Antisense RNA inactivation of gene expression of a cell-cell adhesion protein (gp64) in the cellular slime mold Polysphondylium pallidum

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SUMMARY

The gp64 protein of Polysphondylium pallidum has been shown to mediate EDTA-stable cell-cell adhesion. To explore the functional role of gp64, we made an antisense RNA expression construct designed to prevent the gene expression of gp64; the construct was introduced into P. pallidum cells and the transformants were characterised. The antisense RNA-expressing clone L3mc2 which had just been harvested at the growth phase tended to re-form in aggregates smaller in size than did the parental cells in either the presence or absence of 10 mM EDTA. In contrast, 6.5-hour starved L3mc2 cells remained considerably dissociated from each other after 5 minutes gyrating, although aggregation gradually increased by 50% during a further 55 minutes gyrating, in the presence of 10 mM EDTA. Correspondingly, L3mc2 lacked specifically the cell-cell adhesion protein, gp64. We therefore conclude that the gp64 protein is involved in forming the EDTA-resistant cell-cell contact. In spite of the absence of gp64, L3mc2 exhibited normal developmental processes, a fact which demonstrates that another cell-cell adhesion system exists in the development of Polysphondylium. This is the first report in which an antisense RNA technique was successfully applied to Polysphondylium.

Key words: Cellular slime mold, Polysphondylium pallidum, Cell-cell adhesion protein, gp64, Antisense RNA

INTRODUCTION

The cellular slime mold Polysphondylium pallidum is a eukaryotic microorganism with a life cycle that alternates between monocellular and multicellular stages. Starvation triggers a developmental program in which less than 10^5 cells stream together to form a multicellular aggregate that proceeds through several stages and culminates in the formation of a branching fruiting body. Cell to cell contact is important in the formation of streams of cohering cells.

A related genus, Dictyostelium discoideum, has two types of contact sites: contact site A and contact site B. EDTA-resistant adhesion sites specific for aggregation are blocked with adhesion-blocking antibody fragments (Fab) against contact site A (Beug and Gerisch, 1972; Mueller and Gerisch, 1978), while contact site B is an EDTA-sensitive site and is expressed in growth and aggregation phase cells (Gerisch et al., 1980). The sequence by which a cDNA encodes contact site A has been determined (Noegel et al., 1986; Kamboj et al., 1988). This protein is anchored in the membrane by a glycosylphosphatidyl (Stadler et al., 1989), and gp80 homophilic binding has been determined by Kamboj et al. (1989).

A putative cell-cell adhesion protein (referred to as gp64) of Polysphondylium pallidum has been shown to mediate EDTA-resistant cell-cell binding (Bozzaro and Gerisch, 1978; Bozzaro et al., 1981). A monoclonal antibody (mAb293) raised against gp64 completely blocked cell-cell adhesion of P. pallidum, although the antibody will react with many antigens (Toda et al., 1984a). The inhibitory effect can be cancelled with an excess of L-fucose, a probable terminal sugar of the cell-surface carbohydrates, suggesting that mAb293 recognizes a sugar moiety of gp64 (Toda et al., 1984a,b). Recently, we cloned a cDNA for gp64 (1.1 kb), which also showed extensive expression of its mRNA in the late vegetative stage, accumulation of the protein during aggregation, and a decrease at later stages (Manabe et al., 1994). Moreover, we have found that gp64 possesses both a glycolipid anchor (Saito and Ochiai, 1993) and Sushi domains which seem to be involved in protein-protein interactions (Saito et al., 1994).

The use of antisense RNA to block the expression of selected genes is a powerful tool (Crowley et al., 1985; Knecht and Loomis, 1987; Colman, 1990; Fang et al., 1993). The antisense technique should be particularly useful for working with P. pallidum, since we lack the established technique to knock out genes by homologous recombination which is available for use with Dictyostelium. We here report the use of the antisense RNA technique to block the expression of the gp64 gene in P. pallidum cells, and describe the characterization of a mutant cell line with respect to adhesiveness and morphology during development.

MATERIALS AND METHODS

Strain and cell culture
The P. pallidum strain WS320 was grown according to the method of
Manabe et al. (1990). To cultivate it axenically, cells were grown in A-medium (Sussman, 1963, 1987). To cultivate it on solid agar plates, cells were grown on nutrient-agar plates (LP) in association with *Escherichia coli* B/r (Francis et al., 1991). All the transformants were grown under the selective conditions of the aminoglycoside antibiotic G418 (at 100 μg/ml in suspension culture, and 200 μg/ml on agar plates), unless otherwise noted.

**Antisense-RNA construct and transformation**

An antisense RNA construct, pLDL3, was created to inhibit the expression of the mRNA of gp64. pLDL3 was based on pLD-1 (the gift of Dr Lucy Drury) which contained a replication origin of the *Dictyostelium* plasmid Ddp2 and a neomycin phosphotransferase gene from Tn5. A 0.8 kb fragment (XbaI digested, blunt ends) and an 0.7 kb fragment (HindIII digested, blunt ends) from pDneo (Witke et al., 1987; the gift of Dr W. Nellen), which contains, respectively, an actin 6 promoter and an actin 8 terminator, were inserted into the XbaI site of pLD-1 after their ends had been blunted (this was named the parental vector pLDL1). To make a gp64 antisense RNA construct, we inserted a cDNA fragment of gp64 downstream of the actin 6 promoter in an antisense orientation relative to the actin promoter (named antisense RNA construct pLDL3). The resultant plasmids were finally selected by confirming the occurrence of the expected cDNA fragments cleaved with restriction enzymes.

*P. pallidum* transformation was carried out by electroporation (Nellen et al., 1984; Howard et al., 1988; Dynes and Firtel, 1989). Namely, *P. pallidum* cells were grown in shaken suspension up to 1×10⁶ cells/ml, washed free of bacteria by centrifugation, and resuspended in an electroporation buffer at 1×10⁶ cells/ml. The supercoiled DNA of the constructs were added to the cell suspension to a final concentration of 100 μg/ml. The resultant mixture was transferred to a 2 mm electroporation cuvette, incubated on ice for 10 minutes, then transferred onto plastic dishes containing HL-5 medium. After 6 hours, the cells were harvested and plated in the presence of G418 at 200 μg/ml on lawns of G418-resistant *E. coli* (Nellen et al., 1984; Hughes et al., 1992; Vocke and Cox, 1992). G418-resistant *E. coli* was obtained from Dr D. L. Welker (Utah State University). After several days, transformants appeared as plaques and were recloned.

**Southern and northern blot analysis**

Genomic DNA was digested with both EcoRI and PstI. The resultant DNA fragments were separated on a 1% agarose gel, transferred to Hybond N+ (Amersham Co., Japan) and probed with a 1.1 kb gp64 DNA fragment labelled with horseradish peroxidase or a 0.7 kb fragment (HindIII digested, blunt ends) from pDneo (Witke et al., 1987; the gift of Dr W. Nellen), which contains a replication origin of the *Dictyostelium* plasmid Ddp2 and a neomycin phosphotransferase gene from Tn5. Total RNA (3 μg) was separated in an 1.2% agarose gel containing 2.2 M formaldehyde and transferred to Hybond N+. The sense and antisense transcripts of gp64 were detected with the gp64 cDNA probe. The hybridization procedure was performed by means of the ECL™ gene-detection system (Amersham Co., Japan).

**SDS-PAGE and immunoblotting**

Proteins from crude membrane fractions (equivalent to 4×10⁵ cells) were electrophoretically separated on 7.5% SDS-polyacrylamide gels (Laemmli, 1970) and blotted on a PVDF filter (Nihon Millipore Ltd, Japan). gp64 was detected with mAb1E5, used to recognize a peptide moiety of gp64 glycoprotein. Carbohydrates on gp64 and other glycoproteins were probed with mAb293, specific for a fucose-containing structure (Toda et al., 1987). The epitope was indirectly detected with ECL™ western blotting detection reagents (Amersham Co., Japan).

**Measurement of cell adhesion**

Cell-cell adhesion was measured by a combined modification of methods (Knecht et al., 1987; Brar and Siu, 1993; C.-H. Siu, personal communication). Cells were cultured with bacteria in shaken suspension, harvested in late growth phase, and washed free of bacteria; the washed cells were adjusted to 1×10⁷ cells/ml with a 17 mM phosphate buffer, some were immediately used to measure cell-cell adhesion (t₀ cells), and the residual cells were allowed to starve for 6.5 hours in the buffer (t₆.₅ cells). The cells were diluted to 2.5×10⁸ cells/ml and cell aggregates were completely dissociated by vortexing for 5 seconds. A suspension (0.6 ml) was gyrated at 180 rpm in a silliconized scintillation vial to allow cells to re-form aggregates in the presence or absence of 10 mM EDTA. At appropriate time intervals, the number of nonaggregated cells, including both singlets and doublets, was counted by means of a hemocytometer.

**Development of transformants on filters**

Cells were harvested in late growth phase, and washed free of bacteria. The washed cells were put on filters at a cell density of 5.4×10⁶ cells per mm², and allowed to develop.

**RESULTS**

**Cells transformed with the gp64 antisense RNA vector**

To generate transformants expressing antisense RNA of gp64 mRNA, we used the antisense construct pLDL3 (Fig. 1). This vector was based on pLD-1, which contained a replication origin of *Dictyostelium* plasmid Ddp2. The choice of this vector is basically due to two reasons: first, the copy number of Ddp2 in the haploid genome is high (300 in *Dictyostelium*; Leiting et al., 1990), by which overwhelming antisense RNAs would be produced over the endogenous target RNA in *Polysphondylium*. Second, by the use of an extrachromosomal vector harmful positional effects of random DNA integration into the genome would be avoidable. To estimate the copy number and stability of the extrachromosomal vector, we inserted a cDNA fragment of gp64 downstream of the actin 6 promoter in an antisense orientation relative to the actin promoter (named antisense RNA construct pLDL3). The resultant plasmids were finally selected by confirming the occurrence of the expected cDNA fragments cleaved with restriction enzymes.

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Genomic DNA was digested with both EcoRI and PstI. The resultant DNA fragments were separated on a 1% agarose gel, transferred to Hybond N+ (Amersham Co., Japan) and probed with a 1.1 kb gp64 cDNA fragment labelled with horseradish peroxidase or a 0.9 kb PstI fragment of the neomycin phosphotransferase gene, Tn5.

Total RNA (3 μg) was separated in an 1.2% agarose gel containing 2.2 M formaldehyde and transferred to Hybond N+. The sense and antisense transcripts of gp64 were detected with the gp64 cDNA probe. The hybridization procedure was performed by means of the ECL™ gene-detection system (Amersham Co., Japan).

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Cells were harvested in late growth phase, and washed free of bacteria. The washed cells were put on filters at a cell density of 5.4×10⁶ cells per mm², and allowed to develop.

**Antisense gp64 construct pLDL3**

This vector is based on pLD1, consisting of a replication origin of *Dictyostelium* plasmid Ddp2 and the Tn5 neomycin phosphotransferase gene driven from the *Dictyostelium* actin 6 promoter. This vector confers resistance to the antibiotic G418 on *Polysphondylium*. To express complementary transcripts against endogenous gp64 mRNA, the complete gp64 coding unit is inserted downstream of the actin 6 promoter of the vector in an antisense orientation. The antisense gp64 RNA is terminated by a *Dictyostelium* actin 8 terminator.
grown in G418 at 100 m g/ml; L3mc2(-G418), DNA from an antisense RNA transformant carrying construct pLDL3 from the vector control carrying vector pLD1 alone; L3mc2, DNA from parental P. pallidum cells; L131, DNA from the vector control carrying vector pLD1 alone; L3mc2, DNA from a gp64 antisense RNA transformant carrying construct pLDL3 grown in G418 at 100 µg/ml; L3mc2(-G418), DNA from an antisense RNA transformant grown for 20 generations in the absence of G418. After digesting genomic DNA of the L3mc2 clone with EcoRI and PstI, both 5.1 kb (containing endogenous gp64 gene) and 1.1 kb (containing gp64 cDNA) fragments were detected with a gp64 cDNA probe (a). When these fragments were compared with their intensities, the L3mc2 seems to contain >10 copies of the construct pLDL3 per haploid genome. A 0.9 kb fragment was detected in the transformants with a Tn5 probe (b). In the case of L3mc2(-G418), however, neither gp64 cDNA nor Tn5 sequences were detected, indicating that the construct pLDL3 had been deleted from the transformant by cultivating the L3mc2 clone without G418 for 20 generations.

Fig. 2. Southern blot analysis of DNA from transformants. Genomic DNA was digested with EcoRI and PstI, and the resulting fragments were first separated in a 1% agarose gel, then transferred to nylon membrane Hybond N+. The filters were probed with a gp64 cDNA probe prepared by the ECL gene-detection system (Amersham, Japan). Lanes: pLDL3, DNA from the gp64 antisense RNA construct; P. pal, DNA from parental P. pallidum cells; L131, DNA from the vector control carrying vector pLD1 alone; L3mc2, DNA from a gp64 antisense RNA transformant carrying construct pLDL3 grown in G418 at 100 µg/ml; L3mc2(-G418), DNA from an antisense RNA transformant grown for 20 generations in the absence of G418. After digesting genomic DNA of the L3mc2 clone with EcoRI and PstI, both 5.1 kb (containing endogenous gp64 gene) and 1.1 kb (containing gp64 cDNA) fragments were detected with a gp64 cDNA probe (a). When these fragments were compared with their intensities, the L3mc2 seems to contain >10 copies of the construct pLDL3 per haploid genome. A 0.9 kb fragment was detected in the transformants with a Tn5 probe (b). In the case of L3mc2(-G418), however, neither gp64 cDNA nor Tn5 sequences were detected, indicating that the construct pLDL3 had been deleted from the transformant by cultivating the L3mc2 clone without G418 for 20 generations.

Differential expression of gp64 gene in transformant L3mc2 grown in different conditions

We examined the amounts of endogenous sense and antisense RNA transcripts in L3mc2 grown under three different growth conditions (Fig. 3). In all growth conditions, namely in shaken suspension in association with bacteria, on nutrient agar plates in association with bacteria or in suspension in an axenic medium, all wild-type cells expressed gp64 mRNA at approximately equal levels. Similarly, transformant L3mc2 expressed the gp64 antisense transcript at equal levels under these conditions. Surprisingly, however, the amount of gp64 mRNA in the antisense transformants varied dramatically depending on the growth medium: while the message was reduced in the presence of bacteria by a factor of about 10 (we measured the relative intensity of the sense messages by densitometric tracings), it was almost unaffected in cells grown in the axenic medium. This indicates a differential efficiency of antisense mediated gene silencing that depends on the physiological state of the cell, as Sadiq et al. (1994) have previously suggested. In addition, our data show that the actin 6 promoter in P. pallidum works well in growth-phase cells grown with E. coli, although it is not so active in D. discoideum under these conditions (Knecht and Loomis, 1987).

To estimate the amounts of gp64 synthesized in L3mc2 cells grown under the different conditions (as described above), we measured the amounts of gp64 using mAb1E5 specific for the peptide moiety of gp64 (Fig. 4a). As described above, gp64 mRNA had been reduced in L3mc2 grown with bacteria and correspondingly gp64 was reduced to about one-tenth of the amount in the parental strain or control transfor-
mant L131 (these cells were pre-cultured in A-medium, after which the cells were transferred to a large-scale culture and cultivated for 5 generations; thus, one-tenth of the residual gp64 would be the gp64 which had been synthesized in cells pre-cultivated in A-medium), while in L3mc2 cells grown in axenic medium gp64 did not decrease significantly. This reflected the observations made of the RNA level and demonstrated again that inhibition by antisense RNA failed when cells were grown in the axenic medium. To see if only the gp64 target or other glycoproteins were also affected, we stained the same blot with mAb293. As Fig. 4b shows, no other proteins were significantly affected. In addition, the overall pattern of glycosylated proteins in the cells grown in axenic medium were denser than those of glycosylated proteins in the cells grown in association with bacteria. This difference can probably be ascribed to the size of the cells grown under the different conditions.

**Cell adhesiveness on L3mc2**

To examine whether gp64 is involved in cell-cell adhesion, we measured cell adhesiveness of L3mc2 cells. Transformant L3mc2 was grown in association with *E. coli*, in which conditions L3mc2 had the lowest gp64 content, harvested during the growth phase, washed free of bacteria and the cell suspensions were adjusted to 2.5×10⁶ cells/ml with a 17 mM phosphate buffer. Alquots of the cell suspension were removed at the indicated times (5 minutes, 15 minutes, 25 minutes, 40 minutes and 60 minutes) and their cell adhesiveness was measured as described in Materials and Methods.

**Fig. 4.** Reduction of gp64 on the transformed cells grown on nutrient-agar LP plates in association with *E. coli* or grown in shaken phosphate buffer in association with *E. coli*. Crude membrane fractions equivalent to 4×10⁵ cells were subjected to 7.5% SDS-PAGE, and then transferred to PVDF membrane (Nihon Millipore Ltd, Japan). The blot was caused to react with mAb1E5, specific for a gp64 peptide moiety, to detect gp64 proteins (a), and with mAb293, specific for a common carbohydrate antigen of glycoproteins, to see the influence of transformations on other glycoproteins (b). Lanes: L131 (-G418), the transformed clone carrying a vector control alone, followed by cultivation without G418 for 20 generations; L3mc2 (-G418), the transformed clone carrying pLDL3 construct, followed by cultivation for 20 generations without G418; other abbreviated names are the same as those in Fig. 2.

**Fig. 5.** Differential kinetics of cell adhesion between the control strains and the transformants. Cells were grown in shaken suspension in association with *E. coli* B/r, harvested at late growth phase, and cell density was adjusted to 2.5×10⁶ cells/ml with a 17 mM phosphate buffer. Alquots of the cell suspension were removed at the indicated times (5 minutes, 15 minutes, 25 minutes, 40 minutes and 60 minutes) and their cell adhesiveness was measured as described in Materials and Methods.

(a) t₀ cells; (b) t₆.₅ cells. (□,■) *P. pallidum* parental strain; (○,●) the vector transformant; (△,▲) the antisense RNA expression transformant. (□,■, ●, ▲) in the presence of EDTA; (◇,○,△) in the absence of EDTA. Results from separate experiments are mean % cell aggregation. The error bars represent s.e.m. (n=3).
gates at a level of about 65% to 90% during 60 minutes gyrating, whereas, with EDTA, there was 50% to 75% cell aggregation, especially in the parental strain. However, L3mc2 showed about 10% less aggregation than both the control strains in the presence or absence of 10 mM EDTA (Fig. 5a). After starvation for 6.5 hours (t6.5 cells), in the absence of EDTA, the parental and L131 strains immediately re-formed aggregates at rather high levels (60%-90%) during 5 minutes gyrating and maintained almost the same levels during further gyrating, while L3mc2 showed a similar kinetics, but showed the lowest level of aggregation. In the presence of 10 mM EDTA, the control clones re-formed somewhat lower levels of aggregates than those of the same clones in the absence of EDTA. By contrast, L3mc2 showed only a very low level (20%) of aggregation at 5 minutes but this increased gradually to 50% during 5 to 60 minutes gyrating (Fig. 5b).

To confirm whether the smaller aggregation level of L3mc2 is due to the reduction of gp64, we examined the amount of gp64 protein by using a monoclonal antibody (mAb1E5). Although the control clones contained gp64 protein in both t0 and t6.5 cells, L3mc2 did not contain any gp64 at two stages (these cells were cultivated in shaken suspension in association with E. coli in both the steps of the pre- and main-culture) (Fig. 6a). Fig. 6 also demonstrates that all the lanes contain almost the same amounts of mAb293-reacted glycosylated proteins (Fig. 6b). In conclusion, dissociated L3mc2 cells show the least reaggregation ability, especially in the presence of EDTA and correspondingly have no gp64 at a detectable level. This fact implies that gp64 protein is involved in EDTA-resistant cell-cell adhesion.

**Development of transformants on filter**

To investigate the effects of a decreased amount of gp64 on development, L3mc2 cells were grown in shaken suspension with E. coli, and harvested at a late growth phase. The washed cells were transferred onto black Millipore filters and allowed to develop. Under these culture conditions none of the gp64 accumulated in the L3mc2 cells, as described above. Although the developmental processes and the morphology of the fruiting bodies of the transformant were essentially normal (Fig. 7), we did notice minor differences between the parental strain and the transformants (L131 and L3mc2); both the transformants had a tendency to culminate a little faster than the parental cells (Fig. 7, t1.5 cells), and they finally formed normal fruiting bodies (Fig. 7, t2.5). There was no significant morphological difference between any of the strains when they were also developed on agar plates.

**DISCUSSION**

Although the technique of homologous recombination is available for *Dictyostelium* (Knecht, 1989), this technique has so far been unsuccessful for *Polysphondylium*. We therefore used the antisense RNA technique for studying the functional role of gp64. To generate transformants that would silence the expression of gp64, we used an extrachromosomal transformation vector containing a replication origin of the *Dictyostelium* plasmid Ddp2. Although we have not directly examined whether this plasmid is replicating extrachromosomally in *Polysphondylium*, we found that our transformant contained constructs of more than 10 copies per haploid genome. Accordingly, we suppose that this extrachromosomal vector is a feasible agent for such transformation experiments with *P. pallidum*, even though the copy number per haploid genome is not so high, compared to in *Dictyostelium*.

It is interesting to consider here the regulation of the expression of actin genes in the cellular slime molds. In the cases of actin promoters, those in the growth-phase cells are active in an axenic medium, but not in bacterial suspensions (Knecht et al., 1986; Nellen et al., 1986; Knecht and Loomis, 1987). In *P. pallidum*, however, the situation is different: the actin 6 promoter is active in cells grown in both a 17 mM phosphate buffer with *E. coli* and an axenic medium. The data suggest that the regulation system of this actin promoter in the growth-phase cells of *P. pallidum* differs from that of *D. discoideum*.

In previous antisense RNA experiments which inhibited the expression of discoidin I (Crowley et al., 1985) and a myosin heavy chain (Knecht and Loomis, 1987), effective antisense inhibition has been observed in cells grown in HL-5 medium. The sense RNA and the respective proteins were reduced with an excess of antisense RNA (Knecht and Loomis, 1987) and without the detection of antisense RNA (although antisense RNA was detected in a ‘Run-on’ assay; Crowley et al., 1985). Interestingly, in our case, both RNA transcripts were observed
in the transformants under all culture conditions. Especially, in L3mc2 cells grown in the axenic medium, neither the endogenous sense RNA nor the gp64 proteins were substantially reduced in spite of an excess of antisense RNA. This may be explained by a lack of antisense RNA available for interaction with mRNA or by the involvement of some additional mechanisms such as inefficient hybridization or inhibition of degradation. Namely, in the cells grown in an axenic medium, sense to antisense RNA hybridization may not occur as a result of a lack of helper proteins to mediate RNA-RNA interaction, following the failure of effective digestion by a double-strand specific RNase (dsRNase) (Nellen and Lichtenstein, 1993; Munroe and Dong, 1992), while sense-antisense hybrid RNAs may not be digested, because of the possible inactivation of dsRNase (Sadiq et al., 1994).

We examined the effect of the reduced amounts of gp64 in relation to cell adhesiveness. L3mc2 cells which had just been harvested at a late-log phase showed a little less agglutinability than those of the control strains, especially in the presence of 10 mM EDTA. After starvation for 6.5 hours, L3mc2 cells showed considerably less adhesiveness than did the controls, especially in the presence of 10 mM EDTA. Nevertheless, L3mc2 cells remained partly associated, although the accumulation of gp64 protein was completely cancelled in L3mc2 cells. These data indicate that there is another, as yet unexplained, adhesion system (or systems) in the early developmental phase of Polysphondylium. Nor were we able to explain what caused such differences of adhesiveness between t0 and t6.5 cells, because it is known that Polysphondylium cells will acquire an additional adhesion component, gp64 II, during the aggregation phase (Bozzaro and Gerisch, 1978; Toda et al., 1987). Nonetheless, the present results demonstrate that gp64 protein is involved in EDTA-resistant cell-cell contact.

Gerisch et al. (1993) recently proposed that ‘the blockage of cell adhesion by Fab of this antibody may simply indicate that one of the proteins carrying this epitope is important for adhesion’, on a reasonable consideration of the procedures used for analysing gp64 up to date. The result of the present experiment suggests that his original idea was probably right, namely that gp64 is likely to be the actual cell-cell adhesion protein of Polysphondylium.

Although Bozzaro and Gerisch (1978) described that in P.

diant formation of polyclonal antibodies against the mercaptoethanol-sensitive structure of a cell-cell adhesion protein of *Polysphondylium pallidum*. J. Biochem. 128, 582-585.


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