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Intracellular ANKRD1 protein levels are regulated by 26S proteasome-mediated degradation

Ileana Badi [a,1], Raffaella Cinquetti [a,1], Michela Frascoli [a], Cinzia Parolini [b], Giulia Chiesa [b], Roberto Taramelli [a,2], Francesco Acquati [a,2,*]

[a] Dipartimento di Biotecnologie e Scienze Molecolari, Università degli Studi dell'Insubria, Via JH Dunant 3 – 21100 Varese, Italy
[b] Dipartimento di Scienze Farmacologiche, Università degli Studi di Milano, Via Balzaretti 9 – 20133 Milan, Italy

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ABSTRACT

The ANKRD1/CARP gene encodes a muscle-specific protein which has been implicated in transcriptional regulation and myofibrillar assembly. Several features at both the mRNA and protein levels define ANKRD1 as a gene whose expression is tightly regulated, and deregulated expression of this protein has been recently associated to human congenital heart disease. It is therefore crucial to define the intracellular pathways that regulate the ANKRD1 protein's steady-state levels. Here, we show that ANKRD1 is a short-lived protein whose levels are tightly regulated by the 26S proteasome. In addition, a critical role for a putative PEST motif was established, although other degrons within the ANKRD1 protein are likely implicated in the control of its intracellular levels.

1. Introduction

Intracellular proteolysis has long been recognized as a widespread mechanism for the regulation of gene function at the protein level [1,2]. Indeed, eucaryotic cells are endowed with sophisticated regulatory mechanisms controlling the turnover rate of a host of proteins, including cyclins and cell cycle-related proteins, structural proteins, cell-surface receptors and transcription factors [3–5]. These observations led to the well established concept that regulated proteolysis plays an essential role in a wide range of biological processes such as cell proliferation, differentiation, apoptosis and development.

Although many distinct intracellular proteases (such as the calpain family members and the lysosome-associated acid proteases) are involved in the regulation of protein intracellular levels [6,7], a major role for the control of protein turnover rates has been assigned to the 26S proteasome. Indeed, this evolutionarily widespread protein degradation machinery is involved in the regulation of the intracellular levels for a wide range of proteins which have been related to all the above-mentioned biological processes [8].

As far as the involvement of controlled proteolysis in developmental processes is concerned, among the candidate proteins underlying such regulatory mechanisms are those involved in the regulation of gene expression. Indeed, several reports have demonstrated in recent years the role of the 26S proteasome in controlling the intracellular stability of a host of transcription factors [9].

Recently, we reported experimental evidences pointing at ANKRD1/CARP, which encodes a cardiac-specific transcriptional repressor, as a candidate gene for Total Anomalous Pulmonary Venous Return (TAPVR), a cardiac developmental anomaly accounting for 1.5% of all human congenital heart diseases [10]. A preliminary characterization of TAPVR-associated mutations led us to envision the tight regulation of intracellular ANKRD1 levels as a critical factor for the execution of heart developmental processes underlying the proper connection of the pulmonary veins to the atrial chambers [11]. Therefore, investigation of the intracellular pathways involved in the regulation of ANKRD1 protein levels might contribute to a better definition of the molecular mechanisms underlying its function in the developing heart.

Here, we provide experimental evidence showing that TAPVR-associated mutations indeed affect the in vivo intracellular stability of this protein, thus suggesting that regulation of ANKRD1 levels represents a critical mechanism for this protein's function.

* Corresponding author. Fax: +39 0332 421500.
E-mail address: francesco.acquati@uninsubria.it (F. Acquati).
[1] These authors contributed equally to this work.
[2] Present address: Department of Anatomy and Embryology, Academic Medical Center, Meibergdreef 15 (L2-104), 1105 AZ Amsterdam, The Netherlands.

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Moreover, we show that the 26S proteasome plays a key role in the above-mentioned process.

2. Materials and methods

2.1. Chemicals

Cycloheximide and NH_4Cl were purchased from Sigma and Merck, respectively. MG-132 and calpeptin (from Calbiochem) were dissolved in DMSO.

2.2. Plasmid constructs

Wild-type ANKRD1 cDNA was tagged with a triple FLAG epitope (3FLAG) by PCR-amplification and cloning into pcDNA3 expression vector (Invitrogen). Triple-FLAG T116 M and Δ-PEST variants were generated by overlap extension PCR. The 1-107 and Δ(5–125) ANKRD1 deletion mutants were assembled by PCR-amplification from wild-type ANKRD1 cDNA and cloning into the same pcDNA3 vector. Untagged or FLAG-tagged wild-type, T116 M, and Δ-PEST ANKRD1 coding sequences were also cloned in pIRESneo3 (Clontech) for stable transfection in HeLa and SK-MES-1 cells. The GFP-PEST construct was assembled by cloning a short cDNA fragment encoding ANKRD1's PEST motif (aa 107–125) in frame with the EGFP coding region from the pEGFP-C3 vector (Clontech). pMT123, a vector expressing influenza virus hemagglutinin HA-tagged ubiquitin, and the empty HA-tagged expression vector were kindly provided by Dr. D. Bohmann (University of Rochester Medical Center). The sequences of all primers used in cloning strategies are available upon request. The pEGFP-N1 plasmid (Clontech) was used to normalize transfection efficiencies.

2.3. Cell culture and transfections

HeLa cells were cultured in DMEM/F12 (Sigma) supplemented with 10% Fetal Bovine Serum (FBS) and 2 mM glutamine. The SK-MES-1 cell line was maintained in DMEM (Sigma) supplemented with 10% FBS and 2 mM glutamine. For stable transfections, 2 μg of recombinant constructs in pIRESneo3 were transfected in HeLa or SK-MES-1 cell lines using Lipofectamine 2000 Reagent (Invitrogen). HeLa and SK-MES-1 clones were selected in 400 or 800 μg/mL G-418 (Gibco), respectively, and expression levels were assayed by immunoblot analysis with M2 anti-FLAG monoclonal antibody (Sigma). HeLa cells were also transiently transfected with pEGFP-N1 vector, pEGFP-C3-PEST and pcDNA/ANKRD1-3FLAG deletion mutants expression vectors at a 1:1.5 ratio, using Lipofectamine 2000 Reagent; salmon sperm DNA was used to keep the total amount of transfected DNA constant at 4.25 μg. For ubiquitin incorporation assays, HeLa cells were transfected with a standard calcium phosphate transfection method.

2.4. Immunoblotting

Cells were harvested by scraping and sonicated in 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40 and 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin and 1 mM benzamidine. Following incubation at 4 °C for 15 min and centrifugation at 12 000×*g* for 15 min at 4 °C, supernatants were collected and stored at −20 °C. Protein concentration was determined with the Bradford protein assay (Bio-Rad). Cell lysates (40 μg) were resolved by SDS–PAGE and transferred to PVDF membranes (Millipore). 3FLAG-tagged ANKRD1 variants were detected with the anti-FLAG antibody at a concentration of 2.5 μg/mL. Loading controls were performed by detecting α-tubulin with anti-α-tubulin DM 1A (Sigma) at a dilution of 1:1000. GFP and HA-tagged ubiquitin were detected with monoclonal anti-GFP and anti-HA (clone 12CA5) antibodies (Roche) diluted at 1:3000 and 1:2000, respectively. Membranes were then incubated with secondary goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) (Pierce) diluted at 1:900. For the detection of endogenous ANKRD1 protein, 80 μg of total cell extracts were subjected to SDS–PAGE and electroblotted onto a PVDF membrane. Protein levels were detected with polyclonal anti-CARP (H-120) antibody (Santa Cruz Biotechnology Inc.) at 1:500 dilution. After washing, the membrane was incubated with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (Pierce), diluted at 1:900. Antibody complexes were visualized with SuperSignal West Dura Extended Duration Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

2.5. ANKRD1 half-life determination

Stably transfected or untransfected SK-MES-1 and stably transfected HeLa cells were plated 24 h prior to treatment with 100 μg/mL cycloheximide. Cells harvesting and immunoblot analysis at the appropriate time points were carried out as described above. Untagged or FLAG-tagged ANKRD1 variants or endogenous ANKRD1 levels were determined by densitometric analysis using ImageJ (rsb.info.nih.gov/ij), following normalization with α-tubulin levels. The rate of protein decay was determined as described in [12] from four to five independent experiments.

2.6. Immunofluorescence

HeLa cells transfected with ANKRD1-3FLAG variants were cultured on coverslips, fixed with 3% paraformaldehyde at room temperature and permeabilized with 0.5% Triton X-100 for 10 min on ice. After blocking with 2% BSA for 60 min at 37 °C, samples were incubated with monoclonal M2 anti-FLAG antibody diluted at 1:50, for 1 h at room temperature. Coverslips were then incubated with FITC AffiniPure Fab Fragment Donkey Anti-Mouse IgG (H + L) (Jackson ImmunoResearch Lab Inc.) diluted at 1:150 for 1 h at room temperature. Filamentous actin was stained with Phalloidin-tetramethylrhodamine B isothiocyanate conjugate (Fluka) at the dilution of 1:150. Images were captured using an Olympus IX51 inverted microscope equipped with a Nikon DXM1200F digital camera and analyzed with Confocal Assistant 4.02 (Cas40) software.

2.7. Ubiquitin incorporation assay

Untransfected or wild-type ANKRD1-3FLAG-expressing HeLa cells were transfected with 15 μg of HA-ubiquitin, HA-K48R-ubiquitin mutant or an empty HA expression vector. Cells were grown for 24 h and DMSO or 20 μM MG-132 was added before culture was continued for 3 h. Cell extracts were prepared as described above and equal protein amounts were immunoprecipitated with EZview Red ANTI-FLAG M2 Affinity Gel (Sigma) according to the manufacturer's protocol. Levels of ubiquitinated proteins and ANKRD1-3FLAG in cell extracts were monitored by immunoblot with anti-HA antibody or anti-FLAG antibody.

3. Results and discussion

3.1. ANKRD1 is a short-lived protein whose intracellular stability is controlled by the PEST motif

We previously reported two independent TAPVR-associated mutations resulting in a significant increase in the expression levels of ANKRD1 at either the transcript or protein levels [11]. In particular, a T116 M missense mutation within a putative PEST motif

in the gene's coding region was found to increase both the in vitro stability and transcriptional repression activity of the ANKRD1 protein [11]. Therefore, we postulated the occurrence of a mechanism for intracellular regulation of ANKRD1 levels by means of PEST-mediated protein degradation.

To investigate this issue, three expression constructs encoding wild-type, T116 M or a PEST-deleted FLAG-tagged ANKRD1 protein were assembled as described in materials and methods and used to investigate the intracellular pathways controlling its turnover rate. To verify that the overall structure of the proteins encoded by these expression constructs was not affected, we evaluated their intracellular localization by immunofluorescence assays following transfection of the above-mentioned constructs into HeLa cells. As shown in Fig. 1, both the intracellular localization and signal intensity were quite similar for all three protein variants, suggesting that ANKRD1 overall structure was not significantly affected by the T116 M and Δ-PEST mutations. Indeed, all three protein variants were localized in the nucleus, as previously described for a functional ANKRD1 protein [13]. Moreover, a functional study carried out by transient co-transfection assays in HeLa cells with a cardiac ANF promoter-driven luciferase-encoding recombinant construct showed that the ANKRD1 T116 M variant was functionally competent as a heart-specific transcriptional repressor (Supplementary Fig. 1). Actually, this mutant ANKRD1 protein proved to be even a slightly more effective transcriptional repressor when compared with the wild-type protein, in keeping with our previous in vitro data showing an increased intracellular stability of ANKRD1 upon replacement of threonine 116 with methionine [11].

We then turned our attention to the stability of wild-type and mutant ANKRD1 proteins in vivo. To this aim, the half-lives of wild-type, T116 M and Δ-PEST FLAG-tagged ANKRD1 proteins were compared in stably transfected HeLa cells following treatment with the protein synthesis inhibitor cycloheximide (CHX). In these experiments, the level of wild-type ANKRD1 protein decreased rapidly upon CHX treatment, with a half-life of 36 ± 5 min (means ± S.E.), showing that ANKRD1 is a short-lived protein (Fig. 2). This finding is in agreement with previous reports suggesting a tight regulation for ANKRD1 expression at both the RNA and protein levels [13].

Moreover, consistent with our previous in vitro studies, the T116 M mutation was found to significantly increase the stability of ANKRD1 in vivo, showing a half-life of 57 ± 8 min (means ± S.E.) (Fig. 2). Thus, replacement of threonine 116 with a "non-PEST" methionine residue apparently confers an increased in vivo stability to the protein, confirming the relevance of the PEST motif for ANKRD1 intracellular turnover. This conclusion was further strengthened by the observation that deletion of the whole PEST motif further increased ANKRD1 stability in vivo, with a half-life of 67 ± 9 min (means ± S.E.) (Fig. 2). The homogeneity of the *b* values (slopes) for the regression lines shown in Fig. 2 was assessed by performing a test of parallelism. By this analysis, the differences in slopes between wild-type ANKRD1 and either T116 M or ΔPEST variants were statistically significant (with *P*-values $4.07 * 10^{-9}$ and $3.1 * 10^{-5}$, respectively), whereas the difference between T116 M and ΔPEST ANKRD1 did not reach statistical significance (*P*-value: 0.58).

Since these experiments were performed with exogenous, FLAG-tagged proteins, we determined whether endogenous ANKRD1 is also a short-lived protein. Due to undetectable ANKRD1 protein levels in HeLa cells, we turned our attention to the lung epithelium-derived SK-MES-1 cell line, whose ANKRD1 protein levels were previously shown to be detectable by western blot

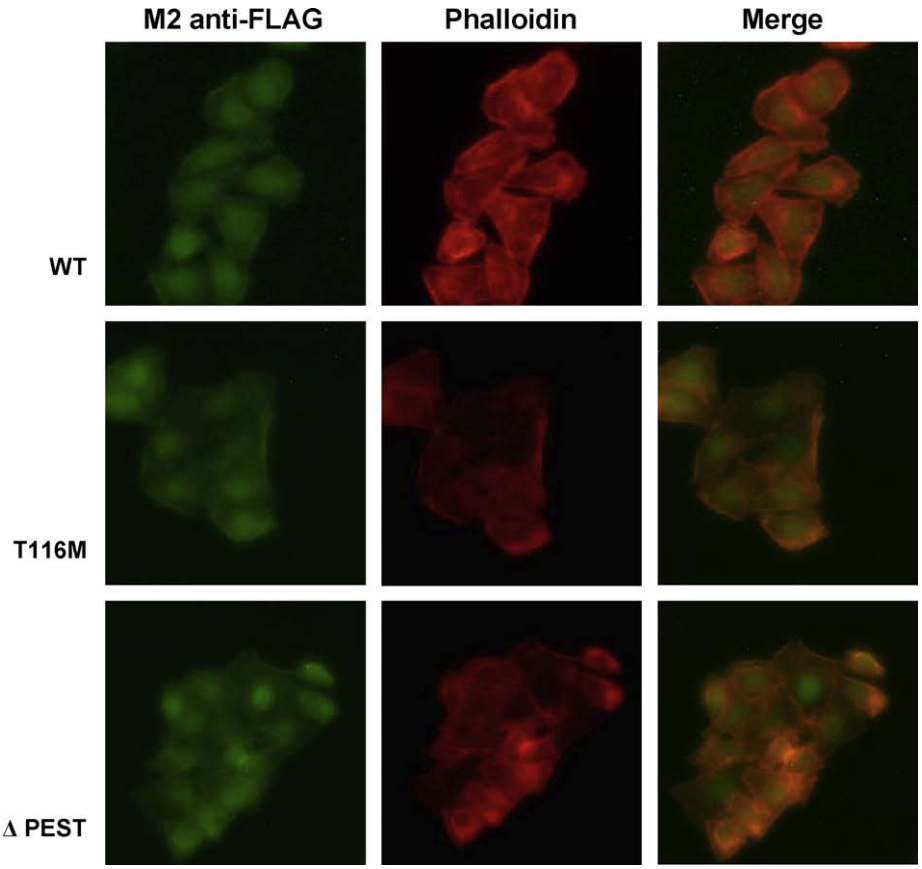

Fig. 1. Immunolocalization of ANKRD1 protein variants. HeLa cells stably transfected with wild-type, T116 M or Δ-PEST ANKRD1-3FLAG expression vectors were subjected to indirect immunofluorescence using an anti-FLAG antibody followed by fluorescein isothiocyanate-conjugated anti-mouse antibody (left column). Filamentous actin was stained with rhodamine-phalloidin (middle column). Merged images reveal that all ANKRD1 variants localize into the nucleus (right column). Magnification 200×.

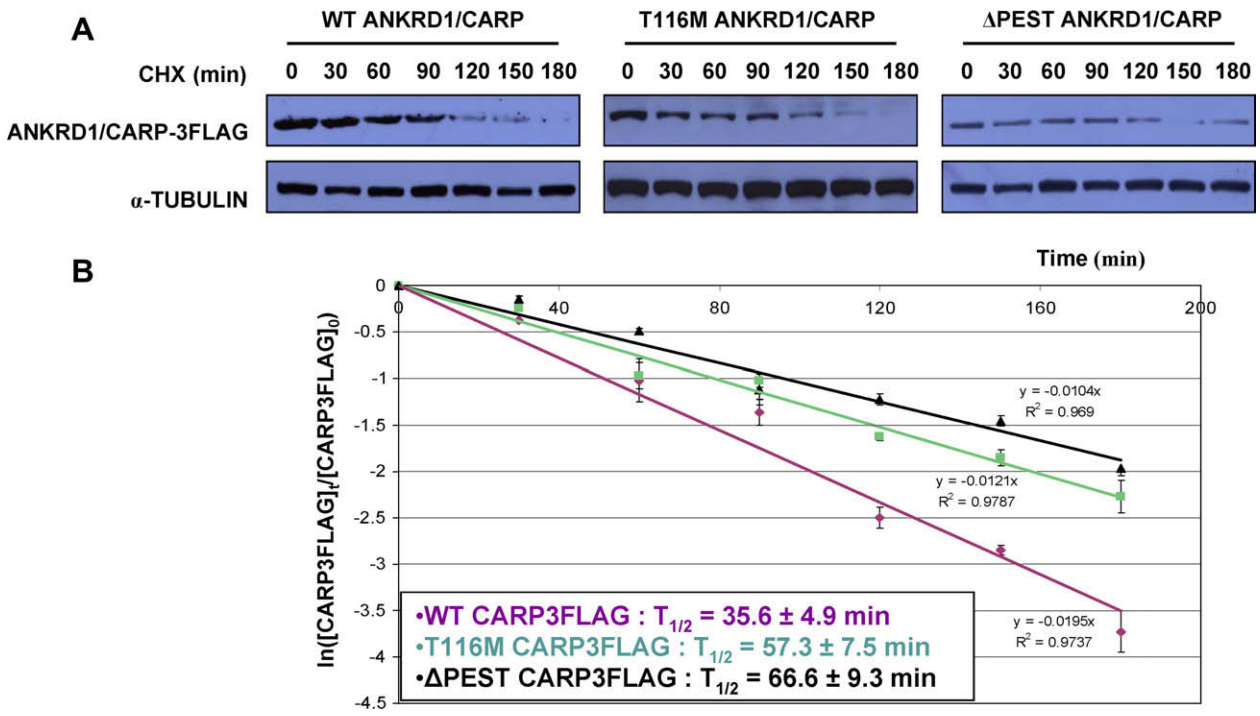

Fig. 2. Determination of ANKRD1 variant's half-life. (A) The half-life of recombinant ANKRD1-3FLAG protein variants was determined in stably transfected HeLa cells following CHX treatment at the indicated times. ANKRD1 protein levels were detected by immunoblotting with anti-FLAG antibody, whereas protein loading was monitored with anti-α-tubulin. (B) The rate of decay was determined by linear regression analysis as described in [12]. Significance of the *b* value (slope) for each regression line was tested by ANOVA: the corresponding *P*-values were $3.08 * 10^{-8}$ for wild-type CARP, $1.52 * 10^{-6}$ for T116 M CARP and $7.28 * 10^{-12}$ for ΔPEST CARP, respectively. Values are indicated as means ± S.E.

analysis. Treatment of SK-MES-1 cells with CHX confirmed a high turnover rate also for endogenous ANKRD1, with a half-life of approximately 18 ± 1 min. (means ± S.E.) in this cell line (Supplementary Fig. 2). Moreover, when half-lives were compared for ANKRD1 proteins which were matched for the presence or absence of the FLAG-tag, most of the variability between SK-MES-1 and HeLa cell lines actually disappeared. Indeed, when untagged ANKRD1 proteins were analyzed in HeLa and SK-MES-1, they turned out to be degraded quite rapidly with a similar half-life in both cell lines, in keeping with our hypothesis of ANKRD1 being a short-lived protein (Supplementary Figs. 2 and 3).

3.2. ANKRD1 is ubiquitylated and processed by the 26S proteasome

We then turned our attention to the intracellular pathway(s) responsible for the rapid ANKRD1 turnover. The contribution of the lysosome/endosome, calpain and 26S proteasome pathways was therefore assessed by transfection of HeLa cells with FLAG-tagged wild-type ANKRD1, followed by incubation of the cells with CHX in the presence or absence of specific inhibitors of the aforementioned proteolytic pathways. As shown in Fig. 3A, the decrease in ANKRD1 protein levels observed 180 minutes after CHX treatment was not affected by NH_4Cl, thus ruling out involvement of the lysosomal degradation pathway in the intracellular ANKRD1 turnover. The same conclusion was drawn from the experiments performed with the calpain pathway's inhibitor calpeptin (Fig. 3A).

However, when the same assay was carried out with the 26S proteasome inhibitor MG-132, a significant stabilization of the wild-type ANKRD1 protein was observed either in the presence or absence of CHX (Fig. 3A, right). Indeed, MG-132 treatment almost completely prevented the CHX-mediated decay of ANKRD1 levels, suggesting that intracellular turnover of wild-type ANKRD1 is mainly carried out by the 26S proteasome. To further verify this hypothesis, we asked whether ANKRD1 might serve as a substrate for ubiquitylation. To this end, FLAG-tagged wild-type ANKRD1-expressing HeLa cells were transiently transfected with a HA-tagged ubiquitin expression vector and immunoprecipitation assays were carried out as described in material and methods. As shown in Fig. 3B, western blot analysis of the immunoprecipitated proteins showed that, whereas no ubiquitinated protein bands could be detected in the absence of MG-132, preincubation of the cells with this proteasomal inhibitor clearly led to the accumulation of ANKRD1-ubiquitin conjugates. Therefore, ANKRD1 is targeted to the 26S proteasome for degradation by means of the classical ubiquitylation pathway. Moreover, the extent of ANKRD1 ubiquitination was decreased when a HA-tagged K48R mutant replaced wild-type ubiquitin in the immunoprecipitation assay (Supplementary Fig. 4). This result suggested a direct role for lysine residue 48 in ANKRD1 ubiquitination. However, residual ANKRD1 ubiquitination was observed in the presence of the K48R mutant form of HA-tagged ubiquitin, suggesting that other lysine residues besides K48 might represent additional targets for ubiquitination.

Since the data described above were obtained with exogenously expressed ANKRD1 protein, in order to confirm that endogenous ANKRD1 also undergo 26S proteasome-mediated processing we investigated its intracellular degradation pattern in the above-described SK-MES-1 cell line. As shown in Fig. 4, endogenous ANKRD1 was also clearly susceptible to 26S proteasomal degradation, since its intracellular levels were significantly increased by MG-132, either in the presence or absence of CHX. Therefore, either endogenous or exogenous ANKRD1 is degraded by the 26S proteasome.

3.3. Several degrons independently target ANKRD1 to the 26S proteasome

Our previous results suggested that the PEST motif might represent a destabilization domain for ANKRD1. We thus decided to investigate its role in proteasomal-mediated degradation. To this

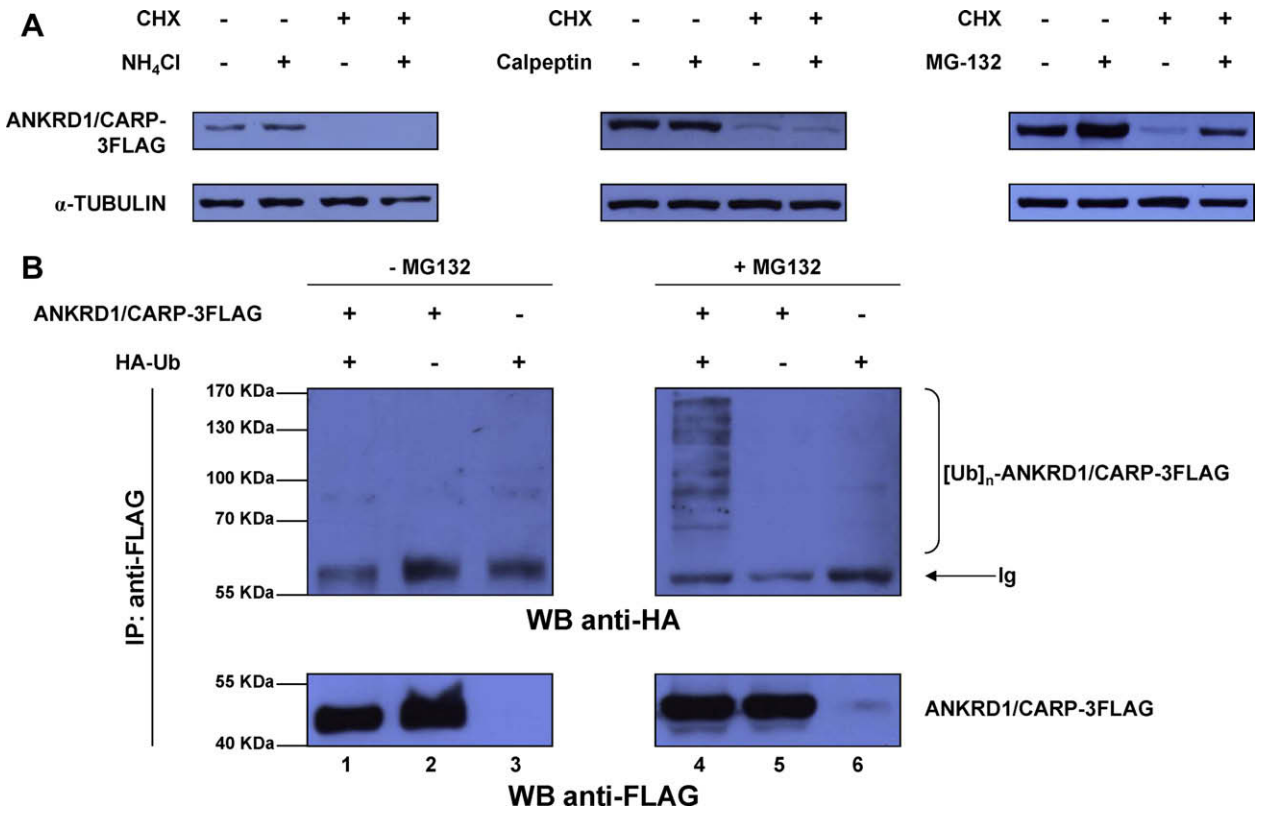

Fig. 3. The ubiquitin–proteasome system is involved in ANKRD1 degradation. (A) HeLa cells expressing wild-type ANKRD1-3FLAG were treated with 20 μM NH_4Cl, calpeptin or 20 μM MG-132 along with 100 μg/mL CHX for 180 min. ANKRD1 protein levels were determined by immunoblotting with anti-FLAG antibody; protein loading was monitored by immunoblotting with anti-α-tubulin. (B) The ubiquitin incorporation assay shows that ANKRD1 is ubiquitinated in vivo, since polyubiquitinated ANKRD1 accumulates in the presence of MG-132 (lane 4).

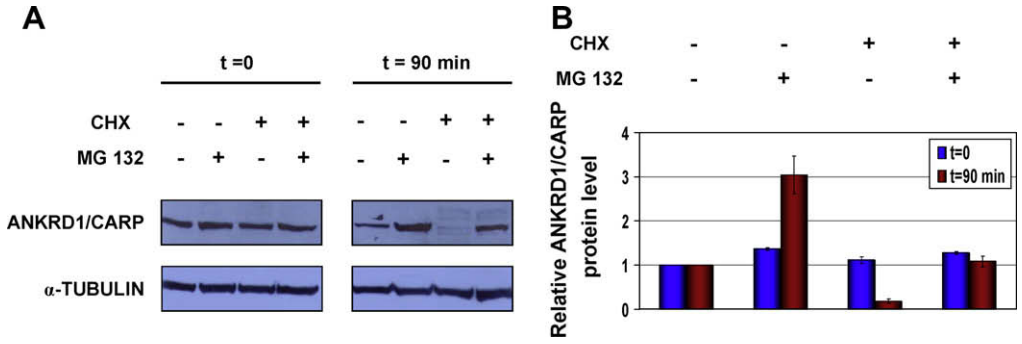

Fig. 4. Endogenous ANKRD1 is degraded by the 26S proteasome. (A) SK-MES-1 cells expressing endogenous ANKRD1 were treated with MG-132 and CHX for 90 min as described in Fig. 3. Cell extracts were then subjected to immunoblotting analysis with anti-CARP (H-120) and anti-α-tubulin antibodies. (B) The bar graph on the right side shows the relative endogenous ANKRD1 levels, normalized by using the α-tubulin signal as an internal reference.

aim, stably transfected HeLa cells expressing wild-type, T116 M and Δ-PEST FLAG-tagged ANKRD1 were incubated for 180 min with CHX in the presence or absence of MG-132 and protein levels were detected by immunoblot analysis. As shown in Fig. 5A, treatment of the cells with CHX alone led to a decrease in the protein levels for either wild-type or PEST-mutated ANKRD1. Thus, although mutations or deletion of the PEST motif can provide some stabilization upon ANKRD1, the protein can still be degraded in the cell. Moreover, treatment with MG-132 alone led to a significant increase of ANKRD1 levels not only for the wild-type but also for the two protein variants tested, suggesting that ANKRD1 can be targeted to the 26S proteasome even in the absence of a functional PEST motif (Fig. 5A). These results suggest the occurrence of other degrons besides the PEST motif which can target ANKRD1 to the 26S proteasomal pathway.

When CHX and MG-132 treatments were combined, the protein levels of wild-type and T116 M ANKRD1 variants almost completely returned to those observed in untreated cells (Fig. 5A), again suggesting the crucial role of the 26S proteasome in ANKRD1's intracellular turnover. Noteworthy, MG-132 could only partially restore the protein levels of Δ-PEST ANKRD1 to those observed in untreated cells (Fig. 5A). This result suggests that ANKRD1 is at least in part targeted to the 26S proteasome through the PEST motif, although a different pathway for ANKRD1 degradation might also be called into action following removal of the PEST motif.

To further evaluate the role of the PEST motif in mediating ANKRD1 degradation by the 26S proteasome, we investigated its ability to affect the intracellular stability of a heterologous stable protein. As shown in Fig. 5B, the intracellular stability of the Green Fluorescent Protein (GFP) protein was significantly reduced following the addition at its C-terminus of an isolated ANKRD1's PEST motif. Such an effect was clearly dependent on the 26S proteasome, since the specific inhibitor MG-132 could easily restore CHX pre-treatment expression levels of the GFP-PEST protein. This experiment provided a very strong support for a role of the PEST motif in the control of ANKRD1 turnover.

Thus, the short half-life of ANKRD1 in vivo is attributable to a large extent to the 26S proteasome, and mutations or removal of the PEST motif might significantly affect both the nature of the underlying degradative pathways acting upon ANKRD1 and the extent to which they effectively process the protein inside the cells, changing its half-life accordingly.

Our data also suggest that targeting of ANKRD1 to the 26S proteasome is not entirely dependent on the PEST motif, since proteasomal degradation could be observed in the presence of either a "weak" PEST-bearing or a completely PEST-null protein. Thus,

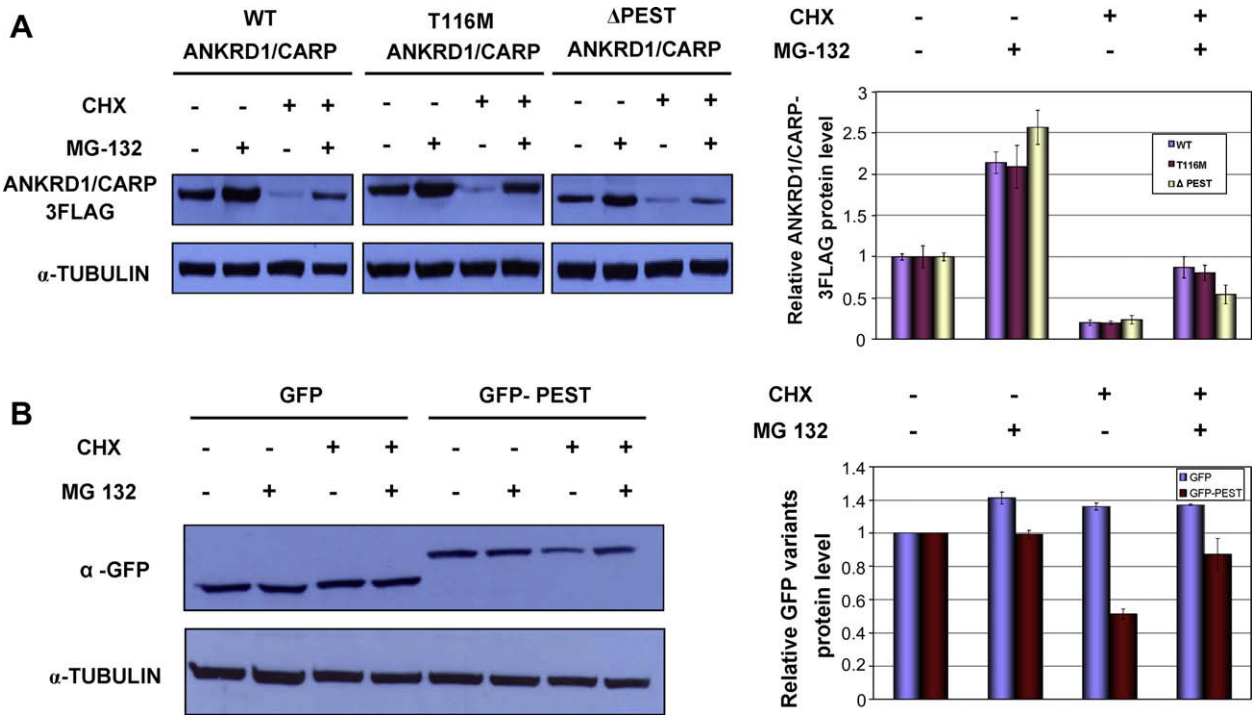

Fig. 5. The PEST motif is involved in the proteasomal degradation of ANKRD1, but other degrons occur besides this motif. (A) HeLa cells expressing ANKRD1-3FLAG protein variants were treated with 20 μM MG-132 along with 100 μg/mL CHX for 180 min. Cell extracts were subjected to immunoblotting analysis with anti-FLAG and anti-α-tubulin antibodies. The bar graph (right side) shows the relative levels of the different ANKRD1-3FLAG variants, normalized to those of α-tubulin. (B) HeLa cells were transiently transfected with the pEGFP-C3-PEST or the pEGFP-N1 expression vectors and treated with MG132 and CHX as indicated in material and methods. Cell extracts were subjected to immunoblotting analysis with both anti-GFP and anti-α-tubulin antibodies. The bar graph on the right shows the relative GFP or GFP-PEST levels, normalized with α-tubulin.

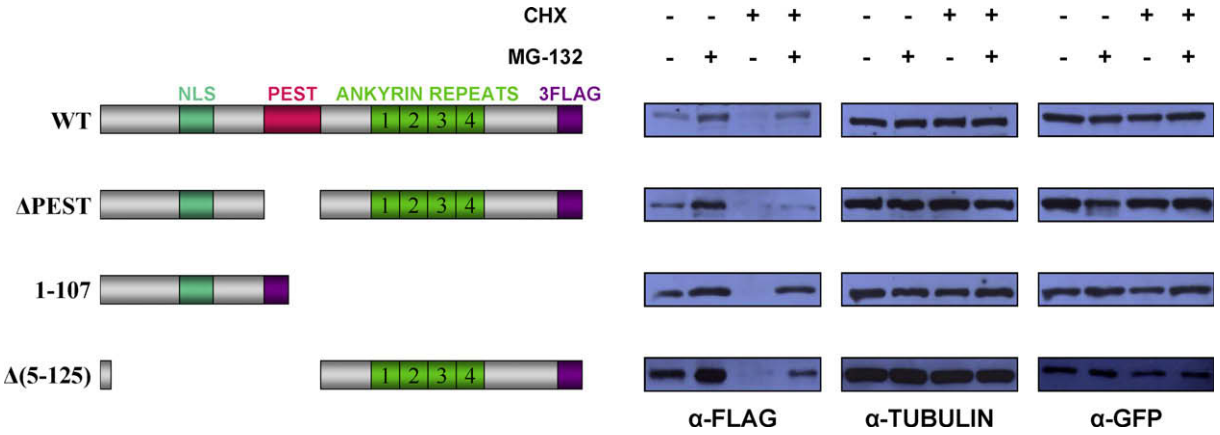

Fig. 6. Multiple degrons exist in ANKRD1. HeLa cells transiently transfected with ANKRD1-3FLAG deletion mutants expression vectors were treated with 20 μM MG-132 along with 100 μg/mL CHX for 180 min. ANKRD1 protein levels were determined by immunoblotting with anti-FLAG antibody, whereas protein loading was detected with anti-α-tubulin and transfection efficiency was monitored by immunoblotting with anti-GFP. The first four N-terminal residues of ANKRD1 were included in the Δ(5–125) deletion mutant to avoid inconsistent results due to the N-end rule.

ANKRD1 could be targeted to the 26S proteasome through multiple independent degrons.

In a preliminary attempt to define other regions involved in proteasomal degradation, two truncated ANKRD1 proteins, spanning either the N-terminal or C-terminal moieties with respect to the PEST motif, were expressed in HeLa cells to evaluate their sensitivity to 26S proteasome-mediated degradation. As shown in Fig. 6, both truncated proteins were rapidly degraded in the presence of CHX, whereas the proteasomal inhibitor MG-132 protected both proteins from degradation, clearly suggesting that multiple domains can independently target ANKRD1 for 26S proteasome-mediated degradation.

In conclusion, we have demonstrated that the cardiac transcriptional repressor ANKRD1 is a short-lived protein whose intracellular levels are regulated by processing through the 26S proteasome. Despite several features of the ANKRD1 gene, such as the presence of mRNA decay elements in the 3′-UTR region and the occurrence of a PEST motif within the encoded protein [13,14], have long suggested that ANKRD1 undergoes a tight intracellular regulation, this is to our knowledge the first report defining the role of a particular degradation pathway in ANKRD1 turnover. Moreover, we have shown that the putative PEST motif of ANKRD1 is indeed involved in controlling its stability, although we also obtained preliminary evidence suggesting that other regions besides the PEST motif are also involved in the regulation of the intracellular levels of this protein. Further investigations are surely warranted to better address this issue.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.07.001.

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