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Biochimica et Biophysica Acta 1463 (2000) 477–482

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Short sequence-paper

Expression and subcellular localization of a membrane protein related to Hsp30p in *Saccharomyces cerevisiae*

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Received 19 August 1999; received in revised form 14 December 1999; accepted 22 December 1999

Abstract

The *Saccharomyces cerevisiae YDR033w* gene product is homologous to Hsp30p and Yro2p, both of which are induced during heat shock. To investigate the subcellular localization of the *YDR033w* gene product, hemagglutinin (HA) epitope-tagged protein was expressed, detected on immunoblots, and localized by immunofluorescence to cell membranes, primarily the plasma membrane. A punctuate immunofluorescence pattern was observed within cell buds. The nuclear envelope, but not the vacuole or mitochondrial membranes, were also immunostained. We refer to *YDR033w* as *MRH1* to denote that it encodes a membrane protein related to Hsp30p. © 2000 Published by Elsevier Science B.V. All rights reserved.

Keywords: Heat shock protein; Plasma membrane; Yeast

Members of the 30 kDa heat shock protein (HSP30) family in *Saccharomyces cerevisiae* are highly hydrophobic and characterized by seven predicted membrane spanning domains [1,2]. The product of the *HSP30/YRO1/YCR021c* gene is not essential for growth and has been shown to be a membrane protein present in a plasma membrane (PM)-enriched fraction [1,2]. *HSP30* expression is induced by heat shock, glucose limitation, and exposure to either alcohol or sorbic acid [1–4]. Hsp30p functions to regulate, either directly or indirectly, the activity of the PM H^+-ATPase, which is encoded by *PMA1* [4]. Cells lacking Hsp30p show increased PM H^+-ATPase activity induced by heat shock or by weak organic acid stress [4]. Homologues of Hsp30p have been identified in *S. cerevisiae* by sequence analysis and are encoded by *YRO2* [5] and *YDR033w* (GenBank accession Z74329). *YRO2* was named on the basis of homology with *HSP30/YRO1* [5], whereas *YDR033w* has not been given a gene name to date. *YDR033w* has not been formally confirmed to be protein encoding by in vivo identification of its protein gene product. During the course of studies to characterize mutants that exhibit synthetic lethality with a temperature sensitive allele of *nop2*, we cloned and localized the YDR033 protein in *S. cerevisiae*. As a result of these studies, we refer to *YDR033w* as *MRH1*, to convey that it encodes a membrane protein related to Hsp30p.

MRH1 is predicted to encode a protein of 320 amino acids, of molecular weight 36 188. Alignments of the amino acid sequences of Mrh1p, Yro2p, and Hsp30p reveal significant conservation of primary

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0005-2736/00/$ – see front matter
PII: S0005-2736(99)00255-2

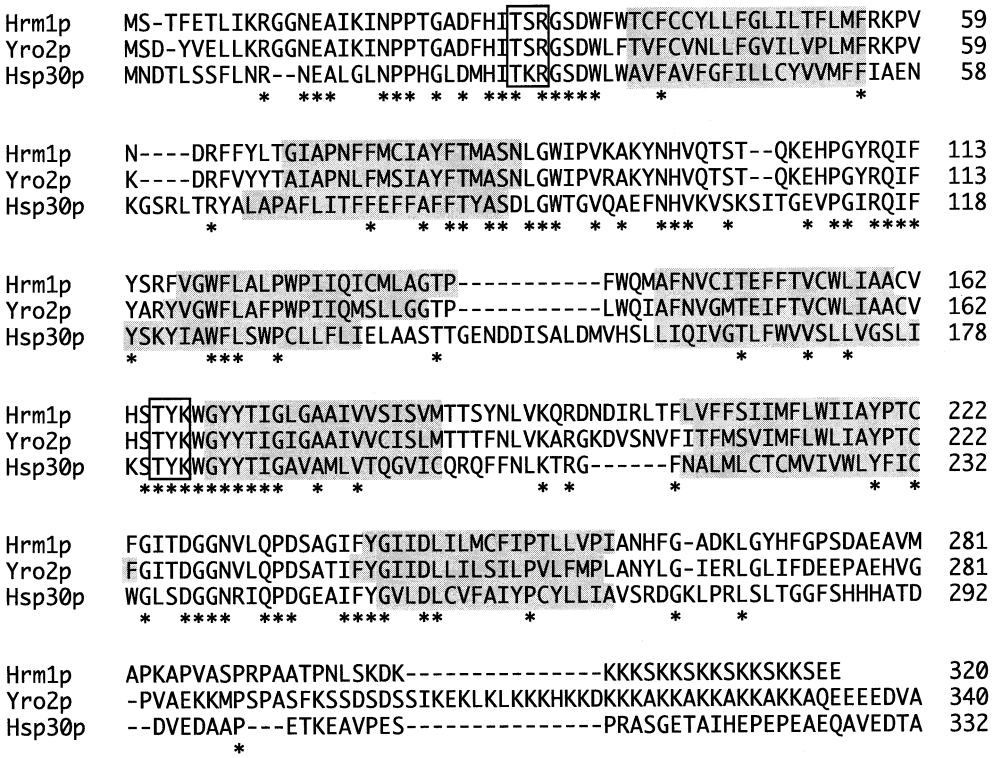

Fig. 1. Multiple sequence alignment of *S. cerevisiae* Mrh1p, Yro2p and Hsp30p. The alignment was generated by CLUSTAL W method [14] with BLOSUM30 protein weight matrix. Amino acid numbers are indicated on the right. Dashes denote gaps introduced to optimize the alignment. Asterisks indicate consensus residues. Putative protein kinase C phosphorylation sites are boxed. Shaded sequences are predicted to be transmembrane helices [15,16]. Residues 341–344 (TDSE) in Yro2p are not shown.

structure within this group of proteins, which provides an important basis for considering them as members of a family of related proteins (Fig. 1). The amino acid sequence of Mrh1p shows 67% identity and 78% similarity to Yro2p. Such a high level of similarity is consistent with a common origin in the form of an ancestral gene that underwent a duplication event. Wolfe and Shields have pointed out that *MRH1* on chromosome IV and *YRO2* on chromosome II are contained within chromosomal duplication block 3 [6]. Mrh1p exhibits 34% identity and 50% similarity to Hsp30p (Fig. 1). Unlike *MRH1* and *YRO2*, *HSP30* has not been found in a chromosomal duplication block [6]. An additional ORF with a lower level of sequence similarity to *MRH1* is *YDR090c* (Smith-Waterman score of 98; 22% identity in 100 amino acid overlap), which is predicted to encode a 34.6 kDa protein with six putative transmembrane domains. An Hsp30p-related protein from *Coriolus versicolor* and a conserved hypothetical protein from *Schizosaccharomyces pombe* also share significant sequence similarity with Mrh1p (data not shown).

Two regions in the alignment of Mrh1p, Yro2p, and Hsp30p are highly conserved. The first one is between N13 and W37 in Mrh1p. The second region is between S164 and G174. A motif search [7,8] revealed that both regions contain a putative protein kinase C phosphorylation site conserved in all three proteins (Fig. 1, boxed tripeptide). Furthermore, the predicted structure of all three proteins is very similar. They are predicted to be largely alpha helical and to contain seven membrane spanning domains. The positions of membrane spanning domains are highly conserved although the corresponding amino acid sequences are less homologous (Fig. 1, shaded boxes). In contrast, sequences at the C-termini are highly divergent, even between the two most similar homologues Mrh1p and Yro2p. This higher degree of diversity in these regions indicates that these proteins are likely to be functionally specialized rather than functionally redundant.

Fig. 2. Multiple sequence alignment of Mrh1p, Yro2p, archaerhodopsin I (AR I; GenPept accession P19585), and bacteriorhodopsin (BR; GenPept accession 229726). The alignment was generated by the CLUSTAL method. Residues identical to Mrh1p are boxed. Underlined amino acids in BR are present in alpha helices A–F, based on crystal structure data [10]. The alignment does not extend across the region between helices B and C in BR.

BLAST searches revealed that Mrh1p exhibited low homology to certain halobacterial rhodopsins, namely sensory rhodopsin II, archaerhodopsin (AR), and bacteriorhodopsin (BR) (Fig. 2). Consistent with this, 'iterative neighborhood cluster analysis', which performs iterative BLAST searches using a predefined minimum alignment score, identified Yro2p as a sequence distantly related to bacteriorhodopsin [9]. The structure of BR has been the subject of numerous investigations, and has recently been determined by X-ray diffraction at a resolution of 2.5Å [10]. Six of the seven membrane-spanning alpha helical domains in BR are similar to putative transmembrane domains in Mrh1p and Yro2p (Fig. 2, heavy lines lettered A–F). This similarity extends to AR, which shares significant homology with BR. Mrh1p (and Yro2p) diverge from BR and AR in three locations, all of which contain amino acid sequences not found in the halobacterial proteins: (1) the amino terminus of Mrh1p is longer than BR and AR; (2) the corresponding sequence in Mrh1p is longer than the loop connecting transmembrane segments B and C in BR and AR; and (3) sequence corresponding to seventh transmembrane helix G and the following C-terminal region in BR is not related to Mrh1p (data not shown; see Fig. 2). Lysine-267 is located within a short stretch of Mrh1p that similar to a stretch of helix G in BR that includes K216 (which is modified by retinal), but K267 is not predicted to be within the seventh putative transmembrane domain in Mrh1p.

The polymerase chain reaction (PCR) was used to amplify the *MRH1* coding sequence. The 5′ primer contained a *Bam*HI site and the 3′ primer contained sequence that encodes hemagglutinin (HA) epitope tag and a *Bam*HI site. The sequences of these prim-

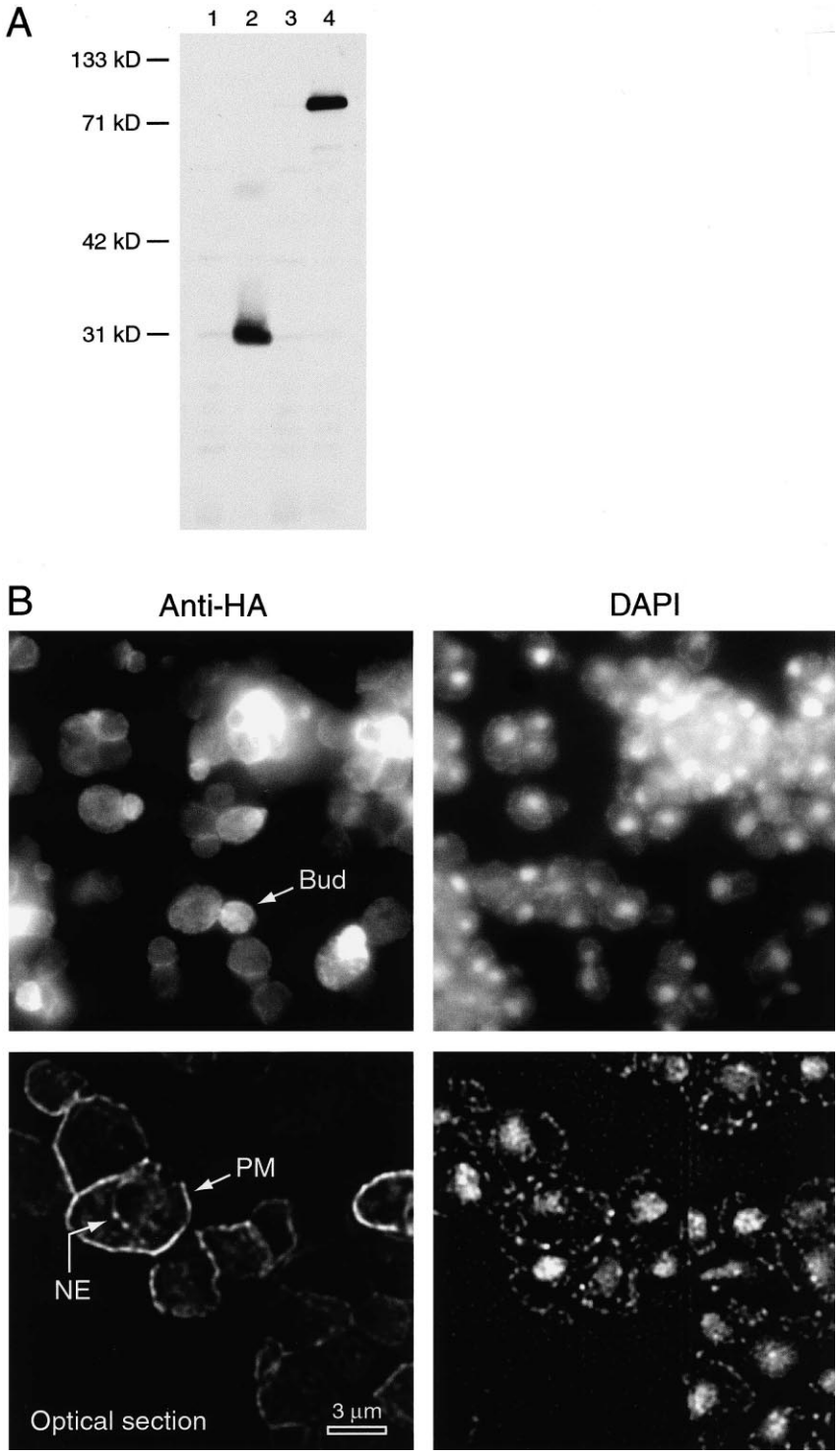

Fig. 3. Expression and localization of HA epitope-tagged Mrh1p. (A) Immunoblot of whole cell protein extracts from strain YKW172 grown in the presence of glucose (lane 1) or galactose (lane 2) probed with anti-HA mAb 16B12. Control extracts were prepared from YJPA8 grown on glucose (lane 3) or galactose (lane 4), which induces expression of the ~90 kDa HA-tagged Nop2p from the *GAL1* promoter. (B) Immunofluorescence localization of epitope-tagged Mrh1p using mAb 16B12 and Cy3-conjugated antimouse secondary antibody. Staining of nuclear chromatin and mitochondrial DNA with DAPI (4′,6-diamidino-2-phenylindole) is shown. (upper panels) From a Zeiss Axiophot epifluorescence microscope equipped with a cooled-CCD camera. (lower panels) Show an optical section obtained using cooled-CCD multi-dimensional deconvolution microscopy.

ers are as follows: 5′ primer: GC**GGATCC**ATATCTAATCATCTTCCTTTAACCCACA; 3′ primer: GA**GGATCC**CTAAGCATAATCAGGAACATCATATGGATATTCTTCAGACTTCTTAGACTTCTTAGATTT. The PCR product was cloned into pRD53 [11], which contains the *GAL1* promoter and *CEN6*, to give pKW20. pKW20 was transformed into a W303 diploid strain to yield strain YKW172. Growth of YKW172 on galactose-containing medium induced expression of epitope-tagged Mrh1p from the *GAL1* promoter (Fig. 3A). The apparent size of HA-tagged Mrh1p revealed by SDS-PAGE is approximately 7 kDa smaller than that predicted by the sequence of HA-tagged Mrh1p (37.3 kDa). A similar phenomenon was also observed in studies of Hsp30p [2], which supports that notion that Mrh1p shares similar biochemical properties with Hsp30p.

Indirect immunofluorescence (IF) was performed on whole yeast cells to define the intracellular distribution of HA-tagged Mrh1p. Staining with monoclonal antibody (mAb) 16B12 (BAbCo, Richmond, CA 94804, USA) directed against the HA tag revealed a range of signal intensity. This variation of signal intensity is primarily due to the effects of a strong *GAL1* promoter coupled with small differences in plasmid copy number. Cells overexpressing Mrh1p show signal distributed throughout the cell. Because overexpression of Mrh1p may lead to an anomalous intracellular distribution, cells expressing low to moderate levels are the most reliable indicators of localization. Cells expressing low to moderate level of epitope tagged Mrh1p protein show a predominantly PM localization pattern (Fig. 3B). PM staining is relatively homogeneous, with small variations in signal intensity apparent over the cell surface (also see optical section data discussed below). A punctuate staining pattern appears in small- and medium-sized cell buds, which are sites of rapid PM expansion during cell growth throughout S and early G2 phases of the cell cycle (Fig. 3B). Cells with large buds, in late G2 and M phases, show a less intense punctuate staining pattern. YKW172 grown on glucose do not produce an IF signal with mAb 16B12, nor do YKW172 cells probed with Cy3-conjugated secondary antibody alone (data not shown). Although cells permeabilized with Tween 20 or Triton X-100 (after formaldehyde fixation) gave indistinguishable IF staining patterns, cells treated with SDS (after formaldehyde fixation) showed very low signal intensity and no PM or nuclear envelope (NE) staining (data not shown). The solubilization and extraction of epitope-tagged Mrh1p by SDS in formaldehyde fixed specimens argues that Mrh1p is a membrane-integral protein, and supports the prediction of a transmembrane topology based on the sequence analyses discussed above.

Conventional IF microscopy revealed a diffuse cytoplasmic staining pattern and a faint circular distribution pattern that surrounded the nucleus, which was counter stained by DAPI (Fig. 3). To better resolve the intracellular staining pattern, IF localization was done using three-dimensional data collection and computational (deconvolution algorithm) removal of out-of-focus information as implemented on a cooled CCD-based imaging workstation [12]. Optical sections of 0.2 μm clearly show staining of the PM and NE, along with a faint, heterogeneous cytosolic staining pattern, which may correspond to secretory pathway organelles and transport intermediates (Fig. 3). Mitochondria, which contain DNA stained by DAPI, and the vacuole, which is the largest intracellular membrane-bound compartment, were not stained by mAb 16B12 (Fig. 3). Considering that the outer membrane of the NE is contiguous with the endoplasmic reticulum membrane, which is difficult to observe by optical microscopy in yeast, it is likely that the NE staining pattern represents Mrh1p at an early step in its transport to the PM. IF staining of the NE, diffuse cytoplasmic staining, and punctuate staining of the cell bud can be interpreted as the visualization of different stages of delivery of Mrh1p to the PM. Alternatively, Mrh1p may have a functional role within these compartments of the secretory pathway as well as at the PM, where it is more abundant.

This report is the first detailed study on the expression and subcellular localization of Mrh1p, as well as the first detailed study of the localization of an Hsp30p-like protein in *S. cerevisiae* using immunofluorescence analysis. The localization of Mrh1p to the PM along with Hsp30p suggests that Mrh1p may participate in similar cellular processes as Hsp30p. The function of Yro2p is unknown. Among those proteins with more limited homology to Mrh1p are AR and BR, both of which are proton transport

proteins with seven transmembrane domains. Conserved residues important for proton transport have been identified in these proteins [10]. However, comparison of their amino acid sequences with that of Mrh1p suggest that while these proteins may share a similar membrane topology, they are unlikely to be functionally related. Thus, Mrh1p is unlikely to be involved in proton transport, although it may participate in the regulation of the Pma1p PM H^+-ATPase, based on its similarity to Hsp30p. The further elucidation of Mrh1p function begs characterization of this protein in the following respects. First, the expression profile of Mrh1p protein should be examined. Interestingly, results from whole genome mRNA quantitation assays of *S. cerevisiae* indicate that levels of Mrh1p mRNA actually decrease in response to heat shock [13], suggesting a function different from Hsp30p. Further detailed analysis of the level of both *MRH1* mRNA and protein under different stress treatments will be required to compare the pattern of expression of Mrh1p with that of Hsp30p. Second, characterization of a Mrh1p deletion strain should provide answers to whether this gene is essential and what the potential defects may result from its absence in vivo.

Acknowledgements

We wish to express our thanks to Grazyna Zimowska and Michael R. Paddy, for their assistance with high resolution optical microscopy analysis, and to J. Michael Thomson, for helpful discussions and assistance with figure preparation.

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