View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Volume 297, number 1,2, 103–106 FEBS 10666 February 1992
© 1992 Federation of European Biochemical Societies 00145793/92/$5.00

# Protein prenylation in *Schizosaccharomyces pombe*

Thomas Giannakouros[a], John Armstrong[b] and Anthony I. Magee[a]

[a]*Laboratory of Eukaryotic Molecular Genetics, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK* and [b]*Membrane Molecular Biology Laboratory, Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK*

Received 3 December 1991

*S. pombe* is shown to be a powerful system for studies concerning attachment of polyisoprenoid moieties to proteins, due to its ability to take up exogenous mevalonic acid efficiently. The fission yeast can take up about 5% of the exogenously added mevalonic acid and incorporate ~10% of this into protein. By contrast, the uptake obtained with the budding yeast *S. cerevisiae* is less than 0.5%. HPLC analysis of total *S. pombe* protein-bound isoprenoids revealed that ~55% of the counts co-migrated with the geranylgeraniol standard, while ~45% of the counts co-migrated with farnesol. We could not detect any effects of mevinolin or other HMG-CoA reductase inhibitors in *S. pombe*.

*S. pombe*; *S. cerevisiae*; Mevalonic acid; Prenylated protein

## 1. INTRODUCTION

The covalent attachment of polyisoprenoid moieties to proteins has recently been observed and is associated with intracellular targeting of those proteins as well as their functional activation [1–3]. Polyisoprenoids such as farnesyl (15 carbon) and geranylgeranyl (20 carbon) are derived from mevalonic acid via a pathway which results ultimately in the production of a wide range of cellular metabolites such as cholesterol, dolichol, ubiquinone and heme [4]. These modifications occur at carboxy-terminal motifs of the general type CAAX (C, cysteine; A, aliphatic; X, any other amino acid). Small and hydrophilic side chains in the X position favour farnesylation of the cysteine residue, while large aliphatic residues favour geranylgeranylation [5]. In addition to polyisoprenylation these sequences are also modified by proteolytic removal of the AAX sequence, and by carboxyl-methylation of the α-carboxyl group [6,7]. In the case of *ras* proteins nearby upstream palmitoylated cysteine residues or a polybasic sequence cooperate to specify plasma membrane binding [8]. Recent data suggest that carboxyl-terminal motifs of the type CC or CXC, found in the *ras*-related family of *rab*/*YPT* proteins, are modified in a related way [9–11]. The subtle variation in these lipid modifications, in concert with protein sequences, very likely contributes to the exquisite selectivity of different members of this family for compartments of the endomembrane system [12].

While all mammalian cells can take up exogenous mevalonic acid with varying efficiencies it has been found that the budding yeast *S. cerevisiae* is poorly suited for studies concerning polyisoprenylation of proteins, due to its inability to take up exogenous mevalonic acid efficiently [2]. In this report we present evidence that, in contrast to *S. cerevisiae*, the fission yeast *S. pombe* has a high capacity for the uptake of mevalonic acid. HPLC analysis of total *S. pombe* protein-bound isoprenoids revealed the existence of both geranylgeraniol and farnesol in similar amounts.

*Correspondence address:* A.I. Magee, Laboratory of Eukaryotic Molecular Genetics, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK. Fax: (44) (81) 906 4477.

*Abbreviations:* HMG-CoA, hydroxymethylglutaryl coenzyme A; MVA, mevalonic acid; SDS, sodium dodecyl sulphate; TCA, trichloroacetic acid.

## 2. MATERIALS AND METHODS

### 2.1. *Isotopic labelling of S. pombe/S. cerevisiae — Preparation of cell extracts*

Wild-type *Schizosaccharomyces pombe* 972h$^-$ or wild-type *Saccharomyces cerevisiae* (CG378-α ade5, can$^R$, leu2–3, 112 trp1–289, ura3–52) were grown at 30°C to mid-logarithmic phase ($OD_{595}$ 0.3–0.4; 6–8 × $10^6$ cells/ml) in complete medium (YE) supplemented with 250 mg/l adenine, histidine, leucine, uracyl and lysine hydrochloride [13–15]. 6.0 $OD_{595}$ units of *S. pombe* or *S. cerevisiae* cells were labelled with 250 μCi [$^3$H]mevalonic acid (triethylammonium salt; New England Nuclear, NET-716; 30.5 Ci/mmol) in a total volume of 3 ml at 30°C. At the appropriate time intervals, 1.2 $OD_{595}$ units of *S. pombe* or *S. cerevisiae* cells were harvested by centrifugation at 5,000 xg for 5 min, washed once with spheroplasting medium (SPI: 1.2 M sorbitol, 50 mM sodium citrate, 50 mM $Na_2HPO_4$, 40 mM EDTA, pH 5.6) and resuspended in 50 μl of SPI. Zymolyase - 20.T (0.4 mg/ml; Seikagaku, Japan) was added and samples were heated at 37°C for 30 min. Spheroplasts were pelleted and then lysed in 40 μl of 1% SDS, 50 mM Tris-HCl, pH 6.8, followed by heating at 100°C for 5 min. The cell extracts, after being clarified by centrifugation at 10,000xg for 5 min, were collected on Whatman 3MM filters and counted directly to measure uptake or after precipitation in 10% trichloroacetic acid (TCA) to measure incorporation. The efficiency of counting of $^3$H when spotted on filters was estimated to be 6.4% using a known

amount of radioactivity. In order to analyze prenylated proteins by polyacrylamide gel electrophoresis TCA-precipitated proteins were collected by centrifugation at 10,000×g at 4°C for 10 min. The protein pellets were extracted three times with 1 ml cold acetone, dried and taken up in 40 μl Laemmli sample buffer. Proteins were resolved by SDS-PAGE [16] on a 12.5% gel, followed by fluorography [17].

2.2 *Prenoid analysis of S.pombe proteins*

20–30,000 cpm of TCA-precipitated $^3$H-labelled proteins were washed three times with acetone until no more counts were released, dried and digested with 100 μg/ml pronase (Calbiochem) in 200 μl 25 mM Tris-HCl, pH 7.5. The resulting peptides were treated with methyl iodide essentially by the method of Casey *et al.* [3] and extracted with chloroform/methanol (9:1). The dried products of the cleavage were dissolved in 100 μl of HPLC solvent B (100% acetonitrile containing 25 mM phosphoric acid). Immediately before injection an aliquot was diluted with an equal volume of water. Analysis was on a Brownlee RP300 cartridge (0.46 × 10 cm) with a 1 cm guard cartridge using a 30 min linear gradient from 100% solvent A (50% aqueous acetonitrile, 25 mM phosphoric acid) to 100% solvent B, followed by 10 min at 100% solvent B, at 0.5 ml/min. One-minute fractions were collected and counted in 6 ml of Ready-Safe scintillation cocktail (Beckman). A Beckman System Gold HPLC was used and standard isoprenoids were monitored at 254 nm. Geraniol and all-*trans* farnesol were from Sigma. All-*trans* geranylgeraniol was a gift of P. Casey (Duke University, N.C., USA), and of Dr. M. Tanomura (Kuraray Co. Ltd., Osaka, Japan).

## 3. RESULTS

Studies on YPT proteins of the fission yeast *S. pombe* have provided the first evidence that *S. pombe* contains proteins that are post-translationally modified by isoprenoid groups (E. Fawell, J. Hancock, T. Giannakouros, C. Newman, J. Armstrong and A.I. Magee, unpublished observations). During these studies we noticed that it was possible to label YPT proteins with [$^3$H]mevalonic acid ([$^3$H]MVA). Considering that

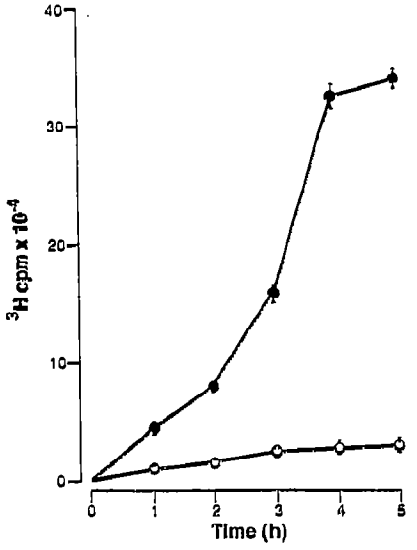


Fig. 1. Uptake of MVA by *S. pombe* and *S. cerevisiae*. *S. pombe* (●) and *S. cerevisiae* (○) cells were labelled and then harvested as described in section 2. Spheroplasts were prepared, lysed in SDS, spotted onto Whatman 3MM filters and counted. The mean and range of duplicate values is shown.

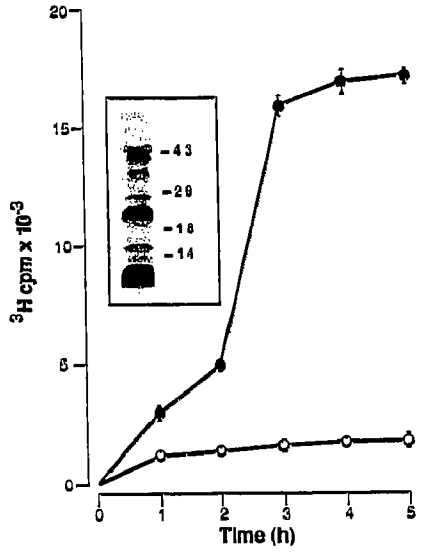


Fig. 2. Incorporation of MVA by *S. pombe* and *S. cerevisiae* into TCA-insoluble material. At the appropriate time intervals cell extracts were prepared (see section 2), collected on Whatman 3MM filters, TCA-precipitated and then counted. SDS-PAGE (12.5% gel) analysis of total labelled proteins of *S. pombe* is shown in the inset. MW markers are given in kDa. Symbols as for Fig. 1.

Schafer et al. [2] have reported that the budding yeast *S. cerevisiae* is unable to take up exogenous mevalonic acid efficiently we decided to determine more precisely the extent to which *S. pombe* and *S. cerevisiae* cells can take up exogenous mevalonic acid and incorporate it into proteins. Cell extracts from [$^3$H]MVA-labelled *S. pombe* and *S. cerevisiae* were prepared at the appropriate time intervals, and counted directly (to measure uptake) or after TCA-precipitation (to measure incorporation into protein). The data presented in Fig. 1 clearly show that *S. pombe* can take up [$^3$H]MVA approximately ten times more efficiently than *S. cerevisiae*. About 10% of the [$^3$H]MVA taken up by *S. pombe* is incorporated into protein (Fig. 2). In the case of *S. cerevisiae* this percentage is even lower. The SDS-

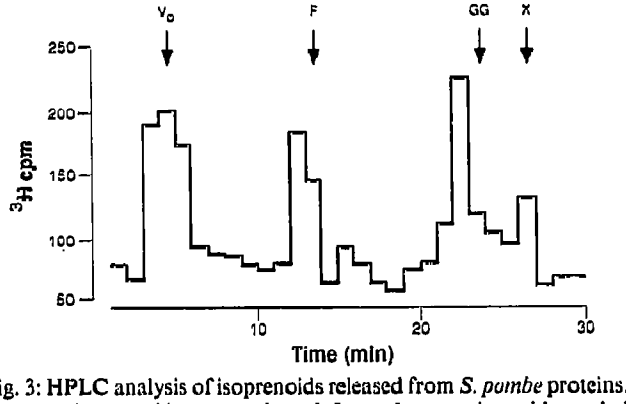


Fig. 3: HPLC analysis of isoprenoids released from *S. pombe* proteins. Labelled isoprenoids were released from the proteins with methyl iodide as described in section 2 and were analyzed by HPLC. The elution positions of authentic geraniol (G), farnesol (F) and geranylgeraniol (GG) are shown. $V_0$ is the void volume of the column. X corresponds to isomers of geranylgeraniol.

PAGE fluorographic profile of the TCA-precipitated proteins from [$^3$H]MVA-labelled *S. pombe* (Fig. 2, inset) was similar to that previously seen in homogenates from a variety of cultured cell lines [18–20] in so far as most of the radioactivity was incorporated into a cluster of proteins between 20–26 kDa. At least two more polypeptides, with apparent molecular masses of 39 and ~10 kDa respectively, were labelled heavily with [$^3$H]MVA, but we have been unable so far to identify any of these proteins. We could not detect any effects of mevinolin or other HMG-CoA reductase inhibitors on any proteins in *S. pombe* (data not shown). Interestingly, an effect of a similar compound, compactin, has been demonstrated in *S. cerevisiae* [2] suggesting either that mevinolin is not taken up by *S. pombe*, or that the *S. pombe* HMG-CoA reductase is not susceptible to this type of inhibitor. Nevertheless, the ability of *S. pombe* cells to incorporate significant amounts of labelled MVA into proteins was exploited to allow an analysis of the chain length of the incorporated label. For that purpose ~20,000 cpm of TCA-precipitated [$^3$H]MVA-labelled proteins, after being extensively washed with acetone, were subjected to methyl iodide cleavage [3] and HPLC analysis (Fig. 3). Material eluting straight through the column in the void volume probably represents uncleaved peptides. A similar peak was obtained in the case of YPT proteins even in mock cleavage reactions without methyl iodide (data not shown). Of the counts which were retained on the column ~55% (average of two analyses: 65.7% and 43.7%) co-eluted with a geranylgeraniol ($C_{20}$ isoprenoid) standard, while ~45% (average of two analyses: 34.2% and 56.3%) of the counts migrated with farnesol ($C_{15}$ isoprenoid). Some label (X) eluted later than geranylgeraniol. This probably corresponds to an isomer of geranylgeraniol [21] and represents less than 15% of the counts. The total amount of geranylgeraniol therefore includes the contribution from this component.

## 4. DISCUSSION

The mechanism of protein prenylation has recently become of interest both because of the wide range of proteins that undergo this modification and the ability of inhibitors of prenylation to suppress some phenotypes of oncogenic *ras* proteins [22,23]. Information on this type of protein modification comes primarily from studies of the processing of mammalian proteins, while the study of yeast proteins has been accomplished either indirectly by using different inhibitors of prenylation, or by expressing them in heterologous systems. The main reason for that is the inability of the budding yeast *S. cerevisiae* to mediate incorporation of [$^3$H]MVA into polyisoprenoid moieties of proteins. Our results clearly demonstrate that this inability is due to the poor uptake of MVA by *S. cerevisiae*. On the contrary the fission yeast *S. pombe* can take up exogenous MVA efficiently. More precisely, *S. pombe* can take up about 5% of the exogenously added MVA and incorporate ~10% of this into protein. This is comparable to results obtained with Swiss 3T3 cells [18]. HPLC analysis of total *S. pombe*-bound isoprenoids revealed that ~45% of the counts co-migrated with the farnesol standard, while ~55% of the counts co-migrated with geranylgeraniol, confirming that the fission yeast can add geranylgeranyl to proteins. The covalent attachment of this latter prenyl group has been established up to now only for higher animal cells [22]. Interestingly the ratio of geranylgeranyl to farnesyl in the case of *S. pombe* (~1.2) seems to differ compared with Chinese hamster ovary and HeLa cells, where the protein bound geranylgeranyl moiety accounts for 80–90% of the total MVA-derived label [25,26].

Incubation of *S. pombe* cells with [$^3$H]MVA results in the labelling of several polypeptides, the most prominent of which migrate in the range of 20–26 kDa on SDS-PAGE and probably correspond to low molecular mass GTP-binding proteins [27]. Our preliminary data suggest that geranylgeraniol is the principal isoprenoid moiety involved in the post-translational modification of the 20–26 kDa proteins (not shown). Significant amounts of radioactivity were also detected in proteins with apparent molecular masses of 39 and ~10 kDa, the identities of which remain to be clarified. We could not detect any effect of mevinolin or other HMG-CoA reductase inhibitors, such as pravastatin, on any of these polypeptides. In conclusion, the fission yeast *S. pombe* proves to be a particularly suitable organism for studying the functional aspects of protein prenylation, as well as the enzymatic mechanisms underlying this type of post-translational modification, due to its ability to incorporate label from [$^3$H]MVA acid into polyisoprenoid moieties of proteins in combination with the powerful genetic tools that can be applied.

*Acknowledgements:* Thomas Giannakouros is a long-term EMBO Fellow. We would like to thank Lee Johnston for kindly providing *S. cerevisiae*, Pat Casey and Dr. M. Tanomura for supplying the geranylgeraniol HPLC standard and Mrs. M. Brennan for her skilful secretarial assistance.

## REFERENCES

[1] Hancock, J.F., Magee, A.I., Childs, J.E. and Marshall, C.J. (1989) Cell 57, 1167–1177.
[2] Schafer, W.R., Kim, R., Sterne, R., Thorner, J., Kim, S.-H. and Rine, J. (1989) Science 245, 379–385.
[3] Casey, P.J., Solski, P.A., Der, C.J. and Buss, J.E. (1989) Proc. Natl. Acad. Sci. USA 86, 8323–8327.
[4] Maltese, W.A. (1990) FASEB J. 4, 3319–3328.
[5] Yamane, H.K., Farnsworth, C.C., Xie, H., Evans, T., Howald, W.N., Gelb, M.H., Glomset, J.A., Clarke, S. and Fung, B.K.-K. (1991) Proc. Natl. Acad. Sci. USA 88, 286–290.
[6] Gutierrez, I., Magee, A.I., Marshall, C.J. and Hancock, J.F. (1989) EMBO J. 8, 1093–1098.
[7] Clarke, S., Vogel, J.P., Deschenes, R.J. and Stock, J. (1988) Proc. Natl. Acad. Sci. USA 85, 4643–4647.

[8] Hancock, J.F., Paterson, H. and Marshall, C.J. (1990) Cell 63, 133–139.
[9] Farnsworth, C.C., Kawata, M., Yoshida, Y., Takai, Y., Gelb, M.H. and Glomset, J.A. (1991) Proc. Natl. Acad. Sci. USA 88, 6196–6200.
[10] Johnston, P.A., Archer, B.T., Robinson, K., Mignery, G.A., Jahn, R. and Südhof, T.C. (1991) Neuron 7, 101–109.
[11] Khosravi-Far, R., Lutz, R.J., Cox, A.D., Conroy, L., Bourne, J.R., Sinensky, M., Balch, W.E., Buss, J.E. and Der, C.J. (1991) Proc. Natl. Acad. Sci. USA 88, 6264–6268.
[12] Chavrier, P., Parton, R.G., Hauri, H.P., Simons, K. and Zerial, M. (1990) Cell 62, 317–329.
[13] Nurse, P. (1975) Nature 256, 547–551.
[14] Gutz, H., Heslot, H., Leupold, U. and Loprieno, N., in: Handbook of Genetics, Vol. 1 (R.C. King, ed), Plenum Press, New York, 1974, pp. 395–427.
[15] Moreno, S., Klar, A. and Nurse, P., in: Methods in Enzymology (C. Guthrie and G.R. Fink, Eds.), Vol. 194, Academic Press, London, 1990, pp. 795–823.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Laskey, R.A. (1980) Methods Enzymol. 65, 363–371.
[18] Schmidt, R.A., Schneider, C.J. and Glomset, J.A. (1984) J. Biol. Chem. 259, 10175–10180.
[19] Sinensky, M. and Logel, J. (1985) Proc. Natl. Acad. Sci. USA 82, 3257–3261.
[20] Repko, E.M. and Maltese, W.A. (1989) J. Biol. Chem. 264, 9945–9952.
[21] Kamiya, Y., Sakurai, A., Tamura, S. and Takahashi, N. (1979) Agric. Biol. Chem. 43, 1049–1053.
[22] Glomset, J.A., Gelb, M.H. and Farnsworth, C.C. (1990) Trends Biochem. Sci. 15, 139–142.
[23] Goldstein, J.L. and Brown, M.S. (1990) Nature 343, 425–430.
[24] Rilling, H.C., Breunger, E., Epstein, W.W. and Crain, P.F. (1990) Science 247, 318–320.
[25] Farnsworth, C.C., Gelb, M.H. and Glomset, J.A. (1990) Science 247, 320–322.
[26] Epstein, W.W., Lever, D.C. and Rilling, H.C. (1990) Proc. Natl. Acad. Sci. USA 87, 7352–7354.
[27] Maltese, W.A., Sheridan, K.M., Repko, E.M. and Erdman, R.A. (1990) J. Biol. Chem. 265, 2148–2155