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Synthesis and farnesylation of a-factor fusion proteins in *Saccharomyces cerevisiae*

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We have generated in-frame fusions between the mouse dihydrofolate reductase (*DHFR*) and parts of the a-factor *MFA1* gene to explore the potential of a-factor as a secretion signal for larger polypeptides. We demonstrated that the fusion proteins are farnesylated by comparing the mobility of fusion proteins prepared from a wild-type strain and a farnesyltransferase mutant (*ste16/ram1*) on SDS-gels and by an in vitro farnesylation assay. In contrast to unmodified DHFR, the fusion proteins could be sedimented from cell extracts by centrifugation. Solubilization experiments indicated that the highly hydrophobic a-factor moiety renders the fusion proteins insoluble, explaining why the fusions are not secreted into the culture medium.

Secretion; Mating; Yeast; Prenylation; ABC transporters

1. INTRODUCTION

Unlike its counterpart α-factor, which is secreted in a classical signal sequence-dependent manner [1], the yeast mating pheromone, a-factor, is secreted by an alternative mechanism [2,3]. Required for its secretion is the Ste6 protein, a member of the ABC (*A*TP *B*inding *C*assette) transporter family [4,5]. The a-factor is synthesized as a larger precursor (pro-a-factor) [6]. Mature a-factor is generated through a series of processing events [7,8]. First, it is farnesylated at a cysteine residue close to the C-terminus, then the last three amino acids are removed and the cysteine is carboxymethylated at the newly exposed hydroxyl-group. These modifications are thought to be required for membrane association of a-factor. After proteolytic removal of the pro-sequence the mature farnesylated dodeca-peptide is released into the medium.

We were interested in finding out whether a-factor fused to the C-terminus of a passenger protein could serve as a secretion signal directing the secretion of the fusion protein. To analyze the localization of a-factor fusions we used the mouse dihydrofolate reductase (DHFR) as a passenger protein. Here we present results showing that the C-terminal modifications of a-factor do occur with the fusion proteins whereas secretion seems to be prevented, perhaps due to aggregate formation within the cell.

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2. MATERIALS AND METHODS

2.1. *Media and strains*

The strains were grown in minimal medium (SD) containing 0.7% yeast nitrogen base without amino acids (Difco), 1% casamino acids (Difco) and 2% glucose. The genotypes of the strains used are: DBY2063 *MATa leu2 ura3*, K91-3b *MATa cryR his4 leu2 lys2 ste16 trp1 tyr1 ura3*.

2.2. *Plasmid constructions*

The plasmids encoding the DHFR–a-factor fusions were constructed by inserting PCR fragments of the *DHFR* and *MFA1* genes into the expression plasmid, pBM272 [9], as indicated in Fig. 1. Standard procedures were used for plasmid construction [10].

2.3. *Farnesylation assay*

Cells were resuspended in farnesylation buffer (50 mM Tris-Cl, pH 7.5, 50 μM zinc acetate, 20 mM KCl, 1 mM DTT) containing 1 mM PMSF, and lysed by agitation with glass beads. The extracts were diluted to a protein concentration of 5 μg/μl. 10 μl of extract (= 50 μg of protein) were mixed with 14 μl farnesylation buffer and 1 μl [^{3}H]farnesylpyrophosphate (Amersham, 20 pmol, 0.2 μCi) and incubated at 37°C for 1 h. After the addition of 10 μl of 4× sample buffer (4% SDS, 250 mM Tris-Cl, pH 6.8, 40% glycerol, 0.04% bromphenolblue) the samples were heated to 95°C for 3 min and separated on a 15% SDS gel. The gel was fixed in 7% acetic acid/20% methanol, soaked in amplify (Amersham), dried and exposed to Kodak XOmat AR film.

3. RESULTS

3.1. *Expression of DHFR–a-factor fusions*

To be able to decide whether the pro-sequence is required as a signal for the maturation or export of a-factor we constructed a fusion where the entire pro-a-factor was fused to the *DHFR* gene (pRK63) and another fusion where only the sequence coding for the mature part of a-factor, including the additional three C-terminal residues, was fused to *DHFR* (pRK47). In another construct, the pro-sequence of a-factor was

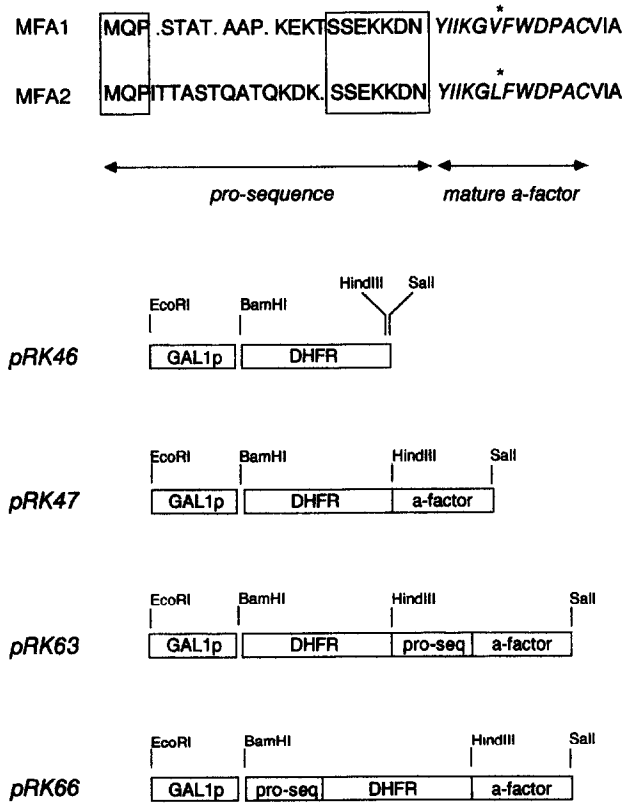

Fig. 1. Structure of the DHFR–a-factor fusions. The two pro-a-factor proteins encoded by the *MFA1* and *MFA2* genes are shown at the top of the diagram. Homologous regions in the pro-sequence are boxed. The two different a-factor variants differ in one amino acid marked by an asterix. Precise in-frame fusions were generated using PCR fragments. Suitable restriction sites were incorporated at the ends of the fragments to facilitate cloning.

placed at the N-terminus of the fusion while the mature a-factor sequence was at the C-terminus (pRK66).

The fusions were expressed from a centromer plasmid under the control of the *GAL1* promoter. Cell extracts were prepared and analyzed by SDS-PAGE and Western blotting. The different proteins were clearly detectable in cell extracts (Fig. 2). The mobility of the fusion proteins approximately corresponded to the size of the proteins derived from the DNA sequence (M_r of DHFR = 21.5 kDa, M_r of pro-a-factor = 3.9 kDa, M_r of mature a-factor = 1.4 kDa). Faster migrating bands observed for pRK66 are probably processed forms of the protein.

To assay for the presence of fusion proteins in the culture supernatant, proteins from 5 ml of the cell-free culture supernatant were TCA-precipitated, separated on a SDS-gel and analyzed by Western blotting with anti-DHFR antibodies. Using this regimen no specific immunoreactive bands could be detected (limit of detection: about 10–20 ng of DHFR protein). a-Factor has a tendency to stick to glass surfaces, therefore we rinsed the culture flask with propanol [2], but we were not able to detect the fusion proteins in the propanol wash. There is the possibility that the internal a-factor is competing with the export of our recombinant fusion proteins. Therefore, we expressed a DHFR–a-factor fusion protein from a construct equivalent to pRK63 in the a-factor deletion strain, SM1229 (*MATα Δmfa1 Δmfa2*) [11]. Even with this strain, however, we were not able to detect the fusion proteins in the culture supernatant. Therefore, we have to conclude (within the limits of detection) that our fusion proteins are not secreted.

3.2. *The DHFR–a-factor fusion proteins can be sedimented by centrifugation*

Cell extracts containing the DHFR–a-factor fusions were prepared by lysis of spheroplasts and spun at 100,000 × *g* for 1 h. All three DHFR–a-factor fusion constructs behaved similarily in that about 90% of the fusion protein was found in the pellet fraction (Fig. 3). In contrast, the DHFR protein without the a-factor extension was found in the supernatant (not shown). To find out whether the fusion proteins are associated with membranes or contained within an organelle we performed a solubilization experiment. As can be seen from Fig. 3 the DHFR–a-factor fusion protein is not solubilized by treatment with 1 M NaCl, 0.2 M Na_2CO_3, pH 11, and 2% Triton X-100. Only 10 M urea was able to solubilize about half of the protein. As a control we used antibodies against the β-subunit of the mitochondrial F_1ATPase which is a pheripheral membrane protein. As expected, this protein can be solubilized in the same extracts by treatment with 0.2 M Na_2CO_3 pH 11, and 2% Triton X-100. It was also partially solubilized by urea treatment. From these results we conclude that the fusion protein is not membrane associated. The most likely explanation for the sedimentation of the proteins is aggregate formation due to the highly hydrophobic a-factor moiety.

3.3. *The DHFR–a-factor fusions are farnesylated*

To see whether farnesyltransferase is able to recognize the DHFR–a-factor fusions as a substrate, we ana-

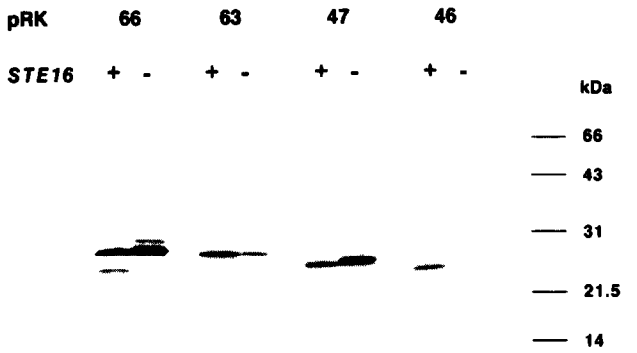

Fig. 2. Farnesylation of DHFR–a-factor fusions. The mobility of the fusion proteins prepared from the wild-type strain, DBY2063(+), and from the *ste16* strain, K91-3b (−), was compared on a 12.5% SDS-gel. Immunoreactive bands were detected by Western blotting using anti-DHFR antibodies.

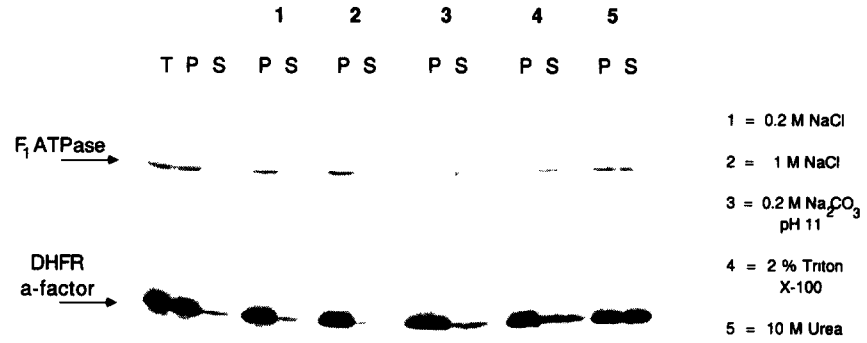

Fig. 3. Solubilization experiment. Extracts from DBY2063 containing the plasmid, pRK47, were incubated for 30 min at 4°C under the conditions indicated (lanes 1–5). Then the extracts were centrifuged for 1 h at 100,000 × *g*. Equal portions of the supernatant (S) and pellet (P) fractions were loaded onto a 12.5% SDS-gel (T = total extract). Immunoreactive bands were detected by Western blotting using antibodies against the β-subunit of F_1ATPase and against DHFR.

lyzed the fusions for the presence of C-terminal modifications. First, we compared the gel mobility of fusions prepared from a wild-type strain and from a strain deficient in farnesyltransferase activity (*ste16/ram1*) [12,13]. The proteins extracted from the *ste16* strain migrated slightly slower on SDS-gels than the proteins prepared from the wild-type strain (Fig. 2), an indication that these proteins are modified. The control protein without an a-factor extension (pRK46) and the fusion encoded by pRK63 did not show an altered mobility in the *ste16* strain.

To demonstrate more directly that the fusions are farnesylated, we used an in vitro farnesylation assay. Cell extracts prepared from the *ste16* strain expressing the different (unmodified) fusion proteins were mixed with extracts from a wild-type *MATα* strain furnishing the farnesyltransferase activity. The mixed extracts were incubated with [^{3}H]farnesylpyrophosphate. The proteins were separated on a SDS-PAGE gel and analyzed by autoradiography (Fig. 4). Four protein bands of apparent sizes of 34, 44, 50 and 70 kDA were constantly detected, irrespective of the type of DHFR fusion protein present in the extract. No farnesylated proteins could be detected using either of the two extracts alone (not shown). This indicates that farnesylation occurs very rapidly so that there are no proteins left in the wild-type extract that can be labeled with the radioactive substrate.

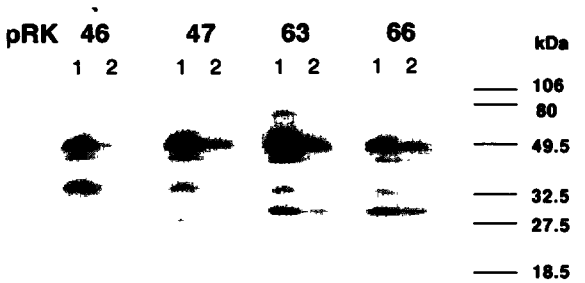

Fig. 4. In vitro farnesylation of the DHFR–a-factor fusion proteins. Extracts from the plasmid containing *ste16* strain K91-3b and from the wild-type strain, DBY2063, were mixed and incubated with [^{3}H]farnesylpyrophosphate. The labeled proteins were separated on a 15% SDS-gel and analyzed by autoradiography. Lanes 1, 5 μl *ste16* + 5 μl wild-type extract; lanes 2, 1 μl *ste16* + 9 μl wild-type extract.

The mobility of one band, however, was dependent on the type of fusion protein present in the extracts. This band could not be detected in the extracts containing the DHFR protein without a-factor extension. The fusion proteins carrying the pro-sequence of a-factor in addition to the mature sequence (pRK63 and pRK66) gave rise to a slower migrating band compared to the fusion, which only has the mature a-factor sequence at its C-terminus (pRK47). From these results it is clear that all fusions carrying a-factor sequences at their C-termini are farnesylated. Thus farnesyltransferase is able to accept the heterologous fusion proteins as a substrate. The pro-sequence is not required as a signal for farnesyltransferase as the 15 C-terminal amino acids are sufficient to direct the farnesylation of the fusion protein (pRK47).

The pRK63 fusion protein is farnesylated, as demonstrated by the in vitro experiment. This protein, however, had the same gel mobility when it was prepared form a *ste16* and from a wild-type strain. What exactly causes the mobility shift in the other fusions is not clear (but the same shift in mobility was also observed for the Ras2 protein). It is difficult to predict how the addition of a farnesyl moiety affects the mobility. The removal of the last three amino acids, however, should lead to a slightly faster migrating protein. One possible explanation is that the observed shift in mobility is mainly caused by the removal of the last three amino acids and by the loss of one negative charge due to the carboxymethylation. The results obtained with the pRK63 fusion could be explained by the assumption that only farnesylation occurs while further processing steps are prevented e.g. due to improper folding of this fusion protein.

4. DISCUSSION

We have examined the potential of a-factor to serve as a secretion signal for heterologous proteins. Although we were able to demonstrate that the first step in the maturation and export pathway of a-factor, the farnesylation, occurs with the DHFR–a-factor fusions, we could not detect the proteins outside the cell. There are several possibilities to explain these findings: (i) the Ste6 translocator is not able to accomodate a polypeptide larger than a-factor; (ii) the farnesylation of the DHFR–a-factor fusions is not sufficient for anchoring the protein in the membrane and therefore the probability of a productive interaction with the Ste6 translocator may be too low; (iii) the folding of the DHFR-fusions is not compatible with membrane translocation by Ste6. The translocation of the DHFR protein through the membrane seems to be very sensitive to alterations in the folding of the protein. Binding of methotrexate to DHFR, which presumably stabilizes the native structure of the protein, inhibits mitochondrial import [14]. In line with this notion is the finding that the fusion proteins probably form protein aggregates in the cell. This is an indication that the folding of the protein is altered.

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