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Analysis of the BiP gene and identification of an ER retention signal in *Schizosaccharomyces pombe*

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We have cloned the gene for the resident luminal ER protein BiP from the fission yeast, *Schizosaccharomyces pombe*. The predicted protein product is equally divergent from the budding yeast and mammalian homologues. Disruption of the BiP gene in *S.pombe* is lethal and BiP mRNA levels are regulated by a variety of stresses including heat shock. Immunofluorescence of cells expressing an epitope-tagged BiP protein show it to be localized to the nuclear envelope, around the cell periphery and in a reticular structure through the cytoplasm. Unexpectedly, we find the BiP protein contains an N-linked glycosylation site which can be utilized. The C-terminal four amino acids of BiP are Ala-Asp-Glu-Leu, a new variant of the XDEL sequence found at the C-termini of luminal endoplasmic reticulum proteins. To determine whether this sequence acts as a sorting signal in *S.pombe* we expressed an acid phosphatase fusion protein extended at its C-terminus with the amino acids ADEL. Analysis of the sorting of this fusion protein indicates that the ADEL sequence is sufficient to cause the retention of proteins in the endoplasmic reticulum. The sequences DDEL, HDEL and KDEL can also direct ER-retention of acid phosphatase in *S.pombe*.

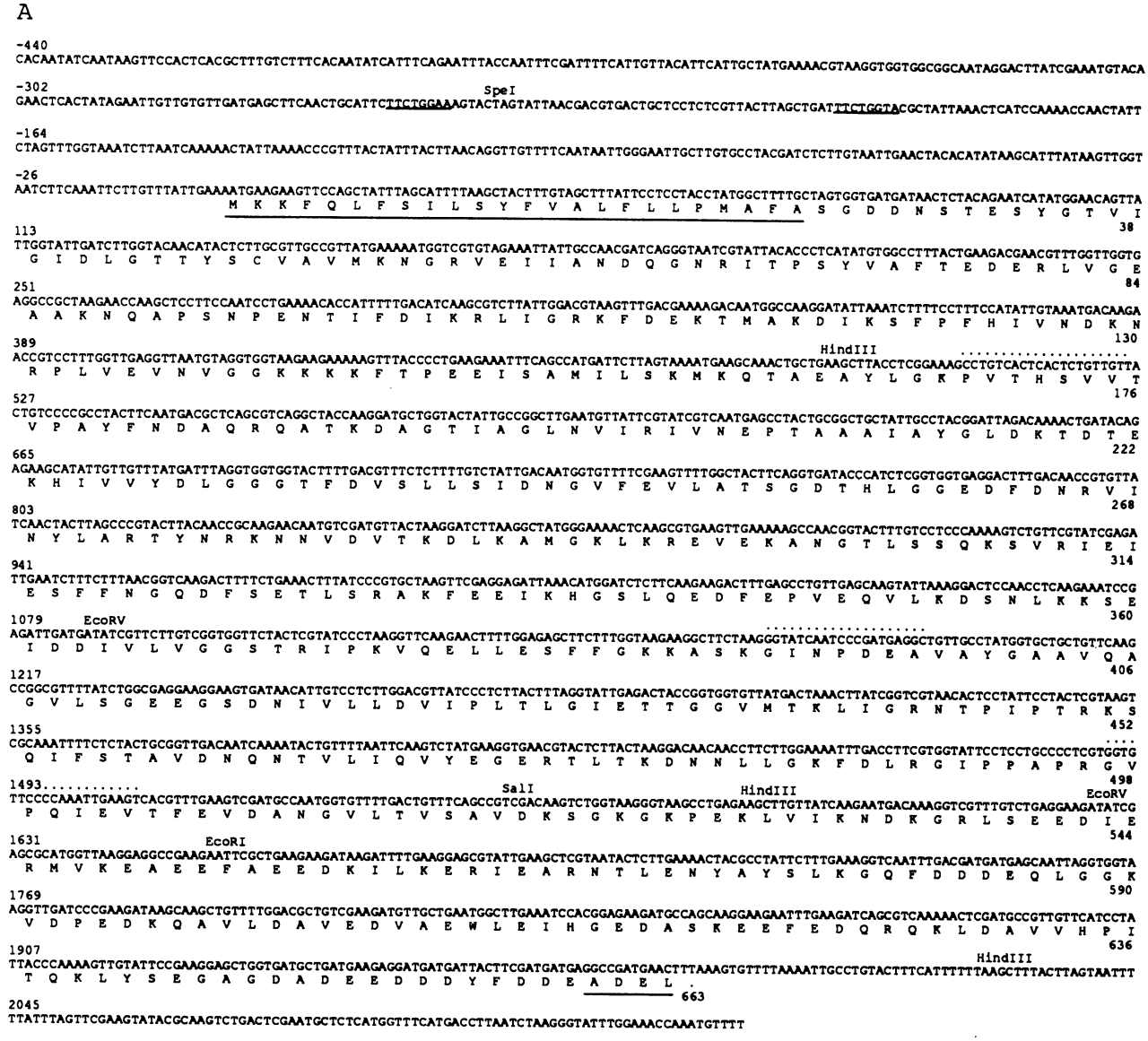
Key words: BiP/endoplasmic reticulum/fission yeast/glycosylation/retention

Introduction

Proteins destined for secretion are synthesized at the endoplasmic reticulum (ER) where they enter the secretory pathway. In addition to glycosylation, the ER is responsible for disulphide bonding, folding and oligomeric assembly of newly synthesized proteins. A number of proteins thought to be involved in these processes are residents of the ER lumen and include protein disulphide isomerase and a protein known as BiP or GRP78. BiP was originally identified in pre-B cells, where it binds to and prevents the secretion of immunoglobulin heavy chains in the absence of light chain synthesis (Haas and Wabl, 1983). It is now known to be ubiquitous and has been observed bound stably to a variety of mutant, misfolded, incompletely glycosylated or partly assembled proteins (Gething *et al.*, 1986; Dorner *et al.*, 1987; Kassenbrock *et al.*, 1988; Machamer *et al.*, 1990). BiP has also been found in transient association with a number of normal secretory and membrane proteins (Bole *et al.*, 1986; Ng *et al.*, 1989; Blount and Merlie, 1991) and hence is thought to be required for the correct folding and assembly of these proteins in the ER. Complexes of BiP with immunoglobulin heavy chain can be disrupted by addition of ATP (Munro and Pelham, 1986). ATP hydrolysis by purified BiP *in vitro* has been shown to be dependent upon peptide binding and necessary for the subsequent release of bound peptide (Flynn *et al.* 1989). It is assumed that ATP hydrolysis occurs during BiP's participation in protein assembly *in vivo*. Genetic analysis of BiP has been facilitated by the identification of its homologue *KAR2* in *Saccharomyces cerevisiae* (Normington *et al.* 1989; Rose *et al.* 1989). *KAR2* is required for karyogamy and additionally is involved in translocation of nascent polypeptides across the ER membrane (Vogel *et al.*, 1990). As yet it is unclear whether BiP performs such roles in mammalian cells. BiP shares a high degree of homology with the HSP70 heat shock proteins, although BiP itself is not induced by heat shock in mammalian cells (Munro and Pelham, 1986). Perturbations of the ER such as the inhibition of glycosylation or treatment with calcium ionophores induce expression of BiP (Welch *et al.*, 1983). These treatments are thought to cause an accumulation of misfolded proteins in the ER, though the mechanism by which this signal is transduced to the nucleus remains a subject for speculation.

BiP and protein disulphide isomerase are members of a class of resident ER proteins which are soluble in the lumen but are not secreted. This localization is mediated by a C-terminal tetrapeptide which is both necessary and sufficient for ER retention (Munro and Pelham, 1987). This sequence is usually KDEL in mammalian cells (Pelham, 1990) and HDEL in *S.cerevisiae* (Pelham *et al.* 1988). Several variants have been identified at the C-termini of luminal ER proteins from other species, including DDEL in *Kluyveromyces lactis* (Lewis and Pelham, 1990a) and SDEL in *Plasmodium falciparum* (Kumar *et al.*, 1988). In *S.cerevisiae*, HDEL-bearing proteins can leave the ER but are selectively retrieved from a subsequent compartment (Dean and Pelham, 1990). A genetic approach has identified the gene for the HDEL receptor, *ERD2* (Lewis *et al.*, 1990; Semenza *et al.*, 1990). A human *ERD2* homologue has also been cloned and the 26 kDa protein product localizes to the Golgi (Lewis and Pelham, 1990b) though it has not yet been shown to cause the retention of KDEL proteins. An additional candidate for the KDEL receptor has been identified in mammalian cells using anti-idiotypic antibodies. A fragment of this 72 kDa protein binds KDEL peptides *in vitro* (Vaux *et al.*, 1990) but the protein is apparently unrelated to human erd2. It is assumed that the receptor will bind escaped ER proteins, return them via a vesicle-mediated pathway to the ER and from there release them in response to an unknown signal or change in environment.

Thus several issues concerning the functions, regulation and localization of BiP remain to be clarified. The fission yeast


A
SpeI
HindIII
EcoRV
SalI
HindIII
EcoRV
EcoRI
B

S.pombe combines the genetic tractability of budding yeast with functional and morphological features which may make it a better model of the secretory pathway of higher cells. For example *S.pombe* adds terminal galactose to its glycoproteins (Moreno *et al.*, 1985) and stacked Golgi structures are readily seen by EM in wild-type cells (Smith and Svoboda, 1972). In addition, several members of the ypt family in *S.pombe* are highly homologous to their mammalian counterparts (Fawell *et al.*, 1989, 1990; Haubruck *et al.*, 1990; Hengst *et al.*, 1990; Miyake and Yamamoto, 1990).

We describe here the isolation and analysis of the BiP gene from *S.pombe*. The gene is essential for viability and its expression is regulated by a variety of stresses. *S.pombe* BiP can be partially glycosylated, unlike BiP from other species. Immunofluorescence of cells expressing an epitope-tagged BiP protein has allowed visualization of the ER of *S.pombe* and shown it to have some reticular structure. The ADEL C-terminal sequence is a new variant of the ER-retention signal and can direct the ER retention of the secretory protein acid phosphatase. In addition, ER-retention signals from other species, including the mammalian sequence KDEL, can also function to some extent in *S.pombe*.

Results

Cloning of the BiP gene

Sequences of rat (Munro and Pelham, 1986) and *S.cerevisiae* (Normington *et al.*, 1989; Rose *et al.*, 1989) BiPs were compared in order to identify regions which are conserved between BiP proteins but not found in other members of the HSP70 family. Degenerate oligonucleotides 1 and 2 were designed as primers as described in Materials and methods. Oligonucleotide 3, an alternative 3′ primer, represented a sequence conserved among HSP70 and BiP proteins. Polymerase chain reaction (PCR) amplification of *S.pombe* genomic DNA using oligonucleotides 1 and 2 yielded one major product of 700 bp. This fragment encoded a sequence with extensive amino acid homology to rat and *S.cerevisiae* BiP. An overlapping product of 1 kb was generated by PCR with oligonucleotides 1 and 3. This fragment was used to screen genomic and cDNA libraries. The resulting clones gave overlapping sequences encoding the entire BiP gene (Figure 1A).

In the upstream region of the BiP gene there are two TATA box sequences within 50 nt of the initiator ATG. The BiP gene contains no introns through a coding region of 2.2 kb. Translation of the DNA sequence predicts a protein product of 73 kDa which is 67% identical to *S.cerevisiae* BiP and 66% identical to rat BiP, as shown in Figure 1B. There is a putative signal peptide at the N-terminus and a probable cleavage site between amino acids 24 and 25 (von Heijne, 1983). The sequence at the extreme C-terminus is Ala−Asp−Glu−Leu (ADEL), which differs from both the mammalian and *S.cerevisiae* XDEL motif involved in sorting of luminal ER proteins (Pelham, 1990). An unexpected feature of the amino acid sequence is the existence of a potential N-linked glycosylation site at position 29.

Disruption of the BiP gene

To determine whether the BiP gene was essential in *S.pombe*, a gene disruption experiment was performed. Approximately 80% of the coding sequence of BiP was replaced by the *S.pombe ura4* gene. Flanking sequences of 0.7 kb on the 5′ side and 1.7 kb on the 3′ side were retained in order to direct homologous recombination upon transformation of the linear fragment into the diploid *S.pombe* strain 611. Ura^+ transformants and the parental strain were analysed by Southern blotting of *Sal*I digested genomic DNA with a probe from outside the region used for transformation (Figure 2A). One out of ~100 transformants tested showed the predicted band of 4.2 kb in addition to the wild-type band of 2.7 kb (Figure 2B, lanes 1 and 2). A *ura4* probe also hybridized to the 4.2 kb band in the disruptant as predicted (Figure 2B, lane 4). The heterozygous BiP-disrupted diploid strain, BKO-8, was sporulated and subjected to random spore analysis. None of the 80 haploid progeny tested were ura^+, indicating that disruption of the BiP gene is lethal.

Regulation of BiP message

In mammalian cells, BiP mRNA is induced by various treatments which cause an accumulation of underglycosylated or misfolded proteins in the ER but it is not induced by heat shock (Welch *et al.*, 1983; Munro and Pelham, 1986). In contrast, *S.cerevisiae* BiP mRNA is induced by heat shock as well as these other stresses (Normington *et al.*, 1989; Rose *et al.*, 1989). To examine the regulation of *S.pombe* BiP, cells were subjected to various treatments and their RNA was analysed by Northern blotting, using a probe which would not hybridize with other members of the HSP70 family. In unstressed cells a single message of 2.5 kb was present (Figure 3, lane 1). Within 10 min of a shift from 30°C to 39°C the level of this message increased several-fold (lane 2). After 30 min at 39°C an additional smaller message of 2.4 kb was present and the level of the larger message was less than at the 10 min time point (lane 3). A similar pattern of BiP messages was observed after 1 h at 39°C (lane 4). Treatment with concentrations of tunicamycin (an inhibitor of N-linked glycosylation) as low as 0.1 μg/ml for 2 h caused the disappearance of the 2.5 kb message and an induction of the 2.4 kb message (lane 5). An even higher level of this message was present in cells when greater concentrations of tunicamycin were used (data not shown). Cells treated for 2 h with the calcium ionophore A23187 at 100 μM (lane 6) or 2-deoxyglucose at 10 mM (lane 7) contained both BiP messages.

No sequences matching the heat shock element (HSE; Pelham, 1985) consensus sequence (5′-CNNGAANNTTC-NNG-3′) could be found in the upstream region of the *S.pombe* BiP gene. A 10 bp consensus sequence (5′-NTT-CNNGAAN-3′) derived from the overlap of two half-HSEs has been identified upstream of the *Drosophila* HSP70 gene and as a dimer acts as a heat shock regulatory element (Xiao and Lis, 1988). Single copies of a similar sequence (consensus 5′-TTCTAGAA-3′) are found upstream of several *S.cerevisiae* heat shock genes (Tuite *et al.*, 1988)

Fig. 1. **(A)** The nucleotide and deduced amino acid sequence of the *S.pombe* BiP gene. Relevant restriction enzyme sites are indicated. TATA box sequences are present at nucleotide positions −47 and −37. Putative heat shock elements are underlined. Sequences to which the PCR oligonucleotides annealed are indicated by dotted lines. The putative signal sequence and the C-terminal sequence ADEL are underlined. The potential N-linked glycosylation site at amino acid 29 is indicated by an arrow head. **(B)** Alignment of amino acid sequences of *S.pombe* (P), *S.cerevisiae* (C) and rat (R) BiP. Identities are shown by a vertical line. Spaces have been inserted to achieve optimum alignment. This sequence has been deposited in the EMBL Nucleotide Sequence Data Library under the accession number X64416.

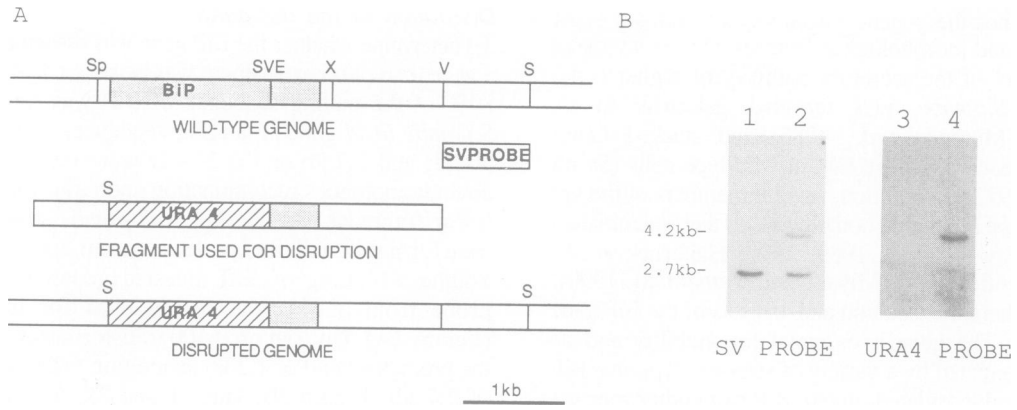


Fig. 2. Gene disruption of *S.pombe* BiP. **(A)** 1.7 kb of BiP coding sequence was effectively replaced by the *S.pombe ura4* gene (1.7 kb). This cloning procedure removed a *Sal*I site in the BiP gene and introduced a new *Sal*I site between the 5′ flanking region and the *ura4* gene. The linear fragment was used to transform *S.pombe* diploid strain 611. Relevant restriction enzyme sites are abbreviated as follows: S, *Sal*I; V, *Eco*RV; Sp, *Spe*I; X, *Xho*I; E, *Eco*RI. **(B)** Southern blot of *Sal*I-digested genomic DNA probed with a *Sal*I–*Eco*RV fragment derived from a region outside that used for the transformation (SV probe). DNA from strain 611 contains one hybridizing fragment of 2.7 kb (lane 1). Strain BKO-8 contains an additional hybridizing band of 4.2 kb (lane 2) indicating homologous integration and disruption of one copy of the BiP gene. This was confirmed by stripping the filter and hybridizing it with a *ura4* probe: 611 DNA (lane 3) does not hybridize as both copies of the *ura4* gene are deleted in this strain. The 4.2 kb fragment alone hybridizes in DNA from the disrupted strain BKO-8 (lane 4).

and the *KAR2* gene (Normington *et al.*, 1989) which is heat inducible. Two sequences (TTCTGGAA and TTCTGGTA) with homology to these consensus elements are found upstream of the *S.pombe* BiP gene (see Figure 1A). Sequences responsible for A23187 induction have been identified in the upstream regions of BiP genes from several species (Resendez *et al.*, 1988) but such sequences are not apparent in the 5′ region of the *S.pombe* BiP gene. These studies reveal that the levels and size of BiP mRNA are affected by treatments which cause an accumulation of misfolded or underglycosylated proteins in the ER.

Localization of epitope-tagged BiP protein

A BiP fusion protein, in which an epitope from the myc protein was inserted 7 aa from the C-terminus, was expressed in *S.pombe* under control of the *S.pombe adh* promoter on a multicopy plasmid. Logarithmic phase cells were fixed and processed for immunofluorescence using the 9E10 monoclonal antibody which recognizes the myc epitope (Figure 4A) and the nuclei stained with DAPI (Figure 4B). The BiP protein is localized to a ring around the nucleus, around the periphery of the cell and in strands throughout the cytoplasm. The level of tagged BiP expression varies considerably from cell to cell which probably reflects variation in the copy number of the expression vector. To visualize this labelling at higher resolution, cells were analysed by confocal microscopy (Figure 4, C–I). A reticulum of stained structures was often observed near the edges of cells and this is particularly apparent in the cell shown in Figure 4I. In sections through a single cell, a reticular pattern is apparent in the peripheral regions of the cell (C and E), whereas nuclear envelope labelling is observed in the middle of the cell (D).

S.pombe BiP can be glycosylated

The existence of an *N*-glycosylation site near the N-terminus of *S.pombe* BiP was unexpected; BiPs in *S.cerevisiae* and mammalian species lack N-linked glycosylation sites whereas such sites occur (but are not used) in cytoplasmic HSP70 proteins. There were a number of possibilities regarding the

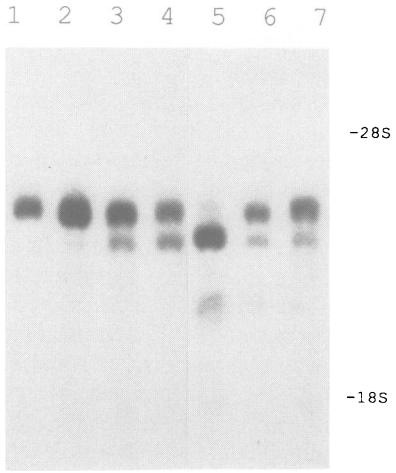


Fig. 3. Northern blot analysis of BiP mRNA. Total RNA from *S.pombe* cultures was separated by electrophoresis in a 1.5% agarose–2.2 M formaldehyde gel, transferred to nitrocellulose and hybridized with a 400 bp *Hin*dIII fragment from the 3′ end of the BiP gene. 8 μg of RNA was loaded per track: lane 1, control cells grown at 30°C; lane 2, 10 min shift from 30°C to 39°C; lanes 3 and 4, shift from 30°C to 39°C for 30 min or 1 h, respectively; lane 5, 2 h in 0.1 μg/ml tunicamycin at 30°; lane 6, 2 h in 10 mM 2-deoxyglucose at 30°C; lane 7, 2 h in 10 μM A23187 at 30°C. Approximate positions of the 18S and 28S ribosomal RNAs are indicated.

potential N-linked glycosylation site in *S.pombe* BiP: the assignment of the site of signal peptide cleavage was incorrect and the NST sequence was removed as part of the signal sequence; the site was present in the mature protein but not utilized; the site was present and glycosylated in the mature protein. As BiP is an ER resident protein, any glycosylation would be expected to be in the form of core sugars.

To determine whether *S.pombe* BiP is glycosylated, epitope-tagged BiP was immunoprecipitated from extracts of metabolically labelled cells and either analysed directly by SDS–PAGE or re-precipitated with concanavalin A bound to Sepharose beads (ConA–Sepharose) before SDS–PAGE analysis. Figure 5A (lanes 1 and 2) shows

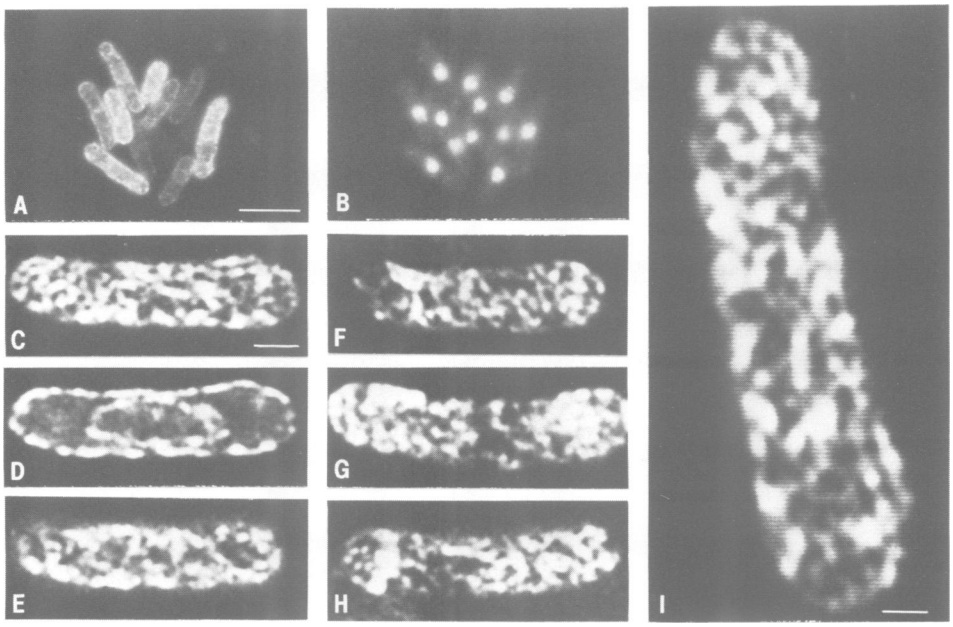


Fig. 4. Immunofluorescence of *S.pombe* cells expressing epitope tagged BiP. Logarithmic phase cells were processed for immunofluorescence as described in Materials and methods. **(A)** Field of cells expressing epitope-tagged BiP, labelled with 9E10 antibody. **(B)** Same field showing nuclear staining with DAPI. **(C–I)** Confocal images of cells labelled with 9E10 antibody. (C–E) sections through a single cell ~1 μm apart. (F–H) Labelling of reticular structures in three different cells. (I) Enlargement of cell in C. The scale bars in A, C and I represent 10 μm, 2 μm and 1 μm, respectively.

immunoprecipitation and immunoprecipitation–ConA precipitation, respectively, from a strain which does not express tagged BiP. A major band of 78 kDa and a minor band of 81 kDa are specifically immunoprecipitated from cells expressing tagged BiP (lane 3); the upper band is precipitated by ConA–Sepharose (lane 4). Lanes 5 and 6 are longer exposures of lanes 2 and 4, respectively. To confirm that this carbohydrate was N-linked, Western blots were performed on extracts from cells expressing the tagged BiP protein. Two species were present, a major band of 78 kDa and a minor band of 81 kDa (Figure 5B, lane 1). When the extract had been treated with endoglycosidase H only the 78 kDa band was apparent (lane 2) indicating that the larger species contains N-linked carbohydrate. These experiments indicate that *S.pombe* BiP is partially N-glycosylated, presumably at the NST sequence near the N-terminus. The difference in mobility between the two species is consistent with the addition of a core sugar moiety of $GlcNAc_2Man_9$. Approximately 10% of the tagged BiP protein is present in the glycosylated form although there was some variation between experiments.

ADEL acts as a retention signal in S.pombe

Pelham and co-workers have shown that C-terminal KDEL in mammalian cells and HDEL in *S.cerevisiae* is necessary and sufficient for retention of proteins in the ER (Munro and Pelham, 1987; Pelham *et al.*, 1988). We wanted to determine whether the ADEL sequence could direct the ER retention of acid phosphatase, which is normally secreted from *S.pombe* cells. *S.pombe* contains two immunologically related acid phosphatases; the *pho1* gene is phosphate-repressible (Maundrell *et al.*, 1985) and the *pho4* gene is thiamin-repressible (Schweingruber *et al.*, 1986a, b; Yang *et al.*, 1990). The *pho4* gene was extended at its 3′ end so that the encoded protein terminated in ADEL or one of two random sequences, DYFD or EKSG. These fusion protein constructs (AP–ADEL, AP–DYFD and AP–EKSG, respectively) were expressed from the *adh* promoter on a multicopy plasmid in strain ES1 which contains a deletion of the *pho1* gene. Cells were grown in media containing 5 μM thiamin. Protein extracts were made from ES1 cells and from cells expressing AP–ADEL, AP–DYFD and AP–EKSG, incubated in the presence or absence of endoglycosidase H and analysed by Western blotting. Under these growth conditions the strain ES1 contains no material cross-reacting with an anti-acid phosphatase antibody (Figure 6, lanes 1 and 2) since *pho1* is deleted and *pho4* is repressed by thiamin. No cross-reacting material was seen in cells expressing AP–EKSG (lane 3) and AP–DYFD (lane 5) unless N-linked oligosaccharides were removed with endoglycosidase H (lanes 4 and 6) to produce a 55 kDa species. In samples not treated with endoglycosidase H, heterogeneous high molecular weight carbohydrate side chains may prevent acid phosphatase from entering the gel, or cause it to migrate as a very diffuse smear which is not detectable with the antibody. Cells expressing AP–ADEL contained an 80 kDa species (lane 7) which represents a core-glycosylated form of acid phosphatase. This was shifted to 55 kDa upon treatment with endoglycosidase H (lane 8). The AP–DYFD and AP–EKSG proteins are subject to heterogeneous high molecular weight modifications associated with passage beyond the ER, whereas the majority of the AP–ADEL protein is present in the core-glycosylated form suggesting that it has been retained in the ER. Although equal amounts of cell extract were loaded in each track, the total cellular acid phosphatase as revealed by endoglycosidase H digestion appears to be greater in the cells expressing AP–ADEL than in those expressing AP–EKSG and AP–DYFD. This effect is probably due to an accumulation of the AP–ADEL protein in the cell due to its ER retention,

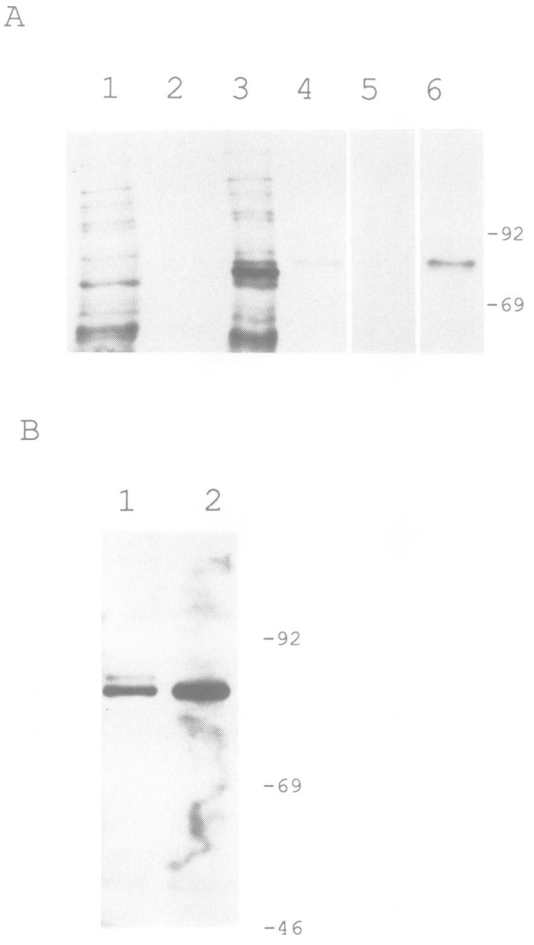


Fig. 5. Glycosylation of *S.pombe* BiP. **(A)** Cells containing plasmids pEVP11 or pEB9 were labelled with [^{35}S]sulphate for 1 h at 30°C. Proteins were immunoprecipitated with the 9E10 mAb (lanes 1 and 3) or immunoprecipitated then ConA precipitated (lanes 2, 4, 5 and 6) and analysed by 8% SDS−PAGE and fluorography. Each lane contains lysate derived from 8×10^7 cells. Comparison of lanes 1 (control cells containing pEVP11) and 3 (cells containing pEB9) indicates that two additional bands of 78 and 81 kDa are present in cells expressing epitope-tagged BiP. The 81 kDa species was precipitated by ConA−Sepharose (lane 4) indicating that it contains carbohydrate. No proteins were re-precipitated by ConA from control cells (lane 2). Lanes 5 and 6 are longer exposures of lanes 2 and 4, respectively. **(B)** Protein extract from cells expressing epitope-tagged BiP was incubated overnight in the presence (lane 2) or absence (lane 1) of endoglycosidase H, separated by 10% SDS−PAGE and analysed by Western blotting using the 9E10 monoclonal antibody. Each track contains lysate derived from 5×10^6 cells. The positions of marker proteins are indicated in kilodaltons.

whereas the AP−DYFD and AP−EKSG proteins are secreted. Although secreted acid phosphatase is thought to be trapped between the plasma membrane and the cell wall, some of this material would be lost upon conversion of the cells to spheroplasts.

Several variants of the XDEL sequence have been found at the C-termini of luminal ER proteins from other species, including KDEL in mammalian cells, HDEL in *S.cerevisiae* and DDEL in *K. lactis*. To define further the requirements for an ER-retention signal in *S.pombe*, these sequences were fused to the C-terminus of acid phosphatase and analysed as described above. Extracts from cells expressing AP−DDEL, AP−HDEL and AP−KDEL all contained a 80 kDa band (Figure 6, lanes 9, 11 and 13). Upon

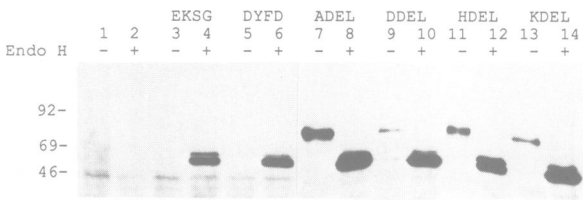


Fig. 6. Sorting of C-terminally extended pho4 fusion proteins. Protein extracts were made from logarithmic phase cells grown in the presence of thiamin and incubated overnight in the presence (lanes 2, 4, 6, 8, 10, 12 and 14) or absence (lanes 1, 3, 5, 7, 9, 11 and 13) of endoglycosidase H and analysed by 8% SDS−PAGE and Western blotting. Each lane contains lysate derived from 3×10^6 cells. Strain ES1 contains no material cross-reacting with the anti-acid phosphatase monoclonal antibody (lanes 1 and 2). Acid phosphatase terminating in EKSG or DYFD is not detectable by the antibody without endoglycosidase H treatment (lanes 3 and 5). Upon endoglycosidase H treatment material appears as a 55 kDa band (lanes 4 and 6). Acid phosphatase terminating in ADEL is present as an 80 kDa band (lane 7) which is shifted down to 55 kDa upon endoglycosidase H treatment (lane 8). Acid phosphatase terminating in DDEL (lanes 9 and 10), HDEL (lanes 11 and 12) and KDEL (lanes 13 and 14) show a similar pattern but the 80 kDa band is much fainter. The positions of marker proteins are indicated in kilodaltons.

endoglycosidase H treatment (lanes 10, 12 and 14), a 55 kDa band of greater intensity than the 80 kDa band was apparent. The relative intensities of the AP−DDEL, AP−HDEL and AP−KDEL 80 kDa bands varied between experiments, but they were consistently weaker than the AP−ADEL 80 kDa band (see lane 7). These results show that all these signals can be recognized in *S.pombe*.

Discussion

Sequence conservation of BiP between *S.cerevisiae* and mammalian cells has enabled us to clone a homologue from the fission yeast *S.pombe* by PCR. The PCR approach was successful in that only BiP sequences were amplified; no other members of the HSP70 family were cloned. The encoded protein has the expected characteristics: extensive sequence homology to the HSP70 family of proteins as well as sequences characteristic only of BiP proteins, a putative N-terminal signal sequence and ER retention signal at the C-terminus (Figure 1). The protein is approximately equally divergent from its mammalian and *S.cerevisiae* homologues, once again underlining the substantial differences between these two yeasts and the potential value of a comparative genetic analysis. However, two features of the sequence were unexpected, namely the presence of a potential glycosylation site near the N-terminus and a new variant of the ER retention signal, ADEL, at the C-terminus.

The BiP gene of *S.pombe*, like that of other species, can be induced by a range of stresses, including the calcium ionophore A23187, the glycosylation inhibitor tunicamycin and 2-deoxyglucose. Tunicamycin appears to have a relatively greater effect on the level of BiP mRNA in *S.pombe* than it does in some mammalian species (Watowich and Morimoto, 1988). In addition, the *S.pombe* gene is induced by heat shock, as is the case with *S.cerevisiae* but not with higher cells. The various stresses cause the appearance of a new BiP mRNA which is shorter than the normal form (Figure 3). The structures of these two mRNAs are unknown; the two putative TATA boxes upstream of the gene are only 10 bp apart which would not account for the 100 bp difference in size between the two messages. It is possible that the message terminates

at a different 3′ position in stressed and unstressed cells, or that the message is differently modified. It has been proposed that an inducing signal common to some or all of these stresses is the appearance of misfolded or aggregated proteins in the lumen of the ER (Kozutsumi *et al.*, 1988), but the pathway for transmission of this signal to the nucleus remains unknown. It will be of interest to discover which elements of such a pathway are conserved between the two yeasts and higher cells.

Immunofluorescence of BiP has allowed us to visualize the ER of *S.pombe* (Figure 4). The pattern includes the elements in which *S.cerevisiae* BiP was observed (Rose *et al.*, 1989), namely the nuclear envelope, filaments radiating from the nucleus and structures beneath the cell surface, but in addition the ER of *S.pombe* has a distinct reticular component in the cytoplasm (Figure 4). In higher cells the reticulum is formed by dynamic interactions between membranes and microtubules (Dabora and Sheetz, 1988). The same may be true in *S.pombe*; if so this might be a useful system to study the nature of these interactions, since both the patterns of microtubule formation (Hagan and Hyams, 1988) and the tubulin genes (Yanagida, 1987) are well characterized in this organism.

Glycosylation of BiP has not been previously observed. The mammalian and *S.cerevisiae* sequences lack sites for *N*-glycosylation, in contrast to the other members of the HSP70 family, which contain potential sites but are never exposed to the glycosylation machinery within the ER. It has been argued that glycosylation would hinder the folding or assembly functions of BiP (Normington *et al.*, 1989). However, *S.pombe* BiP contains a site for *N*-glycosylation which is clearly used, if only in a small proportion of the protein (Figure 5). The significance of this partial modification is unclear. It seems unlikely that glycosylation is required for any activity of the protein, and it is even possible that the glycosylated form is inactive. The *K.lactis* BiP sequence also contains a potential N-linked glycosylation site, near the C-terminus (Lewis and Pelham, 1990a) but it is not known whether it is utilized.

The sequence ADEL is found at the C-terminus of *S.pombe* BiP. This sequence can function as an ER-retention signal when attached to an otherwise secreted protein. Sequences which vary at the −4 position (DDEL, HDEL, KDEL) can also direct ER-retention in *S.pombe*, though apparently with less efficiency than ADEL. This is in contrast to *S.cerevisiae* which does not retain KDEL or DDEL and mammalian cells which do not retain HDEL. The C-terminal sequences of other *S.pombe* luminal ER proteins are as yet unknown, but it is clearly possible that their retention signals differ from that of BiP. The process of ER 'retention' in fact appears to involve selective retrieval of proteins to the ER from a subsequent secretory compartment, probably the early Golgi complex (Pelham, 1988; Dean and Pelham, 1990). Since *S.pombe*, in contrast to *S.cerevisiae*, contains well defined and readily detectable Golgi stacks (Smith and Svoboda, 1972), this organism may prove useful for a combined genetic and morphological analysis of the mechanism of recycling to the ER.

Materials and methods

Bacterial and S.pombe strains

E.coli strain JM101 was used for all cloning procedures. Strain NM646 was used as a host for the λdash library and strains Y1088 and Y1090 were used as hosts for the *S.pombe* cDNA library in λgt11. *S.pombe* strains used were 556 (h$^+$ *ade6*-M216 *ura4*-D18 *leu1*-32), 611 (h$^+$/h$^-$ *ade6*-M216/*ade6*-M210 *ura4*-D18/*ura4*-D18 *leu1*-32/*leu1*-32), obtained from Prof Paul Nurse, ES1 (*pho1*-44 *leu1*-32), obtained from Dr Ernst Schweingruber and NCYC 1845 (h$^-$ *ade6*-M210) .

S.pombe media and procedures

The media used for *S.pombe* were YPD (1% Bacto-yeast extract, 2% bactopeptone, 2% glucose) and minimal medium (Moreno *et al.*, 1990). Nitrogen-free medium was used for the sporulation of diploids. NSM (sulphate-free minimal medium, in which all sulphate salts were replaced by chloride salts) was used in metabolic labelling experiments. Minimal medium was supplemented with 5 μM thiamin when required. Standard genetical procedures (Moreno *et al.*, 1990) were used. *S.pombe* transformations were performed by the lithium chloride procedure (Broker, 1987).

DNA procedures

Standard procedures were used for DNA manipulations (Sambrook *et al.*, 1989). *S.pombe* genomic DNA was prepared from 5 ml logarithmic phase cultures (Moreno *et al.*, 1990). PCR was performed as described (Saiki *et al.*, 1988). Genescreen-Plus (DuPont) was used for library screening and Genescreen (DuPont) for Southern transfers. Probes were prepared using the random hexamer-primed method (Feinberg and Vogelstein, 1982) and hybridization performed according to Church and Gilbert (1984). Double-stranded DNA sequencing was performed using Sequenase (USB) according to the manufacturer's instructions.

RNA preparation and analysis

Logarithmic phase cells of strain NCYC 1845 were split into 20 ml aliquots and subjected to various treatments. Control cells were incubated at 30°C for 2 h. For heat shock, cells were shifted from 30°C to 39°C for the last 10, 30 or 60 min of incubation. Alternatively, cells were grown at 30°C in the presence of 0.1 μg/ml tunicamycin, 10 μM A23187 or 10 mM 2-deoxyglucose for 2 h. At the end of the incubation cells were placed on ice, pelleted and total RNA prepared (Moreno *et al.*, 1990). 8 μg of each RNA sample was analysed by electrophoresis through a 1.5% agarose−2.2 M formaldehyde gel (Sambrook *et al.*, 1989). Transfer to Genescreen membrane was achieved by capillary blotting in 20×SSC and hybridizations performed by the method of Church and Gilbert (1984). The probe was made from a 400 bp *Hin*dIII fragment from the 3′ end of the gene (Figure 1A) which is a region of minimal homology between HSP70s and BiPs.

PCR amplification and cloning of the BiP gene

The following oligonucleotides were synthesized: 1, 5′-TACTCTAGAAGG-TNACKCATGCKGTNGT-3′; 2, 5′-TACGCATGCYTCATCMGGATTRA-TNCC-3′; and 3, 5′-TACGGTACCTCRATCTGNGGMACNCC-3′. To facilitate the cloning of PCR products after amplification, restriction enzyme recognition sites were incorporated at the 5′ ends of the oligonucleotides. Oligonucleotide 1 primes towards the 3′ end of the BiP gene and oligonucleotides 2 and 3 prime towards the 5′ end. Oligonucleotide 1 encodes amino acids KVTHAVV. Four of these seven amino acids are common to HSP70s and to BiPs in *S.cerevisiae* and mammalian species, the remainder are specific to BiP. Degeneracy was reduced by not incorporating all possible codons, relying on weak base-pairing of G and T. Oligonucleotide 2 represents the amino acid sequence GINPDEA of which only the glycine is BiP-specific. Oligonucleotide 3 represents the amino acid sequence GVPQIEV which is common to HSP70s and BiPs. Oligonucleotides 1 and 2 and oligonucleotides 1 and 3 were used in PCR reactions to amplify genomic DNA. 2 μg of genomic DNA was heated to 95°C for 5 min in a volume of 100 μl which contained 250 μM of each dNTP, 10 mM Tris−HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 5 μg of each oligonucleotide. Reactions were then incubated at 45°C for 5 min during which time 2.5 units of Taq polymerase (Cetus) was added. This was followed by a 2 min incubation at 60°C. Two cycles of 1 min at 92°C, 1 min at 50°C and 2 min at 70°C were then performed, followed by 37 cycles of 1 min at 92°C, 1 min at 55°C, 2 min at 70°C. PCR products of the predicted size were gel-purified, digested with the relevant restriction enzymes and cloned into Bluescript and Bluescribe vectors (Stratagene). The 1 kb product of oligonucleotides 1 and 3 was used as a probe to screen ~3000 plaques of a λdash (Stratagene) library containing inserts derived from a *Sau*3A partial digest of *S.pombe* genomic DNA (obtained from Dr Alister Craig). Two positive overlapping clones were obtained. Subclones and deletion series were made for sequencing (Yanisch-Perron *et al.*, 1985). An *S.pombe* cDNA library in λgt11 (Young and Davis, 1983) obtained from Dr Viesturs Simanis and Prof Paul Nurse was also screened. None of the clones obtained were longer than 600 bp. Two of these clones were partially sequenced and the sequences were identical to that obtained from the genomic clones.

Gene disruption

The construct used for disruption (see Figure 2a) was made as follows. A 0.7 kb fragment from the region 5′ of the BiP gene was placed adjacent to the *ura4* gene (1.7 kb). The 3′ end of this fragment is 50 bp upstream of the initiator ATG. A 1.7 kb *Eco*RV fragment encompassing 360 bp of BiP coding sequence and 1.35 kb of 3′ sequence was placed on the other side of the *ura4* gene. This procedure effectively deleted 1.7 kb of BiP coding sequence (~80%) including a *SalI* site and created a new *SalI* site at the junction of the 5′ region and the *ura4* gene as shown in Figure 2A. The linear fragment was transformed into the diploid strain 611. DNA from strain 611 and ura$^+$ transformants was digested with *SalI* and analysed by Southern blotting using a *SalI*−*Eco*RV fragment from outside the region used for transformation as a probe (Figure 2A). The heterozygous BiP-disrupted strain, BKO-8, was sporulated on nitrogen-free medium and the mixture of asci and vegetative cells treated with 1:500 dilution of helicase (IBF Biotechnics, Paris, France) in H_2O at 37°C overnight. Liberated spores were washed and germinated on YPD plates. Colonies were replicated onto media with or without uracil to determine the genotype of the progeny.

Expression of epitope tagged BiP in S.pombe

PCR manipulation of the 0.5 kb *Eco*RI−*Xho*I region (see Figure 2A) was used to insert the myc epitope (Munro and Pelham, 1984; Evan *et al.*, 1985), such that the altered C-terminus is *DDYF*LEQKLISEEDLNI*DDEADEL* (the italicized sequences are derived from BiP). The *Spe*I−*Eco*RI fragment encompassing the rest of the gene was then fused to the *Eco*RI−*Xho*I fragment. The resulting construct was cloned as an *Sst*I−*Bam*HI fragment into pEVP11 which contains the efficient *S.pombe adh* promoter (Russell and Nurse, 1986) and the *S.cerevisiae LEU2* marker to form plasmid pEB9. pEB9 and pEVP11 were transformed into strain 556.

pho4 constructs

The *pho4* gene and its sequence (Yang *et al.*, 1990) were obtained from Dr Ernst Schweingruber. PCR was used to create a *Sst*I site 15 bp 5′ of the *pho4* initiator ATG. The 3′ end of the gene was altered so that the C-terminus was extended from TVYY to TVYYLEQKLISEEDLNIDD-E−ADEL, −DDEL, −HDEL, −KDEL, −EKSG or −DYFD. 200 bp of 3′ region from the BiP gene was included downstream of the stop codon. The constructs were cloned into pEVP11 as *Bam*HI−*Sst*I fragments to form AP−ADEL, AP−DDEL, AP−HDEL, AP−KDEL, AP−EKSG and AP−DYFD respectively and transformed into strain ES1. Transformants were grown in minimal media supplemented with 5 μM thiamin to repress the endogenous *pho4* gene. Western blot analysis was performed with the anti-acid phosphatase monoclonal antibody, 7B4 (obtained from Dr Ernst Schweingruber).

Immunofluorescence

Immunofluorescence of cells expressing epitope-tagged BiP was performed as described (Chappell and Warren, 1989) except that antibody incubations were at 4°C. 9E10 ascites fluid (obtained from Dr Tommy Nilsson) was used at a dilution of 1:1000, FITC-conjugated rabbit anti-mouse (TAGO) was used at 1:100. Cells were mounted in Citifluor anti-fade (Agar Aids) containing 1 μg/ml DAPI (4′,6′-diamidino-2-phenylindole dihydrochloride, Sigma). Cells were observed with a Zeiss Axiophot microscope through a 100× objective and photographed with TMAX-400 film (Kodak). Confocal fluorescence microscopy was performed with a BioRad Lasersharp microscope, using a 60× objective. Images were collected with a Polaroid Quick Print video printer on Plus-X 125 film (Kodak).

Western blotting

S.pombe protein extracts were prepared from logarithmic phase cultures. Cells were spheroplasted in SP1 buffer (1.2 M Sorbitol, 50 mM sodium citrate, 50 mM Na_2HPO_4, 40 mM EDTA, pH 5.6) containing 0.4 mg/ml Zymolyase-20T (ICN) at 37°C for 30 min, heated to 95°C for 5 min in 2×sample buffer (2% SDS, 50 mM Tris−HCl pH 6.8, 2 mM EDTA, 10% glycerol, 0.03% bromophenol blue, 2% β-mercaptoethanol) and cell debris pelleted by centrifugation. Protein extracts from 1×10^7 cells were diluted 5-fold in 60 mM sodium acetate pH 5.5 and incubated overnight at 37°C in the presence or absence of 1 unit of endoglycosidase H (Boehringer-Mannheim) and protease inhibitors (2 mM PMSF, 2.1 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin). Three-fifth volume of 2×sample buffer was added and the samples heated to 95°C. Protein from equal numbers of cells was loaded in each track and separated by electrophoresis in 8 or 10% SDS−PAGE gels. Proteins were transferred to Amersham Hybond-C nitrocellulose membranes by semi-dry electro-blotting. Detection of specific proteins was performed using the Amersham Enhanced Chemi-Luminescence system according to manufacturer's instructions. 9E10 ascites fluid was used at 1:5000 dilution, 7B4 culture supernatant was used at 1:10. Horseradish peroxidase conjugated rabbit anti-mouse (TAGO) was used at 1:1000.

Protein labelling and precipitation

S.pombe cells of strain 556 containing plasmids pEVP11 or pEB9 were grown overnight in NSM (containing appropriate supplements) plus 200 μM ammonium sulphate. 2×10^8 cells were washed and resuspended in 1 ml NSM, 420 μCi [^{35}S]sulphate (Amersham) was added and cells were incubated at 30°C for 1 h. The immunoprecipitation protocol was adapted from Dean and Pelham (1990). Labelled cells were placed on ice and NaN_3 added to 10 mM. Cells were spheroplasted in SP1 buffer containing 0.4 mg/ml Zymolyase-20T and 10 mM NaN_3 for 30 min at 37°C. Cells were pelleted gently and lysed by the addition of 0.5 ml of ice-cold 1% TritonX-100 in PBS (containing 2 mM PMSF, 2.1 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin) with vortexing. 0.5 ml of ice-cold PBS was added and cell debris removed by centrifuging twice at 9000 *g* for 5 min at 4°C. Samples were divided into two aliquots of 400 μl and 9E10 ascites added at a 1:100 dilution. After 1 h on ice 2.9 μg of rabbit anti-mouse antibody (Dakopatts) was added and samples incubated on ice for a further hour before addition of 40 μl of a protein-A−Sepharose slurry (Pharmacia) and the mixture was stirred overnight at 4°C. One of each pair of samples was left at 4°C while the other was washed four times with RIPA buffer (0.5 M NaCl, 50 mM Tris−HCl pH 7.4, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) before heating the protein-A beads to 95°C for 5 min in 40 μl 1% SDS. The supernatant was added to 360 μl 0.5% TritonX-100 in PBS and 40 μl of ConA−Sepharose (Sigma) was added. These samples were then incubated at 4°C with stirring for 4 h. All samples were then washed four times with RIPA buffer and heated to 95°C for 5 min in 2×sample buffer before electrophoresis through an 8% SDS−PAGE gel and fluorography.

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