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Minireview

Cis and *trans* acting factors in the regulation of parathyroid hormone (PTH) mRNA stability by calcium and phosphate

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Abstract Calcium and phosphate regulate parathyroid hormone (PTH) mRNA stability through differences in binding of parathyroid proteins to an element in its 3′-untranslated region. One of the proteins is AUF1 (A+U-rich element binding factor 1). An in vitro degradation assay showed that transcripts for PTH and chimeric growth hormone (GH)–PTH 63 nt, but not for native GH, were stabilized by PT proteins from rats on low calcium diets and destabilized by proteins from rats on low phosphate diets, correlating with PTH mRNA levels in vivo. In transfection experiments the 63 nt binding element destabilized mRNAs of reporter genes and this was prevented by overexpression of AUF1. Our results identified a functional *cis* element in PTH mRNA. Differences in protein binding to this element determine PTH mRNA stability and its regulation by calcium and phosphate.

Key words: Parathyroid hormone gene; Calcium; Phosphate; RNA stability; 3′-Untranslated region; Protein–RNA binding; A+U-rich element binding factor 1

1. Introduction

Parathyroid hormone (PTH) has a central role in maintaining normal calcium (Ca^{2+}) and phosphate (Pi) homeostasis as well as bone strength. Dietary induced hypocalcemia markedly increases PTH mRNA levels, secretion and after prolonged stimulation, parathyroid (PT) cell proliferation [1]. A seven trans-membrane G coupled Ca^{2+} sensing receptor on the PT cell membrane senses changes in extracellular Ca^{2+} concentrations [2]. How low Ca^{2+} regulates the levels of PTH secretion, gene expression and PT cell proliferation is still not clear. We have shown that the increase in PTH mRNA levels is post-transcriptional [3]. Pi also regulates PTH secretion, gene expression and PT cell proliferation [4]. Dietary induced Pi depletion dramatically decreases PTH mRNA levels and this is also post-transcriptional [5]. There is a ~60-fold difference in PTH mRNA levels between hypocalcemic and hypophosphatemic rats and we used these dietary models as tools to define the mechanism of the post-transcriptional regulation of PTH gene expression. We have shown that the post-transcriptional regulation by dietary induced hypocalcemia and hypophosphatemia is mediated by protein–RNA interactions involving protein binding to a specific element in the PTH mRNA 3′-untranslated region (UTR) that determine PTH mRNA stability [3]. We suggest that after a low Ca^{2+} diet there is increased binding to the PTH mRNA 3′-UTR that protects the RNA from degradation resulting in increased PTH mRNA levels. After a low Pi diet there is less binding that allows more degradation and leads to the decrease in PTH mRNA levels (Fig. 1).

2. Protein binding regulates PTH mRNA stability

Weanling rats were fed a control diet or diets deficient in Ca^{2+} (low calcium) or Pi (low Pi) for 2 weeks. A low Ca^{2+} diet resulted in a 10-fold increase in PTH mRNA levels and a low Pi diet to a six-fold decrease compared to the rats fed a control diet. Protein binding to the PTH mRNA 3′-UTR was increased by hypocalcemia and decreased by hypophosphatemia by RNA electrophoretic mobility shift assay (REMSA) and UV cross-linking gels, in correlation with PTH mRNA levels (Fig. 1). This binding was regulated by Ca^{2+} and Pi only in the PT and not in other tissues. UV cross-linking of PT proteins to transcripts for the full-length and the 3′-UTR showed three protein–RNA bands. To identify the PTH mRNA-binding proteins we have performed PTH RNA 3′-UTR affinity chromatography. One of the purified proteins was sequenced and was identical to A+U-rich element binding factor 1 (AUF1) protein (hnRNP D) [6] which is known to be important to the stability of other mRNAs [7]. Recombinant AUF1 bound the PTH mRNA 3′-UTR with a single band at 50 kDa, which corresponds to one of three protein–RNA bands found with cytosolic PT proteins by UV cross-linking gels. Brewer et al. have cloned this RNA-binding protein which binds with high affinity to a variety of A+U-rich elements (AREs) in the 3′-UTRs of a number of mRNAs [8]. These include mRNAs for cytokines, oncoproteins, and G protein coupled receptors, where AUF1 is involved in the degradation of these mRNAs [9]. Three classes of AREs have been characterized, two of which contain several scattered or overlapping copies of the pentanucleotide AUUUA [10], [11]. The class III AREs lack the AUUUA motif but

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Abbreviations: PTH, parathyroid hormone; PT, parathyroid; AUF1, A+U-rich element binding factor; LC8, dynein light chain (M_r 8000); REMSA, RNA electrophoretic mobility shift assay; Ca^{2+}, calcium; Pi, phosphate

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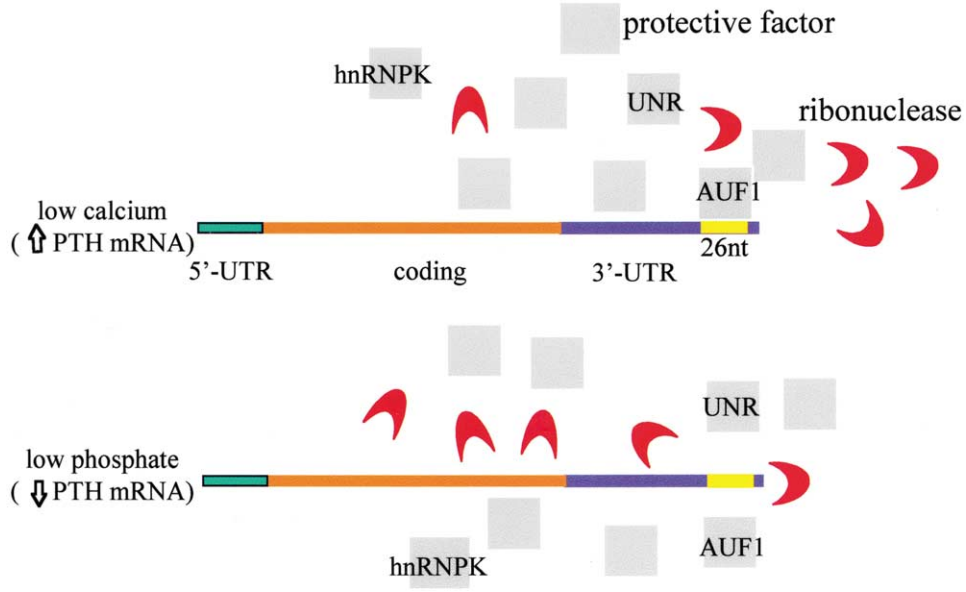

Fig. 1. Regulation of PTH mRNA stability involves PT protective and degrading proteins. Schematic representation of the PTH mRNA with the protective RNA-binding proteins (square) and the degrading ribonucleases that degrade the RNA (packman). Protein binding is increased after a low Ca^{2+} diet resulting in protection of the bound RNA and an increase in PTH mRNA. After a low Pi diet there is less binding, more degradation and a decrease in PTH mRNA levels. The PTH mRNA-binding proteins, AUF1, hnRNP K and UNR are shown and the conserved 26 nt element is indicated.

require a U-rich sequence and possibly other unknown determinants [10]. It has been shown that the AUUUA motif is not required for AUF1 binding [12]. The PTH mRNA 3′-UTRs are rich in A and U ranging from 68 to 74% of the nucleotides [13]. The PTH mRNA 3′-UTR-binding element (see below) is a type III ARE that does not contain any AUUUA sequences [14]. There are four isoforms for AUF1 that are generated by alternative splicing of the AUF1 transcript [15]. The activity of AUF1 may be determined by the presence of other proteins in the binding complex, such as αCP1 and 2 in stabilizing the α-globin mRNA [16]. Two additional PTH mRNA-binding proteins were identified by the affinity chromatography (not shown) as hnRNP K [17,18] and up stream of nRAS (UNR) [19]. These proteins were shown to bind PTH transcripts by binding assays using recombinant proteins. Specific antibodies to each of the three identified proteins led to a super shift in the binding of PT proteins to the PTH mRNA 3′-UTR, demonstrating that they are part of the protein–RNA complex (not shown).

There is no PT cell line therefore we have utilized a cell-free mRNA in vitro degradation assay (IVDA) to demonstrate the functionality of the PT cytosolic proteins in determining the stability of the PTH transcript. This assay has been shown to authentically reproduce cellular decay processes [20]. PT proteins from control rats incubated with a full-length PTH mRNA probe led to gradual degradation of the PTH transcript, which was a result of degrading and protective factors present in the cytosolic extract. The transcript was intact until 40 min with PT proteins from control rats. However, with hypocalcemic PT proteins the transcript was not degraded until 180 min and with hypophosphatemic proteins only until 5 min, correlating with mRNA levels in vivo (Figs. 2A and 3B upper gel) [3]. Therefore, the IVDA reproduces the in vivo stabilizing effect of low Ca^{2+} and destabilizing effects of low Pi on PTH mRNA levels. A transcript that did not include the 3′-UTR or just the 60 nt terminal protein-binding region of the PTH mRNA 3′-UTR was more stable than the full-length PTH transcript and was intact for more than 180 min (Fig. 2B). This suggests that the binding region is an instability element. Moreover, Ca^{2+} (not shown) and Pi (Fig. 2B) did not regulate the stability of the truncated transcript that did not include the protein-binding region [3]. Therefore, the regulation by PT proteins from low Ca^{2+} and Pi rats in the IVDA is dependent upon the protein-binding sequence in the PTH mRNA 3′-UTR.

To study the effect of AUF1 on PTH RNA degradation, recombinant AUF1 was added to the IVDA. Addition of recombinant AUF1 p37 and p40 isoforms stabilized the PTH transcript even by hypophosphatemic proteins, that without AUF1 led to rapid degradation of the full-length PTH transcript [6]. Other control proteins, bovine serum albumin and dynein light chain (M_r 8000) (LC8) [21], which we have shown also binds to the PTH mRNA 3′-UTR, had no effect [22]. This result supports the regulatory role of AUF1 in PTH mRNA stability. The role of the other two PTH mRNA-binding proteins that we have identified, hnRNP K and UNR, in this regulation still remains to be determined.

3. Identification of the minimal protein-binding element in the PTH mRNA 3′-UTR and demonstrating its functionality

Binding and competition experiments by REMSA and UV cross-linking gels identified a minimal sequence of 26 nt that was sufficient for PT protein binding [14]. Sequence analysis of the 26 nt element in the PTH mRNA revealed high conservation of the rat element in the PTH mRNA 3′-UTRs of the murine (23 of 26 nt), human (19 of 26 nt) and canine (19 of 26 nt) species, with human and canine being identical. Such conservation of this sequence that lies outside of the coding region, amongst different species, suggests a functional role for this element.

To study the functionality of the protein-binding element in

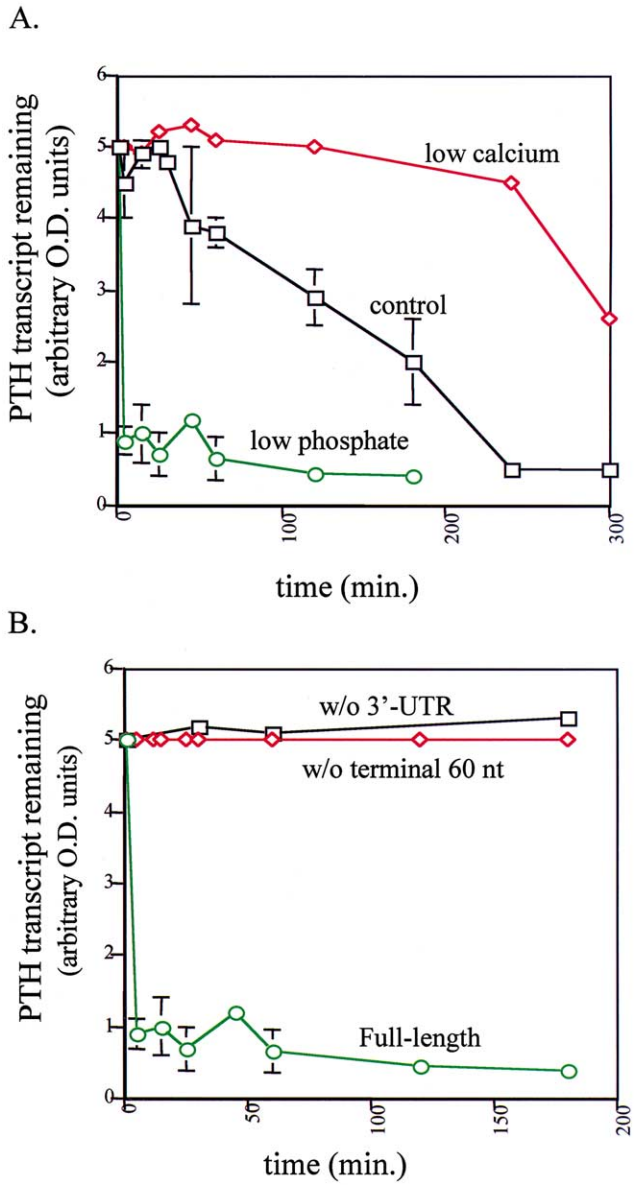

Fig. 2. In vitro degradation of PTH RNA by PT cytosolic proteins is regulated by Ca^{2+} and Pi and dependent on the 60 nt terminal of the PTH mRNA. A: Time–response curve for IVDAs of the full-length PTH transcript incubated with PT proteins from rats fed a normal, low Ca^{2+} (–Ca) or low Pi (–P) diet. Each point represents the mean ± S.E.M. of three or four different experiments, apart from –Ca at 240 and 300 min which is the mean of two experiments. At some points the S.E.M. is less than the size of the graphic symbols. The PTH transcript was stabilized by PT proteins from –Ca rats and rapidly degraded by proteins from –P rats. B: Mapping a region in the PTH 3′-UTR that mediates degradation by PT proteins from –P rats. Time–response curve of the IVDA with transcripts for the full-length and shorter PTH mRNA transcripts incubated with PT proteins from low Pi rats. PTH mRNA probes used are intact (full-length), without the 3′-UTR, and without the 3′-terminal 60 nt of the 3′-UTR. The PTH transcript that did not contain the protein-binding region was not degraded by PT proteins.

the context of another RNA, a 63 bp fragment encoding the 26 nt of the PTH mRNA 3′-UTR and flanking nt, was fused to growth hormone (GH) reporter gene (Fig. 3A). RNAs were transcribed in vitro and transcripts subjected to IVDA with PT proteins. The chimeric GH–PTH 63 nt transcript, as the full-length PTH transcript, was stabilized by PT proteins from rats fed a low Ca^{2+} diet and destabilized by PT proteins of a low Pi diet, correlating with PTH mRNA levels in vivo. The native GH transcript was more stable than PTH and the chimeric RNAs and was not affected by PT proteins from the different diet (Fig. 3B). Therefore, the PTH RNA–protein-binding region destabilized the GH transcript in the presence of PT proteins. Furthermore, this element conferred responsiveness of GH to changes in PT proteins by Ca^{2+} and Pi [14]. The results demonstrate that the protein-binding region of the PTH mRNA 3′-UTR is both necessary and sufficient for determining RNA stability and for the response to Ca^{2+} and Pi.

We then studied the function of the PTH element in cells, using the heterologous cell line HEK293. cDNAs encoding the protein-binding region of the PTH mRNA 3′-UTR were inserted at the 3′-end of two reporter cDNAs. 63 bp of the PTH 3′-UTR were inserted into a GFP construct driven by a CMV promoter and a larger fragment of 100 bp into a GH construct driven by a S16 promoter. The plasmids were transiently transfected into HEK293 cells. At 24 h mRNA levels were measured by Northern blot, and protein levels of GFP by immunofluorescence, and secreted GH by radioimmunoassay. There was a dramatic reduction in the expression of the chimeric genes containing the PTH elements compared to the wild type genes. A truncated PTH 40 nt element had no effect on reporter gene expression (not shown). The 100 and 63 nt transcripts, like the full-length PTH RNA, bound PT proteins by UV and REMSA, but the truncated element did not bind PT proteins. The different constructs for each reporter gene all used the same promoter, therefore these results suggest that insertion of the PTH 3′-UTR element decreased the stability of the reporter transcripts and not their transcription levels. Inhibition of transcription by the addition of DRB to the transfected cells and the measurement of mRNA decay confirmed that the decrease in mRNA levels was post-transcriptional (not shown). These results are in agreement with the decreased stability of the GH chimeric transcript in the IVDA with PT proteins (Fig. 3B). They are consistent with the destabilizing effect of the *cis* element in the PTH mRNA.

Over-expression of AUF1 by cotransfection of the reporter genes together with expression plasmids for myc-tagged AUF1 isoforms [23] prevented the rapid degradation of the chimeric mRNAs containing the PTH element (not shown). This was observed with all the myc-tagged isoforms (not shown). Western blots confirmed the expression of the myc-AUF1 proteins in the cells. These results suggest that the destabilizing affect of the PTH element is due to insufficient amount of AUF1 in the transfected cells and that this is reversed in the presence of increased levels of AUF1. AUF1 therefore inactivates the rapid RNA decay directed by the PTH mRNA 3′-UTR-binding element.

The mechanism of the stabilizing effect of AUF1 is not clear. In the PT low Ca^{2+} and Pi do not elevate the level of AUF1 by Western blots. Preliminary results show differences in post-translational modifications, possibly phosphorylation rather than the amount of AUF1 protein. Recent studies show alterations in RNA–protein binding and RNA stability in response to specific signal transduction pathways. One study showed that activation of p38 mitogen activated protein kinase induced by proinflamatory cytokines inhibited mRNA decay [24]. It is possible that in vivo PTH mRNA turnover is controlled by phosphorylation–dephosphorylation by specific

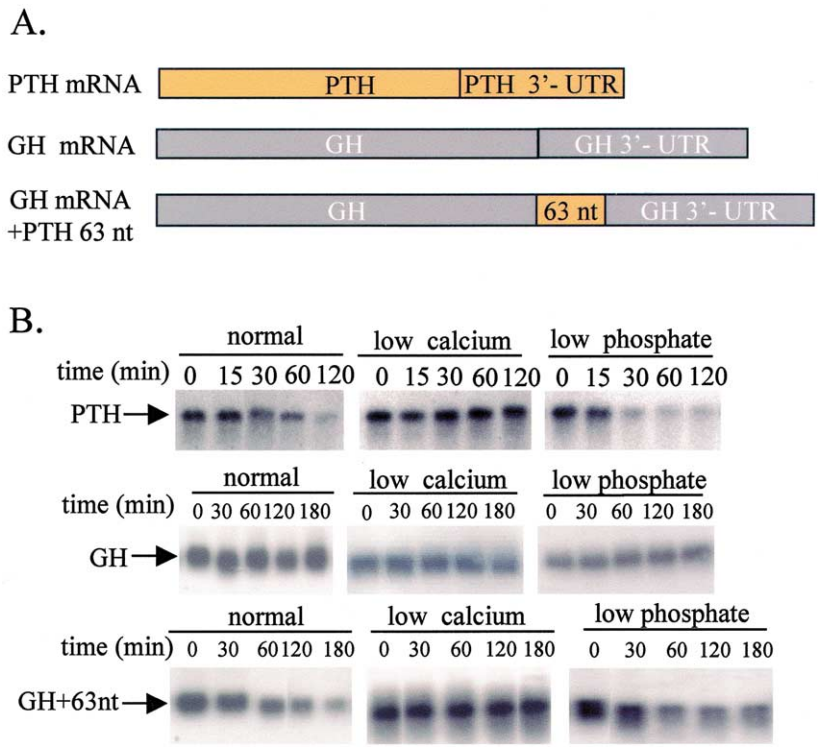

Fig. 3. The PTH mRNA 3′-UTR 63 nt protein-binding region confers responsiveness of GH mRNA to PT proteins from rats fed low Ca^{2+} or low Pi diets in an IVDA. A: Schematic representation of the PTH mRNA (above), GH mRNA and the chimeric GH mRNA containing the PTH 3′-UTR 63 nt element inserted at the end of the GH coding region (bottom). B: Representative gels of IVDAs for labeled transcripts for PTH (top), GH (middle) and GH+63 nt of the PTH 3′-UTR (bottom) with PT proteins from rats fed a normal, low Ca^{2+} or low Pi diet. At timed intervals after protein and RNA incubation samples were removed for RNA analysis. The chimeric GH transcript was less stable than the native GH. The full-length PTH and the chimeric GH–PTH 63 transcripts were stabilized with low Ca^{2+} PT proteins and destabilization with low Pi PT proteins. The PT proteins from the different diets had no affect on the native GH transcript.

kinases and phosphatases that are responsible for the direct modifications. In the experimental models of the IVDA and transfection experiments excess AUF1 compensates for modifications that may occur in vivo and are absent in vitro.

4. The structure of the PTH *cis* acting element

The IVDA and the transfection experiments demonstrate the functional importance of the RNA–protein-binding region in the PTH 3′-UTR. RNA utilizes sequence and structure for its regulatory functions. We have studied the structure of the PTH mRNA 3′-UTR in order to understand the mechanism by which it regulates mRNA half-life both in vitro and in vivo. Using primer extension experiments, RNase H analysis and computer structure modeling we have shown that the 100 nt that include the 26 nt protein-binding core and flanking sequences have a defined loop–stem–loop structure (not shown). This structure is maintained when the full-length transcript is studied. Mutation analysis confirm that this structure is important for protein binding and for the destabilizing effect of the *cis* acting element in transfection experiments with GFP as a reporter gene.

5. Conclusion

Dietary induced hypocalcemia and hypophosphatemia regulate PTH gene expression post-transcriptionally and this is dependent upon the binding of PT cytosolic proteins to instability regions in the PTH mRNA 3′-UTR. The binding of these cytosolic proteins to the PTH mRNA is increased in hypocalcemia and decreased in hypophosphatemia correlating with PTH mRNA levels in vivo. There is no PT cell line and the stability of PTH transcripts was studied by an IVDA. PT proteins from hypocalcemic rats lead to an increase in PTH RNA stability in the IVDA and hypophosphatemic proteins to a marked decrease in stability. One of the PTH mRNA-binding proteins is AUF1 that stabilizes the PTH mRNA. We have identified a conserved 26 nt element in the PTH mRNA 3′-UTR as the minimal protein-binding sequence. The functionality of a 63 nt element that included the 26 nt was studied in reporter RNAs. The stability of the chimeric RNAs was studied in the IVDA with PT proteins of low Ca and Pi rats and in transfection experiments in HEK 293 cells. This element destabilized the reporter genes and was sufficient to confer responsiveness to Ca^{2+} and Pi in the IVDA. Our results demonstrated a functional *cis* element in the PTH mRNA 3′-UTR. Differences in binding of *trans* acting factors to this element determine PTH mRNA stability and its regulation by Ca^{2+} and Pi. The understanding of the mechanism of how Ca^{2+} and Pi regulate PTH gene expression will allow the design of novel treatment strategies for diseases such as the secondary hyperparathyroidism of chronic renal failure.

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