Effect of Tunicamycin on Germ Tube and Yeast Bud Formation in *Candida albicans*

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Tunicamycin is an antimicrobial agent which inhibits the first reaction of the dolichol pathway leading to N-glycosylation of proteins. The effect of tunicamycin on the growth of the dimorphic fungus *Candida albicans* differed depending on the growth phase of the organism. Addition of tunicamycin to stationary phase yeast cells inhibited the resumption of growth of those cells in either morphology, as cultures failed to initiate either yeast bud or germ tube formation. When tunicamycin was added to growing cells, growth was inhibited but not immediately. When it was added to germ tube cultures, nuclear division and septum formation continued for some time before ceasing. Addition of the drug to exponential phase yeast cultures resulted in an approximately 45% increase in cell number before cell division ceased and yeast accumulated in both budded and unbudded stages of the cell cycle. Accumulation of trichloroacetic acid precipitable radiolabelled protein and nucleic acid continued unchanged for some time following addition of tunicamycin; however, after a while a reduced rate of accumulation was noted.

**INTRODUCTION**

Tunicamycin prevents the first reaction of the dolichol pathway leading to N-glycosylation of proteins (Tkacz & Lampen, 1975; Lehle & Tanner, 1976). This pathway functions in all eukaryotic cells including fungi (Takatsuki *et al.*, 1971, 1972; Selitrennikoff, 1979; Speake *et al.*, 1981; Arnold & Tanner, 1982). *Candida albicans* was one of the first micro-organisms shown to be sensitive to this antibiotic. In the presence of the drug, growth was inhibited (Takatsuki *et al.*, 1971) and cells increased in volume (Takatsuki *et al.*, 1972). However, no subsequent analysis of growth inhibition by tunicamycin of this opportunistic fungal pathogen has been reported. The purpose of this study was to examine the effect of tunicamycin on yeast bud and germ tube formation in *C. albicans*.

**METHODS**

*Organism and culture conditions.* *Candida albicans* B311 was maintained on yeast extract/peptone/glucose agar plates. Cultures were grown in a defined minimal medium supplemented with amino acids (Lee *et al.*, 1975). All cultures were grown in a gyratory incubator shaker at 180–200 r.p.m. at 27–28 °C or 37 °C. For experiments in which reinitiation of growth was monitored, stationary phase yeast cells grown in liquid medium were centrifuged and resuspended at 2–5 × 10⁷ cells ml⁻¹ in fresh medium (Chaffin & Sogin, 1976; Mitchell & Soll, 1979a; Chaffin, 1984a). For resumption of yeast growth, cells were resuspended in fresh medium at 27–28 °C, pH 7-4, or at 37 °C, pH 4-5. For germ tube formation, cells were resuspended at 37 °C, pH 7-4. Exponential yeast cultures (approximately 10⁶–10⁷ cells ml⁻¹) were obtained by diluting growing cells into fresh medium. Cultures were incubated for at least one generation or overnight (seven to eight population doublings) before measurements were made.

For monitoring the incorporation of radiolabelled leucine and uracil into cells, cultures were grown as described except that [4,5-³H]leucine (50 Ci mmol⁻¹; 1850 GBq mmol⁻¹) and [2⁻¹⁴C]uracil (57 mCi mmol⁻¹; 2-1 GBq mmol⁻¹) were added at 10 μCi ml⁻¹ and 1 μCi ml⁻¹ respectively when monitoring was initiated.
Radiolabelled compounds were obtained from ICN Pharmaceuticals, Irvine, Calif., USA. At various times 200 μl portions of culture were removed and added to 3 ml ice-cold trichloroacetic acid (TCA) (5%, w/v). After a minimum of 1 h on ice, samples were filtered through glass fibre filters (GF/A filters; Whatman). Filters were washed 3 times with 3–4 ml cold TCA and dried. Filters were counted in Handifluor (Mallinkrodt Inc., St Louis, Mo., USA) with a Packard Tri-carb 460C automatic liquid scintillation system.

Cell number was determined in a Coulter counter. Before counting cells were sonicated for 30 s at 50 W with a Branson sonicator. In experiments in which incorporation of radioactive label into cells was monitored, cell number was determined microscopically in a counting chamber. In other experiments growth was followed with a Klett–Summerson colorimeter. Tunicamycin was obtained from Sigma.

Light and fluorescence microscopy. In germ tube cultures the extent of morphological conversion was monitored by light microscopy as previously described (Chaffin & Sogin, 1976). The criterion for yeast–mycelial transition was the production of a recognizable germ tube whose length was at least one diameter of the parent yeast cell. In treated cultures, some aberrant morphologies were observed, particularly at the apical end of the germ tubes. Such cells were counted as undergoing the morphological conversion if the non-aberrant portion of the structure otherwise met the criterion. In some experiments the proportion of yeast cells with buds was determined by light microscopy. A Zeiss Standard RA microscope was used in all experiments.

Mithramycin (Pfizer) was used to stain nuclei (Slater, 1976). A sample of culture was added to an equal volume of 50% (v/v) aqueous ethanol containing 0.4 mg mithramycin ml⁻¹ and 30 mM-MgCl₂. Cells were stained overnight at 4°C. Cells were viewed with a Zeiss Standard RA microscope equipped for fluorescence. Exciter filters BG38 and UGI and a barrier filter of 470 nm were used. Discrete spots or short streaks were counted as nuclei. A nucleus which appeared to be dividing, or two spots connected by a continuous fluorescent filament, were counted as a single nucleus.

Septa were stained with Calcofluor ST (American Cyanamid). Cells were collected from culture by centrifugation and resuspended in 0.1% Calcofluor ST and counted immediately as previously described (Chaffin, 1984a). A fluorescent line across the germ tube was counted as a septum irrespective of intensity. Approximately 200 or more cells were counted for each microscopic determination.

RESULTS

Effect of tunicamycin on resumption of growth by stationary phase yeast cells

Stationary phase yeast cells of C. albicans can resume growth either as budding yeasts or as germ tubes. The growth phenotype of cells released in fresh medium is dependent on the conditions. The effect of tunicamycin on initiation of growth in each morphology was examined by addition of the drug to the fresh medium in which the cells were resuspended. The presence of the drug prevented bud formation at both 27°C, pH 7.4, and 37°C, pH 4.5 (Fig. 1). Budded cells appeared earlier in untreated cultures growing at 37°C. The presence of the drug also prevented germ tube formation under conditions favouring that morphology (Fig. 1). Both drug concentrations used in this experiment, 3 μg ml⁻¹ and 8 μg ml⁻¹ were equally effective. The effectiveness of lower concentrations in inhibiting yeast growth was examined spectrophotometrically. Concentrations as low as 0.1 μg ml⁻¹ caused some inhibition at 7 h (420 min) but permitted partial or maximum growth at 23 h (1380 min). At 3 μg ml⁻¹ growth was inhibited at 23 h.

Effect of tunicamycin on elongating germ tubes

The effect of tunicamycin on cells which had initiated growth was examined by addition of the drug to cultures at various times after stationary phase cells were resuspended in fresh medium (Fig. 2). Cultures in which germ tubes were not observed at the time of drug addition were inhibited. Cultures in which some germ tubes were present at the time of drug addition showed an increase in the proportion of germ tubes over the fraction present at the time of addition, although maximal conversion was inhibited. This latter observation suggested that inhibition of growth might be delayed after addition of the drug.

The possibility of delayed inhibition was examined by addition of the drug to a culture where the majority of cells were elongating hyphae. Tunicamycin was added at 150 min which permitted nearly maximal conversion in treated cultures but which was early enough to permit observation of individual hyphae in control cultures. Approximately 90% or more of the cells in control and treated cultures formed germ tubes (Fig. 3). The number of septa and nuclei in germ
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Fig. 1. Effect of tunicamycin on resumption of growth by stationary phase yeast cells. Yeasts were grown to stationary phase and resuspended in fresh medium under conditions favouring growth as either yeasts or germ tubes as described in Methods. (a) Cells resuspended under conditions favouring germ tube formation (37 °C, pH 7.4) in the absence of tunicamycin (●) or in the presence of tunicamycin at 3 µg ml⁻¹ (▲) or 8 µg ml⁻¹ (□). (b) Cells resuspended under conditions favouring bud formation and yeast growth. Cells were resuspended at 37 °C, pH 4.5, in the absence of tunicamycin (●) or in the presence of tunicamycin at 3 µg ml⁻¹ (▲) or 8 µg ml⁻¹ (□). Cells were also resuspended in fresh medium at 27 °C, pH 7.4, in the absence of tunicamycin (○) or in the presence of tunicamycin at 3 µg ml⁻¹ (▲) or 8 µg ml⁻¹ (□).

Fig. 2. Addition of tunicamycin to cultures forming germ tubes. Tunicamycin (8 µg ml⁻¹) was added at various times after resuspension of stationary phase cells in fresh medium at 37 °C, pH 7.4. Filled symbols show the percentage of germ tubes in the culture at the time of addition of antibiotic and the corresponding open symbol shows the percentage of germ tubes in the same culture at 240 min.

Fig. 3. Inhibition of germ tube growth by tunicamycin. Stationary phase yeast cells were resuspended under conditions favouring germ tube formation. Germ tube formation was monitored in one culture in the absence of tunicamycin (●) and in a second culture to which tunicamycin (8 µg ml⁻¹) was added 150 min after resuspension as indicated by the arrow (○). Nuclear content and septum formation were determined as described in Methods for both cultures. ▲, Septa in untreated culture; △, septa in treated culture; ■, nuclei in untreated culture; □, nuclei in treated culture.
tubes was determined. In the untreated culture, there was a linear increase in the average number of nuclei per germ tube or hypha. In the presence of tunicamycin there was a similar increase for approximately 100 min before a plateau of two nuclei per germ tube was attained. Formation of septa also showed a linear increase in the control culture. In the presence of tunicamycin, the formation of septa continued to increase for approximately 75 min before reaching a plateau of one septum per germ tube. Following addition of tunicamycin, approximately 80% of the cells completed nuclear division and 65% of the cells initiated a septum. When increases ceased, 85–90% of the cell had two nuclei and one septum.

Effect of tunicamycin on budding yeasts

The antibiotic was added to an exponentially growing yeast culture and the cell number was monitored (Fig. 4). Cell number continued to increase but at a slower rate than before drug addition or in the control culture. After an increase of 45% in cell number over the next 100 min, the increase stopped and the cell number remained unchanged. The average increase for six cultures was 44 ± 4%. A similar experiment was done at 3, 8 and 15 μg tunicamycin ml⁻¹ and growth was monitored spectrophotometrically. At all three concentrations, the treated and control cultures showed a parallel increase for approximately 180 min, and then treated cultures showed a slower rate of increase for the remainder of the observation period. The differences between monitoring growth by cell number and by spectrophotometry suggest that cells may increase in size even after the increase in cell number had ceased.

The pattern of inhibition of increase in cell number (Fig. 4) is typical for a yeast first cycle arrest (Hartwell, 1974). To examine the possibility that the antibiotic inhibited a specific step in the yeast cell cycle resulting in cells accumulating with a uniform morphology, the proportion of budded cells in the population was determined (Fig. 5). Upon addition of the antibiotic to a culture growing at 27 °C there was an initial decrease in the proportion of budded cells. However, the decrease did not continue and cultures in which growth had ceased contained both budded and unbudded yeasts. Consequently, cells did not accumulate in a single stage of the cell cycle as determined by budding. Progress through the cell division cycle was monitored in subsequent experiments by observing bud size and nuclear division. The proportion of cells with
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Table 1. Distribution of cell types in the presence of tunicamycin

The number of cells and distribution of cell types were determined in an exponential phase yeast culture growing at 27 °C, pH 7.4, at the time of addition of 5 µg tunicamycin ml⁻¹, and again after the increase in cell number had ceased. The distributions were normalized to 100 cells at the time of addition of tunicamycin and the percentage of each cell type is given in parentheses.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Unbudded</th>
<th>Small bud</th>
<th>Large bud, one nucleus</th>
<th>Large bud, two nuclei</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>−Tunicamycin</td>
<td>20 (20)</td>
<td>32 (32)</td>
<td>24 (24)</td>
<td>24 (24)</td>
<td>100 (100)</td>
</tr>
<tr>
<td>+Tunicamycin</td>
<td>44 (30)</td>
<td>38 (27)</td>
<td>16 (11)</td>
<td>41 (28)</td>
<td>142† (100)</td>
</tr>
</tbody>
</table>

* Small bud, diameter less than half of parent cell diameter; large bud, diameter greater than or equal to half of parent cell diameter.
† Includes a few aberrant cells not tabulated in the cell types.

large and small buds and the number of nuclei at the time of drug addition and in the inhibited culture are shown for a typical experiment in Table 1. Two cell types, unbudded cells and cells with large buds and two nuclei, increased in proportion while cells with small buds and cells with large buds and one nucleus decreased. In addition some cells appeared aberrant. Two of the most noticeable abnormalities were cells which appeared to have two buds and cells in which both nuclei were in the parent cell.

**Effect of tunicamycin on accumulation of protein and nucleic acid**

Since tunicamycin is not a primary inhibitor of protein or nucleic acid biosynthesis, cells should continue to accumulate protein and nucleic acid for some time following addition of the drug. The possibility was considered that the cessation of changes in the morphological characteristics monitored in the previous experiments coincided with the cessation of the accumulation of protein and nucleic acid. The accumulation of protein and nucleic acid was monitored by determining the incorporation of radiolabelled leucine and uracil into TCA precipitable material. The antibiotic was added to an exponentially growing yeast culture which contained [³H]leucine and [¹⁴C]uracil and incorporation was monitored (Fig. 6). Net accumulation of radiolabelled precipitable material continued essentially unchanged for nearly 200 min and then increased at a reduced rate compared to the control culture for the duration of the observation period. The cell number increased, as previously observed (Fig. 4), for approximately 100 min before ceasing.

The accumulation of protein and nucleic acid in cells resuming growth from the stationary phase in either growth form was examined. Stationary phase cells were resuspended under conditions favouring the resumption of growth as budding yeast. Tunicamycin was added immediately to prohibit growth resumption in the treated culture. Although total accumulation of radiolabelled material was low for the first 200 min, both treated and control cultures showed similar incorporation (Fig. 7a, c). As the incubation continued, although both cultures accumulated radiolabelled material more rapidly, the treated culture did so to a lesser extent than the control culture.

The effect of tunicamycin on germ tube formation and macromolecule accumulation was examined under conditions favouring the formation of germ tubes. Germ tubes did not form in the treated culture, compared with about 95% conversion in the control. During the initial 150 min, a similar low accumulation of labelled leucine and uracil into precipitable material was observed in treated and control cultures (Fig. 7b, d). Subsequently, while the control culture showed a rapid increase in accumulated radiolabelled material, the treated culture showed a slower increase before ceasing. When tunicamycin was added to a culture in which many cells had initiated morphological conversion and were elongating hyphae, accumulation of radiolabelled material was similar in both control and treated cultures for approximately 150 min (Fig. 7b, d). Subsequently, accumulation continued in the treated culture more slowly than in the control culture.
Fig. 6. Effect of tunicamycin on accumulation of radiolabelled protein and nucleic acid in cultures of exponentially growing yeast. $[^{3}H]$Leucine (10 µCi ml$^{-1}$) and $[^{14}C]$uracil (1 µCi ml$^{-1}$) were added to 22 ml exponential phase yeast culture incubated at 28 °C and the culture was immediately divided into two parts and incubation continued. At the time indicated by the arrow tunicamycin (8 µg ml$^{-1}$) was added to one culture. Portions (200 µl) of each culture were removed at various times and the accumulation of TCA precipitable radiolabelled material determined as described in Methods. Accumulation of TCA precipitable material containing (a) $[^{3}H]$leucine and (b) $[^{14}C]$uracil in the absence (○) or presence (●) of tunicamycin.

DISCUSSION

The observations in this study confirmed previous reports that tunicamycin inhibits growth of *C. albicans* (Takatsuki *et al.*, 1971, 1972), and also showed differences in the effect(s) of the antibiotic on resting and growing cells. In the presence of the drug, stationary phase yeast cells were unable to resume growth (Fig. 1). Under appropriate conditions supporting either yeast growth or morphological conversion, cells failed to initiate either buds or germ tubes.

When tunicamycin was added to growing cells, inhibition was not immediately observed. When it was added to cultures in the early stages of morphological conversion, the proportion of converted cells increased although maximum conversion was not reached (Fig. 2). Following addition of tunicamycin to cultures with elongating hyphae, nuclear division and septum formation continued for some time (Fig. 3). Septum formation occurs in stages which are initiated before and after nuclear division (Soll *et al.*, 1978; Mitchell & Soll, 1979b). In the experiments reported here, the stage of septum formation was not determined. However, since the first stage detected by the method used precedes nuclear division (Soll *et al.*, 1978; Mitchell & Soll, 1979b), changes in the number of septa reflect initiation of this pre-nuclear division stage in septal formation. Consequently, increases in septa were observed before increases in nuclei (Fig. 3). These observations were consistent with the majority of cells initiating and/or completing the round of nuclear division and septum formation and becoming inhibited in the next round. Until increases in nuclei and septa ceased, there was no apparent diminution in rate compared to the control culture. The majority of these tunicamycin-inhibited cells (85–90%) had two nuclei and one septum.

Addition of tunicamycin to exponential yeast cultures resulted in an approximately 45% increase in cell number before cell division ceased (Fig. 4). The frequency of budded cells decreased (Fig. 5) but cells in inhibited cultures did not accumulate in a uniform morphological state (Table 1). An examination of the distribution of cell types shown in Table 1 suggested that
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Fig. 7. Effect of tunicamycin on accumulation of radiolabelled protein and nucleic acid in cultures resuming growth in either morphology. Stationary phase yeast cells were resuspended in fresh medium containing [3H]leucine (10 μCi ml⁻¹) and [14C]uracil (1 μCi ml⁻¹). Portions (200 μl) were removed from each culture at various times and the accumulation of TCA precipitable material determined as described in Methods. For resumption of growth in the yeast form, cells were resuspended in 22 ml medium at 28 °C, pH 7.4. The culture was divided into two parts and tunicamycin (8 μg ml⁻¹) was added to one culture immediately as indicated by the arrow. Accumulation of TCA precipitable material containing (a) [3H]leucine and (c) [14C]uracil in the absence (○) or presence (□) of tunicamycin. For resumption of growth in the hyphal form, cells were resuspended in 33 ml medium at 37 °C, pH 7.4. The culture was divided into three parts and tunicamycin (8 μg ml⁻¹) was added to one culture immediately or at 150 min as indicated by the arrows. Accumulation of TCA precipitable material containing (b) [3H]leucine and (d) [14C]uracil in the absence (○) or presence of tunicamycin added at 0 min (○) or 150 min (□).

cells must be able to complete one or more subsequent stages in the cell division cycle before ceasing growth. Since parent cells bud more rapidly following cell division than do daughter cells (Chaffin, 1984b), cells with large buds may complete cell division with many of the parent cells initiating a further bud before growth ceases, while initially unbudded cells may reach the large bud stage before ceasing growth. The distribution of inhibited yeast cells among various cell stages contrasted with the accumulation of most cells initiating germ tube formation in a single type. Tunicamycin addition to an exponential yeast culture of Saccharomyces cerevisiae similarly results in an approximately 50–70% increase in cell number, a decrease in budded cells from 60 to 15% and an accumulation of 85% of the inhibited cells in the unbudded state (Arnold & Tanner, 1982). This accumulation in a uniform type is consistent with inhibition of a cell cycle stage showing first cycle arrest, and Arnold & Tanner (1982) suggest that tunicamycin inhibits the G1/S phase transition in the cell cycle. As expected, stationary phase yeasts are unable to initiate budding in the presence of the antibiotic.

Although some homologues of tunicamycin may inhibit protein synthesis in addition to N-glycosylation of proteins (Duskin & Mahoney, 1982), the effects of the antibiotic are generally attributed to changes in protein glycosylation. The addition of tunicamycin to growing yeast or hyphal cultures did not reduce accumulation of protein and nucleic acid for 150–200 min (Figs 6 and 7). Subsequently, accumulation of radiolabelled TCA precipitable material continued at a reduced rate in treated cultures for the period of observation. When added to cultures of stationary phase cells before the resumption of growth, both treated and control cultures accumulated radiolabelled precipitable material at a similar rate for the first 150–200 min (Fig. 7). When control cultures began to accumulate net precipitable material at an accelerated rate, treated cultures did so at a reduced rate. Only in the treated culture resuming growth in the hyphal form was a plateau reached during the period of observation. Comparison of the changes
in morphological characteristics and the accumulation of protein and nucleic acid reveals some differences. In growing germ tube cultures, septal formation and nuclear division continued at the same rate until reaching a plateau value after about 75-100 min. In yeast cultures, cell division continued at a reduced rate for about 100 min and then ceased. Under similar conditions, the accumulation of radiolabelled precipitable material was unchanged during this period. However, with time, treated cultures showed a reduced rate of accumulation, which continued throughout the observation period. When tunicamycin was added to non-growing cultures, cells in such cultures showed no morphological sign of resuming growth in either form. In contrast, there was no difference in the accumulation of TCA precipitable macromolecules during the first stages of the lag period in treated and control cultures. Subsequently, when control cultures began more rapid accumulation, treated cultures accumulated precipitable material at a slower rate. In addition, differences in growth as monitored by cell number and spectrophotometry suggested the continuance of growth after cell division ceased. These differences suggest that the processes being monitored (cell and nuclear division, bud emergence, germ tube formation and septum formation) ceased before general reduction or cessation of growth. This observation suggested that these processes themselves or a preceding coupled process were directly affected by inhibition of N-glycosylation of proteins. In contrast, general protein and nucleic acid biosynthesis appeared to be less dependent on protein glycosylation.

Arnold & Tanner (1982) postulated that in S. cerevisiae the G1/S phase transition required N-glycosylation of a protein(s) and that the presence of the inhibitor prevented stationary phase cells from emerging from G1 and allowed growing cells beyond G1 to continue into but not past the subsequent G1 phase. Since growth of Burkitt lymphoma cells is also stopped in G1 in the presence of the drug (Nishikawa et al., 1980), Arnold & Tanner (1982) suggested that a role for N-glycosylated proteins in the G1/S phase transition might be a general one. The failure of stationary phase yeast cells of C. albicans to initiate bud or germ tube formation in the presence of the drug is consistent with such a hypothesis. However, the observations on growing yeasts suggested the cell cycle could be blocked at more than one point. The accumulation of unbudded cells, budded cells with a single nucleus and budded cells with two nuclei suggested that cells could be blocked at the G1/S transition, between the S phase/bud emergence and nuclear division, and at cell division respectively. Bud emergence and the S phase begin at the same time (Wain et al., 1976). The accumulation of cells with different sized buds may represent more than one blocked site between bud emergence and nuclear division. As discussed above, each of the stages was traversed by some cells following addition of the drug. Two possible explanations for this observation, consistent with inhibition of N-glycosylation affecting function, are the following. First, sufficient factor(s) remained from the previous cell cycle to permit continuance of the cycle for a period. Second, the synthesis of factors affected by tunicamycin and their execution point are not tightly coupled in the cell cycle, so that the factor(s) being produced at the time of drug addition were required not at the immediate but at a subsequent cell cycle stage. In germ tube cultures it was not possible to distinguish a G1/S block from blockage at multiple sites as observed with yeasts. The majority of germ tubes accumulated at a stage consistent with a G1/S block (or at least before a new round of nuclear division and septum formation). However, the synchrony of the morphogenetic conversion results in a fairly homogeneous population at the time of drug addition. Consequently, even if there are multiple blockage sites as in yeasts, the homogeneity could result in accumulation at one point.

The observations in this report demonstrated that tunicamycin is an inhibitor of growth in C. albicans. General growth, as monitored by accumulation of protein and nucleic acid, was not immediately affected by addition of the drug but continued unchanged for some time before slowing to a reduced rate. In contrast, some processes showed an immediate slowing in rate before ceasing (cell division in yeast) while other processes continued at the same rate before ceasing (nuclear division and septum formation in germ tubes). In any case the inhibited process ceased before the general net accumulation of protein and nucleic acid showed changes. Since the inhibition of the processes probably resulted from failure to produce N-glycosylated
proteins, such proteins may play a fundamental role in the processes. Since only a small number of cellular processes were monitored, the number and identity of the cellular processes dependent on N-glycosylation of proteins for normal function remain to be determined.

REFERENCES


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