Lysine-87 is a functionally important residue in human prothymosin α

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Abstract Human prothymosin α mutants were generated with the aid of random mutagenesis and screened for their ability to inhibit yeast *Saccharomyces cerevisiae* cell growth. Conversion of Lys-87 to Glu resulted in an inactivated prothymosin α mutant, which lost the ability of the wild-type protein to block yeast cell growth. We propose that prothymosin α may possess a bipartite rather than monopartite nuclear localization signal, which includes Lys-87, and that the above mutation destroys one part of the nuclear localization signal, thus preventing efficient nuclear uptake of prothymosin α.

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

1. Introduction

Prothymosin α (ProTα) is a highly acidic small nuclear protein [1–6]. ProTα appears to be an abundant and essential protein in mammalian cells [7,8]. Although the exact function of this protein is unknown, available evidence strongly suggests that ProTα is related to cell proliferation [2,7,9,10]. In an attempt to elucidate the function of ProTα, we began the generation and characterisation of random ProTα mutants with altered properties of the protein. The selection procedure for the mutants was based on the recently observed phenomenon that the wild-type human ProTα, being overproduced in yeast *Saccharomyces cerevisiae*, efficiently inhibited cell growth [11]. Here we report that the point mutation $Lys^{87}Glu$ in human ProTα eliminates inhibitory potential of human ProTα towards yeast cell growth. Possible implications of this fact are discussed.

2. Materials and methods

Escherichia coli JM109 and *S. cerevisiae* 2805 [MATa, *pep4::His3, prb 1*-δ, can1, Gal2, his3δ, ura3-52] strains were used throughout this work. Standard procedures for DNA cloning were employed.

Conditions for PCR-based random mutagenesis of the human ProTα cDNA were essentially as described [12]. Briefly, 5 ng of pHT15 [13] were taken for PCR amplification of ProTα cDNA with 0.2 μg of each direct and reverse pUC19 primers in the reaction buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.1 mg/ml BSA and 5U of *Taq* polymerase in the presence of 250 μM each of dGTP, TTP, dCTP and 50 μM dATP. 25 cycles of PCR were performed with the following temperature profile: 94°C, 1 min; 48°C, 1 min; 72°C, 0.5 min. The PCR products were fractionated in a 1.5% agarose gel, eluted and digested with *Bam*HI and *Eco*RI. The resultant 350 bp long DNA fragments were ligated with the *Bam*HI-*Eco*RI digested pYeDP1/8-2 yeast shuttle vector [14].

Yeast cells were transformed with the plasmids by the DMSO-lithium acetate method [15] and plated on glucose-containing SD medium (2% glucose, 0.67% yeast nitrogen base, 0.1% casamino acids). The resultant colonies were transferred either with a nitrocellulose membrane to galactose-containing plates (2% galactose, 0.67% yeast nitrogen base, 0.1% casamino acids) for clone selection, or to galactose-containing liquid medium for measurement of cell growth parameters.

DNA from yeast cells was isolated by the method described in [16] and used for transformation of bacterial cells for subsequent dideoxy sequencing and rearrangement of plasmid inserts.

The procedure for ProTα isolation from yeast cells was as described [11].

3. Results

In order to identify functionally important amino acid residues in human ProTα, random mutagenesis of ProTα cDNA was performed via error-prone PCR on the pHT15 with direct and reverse primers. pHT15 is a derivative of pUC19, in the *Sma*I site of which a full-length protein-coding region of the human ProTα cDNA was inserted [13]. Mutated PCR products of approx. 430 bp long were purified, digested with *Bam*HI and *Eco*RI, and cloned into the pYeDP1/8-2 shuttle vector under the control of GAL10-CYC1 promoter, which could be induced by growing yeast cells in the galactose-containing medium (Fig. 1).

For selection of ProTα mutants with altered properties of the protein, use was made of the fact that wild-type human ProTα, being overproduced in *S. cerevisiae* cells, efficiently blocks yeast cell growth [11]. Thus, plating the yeast cells bearing pYeDP1/8-2 derivative containing wild-type human ProTα cDNA resulted in colony formation when transcription of the human gene was blocked (i.e. no galactose was present in the medium), but failed to produce colonies when ProTα synthesis was induced by the addition of galactose. In the present approach, yeast cells were transformed with a mixture of plasmids bearing mutated ProTα cDNAs, plated on the galactose-free medium, and the resultant colonies were replica-plated on the galactose-containing medium in order to induce production of human ProTα. A number of yeast clones which gained the ability to grow in the presence of synthesized mutant ProTα were thus selected, and one of them was subjected to detailed analysis.

Plasmid DNA was recovered from this clone and re-transformed into *E. coli* cells, with subsequent sequence determination of the mutated ProTα cDNA. Nine nucleotide differences leading to three amino acid substitutions were identified: S^1T, $N^{39}D$ and $K^{87}E$. To evaluate contribution of a single mutation in the obtained growth phenotype, ProTα cDNA insertion was reconstituted by replacement of mutated *Acy*I-*Eco*RI DNA fragment with the wild-type ProTα cDNA *Acy*I-*Eco*RI fragment leading to production of ProTα with the single S^1T

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Abbreviations: NLS, nuclear localization signal; PCR, polymerase chain reaction; ProTα, prothymosin α

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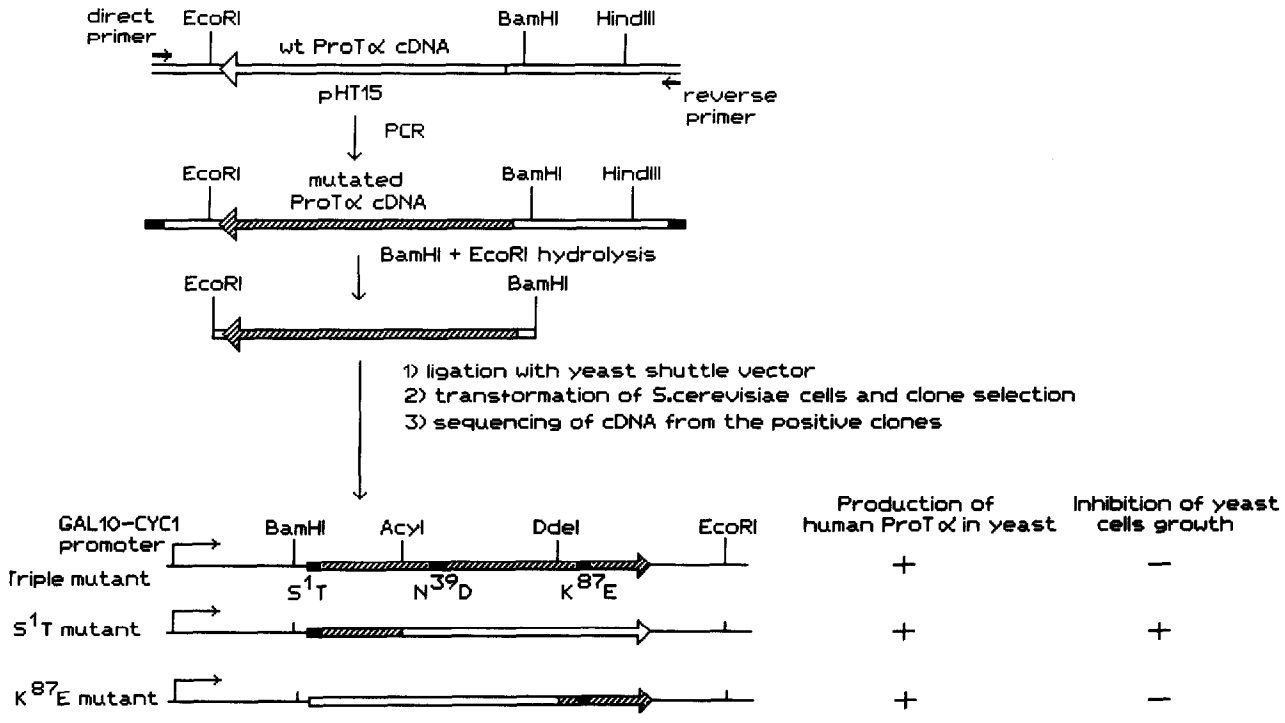

Fig. 1. Schematic representation of the approach aimed at identification of functionally important amino acid residues in human ProTα.

mutation; and by replacement of mutated *Bam*HI-*Dde*I cDNA fragment with the appropriate wild-type DNA fragment leading to production of ProTα with the single $K^{87}E$ mutation (Fig. 1).

Production of both S^1T and $K^{87}E$ ProTα mutants in yeast cells bearing appropriate plasmids was demonstrated by isolation of respective proteins from the cells grown in glucose-containing medium and then shifted to galactose-containing medium for 8 h to induce ProTα synthesis (Fig. 2A, lanes 1–3).

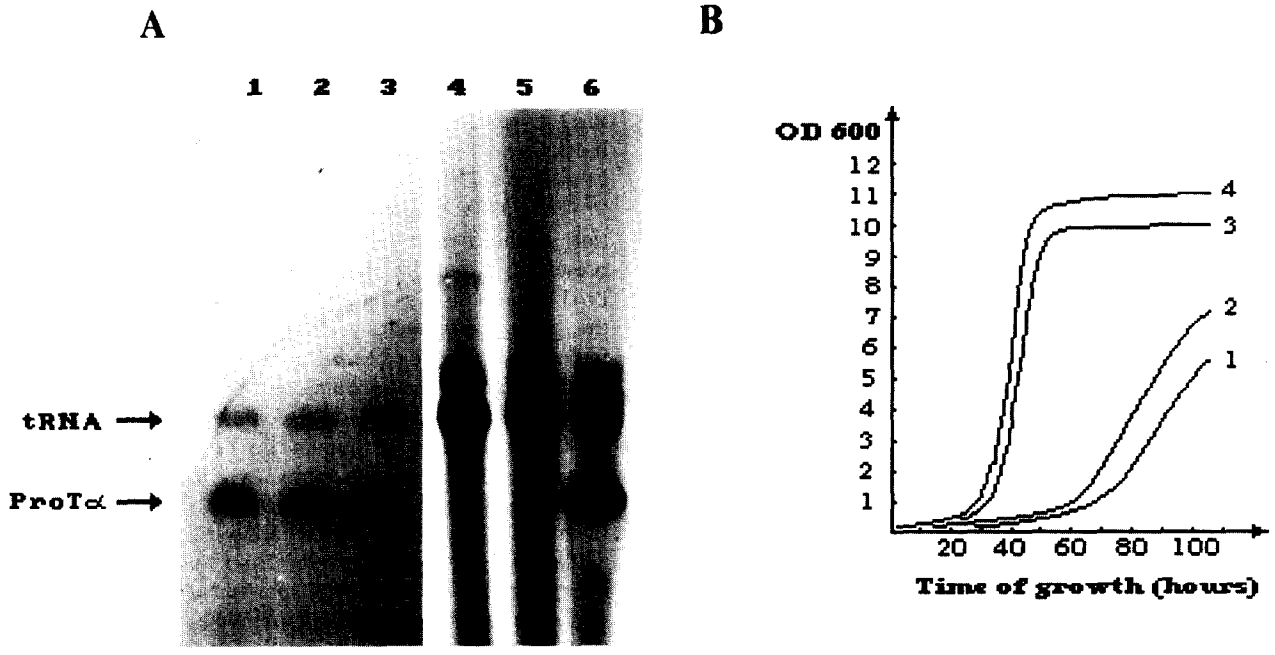

Fig. 2. (A) Synthesis of wild-type human ProTα (lanes 1,4), S^1T mutant (lanes 2,5) and $K^{87}E$ mutant (lanes 3,6) in yeast. Human ProTα was isolated from yeast cells induced with galactose for 8 h (lanes 1–3) or for 72 h (lanes 4–6). The amount of cells taken for ProTα isolation in samples 4–6 was several times higher than that in samples 1–3 in order to visualise the low amount of wild-type ProTα and S^1T mutant if present. The preparations were analysed by 8% polyacrylamide/urea gel electrophoresis followed by methylene blue staining [11]. The positions of ProTα and tRNA are indicated. (B) Growth of yeast cells producing: 1, wild-type human ProTα; 2, S^1T mutant; 3, $K^{87}E$ mutant; 4, no ProTα (vector alone).

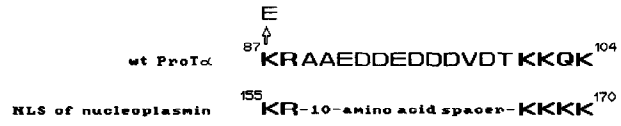

Fig. 3. Alignment of the carboxy-terminal sequence of human ProTα with the bipartite NLS of nucleoplasmin. Basic residues essential for nucleoplasmin NLS functioning and corresponding ProTα residues are in bold. The mutation in ProTα sequence leading to inactivation of the protein is indicated by the arrow.

In order to determine which of these mutations may contribute to the inactivation of ProTα, growth parameters of the yeast cells bearing plasmids encoding wild-type ProTα, S^1T and $K^{87}E$ mutants and vector alone were determined in the liquid medium. While in the absence of inducer (no ProTα production) all four types of cells grew equally well (not shown), synthesis of wild-type ProTα and S^1T mutant in the presence of galactose led to severe inhibition of cell growth (Fig. 2B). In contrast, production of the $K^{87}E$ ProTα mutant exhibited no appreciable effect on yeast cell growth, which was comparable to the growth of the cells containing vector only. It should be mentioned that restoration of yeast cell growth observed at late stages (approx. 70 h post induction (Fig. 2B, curves 1,2) was demonstrated to be due to abrogation of wild-type and S^1T ProTα production by an unknown mechanism. Synthesis of $K^{87}E$ ProTα mutant was still very efficient at this time point (Fig. 2A, lanes 4–6). This observation further strengthens the notion that production of wild-type and S^1T ProTα is deleterious for yeast cells, while $K^{87}E$ mutant lacks this property.

Thus, our results clearly demonstrate that the single $K^{87}E$ mutation inactivates inhibitory action of human ProTα on yeast cell growth, while the S^1T mutant preserves the properties of wild-type protein.

4. Discussion

Generation of ProTα mutants with altered properties of this small nuclear protein appears to be an essential step in deciphering a function of ProTα and structure-function relationship in this protein. Two major questions arise in this regard: which amino acid residues should be mutated, and what assay can be employed for identification of ProTα mutants with biologically significant amino acid substitutions? As for the first question, random mutagenesis of ProTα seems to be preferential, since no fruitful prediction of potential 'active sites' in the protein could be made on the basis of analysis of its primary structure. To this end, we performed PCR-mediated random mutagenesis of the human ProTα cDNA based on the ability of *Taq* polymerase to misincorporate nucleotides at rather high frequency, which could be further increased by the addition of manganese ions (see Section 2) [12].

In order to select ProTα mutants with altered properties of the protein, we made use of the fact that *S. cerevisiae* cells overproducing recombinant human ProTα fail to grow [11]. We have demonstrated recently that deletion of the last nine carboxy-terminal amino acids of ProTα containing putative NLS abolished inhibitory action of the protein [11]. It seemed probable, therefore, that some point mutations could abrogate inhibitory properties of the protein as well, thus permitting identification of the functionally important amino acid residues in ProTα. In this case, a strategy for selection of such mutants appeared to be straightforward: only those mutations which inactivate ProTα will gain the ability of ProTα-producing yeast colonies to form, while the growth capability of all other colonies producing mutated but still active ProTα will still be retarded. Then sequencing of cDNA encoding inactivated ProTα should point to the amino acid residues which are important for ProTα functioning.

As a first example of utilisation of this approach, we have identified Lys-87 of human ProTα as a functionally important amino acid residue. Conversion of Lys-87 to glutamic acid resulted in abrogation of the inhibitory properties of human ProTα on yeast cell growth. Interestingly, because several mutations were simultaneously introduced in one cDNA molecule, it became possible, after recloning, to identify not only the important Lys-87, but also the Ser-1 which appeared to be dispensable for ProTα functioning, at least in the assay employed.

The main question concerning inactivating mutation $K^{87}E$ is whether any biological significance could be inferred from these data. Inactivation of ProTα by this particular mutation is formally similar to abrogation of inhibitory action of ProTα on yeast cell growth resulted from elimination of the last nine carboxy-terminal amino acid residues of ProTα including putative NLS [11]. Although this analogy might well be accidental, it prompted us to take a closer look at the carboxy-terminal sequence of ProTα. The region of the ProTα molecule in question is believed to contain a NLS of this nuclear protein. Although this NLS has never been precisely identified, several lines of evidence suggest that a basic sequence KKQK (residues 101–104) may contribute to the active nuclear import of this protein, in analogy with the canonical continuous basic NLS of SV40 T antigen [3–5,17]. There is yet another type of NLS in nuclear proteins exemplified by the NLS of nucleoplasmin. This NLS is bipartite and is composed of two basic regions separated by a 10 amino acid long spacer (Fig. 3) [18,19]. Both parts of this NLS are essential for the nuclear uptake of the protein. The spacer length and composition can be altered without significant affecting nuclear targeting; however, bulky hydrophobic amino acids should be avoided from this region [18,19].

Inspection of the carboxy-terminal portion of ProTα has revealed that a short basic region KR (residues 87–88) is located 12 amino acid residues upstream from the putative NLS (residues 101–104) (Fig. 3). The spacer region lacks bulky hydrophobic residues and thus conforms to the above-mentioned rules for the bipartite NLS. It is most notable that the identified ProTα-inactivating mutation $K^{87}E$ destroys one part of this putative bipartite NLS.

Thus, it is tempting to suggest that the NLS of ProTα might actually be bipartite rather than monopartite, and that the inability of $K^{87}E$ ProTα mutant to suppress yeast cell growth is due to relaxation of its NLS which prevents efficient nuclear uptake of the protein. This possibility is currently under consideration.

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References

[1] Goodall, G.J., Dominguez, F. and Horecker, B.L. (1986) Proc. Natl. Acad. Sci. USA 83, 8926–8928.
[2] Eschenfeldt, W.H. and Berger, S.L. (1986) Proc. Natl. Acad. Sci. USA 83, 9403–9407.
[3] Watts, J.D., Cary, P.D. and Crane-Robinson, C. (1989) FEBS Lett. 245, 17–20.
[4] Watts, J.D., Cary, P.D., Sautiere, P. and Crane-Robinson, C. (1990) Eur. J. Biochem. 192, 643–651.
[5] Manrow, R.E., Sburlati, A.R., Hanover, J.A. and Berger, S.L. (1991) J. Biol. Chem. 266, 3916–3924.
[6] Clinton, M., Graeve, L., El-Dorry, H., Rodriguez-Boulan, E. and Horecker, B.L. (1991) Proc. Natl. Acad. Sci. USA 88, 6608–6612.
[7] Sburlati, A.R., Manrow, R.E. and Berger, S.L. (1991) Proc. Natl. Acad. Sci. USA 88, 253–257.
[8] Sburlati, A.R., De La Rosa, A., Batey, D.W., Kurys, G.L., Manrow, R.E., Pannell, L.K., Martin, B.M., Sheeley, D.M. and Berger, S.L. (1993) Biochemistry 32, 4587–4596.
[9] Gomez-Marquez, J., Segade, F., Dosil, M., Pichel, J.G., Bustelo, X.R. and Freire, M. (1989) J. Biol. Chem. 264, 8451–8454.
[10] Eilers, M., Schirm, S. and Bishop, J.M. (1991) EMBO J. 10, 133–141.
[11] Pavlov, N., Evstafieva, A., Rubtsov, Y. and Vartapetian, A. (1995) FEBS Lett. 366, 43–45.
[12] Brink, M.F., Nels, R.N.M., Verbeet, M.P. and De Boer, H.A. (1994) J. Mol. Biol. 237, 368–377.
[13] Evstafieva, A.G., Chichkova, N.V., Makarova, T.N., Vartapetian, A.B., Vasilenko, A.V., Abramov, V.M. and Bogdanov, A.A. (1995) Eur. J. Biochem. 231, 639–643.
[14] Cullin, C. and Pompon, D. (1988) Gene 65, 203–217.
[15] Hill, J., Donald, K.A.I.G. and Griffiths, D.E. (1991) Nucl. Acids Res. 19, 5791.
[16] Davis, R.W., Thomas, M., Cameron, J., St.John, T.P., Scherer, S. and Padgett, R.A. (1980) Methods Enzymol. 65, 404–411.
[17] Gomez-Marquez, J. and Segade, F. (1988) FEBS Lett. 226, 217–219.
[18] Robbins, J., Dilworth, S.M., Laskey, R.A. and Dingwall, C. (1991) Cell 64, 615–623.
[19] Dingwall, C. and Laskey, R.A. (1991) Trends Biochem. Sci. 16, 478–481.