Volume 334, number 3, 360–364 FEBS 13274 November 1993

Determination and mutational analysis of the phosphorylation site in the hypusine-containing protein Hyp2p

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Received 27 September 1993

Electrospray mass spectrometry of the purified isoforms of the hypusine-containing protein of *Saccharomyces cerevisiae* Hyp2p suggested a phosphorylation of the acidic isoform, which was confirmed by phosphatase treatment. The phosphorylation site was mapped to the N-acetylated serine residue in position no. 1 by mass spectrometric analysis of enzymatic fragments. Mutation of this serine residue gives rise to only the basic isoform, confirming our protein chemical data. As this mutation has no effect on cell viability or growth rate, the unphosphorylated isoform is sufficient to exert the essential in vivo function of Hyp2p.

eIF-5A; Isoform; Phosphorylation; Acetylation; Amino terminus; Yeast

1. INTRODUCTION

The hypusine-containing protein is so far the only known protein, in which a specific lysine residue is modified by the aminobutyl moiety of the polyamine spermidine. Subsequent hydroxylation of this deoxyhypusine residue results in the unique amino acid hypusine [1]. The protein with its hypusine modification is highly conserved in all eukaryotes (for review see [2]) and was also found in archaebacteria [3,4], but not in eubacteria. Functional analysis in the methionyl puromycin assay suggested a role of this protein in the first peptide bond formation. Therefore, it was originally designated eukaryotic translation initiation factor 5A (eIF-5A) [5]. Nevertheless, very little is known about its function in vivo (for review see [2]).

In the budding yeast *Saccharomyces cerevisiae* the hypusine-containing protein is encoded by two highly homologous genes, one of which is under strict anaerobic control (*HYP1* = *ANB1* = *TIF51B*; [6–8]). In contrast, the transcription of the HYP2 gene (= *TIF51A*) is positively controlled by oxygen and heme. Gene disruption analysis showed that under aerobic conditions *HYP2* expression is essential for viability in yeast [9]. All three forms of the hypusine-containing protein (two isoforms of 21 kDa and a degradation or processing product of 18 kDa) detected under aerobic conditions are *HYP2* gene products [9].

In order to gain some insight into the cellular function of the hypusine-containing protein and the regulation of its activity, we purified the two aerobically expressed isoforms and the degradation product and analyzed their structure in detail.

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Abbreviations: HPLC, high-performance liquid chromatography; Hyp1p, gene product of *HYP1* = *ANB 1* = *TIF51B*; Hyp2p, gene product of *HYP2* = *TIF51A*; *hyp2*, disrupted *HYP2* gene; IEF, isoelectric focussing; pI, isoelectric point; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2. MATERIALS AND METHODS

2.1. *Purification of the Hyp2p isoforms*

Subsequent to the isolation procedure of the Hyp2p [9], the isoforms were separated on an anion exchange HPLC (Aquapore Anion 100 × 2.1 mm, 7.1 μm; Applied Biosystems), which was eluted with 50 mM sodium phosphate (flow rate 0.5 ml/min) with a gradient of 200–300 mM NaCl in 5 min and 300–600 mM NaCl in 30 min. For mass spectrometry, the isoforms were re-chromatographed on a Vydac C_{18} reversed-phase HPLC in a gradient of 10–30% acetonitrile/0.1% trifluoroacetic acid in 10 min and 30–60% acetonitrile/0.1% trifluoroacetic acid in 30 min.

2.2. *Mass spectrometry*

The reversed-phase HPLC fractions were delivered to the sprayer of the atmospheric pressure ionisation source, connected to the tandem quadrupole instrument API III (Sciex, Thornhill, Ontario, Canada), with a flow rate of 5 μl/min by a syringe infusion pump (Model 22, Harvard Apparatus, South Natick, USA). The instrument m/z scale was calibrated with ammonium adduct ions of polypropylene glycol. The average molecular masses of the peptides were calculated from the *m/z* peaks of the charge distribution profiles of the multiply charged ions [10,11].

2.3. *Alkaline phosphatase treatment*

The isoforms were precipitated in 10% trichloroacetic acid (w/v) on ice for 30 min. After centrifugation (16,000 × *g*/4°C/15 min), the pellet was washed with ice-cold aceton, centrifuged, air dried and dissolved in 100 μl 50 mM Tris-HCl, 0.1 mM EDTA, pH 8.5. After incubation for 1 h at 37°C with 0.1 unit calf intestine phosphatase (Boehringer,

FRG) per μg protein [12], the samples were precipitated as described above.

2.4. *Enzymatic cleavage*

For tryptic digestion, the isoforms were precipitated as described above, resuspended in 100 μl 0.1 M Tris-HCl pH 8.0 and incubated with 0.025 μg trypsin per mg protein at 37°C. The same amount of trypsin was added after 2.5 h and 5 h. After 8 h the reaction was stopped with 1% trifluoroacetic acid. Peptides were separated on a Supersphere RP60 Select B reversed-phase HPLC column (125 × 2 mm/4 μm; Merck, FRG) using a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.3 ml/min.

Further cleavage of the N-terminal peptide with endoproteinase Glu-C (Boehringer, FRG) was performed in 50 mM ammonium acetate pH 4.0 with 0.05 μg endoproteinase Glu-C per μg protein for 20 h at room temperature. Peptides were isolated as described above.

2.5. *Miscellaneous methods*

Two-dimensional gels (isoelectric focussing on immobilized pH gradients and SDS-PAGE) and Western blots were performed according to [13] using a 1:250 dilution of the crude α-Hyp2p polyclonal antibody and a 1:4000 dilution of the alkaline phosphatase coupled α-chicken IgG (Sigma, St. Louis).

Ortho-phthaldialdehyde amino acid analysis was performed as described earlier [9]. For amino acid sequence analysis an Applied Biosystems 477A gas phase sequencer connected with a 120A PTH amino acid analyzer was used according to the manufacturer's instructions.

2.6. *Construction of GAL1/HYP2 fusion genes*

PCR amplification was used to clone the coding region of the wild-type gene *HYP2* and the mutated *HYP2[Ala1]* as a 480 bp *Bam*HI/*Xba*I fragment into pRSGAL, which is a single-copy plasmid containing the yeast *GAL1* promoter [9]. The strategy for the design of the primers, amplification and cloning of the PCR products was described previously [14]. The oligodeoxynucleotide used for the construction of pRSG-HYP2[Ala1] led to a change of the triplet immediately following the ATG startcodon from TCT (coding for serine) in the wild-type gene to GCT (coding for alanine). The sequence of the cloned PCR fragment was verified by DNA sequence analysis [15]. Transformation of pRSG-HYP2 or pRSG-HYP2[Ala1] into yeast was performed according to [16]. Yeast cells were either grown on semisynthetic medium (with 3% galactose or 2% glucose) or yeast peptone dextrose (YPD) and yeast peptone galactose (YPGal) media, respectively [17].

3. RESULTS AND DISCUSSION

Under aerobic conditions two isoforms and a degradation product thereof, lacking the first ten amino acids, were identified as gene products of *HYP2* [9]. As a basis for investigating the in vivo function of the Hyp2p forms, we now analyzed their protein chemical structure in detail. In the final purification step, which separates these three proteins, the degradation product, the basic and the acidic isoform eluted from the anion exchange HPLC at 420–450 mM NaCl, 460–490 mM NaCl, and 520–580 mM NaCl, respectively (Fig. 1). The purified proteins were subjected to mass spectrometry.

Electrospray mass spectrometric analysis of the degradation product revealed a mass of 15,921.9 ± 1.9 Da. This value matches the average molecular mass deduced from the amino acid sequence including the hypusine modification (15,922.17 Da). Also the mass of the basic

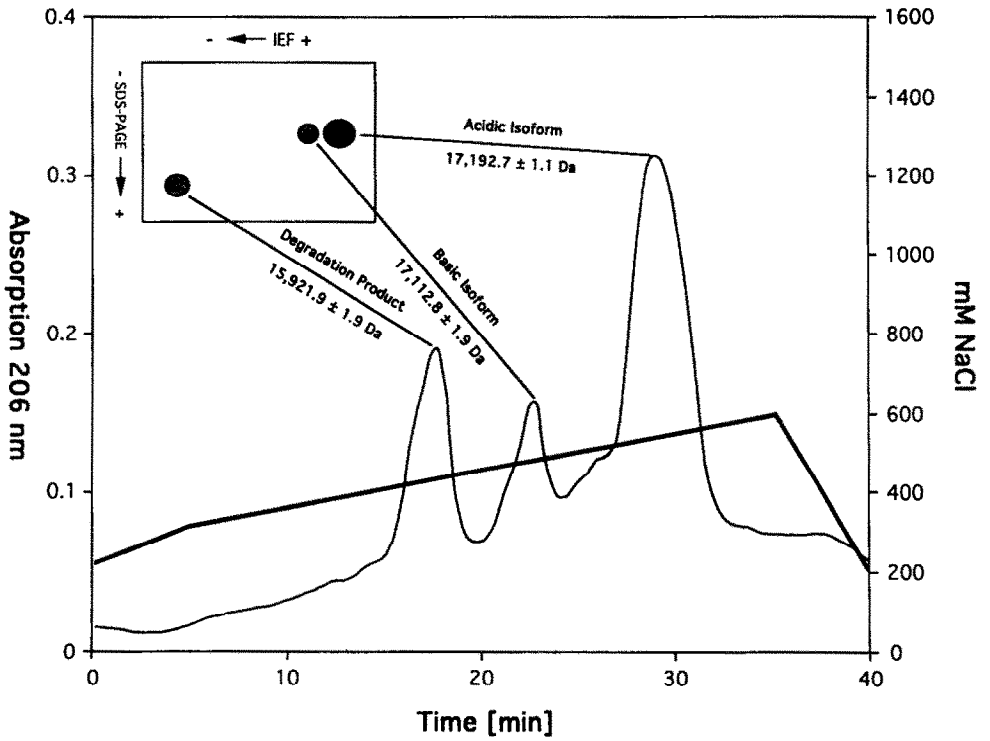

Fig. 1. Chromatogram of the separation of the Hyp2p forms by anion exchange HPLC. The insert shows a schematic two-dimensional gel of the Hyp2p. Correspondence of the protein spots with the absorption peaks together with the respective determined molecular masses of the three Hyp2p forms is indicated by connecting lines.

isoform of 17,112.8 ± 1.9 Da matches the calculated value of 17,111.33 Da (without the N-terminally encoded methionine, but including the hypusine-modification and the N-terminal acetylation). Consequently, also this isoform carries no further modification. In contrast, the mass of the acidic isoform (17,192.7 ± 1.1 Da) is about 80 Da higher than the determined molecular mass of the basic isoform. This led to the conclusion, that the acidic isoform is additionally modified either by sulfatation or by phosphorylation (mass difference of 80.06 Da or 79.08 Da, respectively).

By treatment of a Hyp2p preparation (without prior separation of the isoforms) with calf intestine phosphatase the acidic isoform disappears, whereas the amount of the basic isoform profoundly increases (Fig. 2). The degradation product does not shift in position or intensity (not shown). By this result the additional modification of the acidic isoform is identified as a phosphorylation. Also the mass spectrometric data that neither the degradation product nor the basic isoform are phosphorylated are confirmed.

For identification of the phosphorylation site, the tryptic fragments of the Hyp2p acidic isoform were isolated by reversed-phase HPLC and identified by amino acid analysis (data not shown) and mass spectrometry. With the exception of the N-terminal fragment, the molecular masses of all other fragments were in good agreement with the calculated values. The N-terminal fragment T_1 from serine in position no. 1 to arginine in position no. 26 (Fig. 3) was identified by amino acid analysis and the fact that this fragment is blocked to direct N-terminal sequencing. The molecular mass of this peptide differs from the calculated mass (2847.91 Da) by about 80 Da (determined mass: 2926.5 ± 0.2 Da). As this difference in molecular weight was also found for the intact isoforms, the phosphorylation, which generates the acidic isoform, must be located within this N-terminal tryptic peptide.

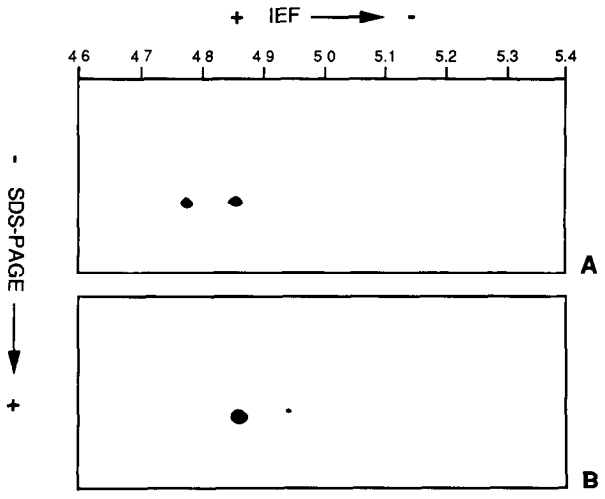

Fig. 2. Silver stained two dimensional gel of 2 μg of Hyp2p isoforms before (A) and after (B) treatment with calf intestine phosphatase, which leads to the loss of the acidic isoform and concomitant increase of the basic isoform. The pH gradient of the isoelectric focussing in the first dimension is indicated on top.

In order to distinguish between the different possible phosphorylation sites, we further digested the N-terminal tryptic fragment of the acidic isoform with endoproteinase Glu-C, an enzyme cleaving glutamyl bonds. The expected and obtained fragments are shown in Fig. 3. Fragment E_4 yielded a mass of 1829.7 ± 0.7 Da (calculated mass: 1830.04 Da), leading to the conclusion that the site of the phosphorylation lies within the remaining eight N-terminal amino acid residues. The mass of 661.4 ± 0.3 Da corresponds to peptide E_{2-3} and thus excludes the threonine residue in position no. 6 as the phosphorylation site. In contrast, the peptide E_{1-2} yielded a mass of 1115.5 ± 0.9 Da, which again lies about 80 Da above the calculated mass of 1035.91 Da (including the acetylation on the fist serine residue). Fragment E_1 was not detected, presumably, because it elutes in the flow through of the reversed phase HPLC, which was not analyzed due to its high salt concentration. These data clearly map the phosphorylation to the first serine residue. Thus in the acidic isoform the N-terminal serine residue is both acetylated and phosphorylated. To our knowledge, this type of double modification of the N-terminus has not been described so far.

This result was also confirmed by direct N-terminal sequencing of the Hyp2p isoforms prepared from the AMR 1 (= W303-1A *nat1–5*) strain of *Saccharomyces cerevisiae*, which is deficient in the N-terminal acetyl transferase Nat1p [18]. The Hyp2p isoforms prepared from this strain are not N-terminally acetylated and are thus accessible to Edman degradation, as we published earlier [9]. Sequence analysis of both isoforms showed a serine residue in position no. 1, but the conversion of serine to its β-elimination product dehydroalanine was almost quantitative in the acidic isoform. This strongly supports a modification of this residue [19].

The acetylation does not contribute to the essential function of the Hyp2p, as can be seen by the viability of the AMR 1 strain. To investigate whether the phosphorylated form of Hyp2p is essential for the in vivo function, we expressed a Hyp2p carrying a serine-1 to alanine-1 mutation in yeast and tested, if it is able to complement a *hyp2* disruption devoid of endogenous wild-type Hyp2p. For this purpose, the following plasmid shuffle experiment was employed: a haploid *hyp2* mutant strain containing the wild-type *HYP2* gene under the control of its own promoter on a multi-copy plasmid [9] was transformed with the centromere plasmid pRSG-HYP2[Ala1] or pRSG-HYP2 producing either the mutant protein HYP2[Ala1]p or the wild-type Hyp2p, respectively. The multi-copy vector YEp-HYP2 harbors the *URA3* gene, the centromeric pRSG-derived plasmids contain the *HIS3* gene as a selective marker. The yeast double transformants were then grown in

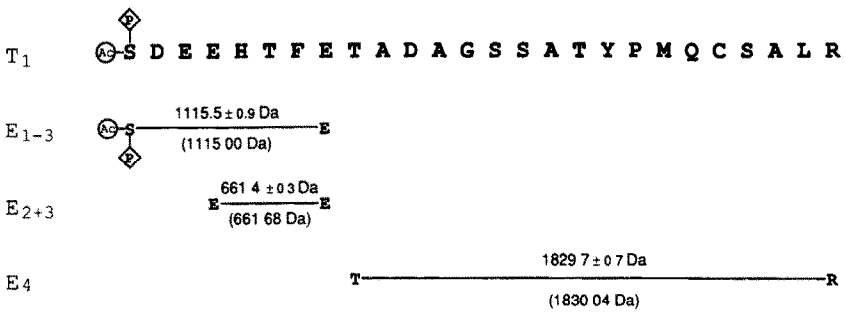

Fig. 3. Schematic representation of the peptides (E_{1-3}, E_{2+3}, E_4) obtained from digestion of the N-terminal tryptic peptide T_1 with endoproteinase Glu-C. Their determined and calculated (in brackets) molecular masses are given above and below the schematic peptide drawings, respectively.

non-selective medium with galactose as the carbon source for five days (medium change every 36 h). Under such conditions, a spontaneous loss of a non-essential plasmid is likely to occur due to its mitotic instability [20]. In contrast, a plasmid encoding an essential function should be strictly retained. The double transformant strain containing YEp-HYP2 and pRSG-HYP2 retained the *URA3* marker in 53% and the *HIS3* marker in 68% of the tested cells. A very similar result was obtained with the strain transformed with pRSG-HYP2[Ala1] instead of pRSG-HYP2 (58% *URA3* / 69% *HIS3*). In both cases, a simultaneous loss of both plasmids in any single cell was never observed, underlining the essential function of Hyp2p under aerobic conditions. When the same experiment was performed with glucose as the carbon source (under these conditions the transcription of the pRSG-encoded *GAL1/HYP2* fusion genes is repressed, whereas the synthesis of Hyp2p encoded by YEp-HYP2 is carbon source-independent), the YEp-HYP2 plasmid was strictly retained in both cases, but the *HIS3* marker was again lost with high frequency. Since in both strains the rate of spontaneous loss of either the YEp-HYP2 or the pRSG-derived plasmids was comparable with galactose as the carbon source, we conclude that the wild-type Hyp2p and the mutant protein Hyp2[Ala1]p are functionally equivalent in vivo. This result was further confirmed by measuring the growth rates in liquid medium. *hyp2* mutant strains, only expressing either *HYP2* or *HYP2[Ala1]* under the control of the *GAL1* promoter reveal almost identical doubling rates in galactose complete medium.

By two-dimensional Western blot analysis of soluble cellular protein extracts we finally checked the isoform pattern of the strain carrying the mutant pRSG-HYP2[Ala1] in comparison with the strain complemented with the wild-type pRSG-HYP2. As is clearly shown in Fig. 4, two isoforms and the degradation product can be detected in the pRSG-HYP2 containing strain. As expected, the extracts of the pRSG-HYP2[Ala1] mutant contain only one isoform of Hyp2p, which was identified as the basic isoform by its relative position in silver stained two dimensional gels (not shown). A degradation product was hardly detectable in this strain (Fig. 4). These data obtained from in vivo experiments confirm our mapping of the phosphorylation site to the serine residue in position no. 1. Furthermore, the perfect viability of the cells devoid of the acidic isoform of the Hyp2p clearly demonstrates that the basic isoform alone is sufficient to fulfill the essential function of this protein in vivo.

During the preparation of this manuscript, Kang et al [21] also published the isolation of the Hyp2p isoforms by Mono S/Mono Q ion exchange chromatography, confirming our observation that more acidic than basic isoform could be obtained. This is in line with the in vivo labeling data presented by Kang et al. [21] that the progression of the basic to the acidic isoform is very rapid with a half-life of about 15 min for the basic isoform.

By in vivo phosphorylation and thin-layer chroma-

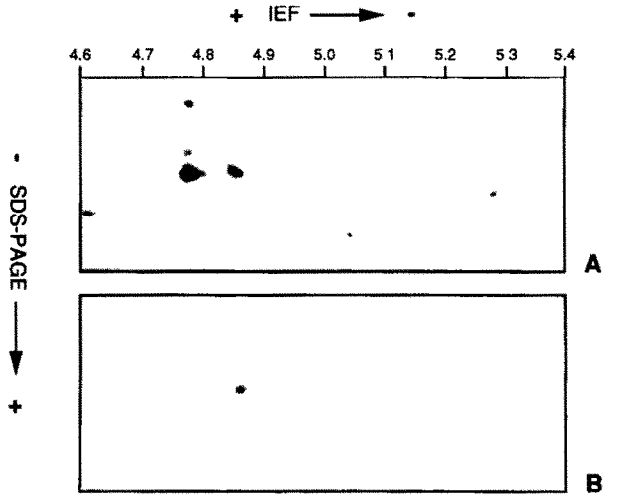

Fig. 4. Two-dimensional Western blot of total soluble extract from the strain carrying the pRSG-HYP2 (A) or the pRGS-HYP2[Ala1] plasmid (B). The cells expressing the wild-type *HYP2* gene show two isoforms (pI = 4.76 and pI = 4.85) and a degradation product (pI = 5.28) thereof. In contrast, in extracts from cells expressing the mutated gene *HYP2[Ala1]* no acidic isoform and only very little degradation product is detectable. The pH gradient of the isoelectric focussing in the first dimension is indicated on top.

tography of total amino acid hydrolysates, these authors were able to characterize the isoform difference as a serine phosphorylation. In addition to the identification of this phosphorylation, we were further able to locate its site to the serine residue in position no. 1 of the acidic isoform by amino acid sequence and composition analysis and mass spectrometry of the Hyp2p forms and their enzymatic fragments. Since the N-terminus of the Hyp2p is in addition blocked by acetylation [9], the double modification of this residue is beside the hypusine modification another odd and possibly unique feature of the Hyp2p. A modification of the threonine residue no. 48 was proposed for the hypusine-containing protein of CHO cells [22,23]. As the only observed deviation of the determined masses of the Hyp2p isoforms and their enzymatic fragments from the calculated values is explained by the phosphorylation of the first serine residue, this clearly excludes further modifications of these purified Hyp2p isoforms in this strictly conserved threonine-48 or any other part of the molecule.

Kang et al. [21] failed to detect the third more basic and smaller Hyp2p form, which we identified as a degradation product [9], in detergent free cellular extracts. In contrast to that we found this degradation product in all extracts of aerobically grown cells independent from procedure, buffer composition or use of detergent ([9,13], Magdolen et al. manuscript in preparation]). In addition, we showed that under identical experimental conditions extracts of the *HYP2[Ala1]* expressing strain, which produces no acidic isoform, contain less degradation product than extracts of the wild-type *HYP2* expressing strain. Therefore we consider the degradation product as a naturally occurring in vivo form, the concentration of which might depend on the amount of the acidic isoform. Nevertheless, more degradation product can be obtained after cell lysis, if protease inhibition is insufficient. Further functional analysis of this degradation/processing product is in preparation. Since we finally demonstrated by mutation of the N- terminal serine residue to alanine that the basic (unphosphorylated) isoform alone can carry out the essential function of the Hyp2p in vivo, it is tempting to speculate that the phosphorylation might result in an inactivation of the protein or even tag the protein for degradation.

Acknowledgements: We thank T. Gorr, A. Hermann and Dr. M. Meyer for critical reading of the manuscript.

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