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Identification of a Region within the Placental Alkaline Phosphatase mRNA That Mediates p180-dependent Targeting to the Endoplasmic Reticulum*

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Background: Certain mRNAs are anchored to the endoplasmic reticulum (ER) by p180.
Results: The placental alkaline phosphatase mRNA requires its transmembrane domain coding region to be anchored by p180.
Conclusion: Translational-independent ER targeting of mRNA by p180 can be mediated by regions of the ORF that encode hydrophobic peptides.
Significance: Translational-independent targeting of mRNA to membranes may be mediated by a mechanism conserved between prokaryotes and eukaryotes.

In both eukaryotic and prokaryotic cells, it has been recently established that mRNAs encoding secreted and membrane proteins can be localized to the surface of membranes via both translation-dependent and RNA element-mediated mechanisms. Previously, we showed that the placental alkaline phosphatase (*ALPP*) mRNA can be localized to the ER membrane independently of translation, and this localization is mediated by p180, an mRNA receptor present in the ER. In this article, we aimed to identify the *cis*-acting RNA element in *ALPP*. Using chimera constructs containing fragments of the *ALPP* mRNA, we demonstrate that the ER-localizing RNA element is present within the 3′ end of the open reading frame and codes for a transmembrane domain. In addition, we show that this region requires p180 for efficient ER anchoring. Taken together, we provide the first insight into the nature of *cis*-acting ER-localizing RNA elements responsible for localizing mRNAs on the ER in mammalian cells.

In eukaryotes, mRNAs encoding secretory, membrane-bound, and most organellar proteins must be targeted to, and then maintained at, the surface of the endoplasmic reticulum (ER).[2] In mammalian cells this localization is mediated by at least two general mechanisms. The first, which is universally conserved across all life, is the co-translational dependent mechanism. In this pathway, mRNAs encoding secreted and membrane proteins are initially translated in the cytoplasm by the free ribosomes. Once the signal sequence exits the ribosome, it recruits the signal recognition particle (SRP), which inhibits further translation and directs the nascent polypeptide chain-ribosome-mRNA complex, to the surface of the ER by binding to the SRP receptor (1, 2). At the ER, the signal sequence is inserted into the translocon, and translation resumes (3, 4). During this process, the mRNA remains anchored on the ER by direct interactions between the translocon and ribosomes (3).

In both prokaryotes and eukaryotes there are alternative mechanisms that act independently of translation, SRP, and ribosomes (5–7). In mammalian cells, this alternative localization pathway is mediated by at least one RNA receptor, p180 (7). This highly abundant membrane-bound protein resides on the ER surface and contains a large positively charged cytoplasmic domain that can directly interact with RNA *in vitro*, likely by forming nonspecific ionic interactions with the phosphate backbone (7). In addition, other mRNA receptors are probably present in the mammalian ER, as p180 depletion does not abolish translation-independent anchoring of bulk mRNA to the surface of this organelle (7).

Interestingly, it appears that only a subset of mRNAs is able to utilize this translation-independent pathway. Individual examples include the human *GRP94* (6), and calreticulin (7) mRNAs, which encode chaperones that reside in the lumen of the ER, and the human placental alkaline phosphatase (*ALPP*) mRNA (7). In addition, mRNAs encoding certain cytosolic proteins also co-fractionate with the ER (8). Importantly, both the calreticulin gene and *ALPP* require p180 for their translation-independent maintenance at the ER (7). In contrast, certain engineered reporter mRNAs, such as *t-ftz*, and natural transcripts, such as the insulin-like 3 and cytochrome p450 8B1 (*CYP8B1*) genes, predominantly use the translation-dependent pathway for ER localization (7, 9). In light of this, p180 probably collaborates with supplementary proteins, which have motif-discriminating RNA-binding domains.

Although p180 appears to be metazoan-specific, a similar system is present in *Escherichia coli* (5, 10). In particular, it was found that the *Bgl* polycistronic transcript is anchored to the plasma membrane of *E. coli* independently of translation. The anchoring of this transcript required a portion of the *BglF* ORF, which encodes several transmembrane domains (TMDs).

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[2] The abbreviations used are: ER, endoplasmic reticulum; ALPP, placental alkaline phosphatase; CYP8B1, cytochrome P-450 8B1; DMSO, dimethyl sulfoxide; fs, frameshift; HHT, homoharringtonine; SRP, signal recognition particle; SSCR, signal sequence coding region; TMCR, transmembrane domain coding region; TMD, transmembrane domain.

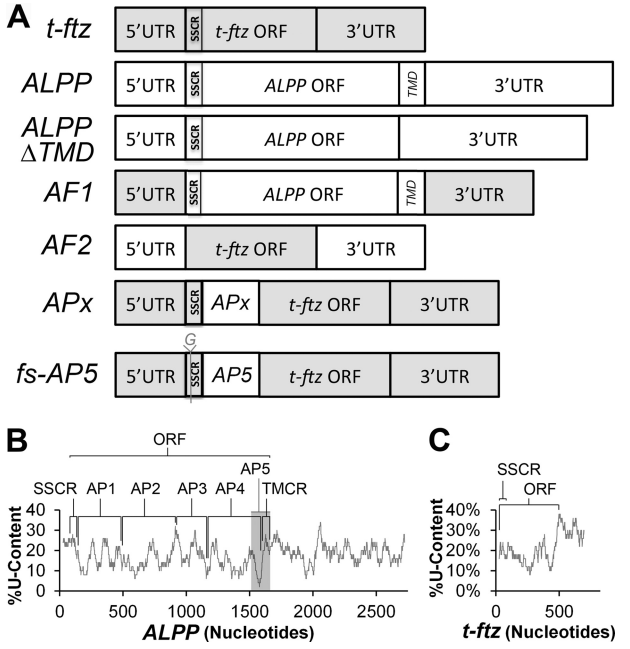

FIGURE 1. **Schematic diagrams of constructs and U content in *ALPP* and *t-ftz*.** *A*, schematic of the chimeric constructs used in this study. All *t-ftz* sequences are depicted in *gray* and *ALPP* sequences in *white*. SSCRs and TMCRs are also indicated. *B*, percentage of U content in *ALPP*, as analyzed using a moving window of 50 nucleotides, plotted against the length of the construct. The SSCR, ORF, TMCR, and various fragments (*AP1–5*) are indicated. Note that the *gray* region represents *AP5*. *C*, percentage U content in *t-ftz*, as analyzed using a moving window of 50 nucleotides.

To better understand this basic cellular process in mammalian cells, it is critical that we identify RNA elements that promote p180-dependent ER localization. Here we identify a region of the *ALPP* mRNA that promotes ER localization. Furthermore, we demonstrate that this RNA fragment uses the p180-dependent pathway.

EXPERIMENTAL PROCEDURES

DNA Plasmid, Construction of Fusion Genes—All chimeric constructs were constructed by restriction free subcloning (11) using full-length *ALPP* in pSPORT6 (7) and *t-ftz* (also known as *MHC-ftz*) in pcDNA3 (12). Briefly, inserts were amplified by PCR, followed by a second vector-based PCR step that either inserts the fragment or uses the fragment to replace a targeted sequence. *AF1* was constructed by replacing the *t-ftz* ORF with the *ALPP* ORF, whereas the converse is true for *AF2* (Fig. 1*A*). *APx* constructs (*AP1*, *AP2*, *AP3*, *AP4*, and *AP5*, Fig. 1*A*) were constructed by inserting various fragments of *ALPP* (as indicated in Fig. 1*B*) between the signal sequence coding region (SSCR) and ORF of *t-ftz*. In particular, *AP1* contains nucleotides 134–480 of the full-length *ALPP* cDNA transcript, *AP2* contains 481–917, *AP3* contains 918–1172, *AP4* contains 1173–1564, and *AP5* contains 1472–1671. Note that *AF1* and *AP1–5* constructs are in pCDNA3, whereas *AF2* is present in pSPORT6. Frameshift *AP5* (*fs-AP5*) was constructed by site-directed mutagenesis using restriction-free subcloning to insert a guanine between the 3rd and 4th nucleotides of the ORF. *ALPP-ΔTMD* was made by removing nucleotides 1504–1594 from the *ALPP* construct using restriction-free cloning. *H1B-GFP* and *M1-ftz* were described previously (7, 13).

Computational Analysis of the Features of Encoded Proteins—The presence of signal sequences or TMDs was predicted using SignalP and TMHMM servers. A Kyte-Doolittle hydropathy plot was computed using ProtScale.

Cell Culture, Permeabilization, shRNA Treatment, Transfection, and Microinjection—Cell culture and transfection were carried out as described previously (7, 9). Depletion of p180 by lentivirus-mediated delivery of shRNA constructs was carried out in U2OS cells as described previously (7). 18–24 h after transfection cells were pretreated with DMEM containing DMSO, 200 μM puromycin (Sigma), or 5 μm homoharringtonine (HHT) (Tocris Bioscience) for 30 min, permeabilized with digitonin (Sigma), and then fixed as described previously (9). To determine the targeting of newly synthesized transcript, plasmids encoding *ALPP*, *t-ftz*, or *AP5* were microinjected into U2OS or COS-7 cells pretreated with DMEM containing either DMSO or 5 μM HHT for 15 min as described previously (7, 14). After injection, cells were incubated with DMEM containing DMSO or HHT for 2 h and then permeabilized with digitonin and fixed as described previously (7, 9, 14).

Fluorescent in Situ Hybridization (FISH), Immunofluorescence, and Imaging—FISH, immunofluorescence, and imaging were performed, as described previously (7, 9, 14). For *ALPP*- and *ftz*-specific probes, see Ref. 7. For *H1B-GFP*, a DNA probe specific for EGFP was used (Alexa Fluor 546, 5′-CCG TCG CCG ATG GGG GTG TTC TGC TGG TAG TGG TCG GCG AGC TGC ACG CTG CC-3′, synthesized by IDT). For Trapα immunofluorescence, a polyclonal primary (15) and Alexa Fluor 647-conjugated secondary (Molecular Probes) were used as described previously (7). Images were analyzed, and subcellular localization was quantified using NIS-element software, as described previously (7, 9, 14).

Cell Fractionation, Western Blotting, and Northern Blotting—U2OS cells were transfected and allowed to express various mRNAs for 18–24 h and then separated into cytosolic and ER fractions as described previously (7). Briefly, cells were trypsinized, washed, and then permeabilized with low levels of digitonin. The nuclear ER fraction was separated from the cytosolic fraction by low speed centrifugation. The pellet was then treated with Triton X-100 detergent to lyse the ER, and the nuclear contents were removed by low speed centrifugation. Cell fractions were separated by SDS-PAGE and immunoprobed with antibodies against α-tubulin (mouse monoclonal DM1A, diluted 1:20,000; Sigma), Aly (rabbit polyclonal (16), 1:1000), Trapα (rabbit polyclonal (15), diluted 1:5000), or p180 (rabbit polyclonal, diluted 1:1000; Sigma). For Northern blots, RNA was isolated from fractions, separated on denaturing agarose gels, and probed using radiolabeled antisense *ftz* probes, as described previously (17).

RESULTS

Efficient Translation-independent Maintenance of ALPP mRNA at the ER Requires Its Open Reading Frame—To identify putative ER-localizing RNA element(s), we created chimera constructs between *ALPP*, which can be targeted and then maintained on the ER in a translation-independent manner, and *t-ftz*, which strictly requires translation for its localization to the ER (7). First, we created two constructs that had UTRs from one gene and the ORF of the other. *AF1* contains the ORF

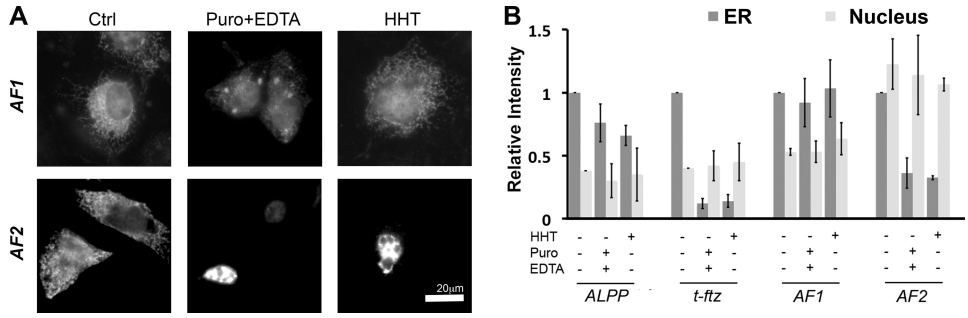

FIGURE 2. **Translation-independent ER localization is mediated by the *ALPP* ORF.** COS-7 cells were transfected with plasmids containing either *ALPP*, *t-ftz*, or the *AF1*, *AF2* fusion constructs and allowed to express mRNA for 18–24 h. The cells were then treated with DMSO (*Ctrl*), puromycin (*Puro*), or HHT for 30 min and then extracted with either digitonin alone, or for puromycin-treated cells, with 20 mM EDTA. The cells were then fixed, stained for mRNA using specific FISH probes (*ALPP* probe for *AF1*, *ftz* probe for *AF2*), and imaged. *A*, representative FISH images of cells expressing *AF1* or *AF2*. *B*, quantification of the fluorescence intensities of mRNA in the ER and nucleus. All data were normalized to the ER staining intensity in the control treated group for each construct. Each *bar* represents the average ± S.E. (*error bars*) of three independent experiments (each experiment consisting of $n>30$ cells). *Scale bar*, 20 μm.

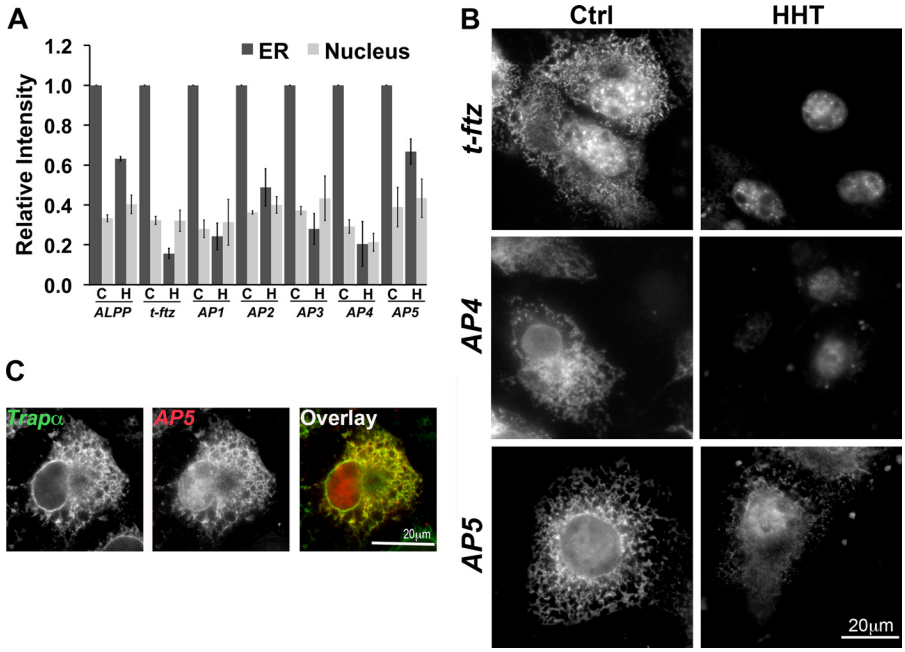

FIGURE 3. **The element responsible for the translation-independent ER localization is present in *AP5*.** COS-7 cells were transfected with plasmid containing either full-length *ALPP*, *t-ftz*, *AP1*, *AP2*, *AP3*, *AP4*, or *AP5* and allowed to express mRNA for 18–24 h. The cells were then treated with DMSO (*C* or *Ctrl*) or HHT (*H*) for 30 min and then extracted with digitonin. Cells were then fixed, stained for mRNAs using specific FISH probes (*ftz* probe was used to detect *AP1–5*), and imaged. *A*, quantification of the fluorescence intensities of mRNA in the ER and nucleus. All data were normalized to the ER staining intensity in the control treated group for each construct. The results were normalized to the ER staining intensities in the control treated group for each construct. Each *bar* represents the average ± S.E. (*error bars*) of three independent experiments (each experiment consisting of $n>30$ cells). *B*, examples of COS-7 cells expressing either *t-ftz*, *AP4*, or *AP5*. *C*, example of a COS-7 cell expressing *AP5* that has been treated with HHT for 30 min then extracted with digitonin. The cell was co-stained for *AP5* using *ftz* FISH probes and Trapα, an ER marker, by immunofluorescence. *Scale bars*, 20 μm.

of *ALPP* and the UTRs of *t-ftz*, whereas *AF2* contains the converse (Fig. 1*A*). These constructs were transfected into COS-7 cells, which were then allowed to express mRNA for 18–24 h. The cells were then treated with either control medium, puromycin, or HHT for 30 min. Puromycin promotes the ejection of nascent polypeptides and can help to disrupt ribosomes when used in combination with an extraction buffer that contains EDTA (7). In contrast, HHT, being an inhibitor of translation initiation, allows ribosomes to complete translation naturally while preventing the association of new ribosomes with the mRNA (18). Note that both drugs completely inhibit translation in <15 min (7). The cells were then treated with digitonin to permeabilize the plasma membrane and extract any cytoplasmic mRNAs that are not ER-bound, as described previously (7, 9). The cells were then fixed, and *AF1* mRNA was detected by FISH using probes directed to the *ALPP* ORF, whereas *AF2* was detected using probes that hybridize to the *ftz* ORF.

Surprisingly, the majority of the *AF1* mRNA was maintained on the ER after either puromycin/EDTA or HHT treatments. In contrast, the ER association of *AF2* mRNA was sensitive to these treatments (Fig. 2). As an internal control we also monitored the amount of nuclear mRNA, which is not affected by digitonin permeabilization (7), and as expected this remained relatively unchanged among different treatment groups (Fig. 2*B*). As published previously (7), *ALPP* remained associated with the ER under all conditions, whereas *t-ftz* required translation for ER association. These results indicated that the ER-localizing *cis*-element was present within the ORF of *ALPP*.

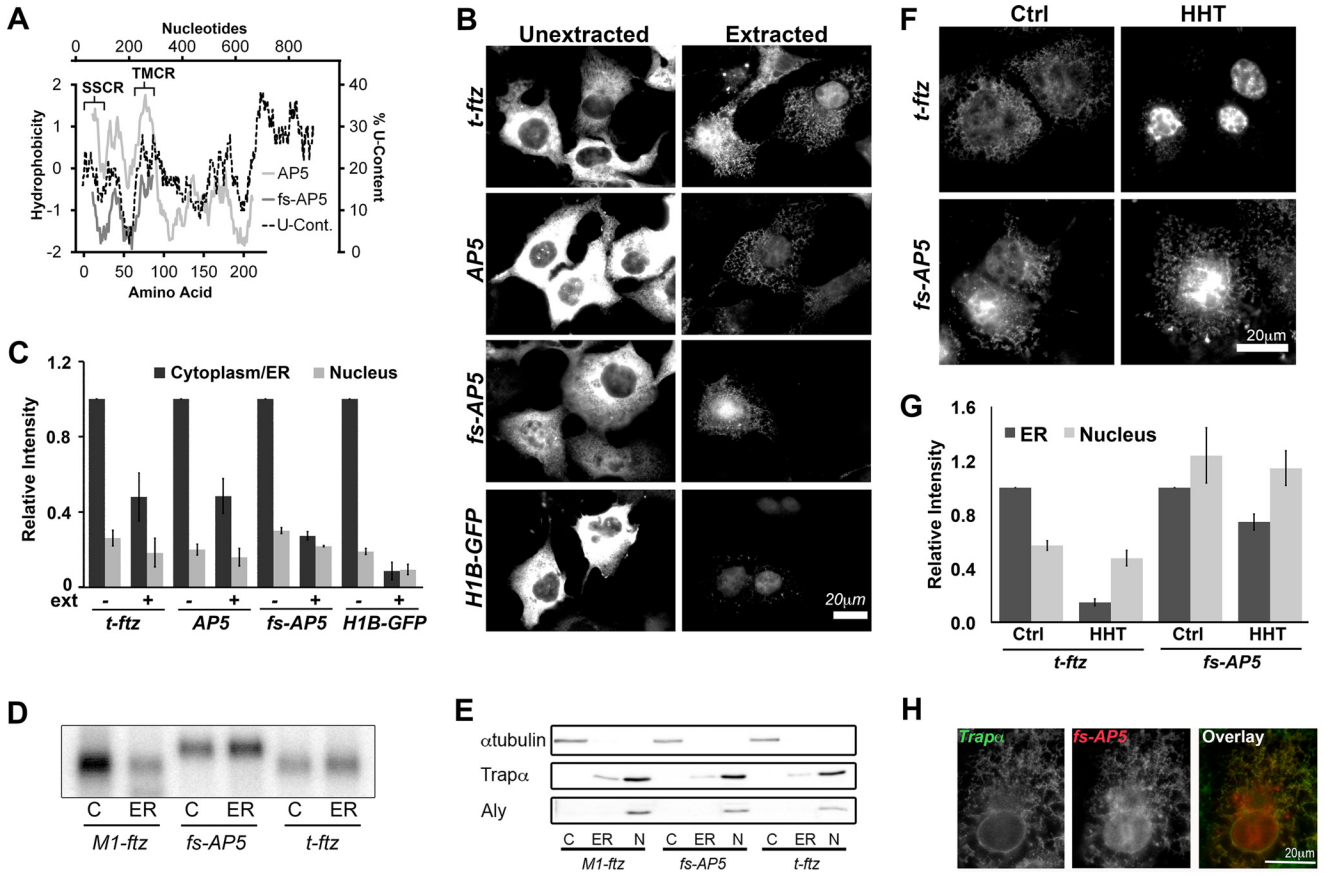

FIGURE 4. **Frameshifted *AP5* can still efficiently be maintained on the ER independently of ribosomes and translation.** *A*, hydrophobicity (*y* axis, *left*) of the polypeptides encoded by *AP5* and *fs-AP5* plotted against the peptide length (*x* axis, *bottom*). The Kyte-Doolittle hydropathy plot was calculated using with a moving window size of 21 amino acids. Note that *AP5* encodes the *t-ftz* signal sequence (residues 1–23 of the AP5 protein), followed by the C-terminal domain of ALPP (including the TMD of ALPP, residues 73–90 of the AP5 protein), and then ends with the hydrophilic ftz protein. In contrast, *fs-AP5* does not encode any hydrophobic region. Also note that the frameshift mutation results in the creation of a premature stop codon and a smaller encoded protein. For comparison, the U content (*x* axis, *right*) along AP5 (*y* axis, *top*) was also plotted. *B* and *C*, COS-7 cells transfected with plasmid containing either *t-ftz*, *AP5*, *fs-AP5*, or *H1B-GFP* and allowed to express mRNA for 18–24 h. Cells were then fixed directly (*Unextracted*) or first digitonin-extracted (*Extracted*) and then fixed. Cells were then stained with specific FISH probes (*ftz* probes were used to for *AP5* and *fs-AP5*) to visualize mRNA distribution. *B*, representative examples. *C*, quantification of fluorescence intensities of mRNA in the cytoplasm/ER and nucleus in unextracted (ext^-) and extracted (ext^+) cells. All data were normalized to the unextracted cytoplasmic/ER staining intensity for each construct. Each *bar* represents the average ± S.E. (*error bars*) of three independent experiments (each experiment consisting of $n>40$ cells). *D* and *E*, U2OS cells transfected with plasmid containing either *t-ftz*, *fs-AP5*, or *M1-ftz*. Cytoplasm (*C*), ER (*ER*), and nuclear (*N*) fractions were collected. *D*, total RNA purified from each fraction. The presence of mRNA was determined via Northern blotting using anti-*ftz* probes. *E*, denatured fractions, separated by SDS-PAGE and analyzed by immunoblotting using antibodies against either Trapα (a resident ER protein), α-tubulin (a cytoplasmic protein), and Aly (a nuclear protein). *F* and *G*, COS-7 cells transfected with plasmids containing the *t-ftz* or *fs-AP5* constructs and allowed to express mRNA for 18–24 h. The cells were then treated with DMSO (*Ctrl*) or HHT for 30 min and then extracted with digitonin. Cells were fixed, stained for mRNA using a FISH probe against *ftz*, and imaged. *F*, representative images. *G*, quantification of the fluorescence intensities of mRNAs in the ER and nucleus. All data were normalized to the ER staining intensity in the control treated group for each construct. Each *bar* represents the average ± S.E. (*error bars*) of three independent experiments (each experiment consisting of $n>30$ cells). *H*, example of a COS-7 cell expressing *fs-AP5* that has been treated with HHT for 30 min and then extracted with digitonin. The cell was co-stained for *fs-AP5* using a *ftz* FISH probe and Trapα, an ER marker, by immunofluorescence. *Scale bars*, 20 μm.

The TMD Coding Region of ALPP mRNA Promotes the Translation-independent Maintenance of mRNA at the ER—The *ALPP* transcript is translated into a protein product that is translocated into the ER and then anchored to the membrane by a carboxyl-terminal TMD. This TMD is then cleaved, and the processed protein becomes covalently linked to a phosphatidylinositol glycan moiety, which retains the protein at the membrane (19). Mature ALPP is then transported through the secretory pathway to the surface of cell.

To determine where the ER-localizing RNA element is located, we inserted five segments of the *ALPP* ORF between the SSCR and ORF of *t-ftz* (*AP1*, *AP2*, *AP3*, *AP4*,and *AP5*) (Fig. 1, *A* and *B*). This allowed us to use the *ftz* FISH probe to detect each of these chimeric mRNAs. Moreover, the presence of the *t-ftz* SSCR ensured that these mRNAs would be properly exported from the nucleus to the cytoplasm (12). Plasmids containing each construct were transfected into COS-7 cells. After 18–24 h, translation-independent ER association was assessed. We found that the *AP5* fusion mRNA was maintained on the ER independently of translation at a level similar to the full-length *ALPP* (Fig. 3 *A* and *B*). In HHT-treated cells, *AP5* mRNA co-localized with Trapα, an ER marker, confirming that this localization is ER-specific (Fig. 3*C*). Of the other chimeras, only *AP2* showed a modest increase in its localization on the ER in HHT treated cells compared with *t-ftz*, suggesting that this fragment may have some limited localization capability. Note that a region of AP5 is also found in AP4 (Fig. 1*B*); however, this later mRNA requires translation for efficient ER anchoring (Fig. 3, *A* and *B*).

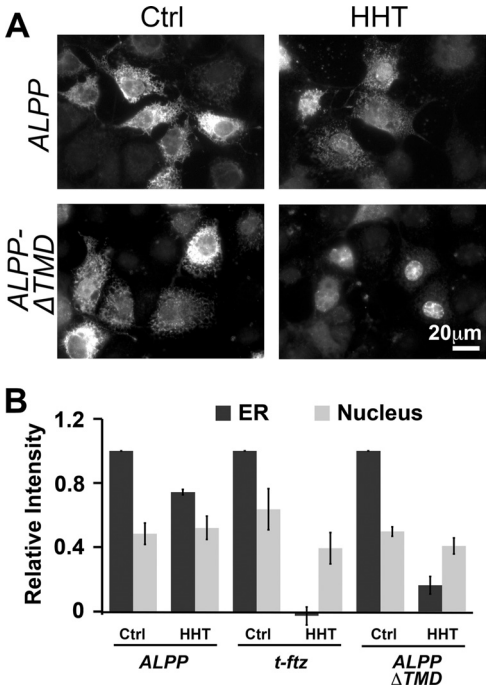

FIGURE 5. **The TMCD is required for the translation-independent ER localization of ALPP.** COS-7 cells were transfected with plasmid containing either *ALPP* or *ALPP-ΔTMD* and allowed to express mRNA for 18–24 h. The cells were treated with DMSO (*Ctrl*) or HHT for 30 min and then extracted with digitonin. The cells were fixed and stained for mRNA using a specific FISH probe against the *ALPP* ORF. *A*, representative examples. *B*, quantification of the fluorescence intensities of mRNAs in the ER and nucleus. All data were normalized to the ER staining intensity in the control treated group for each construct. Each *bar* represents the average ± S.E. (*error bars*) of three independent experiments (each experiment consisting of $n>30$ cells). *Scale bar*, 20 μm.

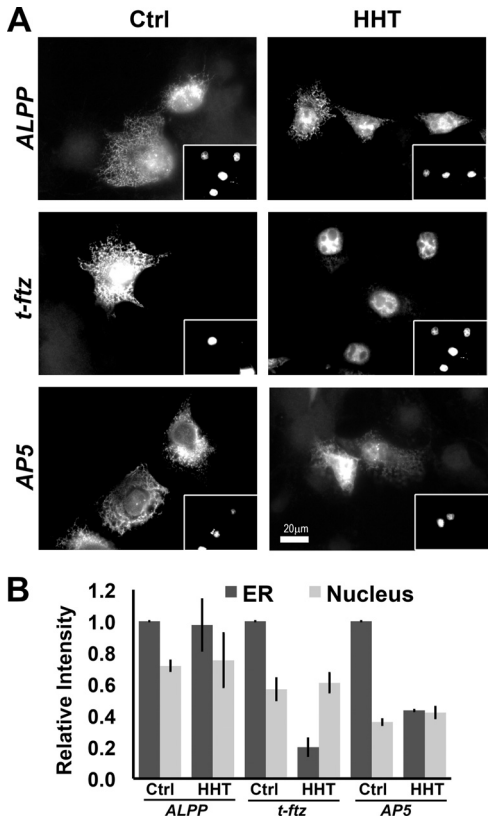

FIGURE 6. **The initial ER targeting of *AP5* can occur independently of translation and ribosomes.** COS-7 cells were pretreated with DMSO (*Ctrl*) or HHT for 15 min, then microinjected with plasmids containing *ALPP*, *t-ftz*, or *AP5*. These plasmids were microinjected with Alexa Fluor 488-conjugated 70-kDa dextran, which marks injected cells and can be seen in the *insets* (*A*). Cells were allowed to express mRNAs for 2 h in the presence of DMSO or HHT, then extracted with digitonin, fixed, and stained with specific FISH probes, and imaged. *A*, representative examples. *B*, quantification of the fluorescence intensities of mRNAs in the ER and nucleus. All data were normalized to the ER staining intensity in the control treated group for each construct. Each *bar* represents the average ± S.E. (*error bars*) of three independent experiments (each experiment consisting of $n>30$ cells). *Scale bar*, 20 μm.

From these experiments we conclude that the *AP5* region of *ALPP* contains an ER-localizing sequence. It is likely that the region responsible for this activity resides in the region that is unique to *AP5* (*i.e.* not found in *AP4*), in particular the TMD-coding region (TMCR).

The Coding Potential of AP5 Is Not Required for ER Localization—To ensure that ER localization of *AP5* was not due to a low level of translation in HHT-treated cells, we inserted a guanine between the 3rd and 4th nucleotides of the ORF of this mRNA, creating frameshifted *AP5* (*fs-AP5*, Fig. 1*A*). This mRNA encodes a polypeptide that does not contain a signal sequence or TMD, as predicted by either SignalP (20) or TMHMM (21) (data not shown). Furthermore, this peptide is mostly hydrophilic as measured by a Kyte-Doolittle hydropathy plot (22) (Fig. 4*A*), and is thus not a likely substrate for co-translational translocation into the ER.

Next, we expressed *fs-AP5* in COS-7 cells and observed its distribution in normal and digitonin-permeabilized cells compared with *AP5* and *t-ftz*. We also monitored *H1B-GFP* mRNA, which is not expected to be at the ER because it encodes a nuclear localized histone 1B-GFP fusion. In the majority of cells the distribution of *t-ftz*, *AP5*, *fs-AP5*, and *H1B-GFP* mRNA was mostly diffuse in the cytoplasm, indicating that a substantial fraction of these transcripts were not ER-bound (Fig. 4*B*). However, after digitonin permeabilization, significant levels of *t-ftz*, *AP5*, and *fs-AP5*, but not *H1B-GFP* mRNA, remained in the cytoplasm in a reticular pattern (Fig. 4*B*; for quantification see 4*C*), that co-localized with Trapα (data not shown). Indeed, we calculated that approximately one quarter of the cytoplasmic *fs-AP5* mRNA is bound to the ER (Fig. 4*C*). In contrast, <10% of the *H1B-GFP* mRNA was ER-associated. We next confirmed this result by analyzing the distribution of various mRNAs in different cell fractions. Transfected U2OS cells were separated into cytoplasm ER and nuclear fractions as described previously (7, 17), and the presence of RNA was determined by Northern blotting. *fs-AP5* and *t-ftz* were present at slightly higher levels in the ER than in the cytoplasmic fraction (Fig. 4*D*). In contrast, *M1-ftz* mRNA, which encodes a cytosolic protein and contains the mRNA nuclear export-promoting element M1 (13), was present predominantly in the cytoplasmic fraction (Fig. 4*D*). To ensure that the fractions were not cross-contaminated, we examined them for various markers. Note that the ER fraction was free of cytosolic proteins, such as α-tubulin, and nuclear factors, such as the RNA-binding protein Aly (Fig. 4*E*). It is likely that the discrepancy in the degree of ER localization for *fs-AP5* between Fig. 4, *B* and *E*, was because COS-7 cells tend to have higher levels of expression than U2OS and thus flood all of the mRNA-binding sites on the ER.

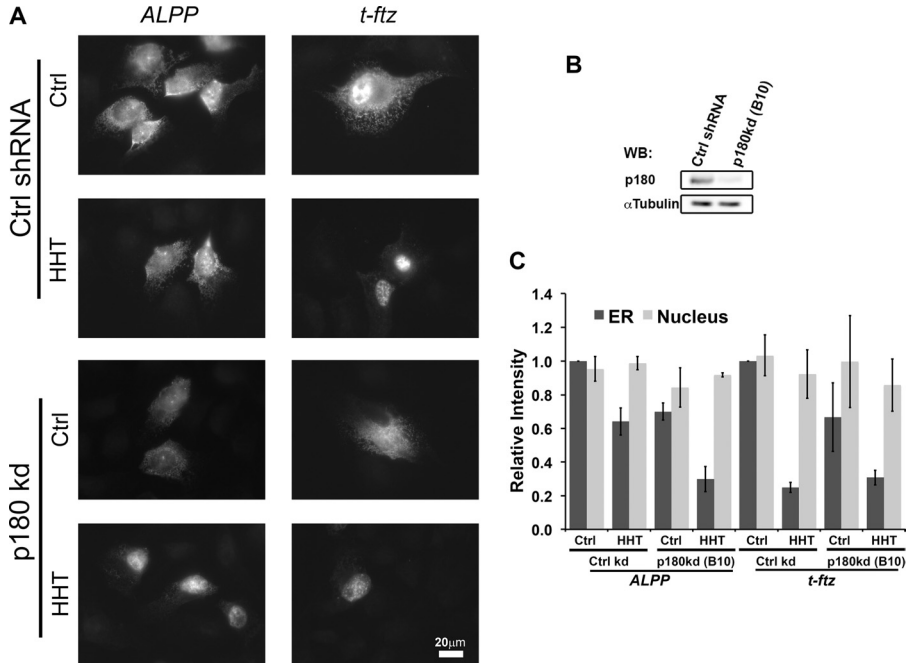

FIGURE 7. **p180 is required for the initial ER targeting of *ALPP*.** U2OS cells were infected with lentivirus carrying control shRNA (*Ctrl shRNA*/*Ctrl kd*) or shRNA clone B10 against p180 (*p180 kd*). The control and p180-depleted cells were pretreated with DMSO (*Ctrl*) or HHT for 15 min, then microinjected with plasmids containing either the *ALPP* or *t-ftz* and allowed to express mRNAs for 2 h in the presence of DMSO or HHT. The cells were then extracted with digitonin, fixed, and stained with specific FISH probes and imaged. *A*, representative examples. *B*, cell lysate collected on the day of injection. The level of depletion was assessed by immunoblotting (*WB*) against p180 and α-tubulin. *C*, quantification of the fluorescence intensities of mRNAs in the ER and nucleus. All data were normalized to the ER staining intensity in the control treated/control shRNA group for each construct. Each *bar* represents the average ± S.E. (*error bars*) of three independent experiments (each experiment consisting of $n>30$ cells). *Scale bar*, 20 μm.

We then assessed whether the localization of *fs-AP5* mRNA required translation. In contrast to *t-ftz*, *fs-AP5* remained ER-associated in COS-7 cells treated with HHT (Fig. 4, *F* and *G*). This ER localization in HHT-treated cells was confirmed by the co-localization of the mRNA with the ER marker Trapα (Fig. 4*H*). These results confirm that *AP5* contains an RNA element that can anchor transcripts on the ER independently of translation.

The TMCD Is Required for the Translation-independent ER Localization of ALPP—We next deleted the last 90 nucleotides of the ORF from the full-length *ALPP*. This construct, *ALPP-ΔTMD*, lacks the TMCR. Although this mRNA localized to the ER in COS-7 cells, we found that it was not retained there after HHT treatment, especially when compared with *ALPP* (Fig. 5). These observations indicate that the ER retention element likely resides in the TMCR.

AP5 Promotes the Efficient Targeting of mRNA to the ER Independently of Translation—Thus far, our data indicate that once localized to the surface of the ER, *AP5* is retained there even after ribosomes have dissociated from the transcript. It is however possible that the initial targeting of this mRNA to the ER is still dependent on translation, with the TMCR mediating its subsequent ER retention independently of translation. To test whether this region can promote the initial targeting to the ER independently of translation, we first treated COS-7 cells with HHT for 15 min to halt all translation, then microinjected plasmids and monitored the localization of the newly synthesized transcripts. In principle, an mRNA should be free of active ribosomes throughout its entire lifetime under these conditions. We found that newly synthesized *AP5* was targeted to the ER in both control treated and in cells pretreated with HHT (Fig. 6). The targeting of *AP5* was less efficient than the full-length *ALPP*, but enhanced compared with *t-ftz* (Fig. 6*B*). These results indicate that *AP5* can both target to, and be retained on, the ER independently of translation.

p180 Is Required for the Efficient Targeting of ALPP mRNA to the ER—Previously, we showed that p180 acts as an mRNA receptor that anchors several different transcripts, such as *ALPP*, on the surface of the ER. Unfortunately, we never tested whether p180 was required for the initial ER targeting of this transcript independently of translation. To test this, we microinjected plasmids containing *ALPP* into p180-depleted U2OS cells (Fig. 7*B*). We observed that in the absence of p180, the ability of the newly synthesized *ALPP* mRNA to target to the ER decreased in both control and HHT-treated cells (Fig. 7, *A* and *C*). In contrast, the ER targeting of *t-ftz* was strictly dependent on active translation and was not affected by p180 depletion. These data indicate that p180 is required not only for ER anchoring, but also for the efficient ER targeting of a subset of mRNA, such as the *ALPP* transcript.

The fact that we can observe a p180 dependence in targeting even in the presence of active translation suggests that p180 also either works in parallel and/or directly enhances SRP-dependent processes. This first model is supported by the fact that p180 is required for enhanced ER targeting even when translation is inhibited. The second possibility is supported by observations that p180 may directly contact ribosomes (23) and/or the translocon (24). Nevertheless, our results strongly indicate that p180 is involved in both the initial targeting and also the anchoring of *ALPP* on the surface of the ER.

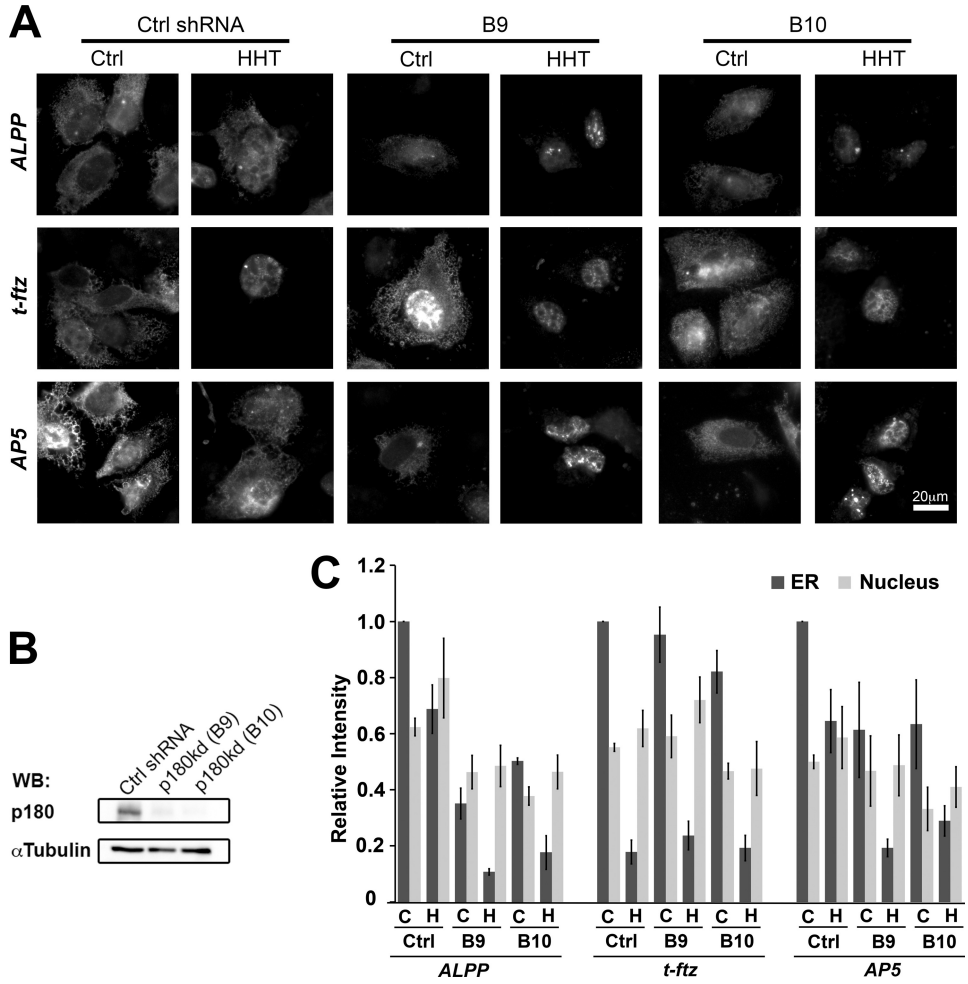

FIGURE 8. **p180 is required for the ER association of *AP5*.** U2OS cells were infected with lentivirus carrying control shRNA (*Ctrl shRNA*) or shRNAs (clones B9 or B10) against p180. The control and p180-depleted cells were transfected with plasmids containing the *ALPP*, *t-ftz*, or *AP5* construct and allowed to express mRNAs for 18–24 h. The cells were then treated with either DMSO (*C* or *Ctrl*) or HHT for 30 min (*H*), digitonin-extracted, fixed, stained with specific FISH probes, and imaged. *A*, representative examples. *B*, cell lysate collected on the day of injection. The level of depletion was assessed by immunoblotting (*WB*) against p180 and α-tubulin. *C*, quantification of the fluorescence intensities of mRNAs in the ER and nucleus. All data were normalized to the ER staining intensity in the control treated/control shRNA group for each construct. Each *bar* represents the average ± S.E. (*error bars*) of three independent experiments (each experiment consisting of $n>30$ cells). *Scale bar*, 20 μm.

ER Maintenance of AP5 mRNA Requires p180—To examine whether *TMCR*-mediated ER localization is p180-dependent, we examined the distribution of *AP5* in U2OS cells depleted of p180 (Fig. 8*B*). Indeed, similar to full-length *ALPP* mRNA, *AP5* showed a significant decrease in its ability to associate with ER in p180-depleted cells (Fig. 8, *A* and *C*). This decrease was seen in both control and HHT-treated cells. Again, the ability of *t-ftz* to be maintained on the ER was strictly dependent on translation and was not affected by the depletion of p180. In conclusion, our results indicate that the *AP5* region of *ALPP*, which includes a TMCR, contains an RNA element that anchors this mRNA to the surface of the ER in a p180-dependent manner.

DISCUSSION

We have identified a region of *ALPP* that contains an RNA element that promotes ER localization. Furthermore, our data indicate that this element requires p180 for its activity. Interestingly, this element maps to a region of *ALPP* that encodes a TMD. This finding suggests that mammalian cells may have a propensity to recognize nucleotide features that are associated with certain protein-coding regions. These inherent biases would be maintained by natural selection primarily to constraints imposed by the encoded polypeptide; however, subsequently these features could be further exaggerated to enhance activities that act at the nucleotide level. For example, the amino acid composition of TMDs is enriched in hydrophobic residues such as Leu, Ile, Met, Val, and Phe. Interestingly, all of these amino acids are encoded by codons that have a uracil at their second position and are thus relatively U-rich (25). Indeed, it has been noted that uracil content is a good predictor of whether any given region within an ORF encodes a TMD or a signal sequence (26). As mentioned in the Introduction, it has also been shown that *E. coli* targets certain mRNAs to the plasma membrane by elements found within TMCRs that are U-rich (5). These results may indicate that the propensity for TMCR to promote the localization of mRNAs to sites of secretory protein production, independently of translation, maybe universally conserved.

This simplistic model, however, does not explain all of our observations. When the uracil content of the *ALPP* transcript

was analyzed, the TMCR contains a relatively high level of uracil compared with other sections of the transcript; however, one can clearly find regions within the ORF and UTRs that have even higher levels (see Fig. 1*B*). Furthermore, regions of the *t-ftz* 3′-UTR (present in both *t-ftz* and *AP5*, see Figs. 1*C* and 4*A*) have levels of uracil that exceed what is found in the TMCR of *ALPP*, yet *t-ftz* does not exhibit much translation-independent ER targeting. We also recently reported that the *CYP8B1* mRNA, which encodes a membrane-bound protein, also requires translation for efficient ER anchoring (9), suggesting that not all TMCRs have this activity. Finally, calreticulin mRNA, which does not encode any TMD, associates with the ER independently of translation in a p180-dependent manner (7). Thus, it is clear that although U-richness in TMCRs may help contribute to this activity, other features are also important. Work in budding yeast has identified other *cis*-elements that are responsible for ER targeting. One example is Pmp1, which contains several UG repeats in its 3′-UTR that can promote ER localization (27). Recently, other yeast mRNAs have been shown to localize to the ER independently of translation in manners that depend on either their ORFs and/or UTRs (28). Thus, it is likely that many different elements may target mRNAs to the ER.

Another element found within ORFs of metazoan mRNAs encoding secretory proteins is the SSCR. It promotes nuclear export (12) and translation (17) of mRNAs. Mutations within the SSCR redirect mRNAs from the ER to stress granules (12), large cytoplasmic aggregates of mRNAs with stalled translation initiation complexes (29). SSCRs also have unusual nucleotide compositions. They are depleted of adenine, enriched in guanine, cytosine, and uracil, and tend to contain CUG repeats (12, 13). SSCRs, however, do not appear to promote translation-independent ER targeting as exemplified by *t-ftz* and insulin-like 3 genes, which despite having SSCRs with high U content, absolutely require translation for efficient targeting and anchoring to the ER (Fig. 3*A*) (7). It is still possible that whereas the SSCR is not sufficient, it may still be required for translation-independent ER targeting.

We have attempted to analyze the degree of conservation in the 90 nucleotides at the end of the *ALPP* ORF; however, this analysis was hampered by the fact that mammals contain numerous *ALPP* paralogs, many of which lack TMCRs. Despite this, we found that these regions had features that are associated with SSCRs. In particular, they have CUG repeats, GC-rich regions and low adenine content. These observations raise the possibility that the number of SSCR-like segments present in a given mRNA impacts the efficiency of the p180-dependent pathway. We are currently identifying p180-associated mRNAs, and through this approach we hope to computationally determine which sequence features and motifs correlate with p180 dependence for ER anchoring.

It remains unclear how p180 promotes the specific anchoring of a subset of mRNAs to the ER. p180 likely binds the RNA backbone through ionic interactions (7) and therefore is not likely to act selectively. In light of this, other RNA-binding proteins, which have classic RNA recognition motifs, may act in conjunction with p180 to selectively retain certain mRNAs. Candidates were previously identified in a mass spectrometry screen of proteins that co-purify with ER-derived polysomes (7). Ultimately, the *ALPP* TMCR could be used to identify these accessory proteins.

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