryonic kidney cells in a specific culture medium. The secreted recombinant vigilin was purified from the serum-free medium to homogeneity by affinity chromatography. The conditioned and purified media were tested for the presence of vigilin by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with specific antibody. An immunoreactive band with an apparent molecular mass of approximately 140 kDa was detected. Immunofluorescence staining of transfected cells with vigilin demonstrated that recombinant vigilin molecules are localized in the nucleus and cytoplasm. In summary, the purified recombinant vigilin will facilitate future studies that address the structure and function of vigilin.

Keywords: SDS-PAGE / vigilin / Immunoblott / Immunofluorescence/ affinity chromatography

Introduction

Ribonucleoprotein complexes (RNPs) are substrates for RNA export from the nucleus to the cytoplasm (Siomi *et al*, 1994; kim *et al*, 1995) through nuclear pore complexes, an energy-dependent process which is mediated by specific saturable factors (Nandabalan and Roeder, 1995). These findings have stimulated efforts to identify RNA-binding proteins involved in RNA export. A number of RNA-binding motifs has been described in recent years, such as the RNP motif, the arginine-rich motif, the RGG box and the heterogenous nuclear ribonucleoprotein K-homologous (KH) domain (Kiledjian *et al*, 1995). The multi-KH-protein vigilin has a nuclear localization sequence, and has been localized in the nucleus and cytoplasm of a wide variety of cells (Kuegler *et al*, 1996).

Analysis of the vigilin KH-domain structure has suggested that the protein consists of 15 KH domains (Kruse *et al*, 1996), rather than the 14 domains described previously. The occurrence of an additional fifteenth N-terminal KH domain is supported by the observation that the exon/intron boundaries also define the structure of this domain, as observed for the remaining 14 domains (Lang and Fridovich-Keil, 2000). Since the KH-domain sequence motif is highly degenerate, and to avoid confusion with the numbering of the 14 KH domains of vigilin described previously, the vigilin tRNA complex has only been characterized from a cytosolic extract (Kruse *et al*, 1998).

Although vigilin is an ubiquitous protein, it should be emphasized that highest expression is observed in those cells which are known to produce high quantities of protein, such as liver parenchymal cells or pancreatic secretory cells (Plenz *et al*,1993; Arning *et al*, 1996). From the increase of vigilin production in cells and tissues with stimulated translational activity, and owing to the fact that

antivigilin antibodies may inhibit the synthesis of proteins during translation *in vitro* (Weber *et al*,1997; Kruse *et al*, 2000), it is reasonable to assume that vigilin may have a, as yet unknown, function in protein translation. The pesent study was designed to investigate the construction, transfection and production of recombanint vigilin in mammalian expression system as secreted protein.

Materials and Methods

Recombinant expression plasmid of vigilin (pCEP-vigilin plasmid)

Vigilin cDNA was amplified by polymerase chain reaction (Mullis *et al*, 1986) with sense primer and anti-sense primer. The amplification product was inserted into restricted expression vector pCEP-Pu (Kohfeldt *et al.*, 1997).

Transfection and Production of Recombinant vigilin

Human embryonic kidney cells were cultivated in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 0.25 mg/ml G418, 0.1 mg/ml penicillin/streptomycin, and 2 mM L-Glutamine. The pCEP-vigilin plasmid (25 µg) was transfected into cells (one million cells/10 cm^2 culture dish) using a calcium phosphate precipitation method (Chen and Okayama, 1987) and a selection period with 0.5 µg/ml puromycin.

Purification of recombinant vigilin by affinity chromatography

The serum-free cell culture media (three liters) containing recombinant vigilin was concentrated to ~50 ml, and dialyzed. The dialyzate was loaded on a Ni^{2+}-nitrilotriacetate superflow column. After being washed with 30 column vol. of wash buffer [50 mM NaH$_2$PO$_4$ (pH 8.0)/1 M NaCl] and 10 column vol. of wash buffer containing 40 mM imidazole, the recombinant vigilin was eluted in the wash buffer containing 80 mM imidazole.

Western Blot (immunoblot) analysis

Proteins were resolved on sodium dodecyl sulfate (SDS) polyacrylamide gels (Laemmli ,1970) and transferred onto nitrocellulose membrane (Li *et al*, 1993). Membranes were incubated for 2 h in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Triton X-100 (TBST) containing 15 % skim milk, rinsed with TBST, and then incubated over night with polyclonal anti-vigilin antiserum FPIII (1:1000 diluted) in TBST containing 0.1 % bovine serum albumin (BSA). Membranes were washed three times with TBST, incubated with anti-rabbit immunglobulins conjugated with alkaline phosphatase (1:1000 diluted) in TBST including 0.1 % BSA for 1 h at room temperature. After incubation in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM MgCl$_2$ (AP-buffer) for 10 min, protein bands were visualized by a color reaction with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate dissolved in buffer until the bands were clearly visible.

Immunofluorescence staining of transfected cells with vigilin

The transfected human embryonic kidney cells with vigilin were grown in Iscoves medium, supplemented with 10 % fetal-calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.05 mg/ml l-ascorbic acid sodium salt and 2mM glutamine. Under these growth conditions briefly 2-4x10^5 cells were grown on round cover slips (22 mm in diameter) for 48 h, washed twice with phosphate buffered saline (PBS, 137 mM NaCl, 8.5 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 3 mM KCL, pH 7.3), fixed with methanol for 10 min and subsequently with acetone for 1 min at –20 °C. Cells were washed again twice with PBS. As primary antiserum and anti-vigilin antibody FPIII (dilution 1:200 in PBS containing 0.1 % BSA) were applied for one hour at 37 °C. For fluorescence staining of cells a Cy3 conjugated secondary anti-rabbit antiserum (dilution 1:400) combined with an AMCA conjugated secondary anti-human antiserum (dilution 1:100).

Results

Construction and expression of recombinant vigilin

cDNA of vigilin was amplified by polymerase chain reaction (PCR) with sense primer 5'-CATGCCATGGGGA-GTTCCGTTGCAGTTTTGACC-3' and anti-sense primer 5'-GAAGATCTTCGT-TTGGGGCCCCAAGGGAG-3' in the presence of dNTPs and PCR buffer. The PCR amplification products were fractionated by gel electrophoresis using 0.8% agarose gel (Fig. 1). DNA band was visualized under ultraviolet light after staining with ethidium bromide (Fig. 1, lane 2). The NcoI-BglII fragment of the amplification product was inserted into the NcoI-BglII restricted expression vector pCEP-Pu containing the signal peptide sequence BM-40 and a Puromycine resistance gene (Fig. 2) resulting in plasmid pCEP-vigilin. This plasmid encodes 1266 amino acids residues (3800bp) which is identical to the whole length of vigilin. The plasmid pCEP-vigilin was resticted by specific enzymes to control the insert PCR fragment into vector (Fig. 3). The NcoI-BglII restricted plasmid show two bands (Fig. 3A, lane 2) and NheI-NcoI-BglII restricted plasmid show three bands (Fig. 3B and C, lane 2). As negative control without restriction, the plasmid show no band (Fig. 3B, lane 3).

pCEP-vigilin plasmid was transfected into human embryonic kidney cells. The transfected cells were grown to confluence, washed twice with phosphate-buffered saline, and switched to serum-free medium. The media were collected every 48 h, cooled, centrifuged to remove cellular debris.

purification of recombinant vigilin

The purification of recombinant vigilin was started with two liter of conditioned serum-free medium. After concentration and dialysis, the dailyzate was purified by affinity chromatography (Fig. 4). All of contaminating proteins eluted at different positions. The elution profile recorded at 230 nm show elution fractions of vigilin from 1-5 (Fig. 4).

Characterization of recombinant vigilin A-SDS-PAGE and Immunobloting analysis of recombinant vigilin

Before purification, the conditioned serum-free medium was tested for the presence of recombinant vigilin by SDS gel electrophoresis using coomassie blue staining and immunoblotting. Compared with nontransfected controls (Fig. 5A, lane 1), the transfected cells secreted approximately 2 □g /ml of recombinant vigilin with an apparent molecular mass of 140 kDa (Fig. 5A, lane2). The same recombinant fragment was identified in immunoblots with specific antibody against vigilin at the same molecular mass of 140 kDa (Fig. 6, lane2).

After purification, The elution fractions were tested for the presence of recombinant vigilin by SDS gel electropho-resis and stained with coomassie blue staining. About 40 % of started protein was recovered and the final purity of highly purified recombinant vigilin was detected to be more than 95 % (Fig. 5B, lanes 1-4). An immunoreactive band with an apparent molecular mass of approximately 140 kDa (Fig. 6, lane3) was detected, while, no immunreactive product was detected in the preparations from control transfected cells (Fig. 6, lane1).

B- Immunofluorescence staining analysis of recombinant vigilin

Immunofluorescence staining served to study the localization of recombinant vigilin molecules in cytoplasm and nucleus of transfected cells (Fig. 7). Transfected cells were immunostained with antibody against vigilin indicating the localization of vigilin molecules in the cytoplasm and nucleus (Fig.7A). On the other hand, the nontransfected cells with vigilin did not show immunostained (Fig.7B) as a negative control.

Fig. 1   Polymerase chain reaction (PCR) analysis. The PCR   products demonstrated the full vigilin fragment (3800 bp) in lane 2. I kb ladder marker was run in lane 1.
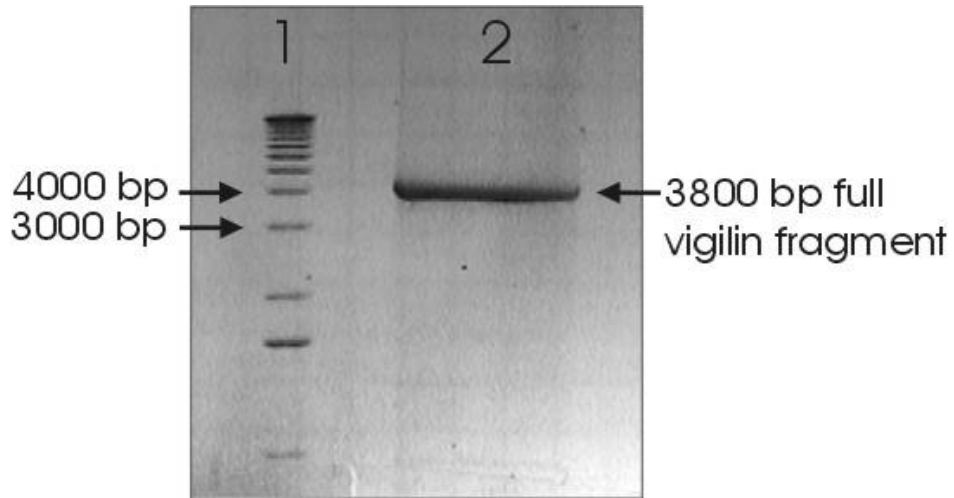

Fig. 2   Construction of recombinant vigilin plasmid. The PCR vigilin fragment was ligated into pCEP-PU vector resulting in pCEP-PU-vigilin plasmid.
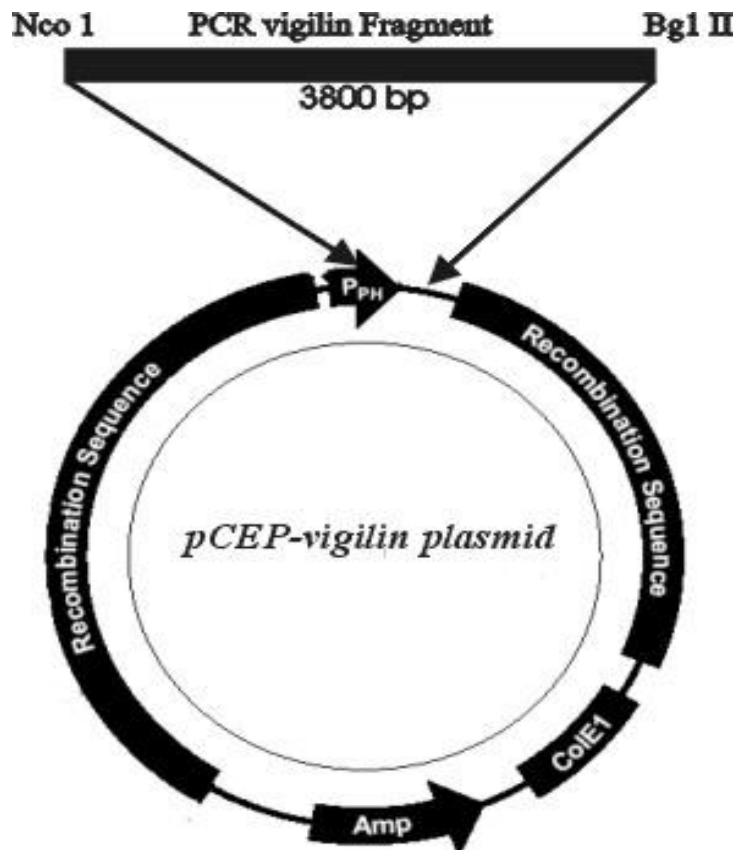
Fig. 3 **The restriction endonucleases digestion of pCEP-PU-vigilin plasmid. Analysis on 0.8% agarose gel, the vigilin plasmid was digested with Nco I- BgI II (panel A; lane2), Nhe I- Nco I- BgI II (panel B; lane2 and panel C; lane2) and negative control without enzymes (panel B; lane3). I kb ladder marker for DNA shown in lane 1.**
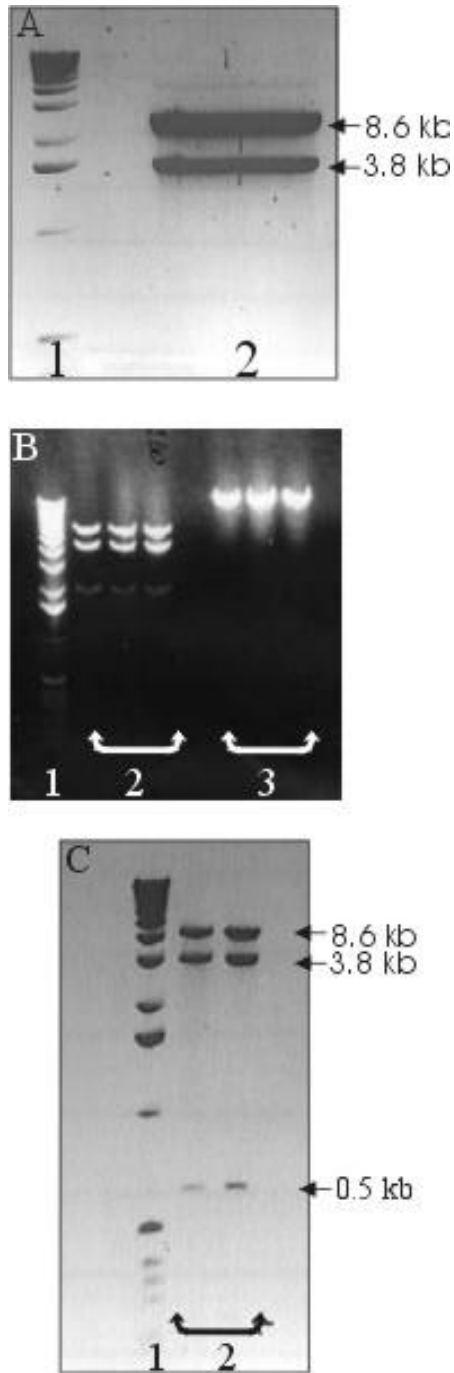
Fig. 4 **Purification of secreted vigilin by affinity chromatography. The elution profile recorded at 230 nm shows the elution fractions of purified vigilin.**
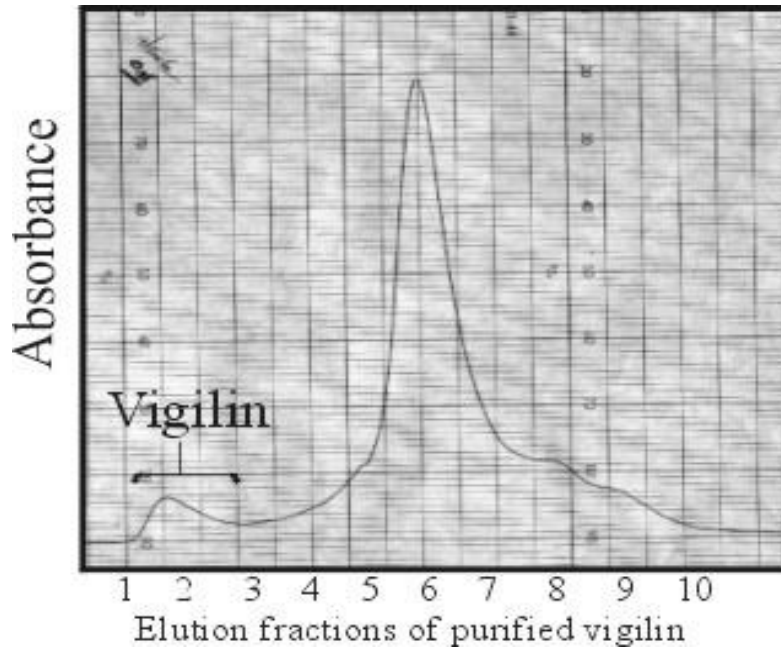

Fig. 5 **SDS- polyacrylamide gel electrophoresis (SDS-PAGE) analysis of recombinant vigilin. The secreted vigilin was purified, separated by SDS-PAGE and stained with Coomassie Blue. A and B, secreted vigilin before and after purification respectively. Control medium from nontransfected cells ((panel A; lane 1) and transfected cells with vigilin (panel A; lane2). Vigilin pattern after affinity chromatography (panel B; lanes 1-4). Positions of globular marker proteins are indicated in kDa.**
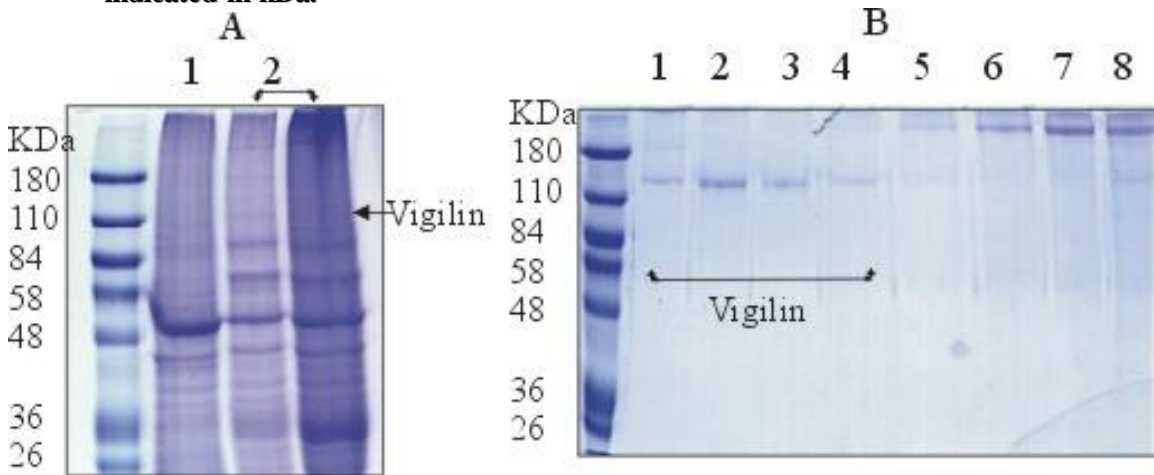
Fig. 6 **Immunoblot analysis of secreted recombinant vigilin. The purified recombinant vigilin separated by SDS gel electrophoresis and electrotransferred to nitrocellulose membranes. The blot was incubated with first antibody against vigilin for 2 hrs and with second antibody for one hour. The conditional free media of secreted vigilin before purification shown in lane 2 and after purification in lane 3. Proteins marker indicated in kDa in lane 1.**
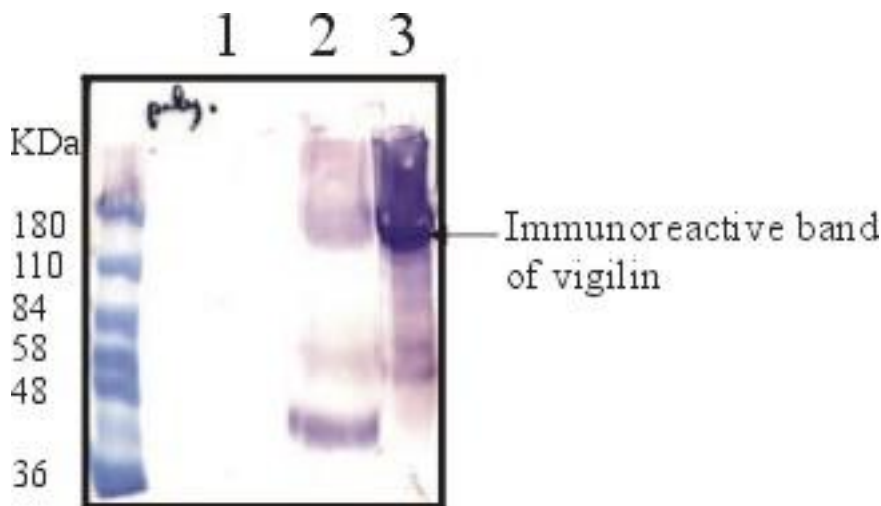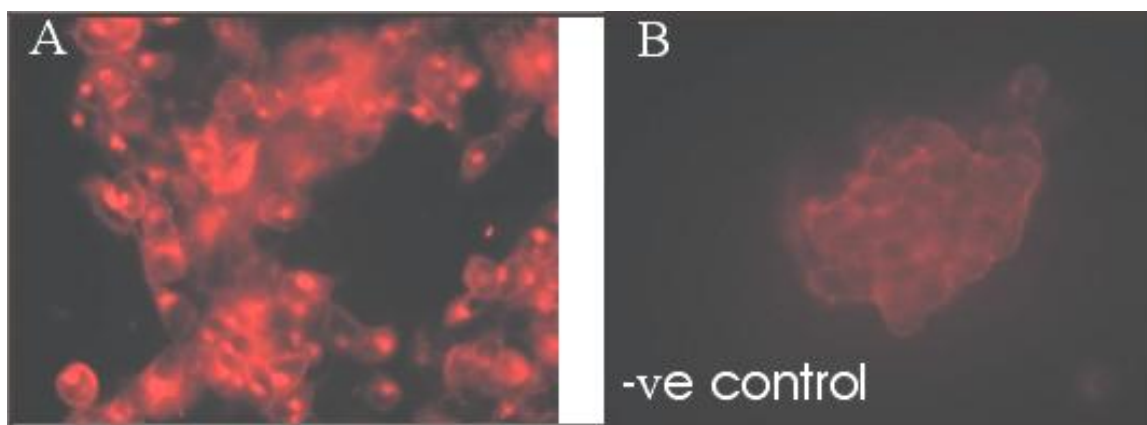

Fig. 7 **Immunofluorescence microscopy of transfected cells with vigilin (A) and non-transfected cells (B) as negative control.**

Discussion

Vigilin, a protein with 15 reiterative putative RNA-binding domains of the KH type (Dodsen and Shapiro, 1997), has been shown to be capable of binding RNA. The observation that vigilin is part of a nuclear as well as a cytoplasmic multi-protein tRNP (Kügler *et al.*, 1996) suggested that one cellular function of the protein might be associated with tRNA binding and/or

export. This idea was supported by the finding that cellular levels of vigilin are always highest under conditions of increased protein synthesis (Mahone *et al.,* 1995), which imply a high demand for tRNA molecules.

Also, the isolated vigilin complexes are composed of a large number of polypeptides, they nevertheless satisfy several criteria of specificity. First, they have been isolated from soluble nuclear and cytoplasmic protein fractions by using

affinity-purified anti-vigilin antibodies, yielding highly reproducible protein patterns with different immuno-affinity matrix preparations and different batches of soluble protein fractions from HEp-2 cells (Schmidt *et al.,* 2002). Secondary, such complexes resist treatment with high salt concentrations (0.8–1.0 M NaCl), which releases the bound tRNA.

The prescnce of l4-1S KH-domains is a distinct structural feature of vigilins since other KH-proteins in general contain only 1-S KH-domains. Also, the presence of KH-domains in such numbcrs as seen in vigilin could bc the reason of a variety at first glance contradictory interaction profiles with different RNA species (Weber et al., 1997). Looking at the motif alignment in vigilin and other KH-proteins shows evident that the tight consecutive arrangement of the 14/I S KH-domains is exceptional as is the localization of both the NLS and NES at unique sitcs within the KH-domains.

A central question is concerned with which protein components of the cytoplasmic and nuclear vigilin-containing tRNPs physically interact with tRNA. Vigilin itself may, in part, fulfil this role, owing to its tandemly repeated KH domains, which have been shown to bind RNA in other proteins. This question was addressed by analysing the binding of radioactive vigilin translated *in vitro* to biotinylated tRNA immobilized on streptavidin-coated magnetic beads (Dodson and Shapiro, 1997). Specificity of tRNA-binding is suggested by competition experiments, since the presence of excess free tRNA over immobilized tRNA inhibited reproducibly vigilin binding to the immobilized tRNA. Thus , the evidence that vigilin may be able to bind a cellular RNA, namely tRNA, which is in line with structural investigations (Draper *et al.*, 2001) which predict an RNA-binding surface on the vigilin KH domain. However, these results do not rule out the possibility that vigilin and other components of our immunoafinity-purified vigilin complexes may interact with tRNA (Kruse *et al.,* 2000). The prominent 140 kDa protein band found to be enriched in both the cytoplasmic and the nuclear vigilin complexes. In conclusion, These experimental strategy of expressing the vigilin as a secreted recombinant protein should facilitate future studies that address the structure and function of this protein.

Acknowledgements

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إنشاء وعدوى وانتاج الفيجيلين المعاد صياغته (المأشوب)

فى أوساط زراعة الأنسجة فى الثدييات

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الفيجيلين هو أحد البروتينات النووية للخلية ؛ يتكون من 15 جزئ من الاحماض النووية المتحدة ببعضها والغير متماثلة فى التركيب حيث تتواجد بوفرة مع البروتينات عالية التسكر . والبحث موضوع الدراسة يتعلق بإنشاء، عدوى وانتاج الفيجيلين المعاد صياغتة فى أوساط زراعة الانسجة فى الثدييات.

وقد تم تضخيم التتابع الكلى للأحماض الأمينية للفيجيلين بإستخدام سلسلة تفاعل أنزيم البلمرة ، ثم بعد ذلك التحام هذه الوحدات من الحمض النووى فى ناقلات الكلونة.

ثم بعد ذلك تم إنتاج هذا البروتين المعاد صياغتة فى خلايا كلوية جنينيه وذلك بنقل بلاذميد مأشوب لهذا النوع من الاحماض النووية. وتم تنقيته من الوسط البروتيني المفرز من تلك الخلايا المحورة وذلك بواسطة طريقة القابلة الكروماتوجراف وقد تم الحصول على بروتين عالي النقاوة.

وقد تم التعرف على هذا البروتين المعاد صياغته بواسطة تقنيات الفصل الكهربائي الجيلاتينى والوسترن بلوت. وأسفرت هذه التقنيات عن أن الوزن الجزيئ لهذا البروتين حوالى 140 كيلودالتون. وبإستخدام تقنية الصبغ المناعى الفلوريسنسى للخلايا الكلوية المحورة المزروع بها الفيجيلين وجد أن جزيئات هذا البروتين متمركزة فى السيتوبلازم والنواة لهذه الخلايا.

ومن خلاصة ما سبق، فقد تم الحصول على الفيجيلين المعاد صياغتة (المأشوب) فى صورة نقية مما يسهل الدراسات المستقبلة علية من حيث التركيب والوظيفة.