Structural analysis of new local features in SECIS RNA hairpins

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ABSTRACT

Decoding of the UGA selenocysteine codon for selenoprotein translation requires the SECIS element, a stem–loop motif in the 3′-UTR of the mRNA carrying short or large apical loops. In previous structural studies, we derived a secondary structure model for SECIS RNAs with short apical loops. Work from others proposed that intra-apical loop base pairing can occur in those SECIS that possess large apical loops, yielding form 2 SECIS versus the form 1 with short loops. In this work, SECIS elements arising from eight different selenoprotein mRNAs were assayed by enzymatic and/or chemical probing showing that seven can adopt form 2. Further, database searches led to the discovery in drosophila and zebrafish of SECIS elements in the selenophosphate synthetase 2, type 1 deiodinase and SelW mRNAs. Alignment of SECIS sequences not only highlighted the predominance of form 2 but also made it possible to classify the SECIS elements according to the type of selenoprotein mRNA they belong to. Interestingly, the alignment revealed that an unpaired adenine, previously thought to be invariant, is replaced by a guanine in four SECIS elements. Tested in vivo, neither the A to G nor the A to U changes at this position greatly affected the activity while the most detrimental effect was provided by a C. The putative contribution of the various SECIS motifs to function and ligand binding is discussed.

INTRODUCTION

Selenocysteine is the major biological form of selenium. Only bacteria, archaea and animals can co-translationally incorporate it into selenoproteins at specific positions. Such a translational mechanism requires the participation of a complex molecular machinery, in particular for designating an internal, in-frame UGA as the selenocysteine-encoding and not a stop codon (for reviews see 1–4). The mechanism leading to selenocysteine biosynthesis and incorporation into selenoproteins has been unravelled in Escherichia coli (3). The general translation elongation factor EF-Tu does not intervene in this process where it is replaced by the specialized translation factor SELB. Indeed, the SELB/GTP/Sec-tRNASec complex binds to a stem–loop structure adjacent to the UGA selenocysteine codon in the mRNA, forming a quaternary complex that directs the charged tRNA3Sec to the A site of the ribosome. In eukaryotes, a hairpin structure called SECIS (SElenoCysteine Insertion Sequence) resides in the 3′-UTR of selenoprotein mRNAs. It is mandatory for recognition of UGA as a selenocysteine codon (1). Different SECIS-binding proteins have been described (5–11), but among these only SBP2 was shown to be effectively required for mammalian selenoprotein translation (11). Since SECIS-binding proteins are not the eukaryotic homologs of the bacterial SELB, it looks as if two (SBP2 and the eukaryotic SELB factor) or more proteins act in concert to accomplish this recoding event, in contrast to bacteria (8,11).

The spacing between the UGA codon and the SECIS element, that can reside in some instances as far away as 5 kb, raises several structural and functional issues regarding how the RNA–protein complex formed at the SECIS hairpin can fulfill its role at a distance. In an attempt to better understand the underlying mechanism, we previously undertook the experimental determination of the secondary structure of the SECIS RNA element. Structure probing experiments combined with sequence comparisons enabled us to propose a structural model consisting of a hairpin comprising the two helices I and II separated by an internal loop (12). Most predominantly, helix II contains a quartet of non-Watson–Crick base pairs constituting a motif essential to mediate selenoprotein translation (13). Using sequence comparisons and site-directed mutagenesis, other investigators have proposed a model similar to ours but with additional base pairing in the apical loop of some of the SECIS elements (14,15). Their findings led to the categorization of the SECIS elements into two forms according to the size of the apical loop: form 1 corresponds to the model described in Walczak et al. (12) and Martin et al. (14), and form 2, containing a larger number of nucleotides in the apical loop, enables intra-loop base pairing.

By virtue of a screen searching SECIS secondary structures in databases, we earlier identified the four novel selenoproteins SelN, SelX, SelZI1 and SelZI2 (16). Also discovered by this approach were the SelD and SelY SECIS elements in the human selenophosphate synthetase 2 and type 2 iodothyronine deiodinase mRNAs, respectively (17,18) that had not been...
reported in the literature at the time of our search. Here, our objective was to determine experimentally the form adopted by these SECIS elements, and also to examine the structures of a few others proposed in the literature to fold into form 2. Arising from this work, structure probing experiments as well as sequence comparisons with SECIS elements in the database support the notion that the form 2 model is representative of a substantial number of SECIS elements. Additionally, both the expansion of the repertoire of SECIS sequences and functional assays led us to establish that replacement of an adenine, considered so far as invariant, is not as detrimental as anticipated.

MATERIALS AND METHODS

EMBL/GenBank/DDBJ data

The accession number for Drosophila melanogaster selenophosphate synthetase 2 homology is AF279253.

Constructs and site-directed mutagenesis

Vectors for in vitro transcription of the various SECIS elements were made as follows. The Bcl–KpnI digested fragments of the pSelN, pSPS2 (also called pSelD), pSelX, pSelY, pSelZ and pSel15 plasmids (16) were introduced into Bcl–KpnI digested pT7BcK (12) vectors, giving rise to pT7BcKSelN, pT7BcKSelD,
pT7BcKSelX, pT7BcKSelY, pT7BcKSelZ and pT7BcKSel15, respectively.

*Xenopus laevis* type 3 iodothyronine deiodinase (XlDI3) was obtained by PCR of genomic DNA. Primers were designed to amplify the sequence spanning positions 1274–1380 (19). The 5′ and 3′ primers incorporated *Bam*H1+T7 promoter and *Eco*RI sites, respectively. An *Eco*RI–*Bam*H1 digest containing the SECIS sequence was inserted into the *Eco*RI–*Bam*H1 cleaved pUC119 plasmid, generating pSEX5.

The rat PHGPx SECIS element was constructed by hybridizing the overlapping oligodeoxynucleotides 5′-GATCACCTTCCACCCCC-3′, 5′-GCACTCATGACGGTCTGCCTGAA-3′, 5′-AACCAACCGCGCTGGGGGCAGTC-3′, 5′-CGAGGCCCTGGCGTGCATCC-3′, 5′-CCGCCCGAGGAAGGGTAC-3′, 5′-GCACTCATGACGGTCTGCCTGAA-3′, 5′-AACCAACCGCGCTGGGGGCAGTC-3′, 5′-CGAGGCCCTGGCGTGCATCC-3′, 5′-CCGCCCGAGGAAGGGTAC-3′, and 5′-GATCACCTTCCACCCCC-3′.
5′-CCCTTCTCAGGGGAGTGACAG-3′, 5′-CCAGGTC-CCTCGAGATGCCC-3′, 5′-CACCAGGCGGTGGTATTTC-AGGCAAG-3′ and 5′-CGTCTAGAGTGCCCCGGGTGGA-AGGT-3′. The resulting fragment (positions 52–147; 20), containing BclI and KpnI sites at the 5′ and 3′ ends, respectively, was cloned into the pT7BcK vector to produce pT7BcKPAGPs.

To linearize DNAs for further transcription by T7 RNA polymerase and to allow reverse transcription of the RNAs, an Xhol site was introduced by site-directed mutagenesis into pT7BcK, 3′ to the universal primer hybridization sequence, yielding pT7BcKX. BclI–KpnI digests of pT7BcKSelX, pT7BcKSelY, pT7BcKSelD and pT7BcKPHGPx were ligated to BclI–KpnI digested pT7BcKX vectors. The BamHI–EcoRI fragment of pSEX5 was inserted into the BamHI–EcoRI cleaved pT7BcKX vector.

An EST (accession no. AA541242) containing the partial sequence of the drosophila RNA polymerase, putatively encoding the selenophosphate synthetase 2 sequence of the drosophila cDNA, putatively encoding the drosophila selenophosphate synthetase 2, was obtained by querying the EST database with the human selenophosphate synthetase 2 (SelD) sequence. Ten micrograms of linearized DNA was incubated for 2 h in 250 mM Tris–HCl pH 8.0, 22 mM MgCl2, 1 mM spermidine, 0.01% Triton X-100, 50 mM NaCl containing 25 mM Na2SO4. Alkaline ladders were performed by incubating 5 µl of 2% LiClO4 in acetone.

In vitro transcription
Vectors were linearized by EcoRI or Xhol prior to in vitro transcription. Ten micrograms of linearized DNA was incubated for 2 h in 250 µl of a buffer containing 40 mM Tris–HCl pH 8.0, 22 mM MgCl2, 1 mM spermidine, 0.01% Triton X100, 5 mM DTE, 24 U RNasin, 4 mM of each NTP, 15 µl of T7 RNA polymerase. To produce 5′ ApG-ending RNAs for 5′-labeling, 5 mM ApG was added and the GTP concentration decreased to 1 mM. After 1 h incubation at 37°C, 1 mM GTP was added and the mixture incubated for a further 1 h. The RNAs were purified on 10% denaturing polyacrylamide gels and electroeluted.

RNA structure probing
Enzymatic cleavage. 5′ ApG RNAs were 5′ end-labeled with [γ-32P]ATP. Digestions were performed for 5–15 min at 20°C in a buffer containing 20 mM Tris–HCl pH 7.5, 5 mM MgCl2, 100 mM KCl, with either 2 × 10−3 U/µg RNase V1, 5 × 10−3 U/µg RNase T2 or 10−3 U/µg RNase T1 (12). Samples were fractionated on 10% sequencing gels.

Alkaline ladders were performed by incubating 5′ end-labeled RNAs in 50 mM sodium carbonate pH 8.9 for 5 min at 90°C. RNAs were precipitated with 600 µl of 2% LiClO4 in acetone.

For RNase T1 ladders, 103 c.p.m. of 5′ end-labeled RNAs were digested with 5 × 10−3 U/µg RNase T1 for 10 min at 37°C in 25 mM sodium citrate pH 5.5, 1 mM EDTA, 8 M urea, 0.025% bromophenol blue, 0.025% xylene cyanol (12).

Chemical probing. Cleavage of 5′ end-labeled RNAs by Pb++ ions occurred in 25 mM HEPES–NaOH pH 7, 10 mM Mg acetate, 50 mM KCl containing 25 × 103 c.p.m. RNA and 8 µg carrier tRNA. Samples were preincubated for 2 min before adding lead acetate to the final concentrations indicated in Figure 2.

Chemical reactions with dimethylsulfate (DMS) and carbodiimide (CMCT) were performed using the standard procedures described in Krol and Carbon (21) and the legend to Figure 3. Detection of the modified bases was accomplished by extension of the 5′ end-labeled 17mer universal primer. The positions of modifications were mapped by dideoxysequencing with reverse transcriptase. The reverse transcripts were separated on 10% sequencing gels.

Transfection of COS-7 cells and glutathione peroxidase assays
COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 0.1 mg/ml gentamycin according to standard cell culture procedures. Transient transfections were carried out as described (13,16) with 5 µg of test DNA, 4 µg of tRNAec expression vector and 1 µg of plasmid CMV-LacZ as the transfection standard. Sodium selenite (10 mM) was added to the culture medium. Prior to glutathione peroxidase activity measurements, β-galactosidase activities were assayed with one-tenth volume of crude cell extract to normalize the results (13,16). Assays were done in triplicate.

RESULTS

Structure probing of form 1 and form 2 SECIS RNAs

We carried out the secondary structure determination of eight SECIS RNAs contained in the mRNAs of the human SelN, SelX, SelZ (SelZf1 and SelZf2 mRNA sharing the same SECIS element), SelD and SelY (16), mouse Sel15 kDa (22), rat phospholipid hydroperoxide glutathione peroxidase (20) and XID3 (19) selenoproteins. In the course of this study, they will be termed hSelN, hSelX, hSelZ, hSelD, hSelY, mSel15, rPHGPx and XID3, respectively. Figure 1 (left hairpins except hSelN) shows the predicted secondary structure models. Seven hairpins have the potential to form the short helix 3 giving rise to the SECIS form 2 displayed on the right. In hSelN, helix 3 could form through complementarity between A26UC and C. In such experiments, lead acetate-induced cleavage was employed to sense single-stranded RNA regions. Representative gels for hSelX and rPHGPx are shown in Figure 2I–J, respectively. Where necessary, chemical probing with DMS (reacts at the N1-A and N3-C positions) and CMCT (reacts at the N1-G and N3-U) was undertaken in an attempt to resolve ambiguities. In such experiments, protection of the chemical groups of the bases against chemical modification is indicative of their participation in base pairing. The reactions were conducted under native or semi-denaturing conditions to provide information about the
stability of the base pairs that might be disrupted in the absence of Mg^{++} and K^{+} ions (Fig. 3A–E). A compilation of the probing data for each SECIS RNA is represented in Figure 1, arising from the gels in Figures 2 and 3 and data not shown. Interpretation of the results generated by combination of the various probes led to the following conclusions. The eight SECIS RNAs can adopt the overall 2D structure model proposed for the SECIS element, with helices 1 and 2 separated by the internal loop 1 of variable size (12). Because helix 1 varies in length, only the top five base pairs were depicted in Figure 1.
Helix 2 falls within a range of 13–15 bp, consistent with the consensus SECIS secondary structure model and other data (12,15 and our unpublished data). Only the mSel15 and XID3 helices II are interrupted by the A51 and U54 bulges, respectively. Similar to the human and rat type 1 deiodinases, and rat glutathione peroxidases SECIS (12), the existence of the quartet of non-Watson–Crick base pairs is supported by the weak RNase T1 susceptibilities of the GpA bond in the GA tandem and the lack of strong Pb⁺⁺ cleavages (Fig. 2A–J). Formation of the quartet is further substantiated by the protection under native conditions of N3-U10, N1-G11, N1-G53 and N1-G55 in hSelD (Fig. 3A), N3-U10, N1-G11, N3-U13, N1-G52, N1-G53 and N3-U55 in hSelY (Fig. 3C), and N3-U12 and N1-G57 in XID3 (Fig. 3E). In hSelN, the apical loop is readily cleaved by
RNases T1 and T2 (Fig. 2B), thus excluding base pairing of A26UC with G32AU. The hSelN SECIS element therefore adopts the form 1 structure with a 14–15 bp helix 2 and a 10–12 nt long apical loop depending on whether the closing G.U base pair does form. For the hSelY, hSelZ, mSel15, rPHGPx and XlDI3 SECIS, analysis of the RNases and Pb++ cleavages enabled us to propose the formation of the short helix 3 (Fig. 1). Indeed, the five RNAs show high susceptibility towards RNase T2 (and T1) and Pb++ in the purine stretch of internal loop 2 and in the apical loop (Fig. 2D–H, J). The occurrence of helix 3 is further supported by the reactivities under native conditions of N1-A25, N1-A26, N1-A27, N1-A28 and N1-G36 in rPHGPx, and of N1-A27, N1-A28, N3-U35, N3-C36, N1-A37, N3-C38, N1-A43, N1-A44 in XlDI3 (Fig. 3C–E).

Additional information could be gained for the rPHGPx helix 1. Figure 3D shows that N1-A6, N3-C7, N1-G64, N3-U65, N1-G66, N1-A68 and N3-U69 are reactive, whereas N1-G4 and N3-C5 are protected under native conditions. These reactivities argue more in favor of the base pairing between CCGGC (positions 1–5) and GCCCG (positions 74–78), than between GCAC (positions 4–7) and GUGC (positions 64–67), as was proposed earlier (20). The base pairing is further supported by the compensatory base pair change C2-G77 to U-A in the porcine PHGPx SECIS (Table 1).

Interpretation of the data is less straightforward for the hSelD and hSelX SECIS. In hSelD, the apical loop is fairly cleaved by RNase T2 between C24 and A28, C32 and A37. In addition, the G38-G39-A40 bonds are readily hydrolysed by RNase T1 (Fig. 2A). Chemical probing indicated reactivities of N1-A27, N1-A28, N3-U31, N3-U32, N3-C33, N3-C34, N1-A35 and N1-A40 are reactive under native conditions (Fig. 3B). RNase T2 and Pb++ cleavages are observed between C24 and C28, and C30 and A35, RNase V1 cuts moderately the C29-C30 bond but RNase T1 cleavages are weak after G36 and G37 (Fig. 2C). Altogether, the data for hSelD and hSelX point to a dynamic structure in equilibrium between an open form of the apical loop and the short helix 3, under our experimental conditions.

In conclusion, hSelY, hSelZ, mSel15, rPHGPx, XlDI3 fold into form 2, whereas hSelN adopts form 1. In the absence of clear-cut data, we propose that the hSelD and hSelX SECIS secondary structures transiently fold into the form 2 model.

**Database search and structure-based sequence alignment of SECIS elements**

Next, we wished to identify and select sequences in the 3′-UTR that can fold into SECIS elements that have not been reported in a structural alignment yet. To this end, we searched cDNAs or ESTs putatively encoding homologs to previously reported selenoproteins in various organisms. Nucleotide databases were queried with TBLASTN at NCBI, using the amino acid

**Figure 2.** (Above and previous two pages) Enzymatic and Pb(II) probing of the SECIS RNAs shown in Figure 1. (A) hSelD; (B) hSelN; (C) hSelX; (D) hSelY; (E) hSelZ; (F) mSel15; (G) rPHGPx; (H) XlDI3. The RNAs were digested with RNases T1, T2 and V1 for 5 and 10 min. (I) hSelX SECIS was cleaved at 20°C with 4, 7.5 and 10 mM Pb(II) acetate for 2 and 5 min (lanes 2–7, respectively). (J) rPHGPx SECIS was cleaved at 37°C for 2 min with 1.25 (lane 2), 2.5 (lane 3) and 5 mM (lane 4) Pb(II) acetate. Lanes marked C are control reactions; L, alkaline ladder; T1, RNase T1 ladder. The numbering corresponds to that in Figure 1.
sequences of the human cellular glutathione peroxidase, human selenophosphate synthetase 2, rat SelW, human thioredoxin reductases, and human type 1, 2 and 3 iodothyronine deiodinases. Except those already indexed as SECIS elements in complete cDNA sequences (Table 1), two zebrafish ESTs were obtained (GenBank accession nos AW232474 and
AW128765) that putatively encode the GPx and SelW homologs, respectively. A SECIS element could be identified in each of the EST sequences by detecting the invariant RUGA-(13–15 nt)-AAR-(23–26 nt)-GA sequence motif, a characteristic structural feature of the SECIS element (12,24). These sequences were manually examined for their ability to adopt either form 1 or form 2. Also, a drosophila cDNA (GenBank accession no. AF279253) was identified as the putative human selenophosphate synthetase 2 homolog. It differs from the previously reported putative drosophila selenophosphate synthetase gene (25) in that it contains an inframe UGA codon and a SECIS element.

Twenty six SECIS are listed in Table 1. They include those found in the search, others (15,16,24,26) aligned for comparison, and SECIS RNAs whose structure was probed in this work. The majority can fold into form 2 and a few of them only adopt form 1, corroborating an earlier observation (15). The alignment and the probing data showed that the length of helix 3 is variable, ranging from 2 (hSelID and mTrxR1) to 7 bp in the chicken type 3 deiodinase. The 3 bp helix 3 in the drosophila and mouse SelID is supported by the compensatory base pair change U-A to G-C. No significant sequence conservation could be inferred in the short apical loop of form 2. Interestingly, the form 2 SECIS RNAs expose the invariant adenine stretch in the internal loop 2. This loop was shown to be very accessible to RNases and chemicals in this work. It is exclusively composed of purines in type 2 deiodinases, X.laevis, chicken and Oreochromis niloticus type 3 deiodinases, TrxR2 thioredoxin reductases and hSelZ.

An adenine is found 5’ to the non-Watson–Crick quartet. It has been tacitly considered as invariant because the sample of the examined SECIS elements was too limited to allow the discovery of variants. However, a G was recently discovered in the SECIS element of the Caenorhabditis elegans thioredoxin reductase (24). Table 1 shows that this also happens in the chicken and O.niloticus type 3 deiodinases and in the second SECIS element of the O.niloticus type 1 deiodinase (O.n.D11b).

The GPx SECIS function can tolerate A to G, C or U changes 5’ to the non-Watson–Crick quartet

The finding that a G can occur in a few SECIS at the position believed to be occupied by an invariant A 5’ to the UGA sequence of the quartet prompted us to examine whether substituting G and also C or U for A at position 1017 position is rather tolerant to mutation. Indeed, only the substitution of C for A led to a substantial drop to 34% residual activity, whereas type 2 and 3 deiodinases and thioredoxin reductases adopt the form 2. The stability of the short helix 3 is apparently too low in the human SelD and SelX to enable detection of form 2, exclusively. Rather, there was likely an equilibrium between forms 1 and 2 under the experimental conditions employed and it may be that it is shifted in vivo toward one form or the other by the presence of SECIS-binding protein(s).

In conclusion, the mutational analysis revealed that a G can substitute for A without significantly affecting SECIS function, in accordance with the alignment shown in Table 1. This was also the case for U, even though no U was found in SECIS sequences so far. Replacement of A by a C, however, was more deleterious but did not abrogate SECIS activity.

DISCUSSION

In this work, we have experimentally analyzed the secondary structures of eight different SECIS elements. We proposed in an earlier work secondary structure models for the mammalian type 1 iodothyronine deiodinase and cellular glutathione peroxidase SECIS RNAs (12). The motivation for investigating the structure of other SECIS RNAs arose from the finding by site-directed mutagenesis that certain SECIS elements with large apical loops could adopt the form 2 structure with intra-apical loop base pairing (15). Using an ensemble of enzymatic and chemical probes, we could show that among the RNAs analyzed, the human SelY and SelZ, rat PHGPx, X.laevis type 3 deiodinase and mouse Sel15 SECIS elements adopt the form 2. The stability of the short helix 3 is apparently too low in the human SelD and SelX to enable detection of form 2, exclusively. Rather, there was likely an equilibrium between forms 1 and 2 under the experimental conditions employed and it may be that it is shifted in vivo toward one form or the other by the presence of SECIS-binding protein(s).

Only one SECIS, human SelN, could adopt form 1. A structure-based sequence alignment with SECIS sequences other than those used previously (12,15) clearly argues in favor of form 2 being predominant. Besides, information emanating from Table 1 and Walczak et al. (12) and Grundner-Culeman et al. (15) indicates that SECIS elements can be categorized according to the type of selenoprotein mRNA they belong to. For example, type 1 deiodinases and cellular GPx fall into form 1 whereas type 2 and 3 deiodinases and thioredoxin reductases belong to form 2. Two other interesting findings could be derived from the data that led to Table 1: (i) the existence of a selenocysteine-containing, in addition to the arginine-containing (25), selenophosphate synthetase (SP52) in drosophilae, like in mammals (17) and a few eubacteria and archaee (3); (ii) the adenosine 5’ to the non-Watson–Crick
quartet is not invariant but rather semi-conserved since a guanine was discovered in four SECIS elements. Another interesting example is provided by the O.niloticus type 1 deiodinase. Apart from the SelP mRNA that contains several selenocysteine codons (4), this is the only reported case of a selenoprotein mRNA containing two SECIS elements. There is no evidence yet for the functionality and need for two SECIS to read a single selenocysteine codon in the O.niloticus type 1 deiodinase. However, the compensatory base changes observed in helices 1 of the two SECIS (U-A, G-C and U-A in the first SECIS to G-C, U-A and C-G in the second, respectively), the A to G change below the quartet, the several pyrimidine or purine transitions in the loops and the C.A to A.A change at the top base pair of the quartet, argue in favour of the maintenance of two functional SECIS structures by selective pressure. The probing data revealed the strong accessibility of the conserved adenine stretch in internal loop 2. Recent data concluded that the SECIS-binding protein SBP2 does not require this region for binding (11). The internal loop 2 and the exposed adenines herein could serve as a signal for the interaction with the specialized translation factor SelB or be part of a more complex, long-range RNA–RNA interaction with upstream regions of the mRNA or the ribosome.

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because of evolutionary advantages. Among the advantages, one could invoke the binding of different proteins, but SBP2 has been shown to bind to both types of SECIS RNAs (10). Another advantage could be correlated to the stretch of conserved adenines at the apex of the SECIS stem-loop, that occurs in a slightly different conformation in the two forms. The As are accessible in form 1 within the apical loop and well exposed and bulging out in the internal loop 2 of form 2. We hypothesize that this duality can provide a differential affinity towards a protein or RNA ligand perhaps leading to the observed distinct efficiencies of form 1 and form 2 SECIS elements in UGA readthrough (27).

The recent characterization of SBP2 and development of an in vitro assay for selenoprotein translation (11) will certainly help define the contribution of the various SECIS motifs to SBP2 binding and selenoprotein translation.

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