THE NUD1 GENE OF YEAST SACCHAROMYCES CEREVISIAE COMPLEMENTS A THERMOSENSITIVE LYTIC MUTATION

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ABSTRACT
Temperature sensitive (ts) yeast strains have been obtained by ethyl methane sulfonate (EMS) mutagenesis. They have a normal growth at low temperature and releases of intracellular proteins in the culture medium at 37ºC. The ts mutation was localized in the NUD1 gene, which codes for a component of the yeast spindle pole body (SPB). Construction of a frame shift mutation in the wild type gene demonstrated that the correct sequence of the NUD1 gene only could complement the ts phenotype. Further analysis showed that the ts mutation deprived the cells of a normal termination of mitosis, thus causing death at restrictive temperature and a consequence cell lysis.

Abreviations: EMS, ethyl methane sulfonate; LRR, Leucine – Rich Repeats; SPB, spindle pole body; ts, temperature sensitive phenotype; ALP, alkaline phosphatase; MEN, mitotic exit network; SDS-PAGE, sodium dodecyl phosphate polyacrilamide gel; Da, Daltons; ATP, adenosine triphosphate

Introduction
Previous studies of our group have led to the isolation of thermosensitive (ts) yeast mutants obtained by ethil methane sulfonate (EMS) mutagenesis. The ts mutant cells showed not only an increased content of alkaline phosphatase (ALP) in the growth medium at 37ºC but also an increased amount of intracellular proteins. Combining traditional genetics methods, gene cloning and the use of yeast artificial chromosomes made it possible to identify the NUD1 gene as complementing the ts mutant phenotype. Sequencing of the defective allele from mutant cells revealed the presence of a point mutation resulting in a single amino acid substitution: glycine with glutamate at position 585 (the nud1-G585E allele) (1).

The NUD1 gene codes for a protein (Nud1p) which is a component of the yeast spindle pole body (SPB) and is required for the exit from mitosis (2, 3). Although most of the SPB components are coiled – coil proteins (4), Nud1p has nine Leucine – Rich Repeats (LRR). LRR motifs have been found in functionally diverse proteins, most of which are involved in protein-protein interactions or signal transduction pathways (5). Nud1p is
also a component of the mitotic exit network (MEN) and may function as a scaffold protein for regulating or facilitating the binding of proteins Tem1p to Cdc15p, thereby enhancing the transduction of the signal that triggers the late events of the cell cycle (2). In budding yeast SPB is a trilaminar structure embedded in the nuclear envelope. It contains an inner plaque that nucleates the nuclear microtubules, the central plaque and an outer plaque that organizes the cytoplasmic microtubules (6). Nud1p is a component of an outer plaque and connects it to the central plaque (2, 4). It has been found that mutations in other SPB components (Spc110p, Cdc31p and Kic1p) resulted in cell lysis (7, 8, 9, 10).

Three different thermosensitive alleles of the NUD1 gene have previously been isolated: nud1-2 with five altered amino acids, one of which only is found in the LRR-2 (6); nud1-44 with nine amino acids substitutions, three of which are located in several LRRs: LRR-1, LRR-4 and LRR-5, and nud1-52, with two changed amino acids, neither of which is found in the LRRs (2). The single amino acid substitution found in the mutant allele nud1-G585E is localised in LRR-3.

Materials and Methods

Strains and Media. The following yeast strains were used in this study: L4B (MATα leu2 his4 ts+); FW-7B (MATα ura3 his3 leu2 ade2-1 trp1-1 can1 ts+), both mutant strains carried the mutant allele nud1-G585E; LS56 (MATα ura3-52 bsk1::URA3 inol) and the wild type strain W303-1A (MATα ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100). Yeast strains were grown on standard yeast medium YEPD – 1% yeast extract, 2% bactopente and 2% dextrose. YEPDS medium – YEPD with added 0.5M sorbitol. Escherichia coli DH5a strain was used as host for maintenance and propagation of plasmids. Bacterial cultures were grown in Luria-Bertani (LB) medium supplemented with 100mg/ml ampicillin (11).

DNA Manipulation. Plasmid preparation, restriction enzyme digestions, agarose gel electrophoresis and ligation of DNA fragments were accomplished using standard techniques (11). E. coli cells were transformed by the method of Inoue et. al with slight modifications (12). To introduce plasmid DNA into yeast cells, a modification of the lithium acetate procedure was followed (13).

Protein analysis. Protein extraction from yeast cells was made according to the method of Horvath et al., (14). Extracellular proteins were concentrated from cell – free medium using Centricon Plus columns (Amicon). Proteins were analyzed by SDS-PAGE and Western blotting as described in Molecular cloning (11). Two different antibodies were used: against Pgk1p (phosphoglycerate kinase) a protein localized in the citoplasm with molecular weight of 44 636 Da; against Gcn20p a protein localized in Gcn1p – Gcn20p ribosomal complex, molecular weight 84 882 Da.

Results and Discussion

In a previous work we have identified the NUD1 gene as the wild type gene complementing the ts mutant phenotype i.e. lack of cell growth and release of intracellular proteins at 37ºC (1). To demonstrate that only the correct sequence of the NUD1 could complement the mutant phenotype a frame shift mutation was introduced into the wild type gene NUD1. Plasmid p30.8.1.2, carrying the wild type allele of the NUD1 gene was kindly provided by Dr. John Kilmartin (MRC, Cambridge). This plasmid referred to here as pNUD1 contains a DNA fragment cloned into XbaI/XhoI sites of the polylinker of the centromeric vector pRS316. The
DNA fragment contains 1330 bp before the ATG codon and 1514 bp after the stop codon of the NUD1 gene. The plasmid pNUD1 was digested with Cla I enzyme which recognizes unique site 1807 bp after the ATG codon in the NUD1 coding sequence. The two overhangs of the plasmid were then filled in by T4 DNA polymerase and ligated. Thus the Cla I site ATCGAT was destroyed and a site for the Nru I ATCGCGAT was generated. The new plasmid was designated as pNfs and the mutated site was confirmed by restriction analysis (Fig. 1). Transformation of the yeast mutant strain FW-7B with the plasmids pNUD1 and pNfs clearly demonstrated that only the wild type NUD1 gene was capable of complementing the ts phenotype (Fig. 2).

Our previous studies have shown that the mutant yeast cells gave not only positive ALP assay result but also released an increased amount of intracellular proteins in the culture medium. The release of the vacuolar protein ALP is usually accepted as an indication for cell lysis. We proved by sensitivity to Zymolyase, Calcofluor White and killer toxins that the mutation nud1-G585E was not related with cell wall defects (15). Using three different ATP inhibitors, we demonstrated also that the increased protein release was not energy dependent (16). Construction of a double mutant strain SN1-1 carrying the mutations sec1 and nud1-G585E showed that the protein release was independent of the SEC1 gene and therefore of the yeast secretory pathway (17). All this suggests that the reason for protein release in the nud1-G585E mutant cells is lysis. Western blot analysis with two antibodies against Pgk1p (phosphoglycerate kinase) and Gcn20p (a ribosomal protein) was applied to prove directly that the increased protein release from the ts mutant cells was due to cell lysis rather than secretion. Mutant strains FW-7B and 1.4B were cultivated in YEPD or YEPDS medium with aeration first at permissive temperature to a density of approximately 0.650 (A650) and then shifted to restrictive temperature of 37°C.

Fig. 1. Restriction analysis of pNUD1 and pNfs1 plasmids: Lanes 1 and 8: molecular marker; Lane 2: pNfs1 restricted by Nru I; Lane 3: pNUD1 restricted by Nru I; Lane 4: pNfs1 restricted by Cla I; Lane 5: pNUD1 restricted by Cla I; Lane 6: pNfs1 native; Lane 7: pNUD1 native.

Fig. 2. Transformation of FW-7B yeast mutant strain with plasmids: pNUD1 (1); pNfs1 (2) and pRS316 (3) as a control.
Samples were taken at different time intervals, cells were removed by centrifugation and the extracellular proteins were either precipitated by ZnCl₂ (18) or concentrated using Centricon Plus columns. Two other yeast strains were used like controls: the wild type strain W303-1A and the mutant strain LS56 (carrying a mutation in a BCK1 gene) were used as negative and positive control for cell lysis respectively. The latter two strains were analysed following the same procedure as above. It was found that the proteins in the supernatants of both FW-7B and LS56 had the same electrophoretic profile (data not shown). Results from the Western blot analysis gave positive signals for the nud1-G585E mutant strains with both antibodies used (Fig. 3). The positive control LS56 showed lower levels of lysis at both 25°C and at 37°C when is cultivated in the presence of sorbitol, which acted as a suppressor of cell lysis (Fig. 3A, lanes 5 and 7). At restrictive temperature the same strain lysed to a greater extend in the absence of sorbitol (Fig. 3A and B, lane 6). It did not produce any visible signals with the antibody against Gcn20p (Fig. 3B, lanes 5 and 7) probably because this antibody was polyclonal and was not capable of detecting low protein concentrations. The wild type strain W303-1A did not react with both antibodies (Fig. 3A and B, lanes 2, 3 and 4).

In conclusion we can state that the NUD1 gene only is responsible for the complementation of the ts mutant phenotype. The increased release of proteins in the culture medium of ts yeast cells at restrictive temperature (37°C) is due to cell lysis. The point mutation nud1-G585E causes cell death at 37°C and the cell lysis is a consequence of it. Two another mutant alleles of NUD1 gene: nud1-42 and nud1-52 have also been observed to die at 37°C (2) which is in support of our hypothesis.

Fig 3. Western blot analysis: A: with antibody against Pgk1p; B: with antibody against Gcn20p; Lane 1: cell extracted proteins (positive control); Lane 2: wild type strain cultivated at 37°C in YEPDS medium; Lane 3: wild type strain cultivated at 37°C in YEPD medium; Lane 4: wild type strain cultivated at 25°C in YEPD medium; Lane 5: LS56 cultivated at 37°C in YEPDS medium; Lane 6: LS56 cultivated at 37°C in YEPD medium; Lane 7: LS56 cultivated at 25°C in YEPD medium; Lane 8: FW-7B cultivated at 25°C in YEPD medium; Lane 9: FW-7B cultivated at 37°C in YEPD medium; Lane 10: FW-7B cultivated at 37°C in YEPD medium; C: with antibody against Pgk1p; Lane 1: 1.4B cultivated at 25°C in YEPDS medium for 24 hours; Lane 2: 1.4B cultivated at 37°C in YEPDS medium for 24 hours; Lane 3: LS56 cultivated at 37°C in YEPD medium; Lane 4: 1.4B cultivated at 25°C in YEPDS medium for 72 hours; Lane 5: 1.4B cultivated at 37°C in YEPDS medium for 72 hours; Lane 6: cell extracted proteins.
REFERENCES