Mutational analysis of human prothymosin α reveals a bipartite nuclear localization signal

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Abstract Mutants of human prothymosin α with impaired ability to inhibit yeast Saccharomyces cerevisiae cell growth were characterized. Two types of prothymosin α-inactivating mutations were observed. Mutations that belong to the first type compromised the nuclear entry of prothymosin α by affecting its nuclear localization signal. Analysis of subcellular distribution of GFP-prothymosin α fusions revealed a bipartite nuclear localization signal that is both necessary and sufficient for nuclear import of the protein in human cells. Mutations of the second type abrogated the inhibitory action of prothymosin α through an unknown mechanism, without influencing the nuclear import of the protein.

Keywords: Prothymosin α; PCR-based mutagenesis; Cell growth inhibition; Nuclear localization signal; Saccharomyces cerevisiae

1. Introduction

Prothymosin α (ProTa) is an abundant highly acidic protein of 13 kDa with an unknown but essential function [1-5]. ProTa was originally isolated from thymus and was believed to serve as a precursor of the putative thymic hormone thymosin α1, hence its name [1]. However, several features of ProTa, in particular its wide tissue distribution [6] and its ability to accumulate in the nucleus [7-10], suggest an intranuclear function of this protein. Accumulating evidence suggests that ProTa may be related to proliferation of mammalian cells. Elevated levels of ProTa were observed in proliferating cells [11,12]. The growth stimulation of quiescent mammalian cells results in an abrupt increase of ProTa gene expression [3]. The levels of ProTa and its mRNA, however, do not appear to vary significantly in the cell cycle [5,13,14]. A unique functional ProTa-encoding gene was characterized in the mammalian cells [15,16]. Its expression was reported to be directly involved in the inhibitory phenotype of ProTa. The function and the mechanism of action of ProTa are unknown. In this work, we functionally dissected human ProTa. We used a recently described yeast genetic screen [20,21] to isolate a panel of novel ProTa mutations that affect the ability of the protein to inhibit yeast cell growth. Our results show that a subset of mutations that impaired the ability of ProTa to inhibit yeast cell growth led to impaired nuclear import by affecting the NLS. This suggests that nuclear localization may be a prerequisite of the inhibitory phenotype of human ProTa in yeast.

2. Materials and methods

2.1. ProTa mutagenesis and clone selection

E. coli JM109 and S. cerevisiae 2805 [MATa, pep4::His3, prb 1-5, can1, Gal1, his3, ura3-52] strains were used throughout this work. Standard procedures for DNA cloning were used. Conditions for PCR-based random mutagenesis of the human ProTa using pHT15 [22] as a template were as described [21]. The PCR products were digested with BamHI and EcoRI, and inserted into the BamHI-EcoRI-digested pYeDP1/8-2 yeast shuttle vector [23] to produce pYeM series of plasmids. Yeast cells were transformed by the DMSO-lithium acetate method [24] and plated on a glucose-containing SD medium (2% glucose, 0.67% yeast nitrogen base, 0.1% casamino acids). For selection, the resultant colonies were transferred on a nitrocellulose membrane to galactose-containing S5 plates (2% galactose, 0.67% yeast nitrogen base, 0.1% casamino acids). DNA from yeast cells that survived in the presence of mutated ProTa was isolated by the method described in [25] and used to transform bacterial cells for subsequent sequencing and rearranging of plasmid inserts utilizing unique Acyl and Ddel sites in the ProTa cDNA [21]. Plasmids with single or double mutations in ProTa cDNA were retransformed into yeast cells, and cell growth parameters were measured in the galactose-containing S5 liquid medium. The procedure for ProTa isolation from yeast cells was described [20].

2.2. Construction of GFP-ProTa fusion proteins

pGFP-C2 (Clontech) was used for constructing GFP-ProTa fusions and their production in human cells. To produce GFP-ProTa fusion proteins in yeast cells, a complete GFP gene with polylinker as a 1100 bp Neol–Mfd fragment of pGFP-C2 (partially digested with Neol to preserve complete reading frame of GFP) was blunt-ended by the Klenow fragment in the presence of dNTP and inserted into the filled-in BamHI site of pYeDP1/8-2 vector to yield pYeGFP-ProTa.
cDNAs with defined mutations were excised from the rearranged pYeM derivatives with BamHI and EcoRI and inserted into the BglII–EcoRI digested pGFP-C2 and pYeGFP in-frame with GFP. cDNAs encoding wild-type and Δ(101–109) ProTα were excised from pHT15 [22] and pYeKHT1 [20], respectively, with BamHI and EcoRI and ligated into pGFP-C2 and pYeGFP in analogous fashion. To construct GFP-(82–109) fusion protein, pGFP-C2 and pYeGFP were digested with EcoRI, filled-in with the Klenow fragment, cut with KpnI and ligated with the NLS-encoding DNA fragment obtained as follows. A BamHI–EcoRI cdNA fragment encoding wild-type ProTα was hydrolyzed with DdeI, filled-in with the Klenow fragment and recleaved with KpnI. The resultant 90 bp fragment was inserted into the vectors in-frame with GFP. The structure of plasmids was verified by sequencing.

2.3. Fluorescent microscopy of the ProTα-producing cells

A human embryonic kidney cell line, 293, was transiently transfected by calcium precipitate method [26]. Plasmids were purified on QIAGEN columns according to the manufacturer’s instructions. Five micrograms of GFP-ProTα expression plasmids was used without carrier DNA per 60-mm gelatin-coated glass-bottom culture dish, and fluorescent microscopy was performed at Day 1 post-transfection. Images were captured on a SenSys CCD camera (Photometrics) mounted on a Zeiss Axiovert 135 TV inverted microscope, using Zeiss Achroplan 40× water-immersion objective lens.

Yeast S. cerevisiae 2005 cells transformed with the ProTα-encoding derivatives of pYeGFP were grown in SD medium overnight at 30°C. Cells were collected by centrifugation, resuspended in S5 medium to induce transcription of the target gene, and grown overnight at 30°C. Cells were removed from the culture and molded in 0.5% low-melting point agarose on a glass slide. Fluorescent microscopy was performed with a Zeiss Photomicroscope 3 equipped with a Neofluar 100× oil-immersion lens.

Aliquots of galactose-induced yeast cells producing various GFP-ProTα fusions were processed for PAGE. Lysates were made in a buffer containing 0.1 M Tris-HCl, pH 8.0, 0.2 M NaCl, 10 mM β-mercaptoethanol, 5 mM EDTA, 20% glycerol and 1 mM PMSF by glass bead lysis, and then centrifuged at 15000Xg for 3 min. Sample buffer was added to the supernatants, a boiling step was performed, and fluorescent microscopy was performed at 302 nm. Positions of GFP-ProTα bands were visualized by UV illumination of the gels at 302 nm.

3. Results

3.1. Amino acid residues essential for yeast cell growth inhibition reside in the carboxy-terminal region of human ProTα

To define functionally important amino acid residues in human ProTα, we used the recently described approach [21] which included random mutagenesis with subsequent production of various mutant forms of ProTα and evaluation of their properties in yeast S. cerevisiae. The randomly mutagenized ProTα cDNAs were cloned into a yeast expression vector under the control of a strong galactose-inducible promoter. The resultant plasmids were introduced into yeast cells for an in vivo genetic screen for mutations that adversely affect ProTα activity. The screen is based on a severe inhibition of yeast cell growth by wild-type human ProTα. By using this approach, a number of mutant ProTα-producing yeast clones that gained the ability to grow were selected. ProTα produced by each of these clones turned out to possess several amino acid substitutions that are listed in Table 1. In order to evaluate the contribution of particular mutations to the observed growth phenotype, single or double mutations were recovered by DNA shuffling. In addition, a ProTα mutant with several substitutions grouped in the amino-terminal portion of the molecule was generated (Table 1). Production of these ProTα mutants in yeast cells was verified by SDS-PAGE (Fig. 1). For each mutant, similar amounts of protein were produced.

To quantitate ProTα inactivation, growth parameters of the yeast cells bearing plasmids encoding the wild-type and mutant ProTα were determined in the liquid medium. The results of this assay are shown in Fig. 2A. While in the absence of the inducer of human ProTα production all types of cells grew equally well (not shown), growth parameters of the strains producing various ProTα mutants in the presence of galactose turned out to be different. As expected, the wild-type ProTα efficiently blocked cell growth. Interestingly, multiple mutations in the amino-terminal portion of ProTα S1P, I11V, K14W, K17E (Fig. 2A, empty circles); S1T; K14E, E24G (not shown), solely or in combination, did not abrogate the inhibitory action of the protein. Replacing Gly76 for Val, which converts the sequence of our wild-type ProTα clone to that described in [2,3] also had no effect (not shown). However, the mutations in the carboxy-terminal part of ProTα resulted in various degrees of inactivation. Thus, the T105A mutant showed a low level of cell growth inhibition. Single E80G and K101R mutations each resulted in approximately 50% reduction, but the respective double mutant was virtually inactive. The double mutant E44G, E50G showed approximately 50% reduction. The results of these experiments, together with our earlier data on K87E inactivating mutation [21] are summarized in Fig. 2B. It should be noted that deletion of the last nine amino acid residues of ProTα resulted in complete loss of the inhibitory phenotype [20]. From these data we conclude that a number of residues essential for cell growth inhibition are located in the carboxy-terminal portion of ProTα, while multiple mutations in the amino-terminal region of the protein are well tolerated.

3.2. NLS of human ProTα is bipartite

We next addressed the mechanism by which the mutations

<table>
<thead>
<tr>
<th>Table 1 Human ProTα mutants generated in this study</th>
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<tr>
<td><strong>Primary mutants</strong></td>
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<tr>
<td>4. S1T, N39D, K87E</td>
</tr>
<tr>
<td>5. E80G, K101R</td>
</tr>
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<td>7. E80G</td>
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Left column: primary mutants with multiple amino acid substitutions obtained via the screening procedure. Right column: single and double ProTα mutants obtained through the rearrangement of cDNAs of primary mutants. *ProTα mutants that were characterized earlier [21].
in ProTa affect cell growth inhibition. Previous studies led us to hypothesize that the nuclear import of ProTa is a prerequisite for its inhibitory action on yeast cell growth and that mutations that affect the putative NLS of ProTa relieve this

Table 2
Analysis of the human ProTa NLS

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino acid sequence</th>
<th>Yeast growth inhibition</th>
<th>Intracellular localization</th>
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<tr>
<td>Nucleoplasmin NLS</td>
<td>-KR - 10 aa spacer - KKKK-</td>
<td></td>
<td>nuc</td>
</tr>
<tr>
<td>Human ProTa</td>
<td>-AESATGKRAEDDEDDVDTKKQKTDDCOK</td>
<td>+</td>
<td>nuc</td>
</tr>
<tr>
<td>K87E</td>
<td>-<strong><strong><strong><strong>E</strong></strong></strong></strong>**********</td>
<td>-</td>
<td>nuc ≥ cyt</td>
</tr>
<tr>
<td>K101R</td>
<td>-******<strong><strong><strong><strong><strong>R</strong></strong></strong></strong></strong></td>
<td>+/-</td>
<td>nuc ≥ cyt</td>
</tr>
<tr>
<td>T105A</td>
<td>-*****************<em><strong><strong>A</strong></strong></em></td>
<td>-</td>
<td>nuc(y)</td>
</tr>
<tr>
<td>Δ (101-109)</td>
<td>-***********************</td>
<td>-</td>
<td>cyt(h)</td>
</tr>
<tr>
<td>NLS (82-109)</td>
<td>****************************</td>
<td>n. d.</td>
<td>nuc(h)</td>
</tr>
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</table>

The amino acid sequence of human ProTa between residues 81 and 109 is shown, aligned with the bipartite nucleoplasmin NLS sequence [28]. Basic residues essential for nucleoplasmin NLS functioning and corresponding ProTa residues are in bold. The sequences of various ProTa mutants are given below; an asterisk marks a residue identical with that in the wild-type protein; substitutions are shown by indicating the new residue. The columns on the right show the ability (+) or inability (−) of the protein to inhibit yeast cell growth, and indicate subcellular localization of the corresponding GFP-ProTa fusions, respectively. (h) and (y) corresponds to protein localization in human and yeast cells, respectively. n.d., not determined.
inhibitory effect [20,21]. ProTa NLS is known to reside in the carboxy-terminal half of the molecule [8,9]. Although it has not been precisely identified, a short basic block (residues 101–104) similar to the canonical continuous NLS of SV40 T antigen is likely to contribute to the active nuclear import of ProTa. In agreement with this model, our present analysis has identified several deleterious mutations located in or next to this basic sequence. However, based on our recent studies of yeast cell growth arrest by K87E ProTa mutant, we have hypothesized that ProTa NLS might be bipartite rather than continuous [21] (Table 2).

To test this possibility, we studied the subcellular localization of the above ProTa mutants to determine the NLS of this protein. For this purpose, we fused the wild-type ProTa and various ProTa mutants (K87E, K101R, Δ(101–109)) to the carboxy-terminus of GFP and expressed the chimeric genes under the control of the CMV promoter. In human 293 cells, subcellular distribution of hybrid proteins was examined by fluorescent microscopy. The human ProTa–GFP fusion was located exclusively in the nucleus (Fig. 3A), as expected from previous studies [9,10]. This suggests that the GFP fusions faithfully reflect the localization of native ProTa. Neither of the mutant ProTa fusion proteins were exclusively nuclear. The K87E and K101R mutants were found both in the nucleus and the cytoplasm with the preference to the nucleus in the majority of cells (Fig. 3C,D, respectively). In all cases of nuclear localization the label was excluded from the nucleoli, in agreement to the previous data on the wild-type ProTa localization by indirect immunofluorescence [10]. Unlike the above ProTa point mutations that inactivate the NLS only partially, the deletion of the last nine amino acids (residues 101–109) containing the second basic block resulted in the nuclear exclusion (Fig. 3B).

The above results demonstrate that mutations in both parts of the putative bipartite NLS of human ProTa impair nuclear uptake of the protein, indicating that both basic blocks are necessary for efficient nuclear accumulation. Comparison of the carboxy-terminal region of human ProTa with that of closely related rat ProTa [29] demonstrates that at least some amino acid substitutions in the spacer region separating
Fig. 3. Localization of GFP-ProTa mutants in human 293 cells. Cells were transfected with the indicated expression constructs and fluorescent microscopy was performed, as described in Section 2. A: Wild-type ProTa. B: Δ(101–109) mutant. C: K87E mutant. D: K101R mutant. E: C-terminal ProTa peptide (residues 82–109). The transfections were done in parallel, and representative fields are shown for each transfection. Two independent experiments were performed, with similar results. Magnification: ×710.

these two blocks are tolerated. Interestingly, K101R mutation in the second basic block of the NLS impaired nuclear accumulation of ProTa, indicating that a simple requirement for a basic amino acid in this position is not sufficient. Taken together, our results imply that the identified bipartite basic sequence represents the NLS of ProTa. To demonstrate that the putative NLS is not only necessary, but also sufficient to direct nuclear import of ProTa, the C-terminal ProTa peptide (residues 82–109) containing both basic regions was fused to GFP, and the localization of the hybrid protein was studied in human cells. This protein was found to be exclusively nuclear (Fig. 3E) which indicates that the residues 82–109 represent the NLS of ProTa.

3.3. Additional functional determinant in human prothymosin α acting independently of nuclear import

The above analysis suggests a correlation between the ability of human ProTa to accumulate in the nucleus of human cells and its ability to cause growth arrest in yeast cells. To further examine this correlation, we studied the same GFP-tagged wild-type ProTa, mutant ProTa (K87E, K101R, Δ(101–109), T105A), and the NLS (residues 82–109) proteins in yeast 2805 cells. Subcellular localization of these proteins in yeast has several common features with the localization in human cells. GFP alone was diffusely spread throughout the whole cell (not shown), whereas GFP-ProTa fusion was localized exclusively in the nucleus (Fig. 4A). Mutations in both parts of the bipartite NLS caused the appearance of the protein in the cytoplasm, cytoplasmic localization being more pronounced for the K87E mutant (Fig. 4C). The K101R and Δ(101–109) mutants were both nuclear and cytoplasmic, with the preference to the nucleus in the majority of cells (Fig. 4B,D, respectively). Interestingly, the GFP-NLS fusion protein was spread throughout the yeast cells (Fig. 4E), in contrast to the exclusive nuclear localization in human cells (Fig. 3E). To check for degradation, resultant in the removal of ProTa residues from GFP, we performed SDS-PAGE of the

Fig. 4. Localization of GFP-ProTa mutants in S. cerevisiae cells. Yeast cells harboring the indicated constructs were examined by fluorescent microscopy, as described in Section 2. A: Wild-type ProTa. B: Δ(101–109) mutant. C: K87E mutant. D: K101R mutant. E: C-terminal ProTa peptide (residues 82–109). F: T105A mutant. Production of the fusion proteins was controlled by the inducible GAL10-CYC1 promoter. GFP-ProTa were synthesized in yeast cells by the overnight growth in the galactose-containing medium. Magnification: ×2200.
above fusion proteins produced in yeast. All hybrid proteins ran as a single band of the correct mobility, and therefore no degradation occurred (not shown). Thus this NLS is sufficient for the nuclear targeting in human cells, but requires additional signals in yeast cells. This putative signal is apparently located upstream of the NLS, since the GFP-ProTα fusion was exclusively nuclear (Fig. 4A).

Another interesting feature of human ProTα was revealed by T105A mutant. This mutant showed a markedly inactivated phenotype when tested for yeast cell growth inhibition (Fig. 2). Since this mutation is located immediately next to the second basic block of the NLS, it appeared likely that the interference with the NLS could be responsible for this effect, by analogy with K87E and K101R mutations. However, T105A mutant turned out to be exclusively nuclear in yeast cells (Fig. 4F). Thus, it appears that ProTα inactivation in this case is not due to impaired nuclear targeting of this protein, but rather to the loss of the Thr residue in this position. It is therefore likely that an additional functional determinant lies next to or overlaps with the NLS, which might be directly responsible for yeast cell growth arrest by human ProTα.

4. Discussion

Until recently, no functional determinants in ProTα have been identified that could indicate possible mechanisms of its function. The primary structure of ProTα does not provide any hint about its mechanism of action. Its highly negative charge (p/-3.5, about half of the amino acid residues are dicarboxylic [2,3]) and the absence of aromatic and sulfuryielding residues makes this 13 kDa protein unusual and may account for an apparent lack of secondary and higher order structures [8,30]. However, an important prediction was made about a short stretch of basic amino acids KKQK (residues 101–104, see Table 2) located close to the carboxy terminus of ProTα molecule. This motif was proposed to represent the NLS of ProTα, based on (i) similarity to the canonical continuous NLS such as that of SV40 T antigen [31], (ii) localization of the nuclear targeting determinant in the carboxy-terminal half of ProTα [9], and (iii) the inability of a truncated ProTα (residues 1–88) to accumulate in Xenopus oocyte nuclei [8]. Consistent with these data, the human ProTα lacking the last nine carboxy-terminal residues, including this putative NLS, failed to inhibit the yeast cell growth [20]. However, the residue K87, located 13 residues upstream from this putative NLS has been shown to be important for the inhibitory phenotype in yeast, leading us to propose that the NLS of ProTα might be bipartite [21], as exemplified by the NLS of nucleoplasmin [28,32]. In this study, we present mutational analysis of human ProTα aimed to delineate its functionally important determinants and, in particular, to define the structure of its NLS. Several mutations that inactivate the inhibitory phenotype of ProTα located in its carboxy terminal part fell into or close to its putative NLS. This raised the possibility that, at least in some cases, a defect in ProTα functioning may be caused by its failure to enter the nucleus. To test this, we attempted to precisely determine the ProTα NLS and to study the effect of the above-mentioned mutations on subcellular localization of ProTα.

To localize ProTα in living cells, wild-type and mutant human ProTα were expressed as GFP fusions in human and yeast cells. Similar pattern of subcellular localization was observed in human cells and yeast cells for the wild-type ProTα and its mutants. Wild-type ProTα appeared to be exclusively nuclear and excluded from the nucleoli, in agreement with the previously reported data [9,10]. Mutations in both parts of the putative bipartite NLS of human ProTα (K87E; K101R) resulted in a marked redistribution of the protein to the cytoplasm in both human and yeast cells, although the nuclear accumulation was still significant. This is expected for a bipartite NLS, where point mutations in each part of the NLS result in partially cytoplasmic localization of the originally nuclear protein, and only multiple mutations abrogate nuclear import completely [28]. Accordingly, deletion of the second basic stretch of the putative NLS (residues 101–109) resulted in cytoplasmic localization and exclusion from the nucleus of human cells. In yeast cells, this mutant appeared to be distributed between the cytoplasm and the nucleus. Thus, both stretches of basic residues are important for nuclear uptake of ProTα. These sequences are sufficient for nuclear import of ProTα since the GFP–(82–109) fusion protein was localized exclusively in the nucleus of human cells. Strikingly, in the heterologous yeast environment, the protein was spread evenly throughout the whole cell. This result was unexpected because the signals and mechanisms governing nuclear import of proteins are believed to be well conserved in mammalian and yeast cells [33]. Furthermore, a yeast SWI5 protein is known to possess a functional bipartite NLS that is similar to that of ProTα [34]. However, a minimal SWI5 fragment, identified as a NLS by nuclear targeting of a heterologous protein, contained extra SWI5 residues in addition to the bipartite basic sequences. Thus the NLS of human ProTα appears to be necessary but not sufficient for nuclear targeting of the protein in yeast cells. This implies that there is an additional signal(s) within the ProTα sequence required for the nuclear localization of this protein in yeast cells.

Whereas the majority of deleterious mutations in ProTα affected its NLS, T105A mutation, though located immediately next to the NLS, has no effect on the nuclear import of ProTα. This result provides evidence of a novel functionally important determinant of ProTα. We speculate that Thr105 is directly involved in the inhibitory phenotype of ProTα. In summary, our results revealed two possible mechanisms responsible for releasing of the inhibitory action of human ProTα on yeast cell growth, which are represented by the two types of mutations: either by interference with the nuclear import, or by abrogating the inhibitory activity without affecting the nuclear import.

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References

[5] Sburlati, A.R., De La Rosa, A., Batay, D.W., Kury, G.L., Man-