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Biochimica et Biophysica Acta 1463 (2000) 267–278

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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The LEA-like protein HSP 12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress

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Received 25 May 1999; received in revised form 11 October 1999; accepted 25 October 1999

Abstract

The LEA-like protein HSP 12 was identified as having a plasma membrane location in yeast. Gold particles, indicative of the presence of HSP 12, were observed on the external side of the plasma membrane when yeast grown to stationary phase were subjected to immunocytochemical analysis. Growth of yeast in the osmolyte mannitol resulted in an increased number of gold particles that were now observed to be present on both sides of the plasma membrane. No gold particles were observed using a mutant strain of the same yeast that did not express HSP 12. A model liposome system encapsulating the fluorescent dye calcein was used to investigate the protection by HSP 12 of membranes during desiccation. HSP 12 was found to act in an analogous manner to trehalose and protect liposomal membrane integrity against desiccation. The interaction between HSP 12 and the liposomal membrane was judged to be electrostatic as membrane protection was only observed with positively charged liposomes and not with either neutral or negatively charged liposomes. The ability of the wild-type and mutant yeast to grow in media containing ethanol was compared. It was found that yeast not expressing the HSP 12 protein were less able to grow in media containing ethanol. HSP 12 was shown to confer increased integrity on the liposomal membrane in the presence of ethanol. Ethanol, like mannitol, was found to induce HSP 12 protein synthesis. However, yeast grown in both ethanol and mannitol showed a decreased HSP 12 response compared with yeast grown in the presence of either osmolyte alone.

Keywords: Liposomes; Ethanol; LEA proteins; Desiccation

1. Introduction

Late embryogenesis abundant (LEA) proteins [1] are a subset of abscisic acid (ABA) responsive proteins, expression of which has been observed to occur during embryo maturation in nearly all angiosperms. LEA proteins are characteristically small hydrophilic proteins that remain soluble at high temperature [2]. Six groups of LEA proteins have been identified based on common amino acid sequence domains [3,4] and at least five groups have been proposed to contribute towards desiccation tolerance in the embryo. Although the actual biological mechanism of action is unclear, roles in ion sequestration and the maintenance of the shell of hydration during desiccation have been proposed [4–6]. Although *lea*

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0005-2736/00/$ – see front matter
PII: S0005-2736(99)00215-1

mRNAs and LEA proteins are rapidly degraded upon germination, accumulation of these proteins has been reported in several plants in response to water deficit or ABA [7–11]. It is proposed that transcription of LEA proteins increases in drought stressed plants [2,12] and that these proteins play a protective role against desiccation-induced cellular damage. Evidence supporting this latter hypothesis is that expression of the barley group 3 HVA 1 LEA protein in rice resulted in increased tolerance to water stress [5]. Moreover, recombinant yeasts expressing the wheat group I LEA Em protein have recently been reported to be less susceptible to inhibition of growth in media of high osmolarity [13].

Yeast HSP 12 was first identified as a putative heat shock protein on the basis of some limited sequence homology between HSP 12 and known heat shock proteins [14]. HSP 12-specific mRNA was moreover observed to greatly increase on entry into stationary phase and after changing the growth temperature from 30 to 37°C. More recently, however, we have suggested that HSP 12 should instead be classified as an LEA-like protein [15]. Not only was HSP 12 extremely hydrophilic and its synthesis in yeast promoted by the osmolytes NaCl and mannitol, but also the concentration of HSP 12 was diminished rather than increased in yeast cultures grown at 37°C compared with those grown at 30°C. Small heat shock proteins (smHSPs), which have been classed as Group VI LEA proteins, are thought to be involved in the acquisition of thermotolerance [16]. These proteins have been categorised into four multigene families based on sequence similarity. Various locations for the different families have been proposed, including locations in the cytoplasm, chloroplasts, the endoplasmic reticulum, mitochondria as well as an association with membranous structures [17–20]. In contrast, the LEA proteins thus far purified are thought to have a cytoplasmic location as they have been found to be solubilised following tissue homogenisation in buffers of physiological pH and ionic strength [8,21,22].

In this report, we have used immunocytochemical analysis to determine the location of the HSP 12 protein in yeast. Since an association with the plasma membrane was found, the protection against desiccation of a model membrane system by HSP 12 was investigated.

2. Materials and methods

2.1. Strains and media

The parent *S. cerevisiae* yeast strain (the 'wild-type') used was the diploid strain 842 (*a*/*x*, *ade2-1*/*ade2-1*, *trp1-1*/*trp1-1*, *leu2-3*/*leu2-112*, *his3-11*/*his3-15*, *ura3*/*ura3*, *can*^{r}*1-100*/*CAN*). A 'knockout' *HSP 12*::*URA3* mutant of the same strain transformed to uracil prototrophy with linearised *HSP 12* cDNA containing a copy of the *URA3* gene inserted into the *Sty*1 site [14] was used as a control. Both strains were the kind gift from Professor Peter Measock, University of Leicester. These strains were grown in sterile YPD medium (1% yeast extract, 2% peptone, 2% dextrose) supplemented with 100 μg/ml streptomycin and 100 μg/ml penicillin and grown at 30°C to late stationary phase (A_{600} = 60/ml). Ethanol was included in the growth medium for some experiments. Growth was monitored by measurement of the 600 nm absorbance. Cells were harvested by centrifugation at 4°C.

2.2. Immunological techniques

Immunoblots using chemiluminescent detection (Amersham) and ELISA were carried out as described [23]. All work was carried out in PBS except where stated.

2.2.1. Antibody affinity purification

Polyclonal antibodies [15], were purified by adsorption onto and release from HSP 12 immobilised on nitrocellulose to prevent any non-specific interactions. HSP 12 (500 μg) in 10 ml 10 mM Tris-HCl was adsorbed to a 6×6 cm square of nitrocellulose (Schleicher and Schuell) at 4°C for 16 h. The nitrocellulose was thoroughly washed with 0.05% Tween-20. Unbound sites were blocked by incubation in 3% BSA (Boehringer Mannheim) at room temperature for 60 min in this same buffer. After washing the nitrocellulose to remove excess unbound BSA (three washes), the nitrocellulose was incubated at room temperature for 2 h together with a 1:10 dilution of the antibody in 5% skimmed milk powder, 0.1% Tween-20. The nitrocellulose was washed with 0.05% Tween-20 and purified antibody was eluted using 1 M Tris-HCl pH 8.5.

2.2.2. Electron microscopy

2.2.2.1. Yeast. Yeast samples were fixed in 2.5% glutaraldehyde at 4°C for 16 h. Post-fixation was in 1% OsO_4 for 90 min. Following ethanolic dehydration, the material was embedded in epoxy resin [24]. The resin was hardened at 60°C and 90-nm slices were sectioned using a Reichert Ultracut-S ultramicrotome and collected on nickel grids. The grids were sequentially floated on 0.02 M glycine and on 1% BSA after which the blocked grids were floated on the affinity purified anti-HSP 12 antibody at 20°C for 16 h. The grids were thoroughly washed in 1% BSA before being floated on goat-anti-rabbit immunoglobulin attached to 5 nm colloidal gold particles (Sigma) at 20°C for 2 h. After washing in PBS, the sections were fixed by sequential floatation for 10 min each on 0.1% glutaraldehyde, 2% uranyl acetate and 1% lead citrate.

2.2.2.2. Liposomes. Carbon-coated copper grids were floated for 10 min on a 20-μl aliquot of the liposome preparation. The grids were then washed five times by floatation on a drop of water before staining with 2% uranyl acetate followed by 1% lead citrate as above.

Samples were viewed and photographed using a Zeiss EM109 transmission electron microscope.

2.3. Protein purification from baker's yeast

HSP 12 was purified from Baker's yeast as described previously [15]. Biological molecular mass analysis of the pure HSP 12 was carried out using a Voyager DE-Pro Matrix Assisted Laser Desorption/Ionisation Time Of Flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems). Ten picomoles of sample were added to 1 μl of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) matrix (10 mg/ml in 50% acetonitrile, 0.03% trifluoroacetic acid in water) for analysis.

2.4. Recombinant protein purification

The polymerase chain reaction (PCR) was used to amplify the HSP 12 gene in vitro using *S. cerevisiae* chromosomal DNA as the template. The sequence specific primers used, 5′-ATGGATCCATGTCTGACGCAGGTAGAAA-3′ and 5′-GTTCTTACTTTGAAAGCCTTAAGTAT-3′, allowed the insertion of the PCR product of 355 bp [25] into the thrombin site of the expression vector pGEX-2T (Pharmacia). This resulted in recombinant HSP 12 (rHSP 12) being synthesised as a fusion protein together with glutathione *S*-transferase. Cloning, bacterial growth, induction of fusion protein biosynthesis and recovery of pure rHSP 12 after binding to the glutathione-Sepharose matrix and thrombin cleavage were carried out as per manufacturer's recommendations.

2.5. Preparation of phosphatidyl choline from egg yolk

Phosphatidylcholine (PC) was isolated and purified from fresh egg yolk by differential solvent extraction and aluminium oxide chromatography [26]. MALDI-TOF molecular mass analysis of purified egg PC using *trans*-3-indoleacrylic acid (IAA) as the matrix. showed that the composition of the purified phospholipid consisted of four species in the ratio 14:4:2:1 ranging from 759 to 833 Da. This compared with an average molecular mass of 770 Da [27]. The species were considered to contain the following acyl chains: species 1, $C_{16:0}$ and $C_{18:1}$; species 2, $C_{18:0}$ and $C_{18:1}$; species 3, $C_{18:0}$ and $C_{20:4}$; and species 4, $C_{20:4}$ and $C_{20:4}$.

2.6. Liposome preparation

Liposomes to include the fluorescent dye calcein (3,6-dihydroxy-2,3-bis(*N*,*N*′-di(carboxymethyl)-aminomethyl) fluoran) in PBS were prepared by reverse-phase evaporation [28]. These liposomes consisted of bilayers containing 50 mol% cholesterol (Sigma) to minimise leakage and stabilise the liposomes, 45 mol% phosphatidylcholine (PC) to minimise non-specific binding and 5 mol% dicetyl phosphate or 5 mol% stearylamine to minimise aggregation [29] and to impart an overall negative or positive charge, respectively. Neutral liposomes were made from PC and cholesterol only in a 1:1 ratio. Liposomes containing calcein could be prepared in advance and stored at 4°C for a limited period. The integrity of the stored as well as the quality of new liposome preparations was determined by transmission electron microscopy (TEM). This ensured that each

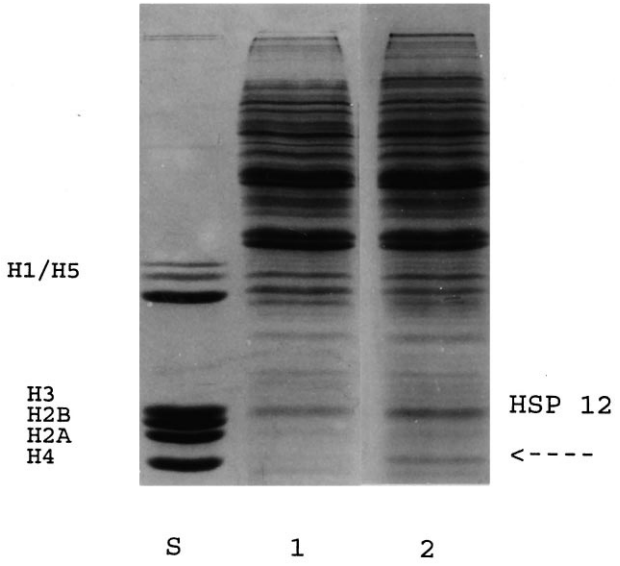

Fig. 1. SDS-PAGE of soluble proteins extracted from knockout (lane 1) and wild-type strains (lane 2) of yeast. The standard (S) is a total extract of chicken erythrocyte histones, the approximate molecular weights (kDa) of which are: H1, 22.5; H3, 15.3; H2B, 13.7; H2A, 14.0; and H4, 11.2. HSP 12 is denoted by the arrow (right).

new preparation had the expected appearance and that fusion and/or disintegration of stored liposomes had not occurred.

2.7. Analysis of liposome membrane integrity after desiccation

Ten nmoles of lipid (approximately 1.2×10^9 liposomes [30] were diluted to 50 μl with PBS containing various concentrations of trehalose or HSP 12 before the sample was dried using a speedy-vac at 30°C. The temperature of the samples in the speedy-vac remained between 28 and 31°C throughout the desiccation process. Water (50 μl) was then added to the dry liposome preparation after which it was diluted to 1 ml with PBS. Calcein fluorescence was determined using an Aminco SPF 500 spectrofluorimeter (excitation wavelength 490 nm, emission wavelength 515 nm). Membrane integrity after desiccation was calculated from the fluorescence of the sample relative to a control non-desiccated sample and the total calcein released from the sample by the addition of Triton X-100 to 0.1%. This concentration was sufficient to lyse all liposomes. Liposome experiments were repeated three times in duplicate using different preparations. Values are quoted as mean ± S.D.

3. Results

3.1. Immunocytochemical analysis of the location of HSP 12

We initially investigated whether any protein present in the knockout strain was recognised by the affinity-purified anti-HSP 12 antibody [15]. SDS-PAGE of total soluble proteins extracted from the wild-type and knockout strains is shown in Fig. 1. Although HSP 12 (arrowed) was present in the wild-type strain, no band representing HSP 12 was present in the knockout strain. A Western blot of this gel (not shown) using the affinity-purified anti-HSP 12 antibody confirmed the presence of HSP 12 in the wild-type strain. The antibody failed to recognise any other proteins present on the gel, demonstrating that the antibody was specific to HSP 12 alone and that the knockout strain expressed no HSP 12.

Thin sections of embedded stationary phase wild-type yeast were probed with an affinity-purified rabbit anti-HSP 12 antibody. The location of this antibody was determined by electron microscopy using a colloidal gold-labelled goat anti-rabbit antibody. The knockout strain was used as a control. Examination of the sections of the wild-type strain (Fig. 2A) showed that gold particles were present on the exterior side of the cell membrane facing the cell wall. No gold particles were observed on the cytoplasmic side of the cell membrane. Gold particles were neither detected within the cytoplasm nor adjacent to membranes present in the cytoplasm. No gold particles whatsoever could be observed in micrographs

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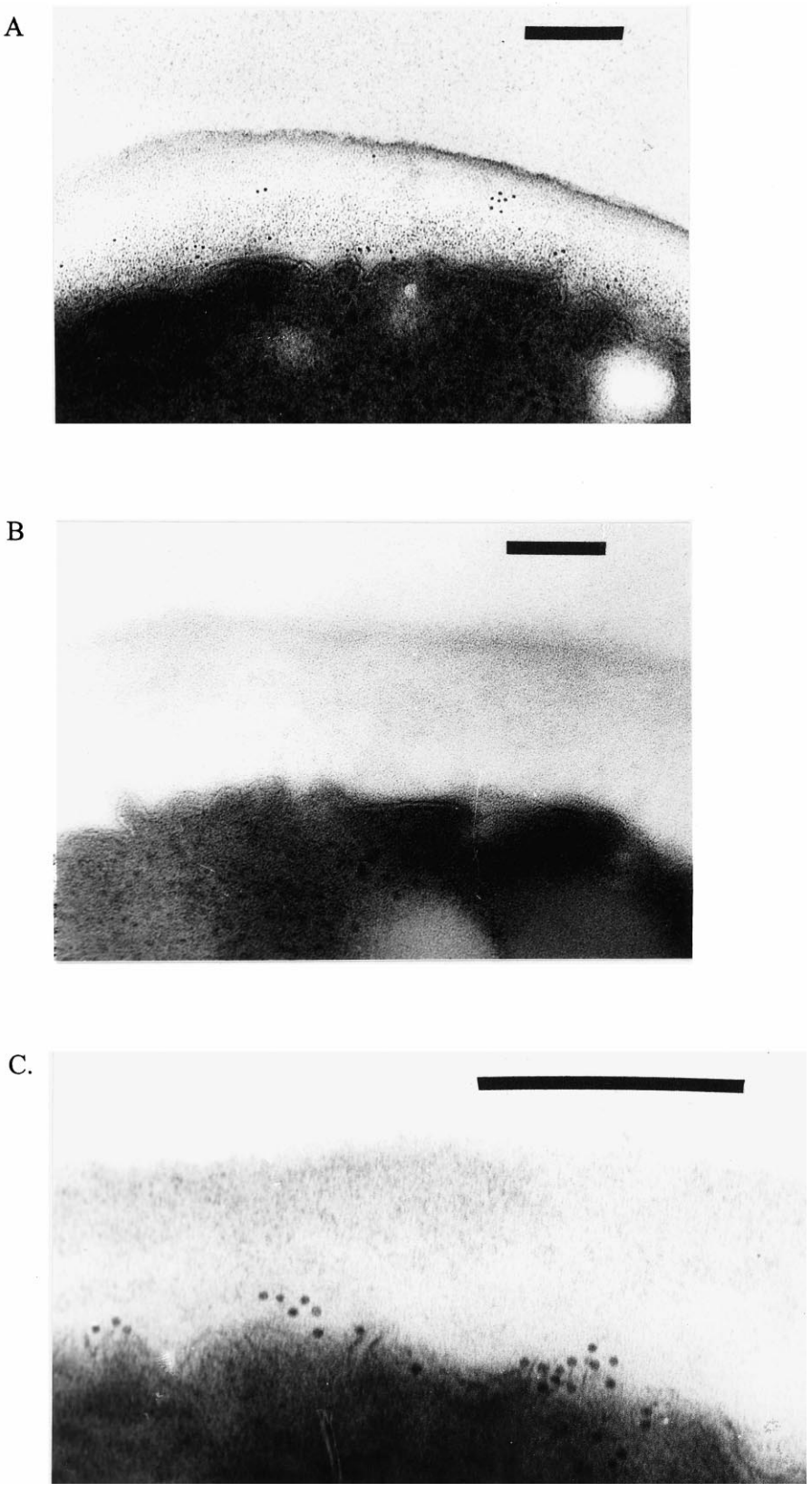
Fig. 2. Electron microscopy of immunogold labelled thin sections of wild-type yeast (A), knockout yeast (B) and wild-type yeast grown in the presence of 1.6 M mannitol (C). The magnifications were ×30 000 (A,B) and ×83 000 (C). The gold particles visible in A and C were 5 nm diameter. Scale bars: 100 nm.


A
B
C.

of the knockout strain (Fig. 2B). Since growth of yeast in 0.8 M mannitol was reported to increase the amount of HSP 12 present on entry into stationary phase [15], the wild-type strain was grown in YPD buffer containing various mannitol concentrations between 0.8 and 2.0 M. It was found (not shown) that the yield of HSP 12 increased upon growing yeast in YPD containing mannitol up to 1.6 M; growth in YPD buffer was inhibited by 2.0 M mannitol. Thin sections of the wild-type strain grown to late stationary phase in the presence of 1.6 M mannitol were similarly examined by electron microscopy. It was found (Fig. 2C) that the number of gold particles associated with the plasma membrane had increased and that, in addition, some gold particles were observed on the cytoplasmic side of the membrane. No gold particles could be detected in any other sub-cellular location. These results suggested an interaction between HSP 12 and the plasma membrane.

3.2. *Maintenance of liposome membrane integrity by trehalose after desiccation and rehydration*

The plasma membrane acts as the principal interface between the extracellular environment and the cytosol [31]. Since membrane systems are considered to be particularly susceptible to dehydration damage [32–36] and since immunocytochemical analysis suggested that HSP 12 was in close proximity to the plasma membrane, we investigated whether HSP 12 protected the membrane against desiccation-induced damage. An artificial liposome membrane system, constructed using egg PC, cholesterol and stearylamine and incorporating the self-quenching fluorescent dye calcein, was used for this study (Fig. 3).

Trehalose, when present on both sides of the lipid bilayer, has been shown to maintain liposome integrity upon lyophilisation [32] suggesting that trehalose substituted for the water of hydration. Liposomes incorporating stearylamine and encapsulating calcein were diluted into PBS containing various concentrations of trehalose and subjected to desiccation before rehydration. Whereas liposomes desiccated in PBS alone showed total loss of membrane integrity upon rehydration, liposomes desiccated in PBS including trehalose maintained up to 70% of membrane integrity (Fig. 4). A linear relationship was observed between the percentage structural integrity maintained after rehydration and the trehalose concentration during desiccation up to a concentration of 12 μg trehalose/μg phospholipid. Liposomes prepared as described, but encapsulating various concentrations of trehalose up to 20 μg trehalose/μg phospholipid and desiccated in PBS (trehalose present only internally during desiccation) showed total loss of membrane integrity. Although encapsulated trehalose had no effect on liposome membrane integrity after desiccation and rehydration, we investigated whether trehalose, if present on both sides of

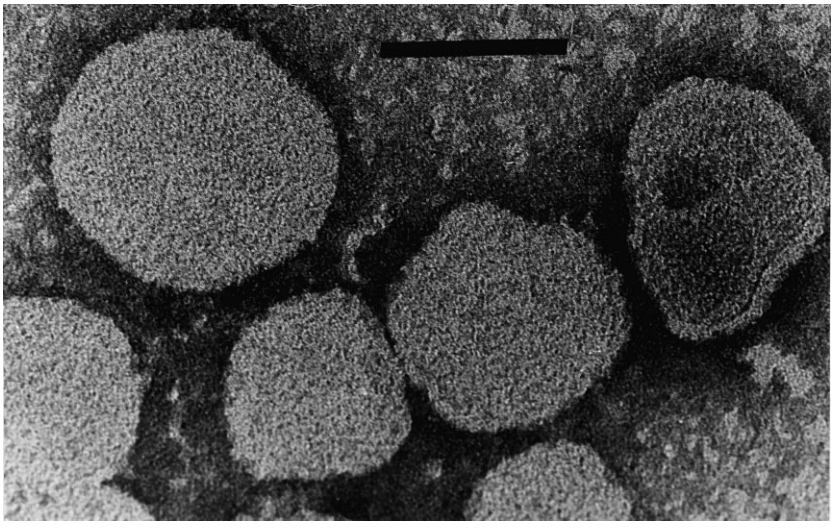


Fig. 3. Electron microscopy of uranyl acetate and lead citrate stained liposomes. Scale bars: 100 nm.

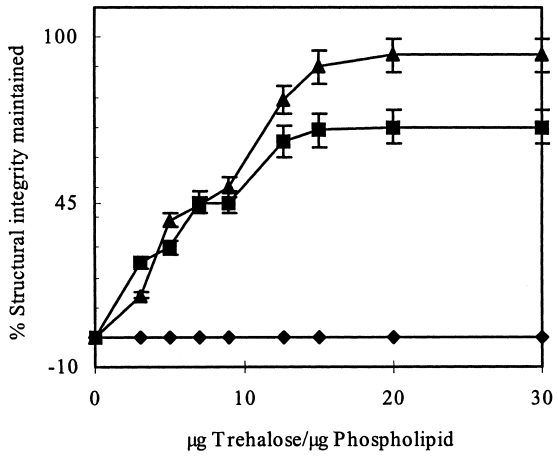


Fig. 4. Effect of the presence of various concentrations of trehalose on the structural integrity of liposomes after desiccation. Structural integrity was assessed by determination of the release of the encapsulated fluorescent dye calcein (3,6-dihydroxy-2,3-bis(*N*,*N*′-di(carboxymethyl)-aminomethyl)fluoran) with total release equivalent to complete loss of integrity. Trehalose was either encapsulated together with the calcein (trehalose only present internally (♦), present externally only (■) or present both internally and externally (▲)).

the liposome membrane, would increase the percentage structural integrity maintained during desiccation and rehydration compared with that observed when trehalose was only present externally. For encapsulation, 20 μg trehalose/μg phospholipid was chosen as this concentration was found to maximally stabilise liposomes when present externally during desiccation and rehydration. Liposomes encapsulating 20 μg trehalose/μg phospholipid desiccated in PBS including trehalose maintained almost complete membrane integrity (Fig. 4). A linear relationship was observed between the percentage structural integrity maintained after rehydration and the trehalose concentration during desiccation up to a concentration of 10 μg trehalose/μg phospholipid. The trehalose concentration required to maximally protect these liposomes against desiccation and rehydration was approximately seven times higher than that of 1.8 g trehalose/g phospholipid reported previously [32]. The liposomes prepared by these authors, however, were composed solely of palmitoyloleoylphosphatidyl choline and phosphatidylserine in a 9:1 ratio.

3.3. Maintenance of liposome membrane integrity by HSP 12 after desiccation and rehydration

We next investigated as to whether HSP 12 could replace trehalose in protecting liposomes against desiccation and subsequent rehydration. Liposomes incorporating stearylamine and encapsulating calcein were diluted into PBS containing various concentrations of HSP 12 and subjected to desiccation before rehydration. Liposomes desiccated in PBS including HSP 12 maintained up to 77% of membrane integrity upon rehydration (Fig. 5). A linear relationship was observed between the percentage structural integrity maintained after rehydration and the HSP 12 concentration during desiccation up to a concentration of 15 μg HSP 12/μg phospholipid. No difference in the relative efficacy of natural HSP 12 and recombinant (rHSP 12) could be detected in that identical protection was observed using either native or recombinant HSP 12 (results only shown for native HSP 12). Since the recombinant form of the protein was far easier to prepare, all future work was performed using rHSP 12. The ability of HSP 12 to protect liposome membranes against desiccation and rehydration when encapsulated internally only and when present on both sides of the liposomal

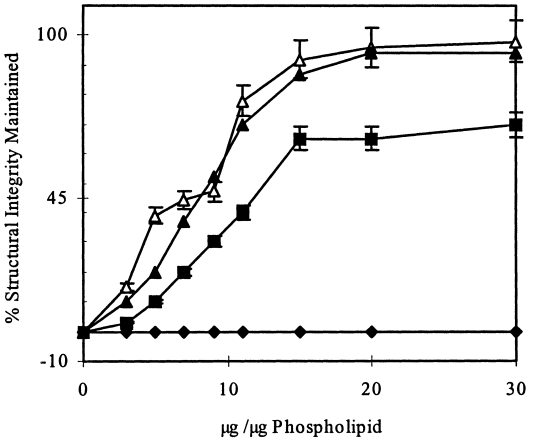


Fig. 5. Effect of the presence of various concentrations of HSP 12 on the structural integrity (assessed as per Fig. 4) of liposomes after desiccation. HSP 12 was either encapsulated together with the calcein (present internally only (♦), present externally only (■) or present both internally and externally (▲)). In addition, the structural integrity after desiccation was determined with trehalose present internally and HSP 12 present externally (△).

membrane was also investigated. It was found that HSP 12 behaved in an analogous manner to trehalose in that no protection was observed when HSP 12 was encapsulated in the liposome, but that complete protection was observed when HSP 12 was present on both sides of the liposomal membrane during desiccation (Fig. 5). Since trehalose and HSP 12 had a similar effect in protecting liposomes against desiccation, we investigated whether these compounds were interchangeable. Liposomes encapsulating 20 μg trehalose/μg phospholipid were diluted into PBS containing various concentrations of HSP 12 and subjected to desiccation before rehydration. It was found (Fig. 5) that encapsulated trehalose could replace the encapsulated HSP 12 with complete protection observed after rehydration. Similarly, liposomes encapsulating 10 μg HSP 12/μg phospholipid were diluted into PBS containing various concentrations of trehalose and subjected to desiccation before rehydration. It was found (not shown) that trehalose could replace HSP 12 present externally with complete protection observed after rehydration.

We wished to investigate whether the protective effect observed with HSP 12 during desiccation was specific to this protein or whether other proteins had a similar effect. Liposomes incorporating stearylamine and encapsulating calcein were diluted into PBS containing various concentrations of either myoglobin (17 000 Da) or cytochrome *c* (12 000 Da) and subjected to desiccation before rehydration. These proteins were chosen because both proteins are of similar molecular size to HSP 12. It was found that myoglobin was ineffective in maintaining the structural integrity of liposomes during desiccation as only 10% protection was observed after rehydration (Fig. 6). Cytochrome *c* was more effective than myoglobin as 35% protection was observed after rehydration (Fig. 6). Since cytochrome *c* is naturally loosely bound to the inner mitochondrial membrane, we postulated that this level of protection might be brought about by non-specific protein:lipid interactions.

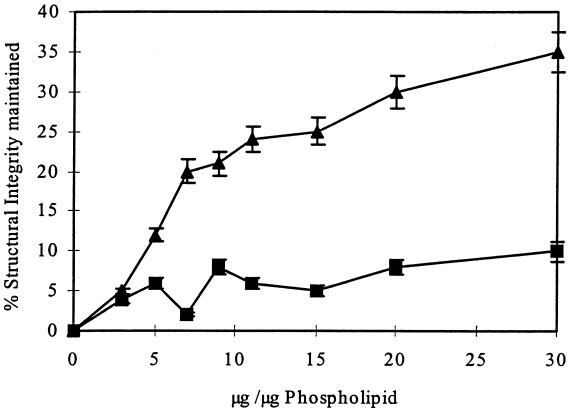


Fig. 6. Effect of the presence of various concentrations of cytochrome *c* (▲) or myoglobin (■) on the structural integrity (assessed as per Fig. 4) of liposomes after desiccation. Cytochrome *c* and myoglobin were present externally only.

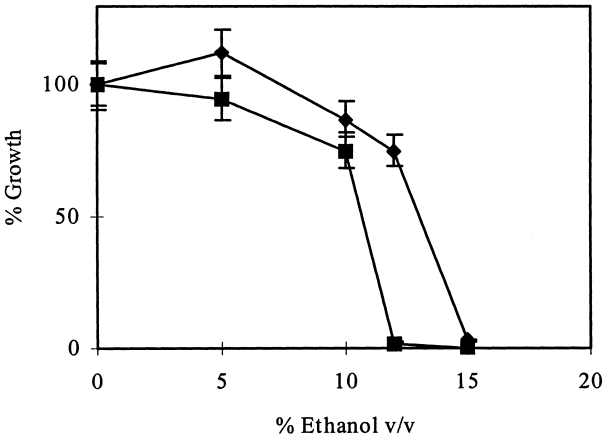


Fig. 7. Effect of ethanol on the survival of the wild-type (♦) and knockout (■) yeast. Yeasts were incubated in YPD medium containing various concentrations of ethanol for 24 h after which the growth (determined from the 600-nm absorption) was compared with growth in the absence of ethanol.

Liposomes incorporating stearylamine were chosen for the experiments reported thus far as HSP 12 is a highly charged protein with almost half (47.8%) of its residues consisting of the amino acids E, K, D, R, H. HSP 12 has an isoelectric point calculated from the amino acid content to be around pH 4.9. The protein only has slight nett negative charge up to pH 9, but rapidly becomes highly positively charged at pH values below the isoelectric point. In order to investigate whether the interaction between HSP 12 and the membrane was electrostatic, the composition of the liposome membrane was altered with either 5% dicetyl phosphate or an additional 5% egg phosphatidylcholine replacing the stearylamine yielding negatively charged or neutral liposomes, respectively. These liposomes encapsulating only PBS buffer and calcein were diluted into PBS containing various concentrations of trehalose or HSP 12 and subjected to desic-

cation and rehydration. Whereas trehalose protection was observed with liposome preparations incorporating either 5% phosphatidylcholine or 5% dicetyl phosphate, no protection of these liposome preparations was observed with HSP 12 (not shown). The level of protection observed was identical to that observed with liposomes incorporating stearylamine. Since only positively charged liposomes were protected against desiccation by HSP 12 suggestive of an electrostatic interaction between HSP 12 and the liposome membrane, we investigated whether this interaction was charge dependent. Liposomes incorporating stearylamine encapsulating calcein only were dialysed against PBS at either pH 7.4, 6.0 or 3.5 before the addition of HSP 12 in the same buffer. The liposome preparation was then subjected to desiccation and rehydration and the structural integrity determined. The structural integrity percentage observed at pH 7.4 was set to 100%. The percentage structural integrity maintained was found to decrease as the pH became more acidic with 60 ± 5% integrity observed at pH 6.0 and 35 ± 3% integrity observed at pH 3.5.

3.4. HSP 12 and ethanol tolerance in yeast

It has been proposed that the lipid composition of the plasma membrane plays an important role in ethanol tolerance in yeast [37]. These authors showed that increased membrane fluidity occurred when yeast were grown in cultures supplemented with ethanol. Furthermore, exposure of yeast to sublethal doses of ethanol induced a heat shock-like response [38]. Since the concentration of HSP 12 increased in yeast during conditions of osmotic stress, the difference in the viability of the wild-type yeast and the knockout strain was investigated after exposure to ethanol, itself an osmolyte. Both strains were grown in YPD media supplemented with ethanol of different concentrations up to 15% and the growth of each determined after 24 h from the 600-nm absorbances of the cultures compared with those of control cultures grown in YPD media without ethanol.

Although no difference in the growth of both strains was detected after 24 h in 5% ethanol (Fig. 7), increasing the ethanol concentration to 10% resulted in a reduced growth rate observed for the knockout strain alone. This strain showed a growth rate of 75% in comparison with growth of this strain in the absence of ethanol. In contrast, the wild-type strain showed no decreased growth rate at this ethanol concentration. Growth in 10% ethanol, however, was unable to discriminate between the two strains due to the errors observed in determination of the rates. Increasing the ethanol concentration to 12% resulted in complete inhibition of growth of the knockout strain, whereas the wild-type strain exhibited a growth rate of 75% relative to growth in the absence of ethanol. This latter strain was unable to grow in medium containing 15% ethanol.

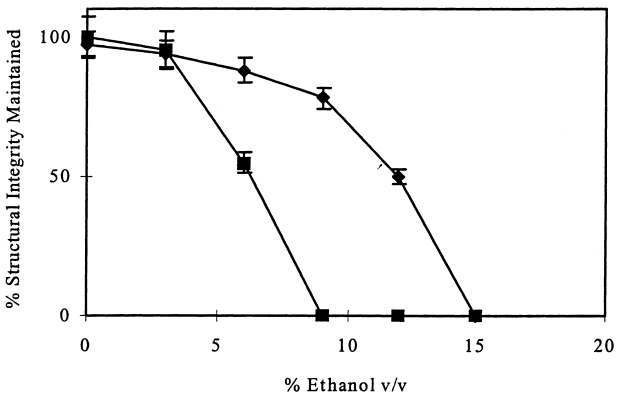


Fig. 8. Effect of HSP 12 on the relationship between ethanol concentration and structural integrity of liposomes. Liposomes were incubated in PBS containing various concentrations of ethanol either alone (■) or together with HSP 12 (◆) and the structural integrity assessed by calcein leakage.

Since these results suggested that HSP 12 played a role in the tolerance of yeast to grow in media containing ethanol, we investigated the ability of HSP 12 to protect membranes against ethanol stress. Liposomes incorporating stearylamine and encapsulating calcein were exposed to ethanol at various concentrations in the presence of 15 μg HSP 12/μg phospholipid and the membrane damage determined by calcein leakage (Fig. 8). It was found that the presence of HSP 12 externally reduced the calcein leakage from liposomes on exposure to ethanol at concentrations greater than 3%. Thus, 88% liposomal membrane integrity was maintained in 6% ethanol in the presence of HSP 12 compared with 55% in its absence. Increasing the ethanol concentration to 9% resulted in 78% membrane integrity maintained in the presence of HSP 12 compared with total calcein leakage in its absence. No membrane integrity

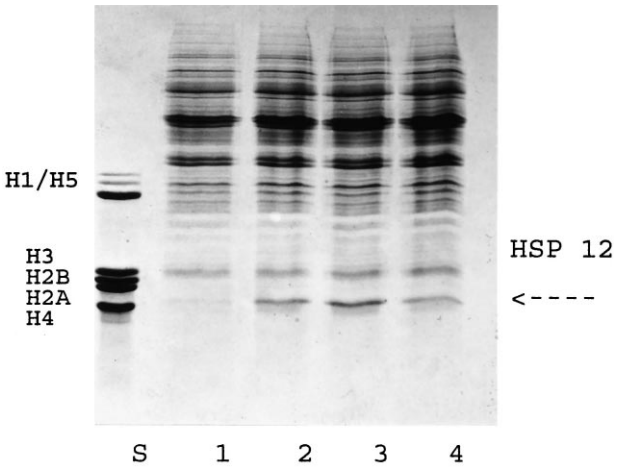


Fig. 9. SDS-PAGE of soluble proteins extracted from wild-type yeast grown in YPD medium alone (lane 1) YPD supplemented with 5% ethanol (lane 2), 1.6 M mannitol (lane 3) or both 5% ethanol and 1.6 M mannitol (lane 4). The standard (S) is a total extract of chicken erythrocyte histones. HSP 12 is denoted by the arrow (right). HSP 12 content was determined by scanning the Coomassie-stained gel.

was observed in 15% ethanol in the presence of HSP 12.

We finally investigated the effect of growth in 5% ethanol on the concentration of HSP 12 in yeast. This concentration was chosen as no effect on yeast growth was visible (Fig. 7). We found (Fig. 9) that yeast grown in the presence of 5% (1.09 M) ethanol had an approximately 10-fold increase in the HSP 12 level compared with the same strain grown in YPD alone. In comparison, yeast grown in the presence of 1.6 M mannitol had an approximately 14-fold increase in the HSP 12 level. This increased response might either be due to different sensitivities to the different osmolytes or to the higher mannitol concentration, 1.6 M compared with 1.09 M. If yeast were grown in the presence of both 5% ethanol and 1.6 M mannitol, however, the HSP 12 level was significantly lower than observed in the presence of either osmolyte alone as only an approximately 3-fold increase was observed. Thus the response to the one osmolyte appeared to be inhibited by growth in the presence of the other.

4. Discussion

Our results suggest that the role of the LEA-like protein HSP 12 in yeast is to protect the plasma membrane against desiccation. Immunocytochemical experiments demonstrated that HSP 12 is located in close proximity to the plasma membrane in yeast. The protein appeared to be specific to this location as no gold particles were observed elsewhere in the cell. Caution must be exercised in the interpretation of the immunocytochemical data. HSP 12 is an extremely water soluble protein, a property that exacerbates the possibility of artefact formation during the manipulations of the yeast cell for viewing under electron microscopy. We believe, however, that it is unlikely that location is artefactual as high solubility would more likely result in a generalised, rather a specific, location. Since the plasma membrane is the principal site of interaction between the cell and the environment, it is perhaps not surprising that a protein synthesised in response to osmotic stress is present in this location. Although growth of yeast in solutions containing ethanol or mannitol resulted in increased HSP 12 levels, interestingly, when ethanol and mannitol were both present in the medium, the HSP 12 concentration failed to show any increase beyond that seen with ethanol alone. This suggests that recognition of the stress caused by the presence of the one osmolyte was inhibited by growth in the presence of the other. It has been previously observed [39] that induction of expression of the HSP 12 gene by a high NaCl concentration resulted in a decreased response when a low concentration of ethanol was present in the medium.

Much work has been performed on the effects of desiccation on lipid bilayers using liposomes as a model membrane system. It has been proposed [35] that adjacent lipid bilayers in liposomes are held apart by the water of hydration associated with the phosphate head groups. Removal and replacement of this water caused alterations in the bilayer configuration, as demonstrated by freeze-fracture, in favour of multilamellar and oligolamellar forms. However, if trehalose were present during lyophilisation, dry liposomes were seen to exist as vesicles embedded in trehalose. These liposomes did not leak their contents into the surroundings, thereby showing preservation of membrane integrity. The liposomes used in this study required a 7-fold higher trehalose concentration to effect a similar level of protection against desiccation. Possible reasons for this discrepancy

might be either that the liposomes used in the two studies were constructed using different phospholipids or that different techniques were used to desiccate the liposome preparations. Crowe et al. [35] used lyophilisation to desiccate liposomes constructed using a 9:1 ratio of synthetic palmitoyloleoylphosphatidylcholine and phosphatidylserine, whereas we used evaporation at 30°C to desiccate liposomes constructed using natural egg lecithin together with cholesterol and stearylamine. Complete protection against desiccation of liposomes was only observed when either trehalose or HSP 12 were present on both sides of the vesicle membrane. Complete protection was not observed when trehalose or HSP 12 was present only externally implying that alterations in the bilayer configuration occurred on desiccation under these circumstances that resulted in an associated leakage of calcein.

Our results suggested that the interaction between the plasma membrane and HSP 12 was largely electrostatic in nature. Only liposomes containing the positively charged lipid, stearylamine, were protected against desiccation by HSP 12. Moreover, changing the pH at which the liposomes were desiccated resulted in decreased protection at more acidic pH values at which HSP 12 would be expected to have a nett positive charge. Complete protection appeared to be unique to HSP 12 as significantly less protection was observed with either of the two other proteins used, myoglobin and cytochrome *c*. Although cytochrome *c* (p*I* 10.6) would be positively charged at pH 7.4, this protein exhibited greater protection than myoglobin (p*I* 7.0) which would have a slight negative charge at this pH. The protection exhibited by these two proteins as well as HSP 12 might therefore be more related to conformation. Both myoglobin and cytochrome *c* are globular proteins that would interact with only small portions of the liposomal membrane. In contrast, LEA proteins have little definitive structure in solutions of low ionic strength [8,40,41] and would therefore be more able to interact with larger stretches of the liposomal membrane. Although trehalose and HSP 12 had an identical effect regarding the protection afforded against desiccation, HSP 12 was effective at approximately a 30-fold lower molar concentration. In addition, we believe that the mechanisms of action of the two molecules are very different. Trehalose is thought to act to replace water by intercalation between adjacent phospholipid head groups [42–44]. During desiccation, glass formation occurs and prevents membrane fusion between adjacent phospholipid bilayers. The interaction between trehalose and the phospholipid head groups also results in a decreased T_m for the phospholipid, thereby stabilising the phospholipid bilayers. Our results, using the liposome model system, suggest that HSP 12 interacts electrostatically with charged groups present on the membrane surface. We would imagine that the negatively charged amino acids, glutamate and aspartate, would ionically interact with the stearylamine amino group and that the positively charged amino acids, arginine and lysine, would interact with the negatively charged phospholipid head groups. Serine and threonine groups on HSP 12 would mimic the action of water by shielding the charge repulsion of any free phosphate groups. Presumably, in yeast, HSP 12 would also interact by hydrogen bonding with membrane proteins and glycolipids and thereby provide a hydrophilic net that would prevent membrane fusion in the desiccated state. The presence of HSP 12 might also result in an increased water content in the dehydrated state as some water molecules might be sufficiently tightly bound to charged groups on HSP 12 to prevent their removal during desiccation. These water molecules would then contribute to stabilisation of the membrane configuration in the desiccated state. Further experiments on whether trehalose and HSP 12 have separate or synergistic roles are in progress.

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