Vigilin, a Ubiquitous Protein with 14 K Homology Domains, Is the Estrogen-inducible Vitellogenin mRNA 3′-Untranslated Region-binding Protein*

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Robin E. Dodson and David J. Shapiro‡
From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

RNA-binding proteins containing KH domains are widely distributed. One KH domain protein of unknown function, vigilin (also known as the high density lipoprotein-binding protein), contains 14 KH domains and is ubiquitous in vertebrate cells. We previously used RNA gel mobility shift assays to describe an estrogen-inducible protein which binds specifically to a segment of the 3′-untranslated region (3′-UTR) of vitellogenin mRNA, an area which has been implicated in the estrogen-mediated stabilization of vitellogenin mRNA. Here we show that the vitellogenin mRNA-binding protein (VitRNABP) is vigilin. The VitRNABP was isolated as a 150–155-kDa protein on a vitellogenin mRNA 3′-UTR affinity column. Peptide microsequencing revealed that the purified protein was vigilin, a conclusion confirmed in Western blot analysis with antibodies to vigilin. Direct confirmation that vigilin is the VitRNABP was obtained from RNA gel mobility shift assays which demonstrated that antibodies to chicken vigilin supershifted the Xenopus VitRNABP band. Xenopus liver vigilin mRNA and the VitRNABP exhibited similar induction by estrogen, providing additional confirmation that vigilin is the estrogen-inducible protein which binds to the 3′-UTR of estrogen-stabilized vitellogenin mRNA. These data support a role for vigilin in the hormonal control of mRNA metabolism.

Post-transcriptional events including nuclear RNA processing and export, localization of RNA within the cell, degradation and stabilization of RNAs, and mRNA translation are increasingly seen as important cellular regulatory sites, whose alteration can contribute to disease states. These processes are largely mediated by RNA-binding proteins (1). One important and widely distributed class of RNA-binding proteins contains K homology (KH) domains (2). This class of proteins includes such biologically significant KH domain-containing proteins as the FMR protein, which is involved in Fragile X mental retardation (3), the Drosophila bicaudal C protein which is important in development (4), and the α-antityp(C)-binding protein which is found in the α-globin mRNA ribonucleoprotein complex (5). One notable but little understood KH domain protein is vigilin (also identified as the human high density lipoprotein-binding protein, HDL-BP), a ubiquitous, highly conserved protein with 14 KH domains (6, 7). Since vigilin has been found in all cell lines and tissues examined (8), it likely plays an important role in RNA metabolism. While vigilin has been used as a model protein in solving the structure of a KH domain uncomplexed to RNA (12), its RNA binding properties have been elusive, and its function(s) have remained obscure.

We have been studying a protein which binds to a segment of the 3′-untranslated region (3′-UTR) of the mRNA encoding the egg yolk precursor protein, vitellogenin. In male Xenopus liver, vitellogenin mRNA levels increase >10,000-fold following estrogen administration (13, 14). The estrogen-mediated induction of vitellogenin mRNA is brought about both by an increase in the rate of vitellogenin gene transcription and by stabilization of cytoplasmic vitellogenin mRNA (13, 14). Hepatic vitellogenin mRNA is degraded with a half-life of 16 h in the absence of estrogen, and 500 h following addition of estrogen to the culture medium (14). The estrogen-mediated stabilization of vitellogenin mRNA requires association of the mRNA with ribosomes (15) and involves the 3′-UTR of the mRNA (16). We identified a protein which binds specifically to a segment of the 3′-UTR of vitellogenin mRNA in an estrogen-inducible manner and named it the vitellogenin mRNA 3′-UTR-binding protein (VitRNABP) (17, 18). Here we describe the isolation of the VitRNABP and demonstrate by several criteria that this protein is Xenopus vigilin.

EXPERIMENTAL PROCEDURES

Protein Isolation, Sequencing, and FPLC—A protein mixture from salt-extracted polysomes from livers of estrogen-treated male Xenopus laevis was prepared as we have described (17). The pB1-15pA used in affinity chromatography was made by cloning the HindIII-DraI fragment of pB1-15 (17) into the HindIII-SmaI site of pSP64pA (Promega). B1-15pA RNA was made by in vitro transcription from the SP6 promoter of the pB1-15pA vector linearized with EcoRI yielding a 146-nucleotide RNA. Poly(U)-agarose beads (Pharmacia Biotech Inc.) were prepared by rinsing (i) once in 4 volumes of water, (ii) once in wash buffer (25 mM HEPES, pH 7.4, 10 mM EDTA, 1 mM EGTA, 0.05 mM NaCl), and (iii) six times in binding buffer (25 mM HEPES, pH 7.4, 10 mM EDTA, 1 mM EGTA, 1 mm LiCl). pB1-15pA RNA (approximately 4 μg of RNA/μl of beads) was added to the beads in 2 volumes of binding buffer and allowed to hybridize for 5–6 h at room temperature. The beads were kept in suspension by rotation. The beads were washed twice in hybridization buffer (HB) (6 mM Tris, pH 7.6, 6% glycerol, 1 mM EDTA, 0.01 mM EGTA, 0.25 mM magnesium acetate) with 50 mM NaCl and once in HB with 50 mM NaCl containing 1 μg/μl yeast tRNA, 1 μg/μl heparin, 0.3 unit/μl RNasin, and protease inhibitors (17). The beads were then incubated with the polysome extract in HB with TRNA, heparin, RNasin, and protease inhibitors with 200 μg of polysome BSA, bovine serum albumin; VitRNABP, vitellogenin mRNA-binding protein; HDL-BP, high density lipoprotein-binding protein; UTR, untranslated region; PCR, polymerase chain reaction; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.
Extraction of RNA and preparation of poly(U)-agarose was performed as described (22). RNA was resolved on a 0.75% formaldehyde-agarose gel, transferred to a Nytran membrane (Schleicher and Schuell) and hybridized to cDNA probes as described (23). cDNA was radiolabeled with [³²P]dCTP by nick translation or random priming using standard methods (20). Ribosomal RNA band intensity was used to control for loading of samples. Intensities of labeled bands were determined using a PhosphorImager (Molecular Dynamics) or a densitometer.

**RESULTS**

**Isolation of the Vitellogenin 3'-UTR RNA-binding Protein**—We described the VitRNABP as an estrogen-inducible protein which bound to a segment of the vitellogenin mRNA 3'-UTR (6). To confirm that the VitRNABP is associated with the 150–155-kDa protein band, we examined the distribution of vitellogenin mRNA 3'-UTR in RNA gel mobility shift assays and in UV cross-linking experiments (17). We used RNA affinity chromatography to isolate the VitRNABP. A 94-nucleotide segment of the vitellogenin mRNA 3'-UTR with a 30-nucleotide poly(A) tail, which is efficiently bound by the VitRNABP, was immobilized on poly(U)-agarose beads by hybridization. A salt extract of polysomes prepared from the livers of 14-day estrogen-treated X. laevis was incubated with the RNA affinity column, and control extracts were incubated with the poly(U)-agarose. Bound proteins were eluted in 500 mM salt and resolved on an SDS-PAGE gel. As shown in Fig. 1A, a predominant protein band of approximately 150–155 kDa, which appeared as a very closely spaced doublet, was eluted from the agarose beads containing the vitellogenin mRNA segment (Fig. 1A, lane 2), but not from control poly(U)-agarose beads (Fig. 1A, lane 3). RNA binding activity and the presence of the 150–155-kDa band were well correlated since the eluate from the B1-15pA RNA beads, but not the eluate from the poly(U) beads, bound RNA in the RNA gel mobility shift assay (data not shown).

We next determined whether the vitellogenin mRNA 3'-UTR binding activity was in the same 150–155-kDa molecular mass range as the protein purified by the RNA affinity column. Since we have been unable to reconstitute mRNA binding after denaturing the protein, we size-fractionated a concentrated polysome extract under native conditions. Proteins eluted from the FPLC sizing column were assayed using the RNA gel mobility shift assay. As shown in Fig. 2, peak binding activity corresponded to a molecular mass range of 145–190 kDa, which was consistent with the 150–155-kDa protein being the VitRNABP.

**Identification of the 150–155-kDa Band as Vitiligin by Microsequencing and Western Blot Analysis**—The 150–155-kDa protein purified by the RNA affinity column was enriched by RNA affinity chromatography (Fig. 1A). The B1-15pA RNA in the RNA gel mobility shift assay (data not shown).

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**Identification of the 150–155-kDa Band as Vitiligin by Microsequencing and Western Blot Analysis**—The 150–155-kDa protein was purified by RNA affinity chromatography (Fig. 1A), size-fractionated on an SDS-polyacrylamide gel, transferred to a membrane, and submitted for microsequencing. Internal sequencing of one of the peptide fragments yielded the sequence; Trp/Ser/Thr/QIR had a 5- out of 6-amino acid match with both the chicken vitellogenin sequence (GenBank™ accession number X65292) (6). Since vigilant and HDL-BP are the independently identified avian and human forms of the same protein, we subse-
Vigilin Binds to the Vitellogenin mRNA 3′-UTR

and human sequences, confirming that the protein sequenced was vigilin.

To ensure that the isolated protein was *Xenopus* vigilin, a Western blot of the protein from the RNA affinity beads was probed with antibodies to a segment of recombinant chicken vigilin, expressed in *Escherichia coli* and affinity-purified (26). In Western blots using crude polysome extracts, the antibody reacts with a single protein (Fig. 1B, lane 1) which is the same size as the protein shown to be vigilin by microsequencing. The protein eluted from the B1-15Pa affinity beads is also specifically recognized by the antibody (Fig. 1B, lane 2) and on shorter exposures can be seen to be a doublet (data not shown). Although this is a polyclonal antibody, in agreement with previous reports (26), we see no cross-reactivity with other proteins. Since the elution pattern was identical in both the SDS-PAGE gels and Western blots, we were confident that the isolated protein was indeed vigilin.

**Antiserum to Chicken Vigilin Binds to the VitRNABP**—While these data demonstrated that the protein purified by affinity chromatography was vigilin, and that vigilin was approximately the same size as the VitRNABP, it was still important to directly establish that the protein bound to the vitellogenin mRNA 3′-UTR in our gel mobility shift assays was vigilin. If the VitRNABP was indeed *Xenopus* vigilin, antibodies to vigilin added to the RNA gel mobility shift assays should alter the mobility of the VitRNABP-RNA complex. In an RNA gel mobility shift assay, the VitRNABP in a polysome extract formed a specific complex with a radiolabeled probe containing a segment of the vitellogenin mRNA 3′-UTR (Fig. 3A, lane 2). Addition of antibody to vigilin supershifted this complex (Fig. 3A, lane 4). Since this is a polyclonal antibody, the number of antibody molecules that can bind to each gel-shifted complex is variable, and some tailing of the supershifted band is observed (Fig. 3A, lane 4 and Fig. 3B, lane 2). This supershift was specific for anti-vigilin, since it did not occur when a control antibody to bovine serum albumin was used (Fig. 3A, lane 6). Control experiments also showed that the antibodies alone did not interact with the RNA probe (Fig. 3A, lanes 3 and 5).

**Vigilin mRNA Is Induced by Estrogen in Xenopus Liver**—Since we had previously reported that the VitRNABP was estrogen-inducible (17), we wanted to determine whether *Xenopus* vigilin mRNA was also regulated by estrogen. We therefore isolated and sequenced a 980-nucleotide *Xenopus* vigilin cDNA clone (see “**Experimental Procedures**”) with a high homology to sequences at the 5′-end of chicken and human vigilin. The *Xenopus* vigilin cDNA showed a 74% and 76% nucleotide identity with the chicken and human vigilin cDNAs, respectively, and an 86% identity to amino acid sequences from both species.

To compare the regulation of *Xenopus* vigilin mRNA levels and vitellogenin mRNA 3′-UTR binding activity, three animals each were treated with vehicle or estrogen, total RNA was isolated from half the liver, and whole cell protein extracts were prepared from the other half. Consistent with our earlier work (17, 18), binding activity in RNA gel shift assays from liver extracts from estrogen-induced *Xenopus* liver was about 3-fold higher than in control extracts (Fig. 4B). In agreement with the RNA gel shift data, Northern blot analysis showed that a predominant 5.7-kilobase vigilin mRNA was induced 2–3-fold after estrogen administration (Fig. 4, A and B).

**DISCUSSION**

Its broad distribution and the presence of 14 KH domains suggested an important, but unidentified, role for vigilin in RNA metabolism. In this work we show that vigilin is the estrogen-inducible protein we previously demonstrated binds to a segment of the vitellogenin mRNA 3′-UTR. This conclusion is supported by our findings that vigilin is retained on a vitellogenin mRNA 3′-UTR affinity column; in gel shift assays, vigilin antiserum binds to the VitRNABP; the mRNA binding

**Fig. 2.** Size fractionation of the vitellogenin mRNA 3′-UTR-binding protein. Proteins from a salt extract of polysomes were separated by size exclusion chromatography on an FPLC. Fractions were assayed using the RNA gel mobility shift assay, and binding was quantified from autoradiograms using densitometry as described under "Experimental Procedures." A plot of relative binding versus eluant volume is shown by solid circles. The elution volumes of molecular weight standards run on the same column are plotted as open circles on a log scale, and the regression line is shown.

**Fig. 3.** Antiserum to chicken vigilin supershifts the VitRNABP-RNA complex in a gel mobility shift assay. A, radiolabeled B1-15 RNA containing a portion of the vitellogenin mRNA 3′-UTR was incubated without protein (PROBE) or hybridized with a salt extract of polysomes (SEP) in the absence of antiserum (CONTROL) or in the presence of antiserum (42 μg) to chicken vigilin (cVIG Ab) or BSA (BSA Ab). Uncomplexed probe is indicated by an arrow at a. The complex formed by VitRNABP and the RNA probe is indicated at b. The supershifted band consisting of VitRNABP, probe, and antiserum is indicated at c. B, an RNA gel mobility shift assay of SEP alone (SEP) and SEP and antiserum (84 μg) to vigilin (SEP + cVigAB) shows an almost complete supershift of band b to band c.
Vigilin Binds to the Vitellogenin mRNA 3′-UTR

In conclusion, we have identified vigilin, a widely distributed protein containing 14 RNA binding domains, as the estrogen-regulated protein which binds to the 3′-UTR of vitellogenin mRNA. Our data provide the first evidence that vigilin can bind a specific mRNA and suggest a role for vigilin in mRNA metabolism.

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REFERENCES

[References list is not included in the natural text.]

Our findings are consistent with reports in several experimental models that vigilin can be regulated by several factors and that increased levels of vigilin correlate with increased protein production (8, 9, 11, 30). Vigilin has been localized immunocytochemically to polysomes (8), and we have extracted vigilin from liver polysomes indicating its association with cytoplasmic RNAs. In a situation such as the estrogen-induced liver in Xenopus, where vitellogenin is 50% of the total cellular mRNA (31), a substantial fraction of intracellular vigilin may be bound to the relatively high affinity binding site in the vitellogenin mRNA 3′-UTR. Our findings are also in agreement with the ubiquitous distribution of vigilin, since we have found vigilin in all Xenopus tissues we have examined and in several cell lines (18).

activity and vigilin are approximately the same size; and both are induced by estrogen. We now refer to the VitRNPB as vigilin.

fig. 4. Vigilin mRNA is inducible by estrogen in Xenopus liver. A: Northern blot analysis of total RNA from livers of Xenopus treated with vehicle (−) or with estradiol (+) and probed with vigilin cDNA. B: comparison of the induction of vigilin mRNA and vitellogenin mRNA 3′-UTR binding activity from livers of Xenopus treated with vehicle (−) or estrogen (+). RNA levels (hatched bars) were determined by Northern analysis as described under “Experimental Procedures.” Relative binding activity of vigilin in liver extracts (solid bars) were determined from RNA gel mobility shift assays. Gel-shifted bands were quantitated using a PhosphorImager. The data represent the average from three animals ± S.E.
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