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Thioredoxin reductase is required for growth and regulates entry into culmination of *Dictyostelium discoideum*

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Summary

The thioredoxin system, consisting of thioredoxin, thioredoxin reductase and NADPH, has been well established to be critical for the redox regulation of protein function and signalling. To investigate the role of thioredoxin reductase (Trr) in *Dictyostelium discoideum*, we generated mutant cells that underexpress or overexpress Trr. Trr-underexpressing cells exhibited severe defects in axenic growth and development. Trr-overexpressing (Trr^{OE}) cells formed very tiny plaques on a bacterial lawn and had a lower rate of bacterial uptake. When developed in the dark, Trr^{OE} cells exhibited a slugger phenotype, defined by a prolonged migrating slug stage. Like other slugger mutants, they were hypersensitive to ammonia, which has been known to inhibit culmination by raising the pH of intracellular acidic compartments. Interestingly, Trr^{OE} cells showed defective acidification of intracellular compartments and decreased activity of vacuolar H^+-ATPase which functions in the acidification of intracellular compartments. Moreover, biochemical studies revealed that the thioredoxin system can directly reduce the catalytic subunit of vacuolar H^+-ATPase whose activity is regulated by reversible disulphide bond formation. Taken together, these results suggest that *Dictyostelium* Trr may be essential for growth and play a role in regulation of phagocytosis and culmination, possibly through the modulation of vacuolar H^+-ATPase activity.

Introduction

The cellular slime mold *Dictyostelium discoideum* is a lower eukaryote capable of forming a multicellular structure. In the vegetative phase, *D. discoideum* amoebae ingest bacteria by phagocytosis or take up nutrients by pinocytosis. When the food source is exhausted, amoebae secrete cAMP and gather by chemotaxis to form a multicellular aggregate. The aggregate elongates and makes a structure called a finger, or standing slug. The slug migrates to the favourable place to disperse spores and finally forms a fruiting body consisting of a stalk and a spore-containing sorus by a process called culmination (Weijer, 2004).

There is a class of mutants which show a prolonged migrating slug stage and delayed entry into culmination (Sussman *et al*., 1978). Such mutants, called sluggers, are excessively sensitive to ammonia (Gee *et al*., 1994). The weak base ammonia, which is produced in large amounts by protein catabolism during development, has been known to inhibit culmination and thereby cause slugs to continue to migrate (Schindler and Sussman, 1977; Gross, 1994). Its effect on the decision between slug migration and culmination can be mimicked by other weak bases and is mediated by raising the pH of the intracellular acidic compartments (Davies *et al*., 1993). Ammonia accumulates as a protonated form in the acidic compartments (Davies *et al*., 1993) and therefore dissipates the proton gradient that is generated by vacuolar H^+-ATPase (V-ATPase). The mutants defective in acidification of intracellular compartments are abnormally sensitive to inhibition by weak base and show a prolonged slug migration (Davies *et al*., 1996). Although the detailed mechanism remains unclear, it is believed that the pH of the intracellular acidic compartments is an important factor for the regulation of culmination.

Regulation of the intracellular redox environment is critical for a variety of cellular processes. The thioredoxin system comprised of NADPH, thioredoxin and thioredoxin reductase, is a ubiquitous redox system present in all living organisms (Holmgren, 1989). It serves as a hydrogen donor system for the reduction of disulphide bonds in proteins and therefore plays important roles in the defence against oxidative stress (Spector *et al*., 1988; Chae *et al*., 1994) and in regulating DNA synthesis (Koc *et al*., 2006), gene transcription (Schenk *et al*., 1994; Hirota *et al*., 1999; Seemann and Hainaut, 2005), cell growth (Powis *et al*., 1994) and apoptosis (Saitoh *et al*., 1998). Thioredoxin reductase is a member of the pyridine nucleotide-

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disulphide oxidoreductase family of flavoenzymes. It catalyses the transfer of reducing equivalents from NADPH, via FAD and the redox active disulphide, to thioredoxin (Dai *et al*., 1996; Arnér and Holmgren, 2000).

Three thioredoxins, Trx1, Trx2 and Trx3, have been previously described in *D. discoideum* (Wetterauer *et al*., 1992a,b). They constitute a highly conserved multigene family and their expressions are developmentally regulated. Their mRNA levels increase strongly with maximal expression during the aggregation stage and decline in later development stage (Wetterauer *et al*., 1992a). Several homologous Trx1 target proteins have been identified more recently (Brodegger *et al*., 2004). Despite potential importance of thioredoxin reductase as a key regulatory enzyme that determines the redox state of thioredoxin system, its physiological role has not yet been studied in *D. discoideum*. To investigate the role of thioredoxin reductase (Trr) in growth and development of *D. discoideum*, we cloned the gene (*trrA*) encoding thioredoxin reductase and generated the mutants underexpressing or overexpressing Trr. In this article, we describe the properties of Trr mutant strains and suggest that Trr may be essential for cell growth and affect phagocytosis and the decision of culmination, possibly through the modulation of V-ATPase activity.

Results

Identification of trrA *gene encoding Trr in* D. discoideum

The unique gene, *trrA*, encoding putative thioredoxin reductase was identified by searching in the *Dictyostelium* genome database (http://www.dictybase.org). The nucleotide sequence of the *D. discoideum trrA* consists of an open reading frame of 960 bp that encodes a polypeptide of 319 amino acids with a calculated molecular mass of 34.2 kDa. *Dictyostelium* Trr belongs to low-molecular-weight thioredoxin reductase (Williams *et al*., 2000), which has been found in prokaryotes and lower eukaryotes, including fungi and plants (Chae *et al*., 1994; Jacquot *et al*., 1994). The deduced amino acid sequence of Trr from *D. discoideum* shares 59%, 54% and 46% sequence identity with those of thioredoxin reductases from *Saccharomyces cerevisiae* (P29509), *Arabidopsis thaliana* (Q39243) and *Escherichia coli* (P09625) respectively. Like other thioredoxin reductases, the redox active C-X-X-C, FAD- and NADPH-binding domains are well conserved in *D. discoideum* Trr (Fig. 1).

Antisense RNA-mediated inhibition and overexpression of Trr in D. discoideum

To understand a role of Trr in *Dictyostelium*, we generated Trr-underexpressing (MBTRA) or Trr-overexpressing (Trr^{OE}) mutant cells. Despite numerous attempts, we have failed to obtain stable *trrA* null cells by homologous recombination or knock-down cells by constitutive antisense-mediated inhibition. Thus, we generated MBTRA cells, which underexpress Trr conditionally, using tetracycline (Tc)-regulated expression system (see *Experimental procedures*). Tc prevents a transcriptional activator from binding to the inducible promoter, which becomes inactive (Blaauw *et al*., 2000). In the absence of Tc, the transcriptional activator binds to the promoter, leading to the expression of *trrA* antisense RNA. When cultured in the absence of Tc, MBTRA cells showed significant decrease in the expression level and enzyme activity of Trr, compared with KAx3 cells as well as with the MBTRA cells cultured in the presence of Tc (Fig. 2). For overexpression, the construct, in which a full-length *trrA* cDNA was fused behind the constitutively active actin15 promoter, was introduced into KAx3 cells. Overexpression of Trr was confirmed by Western blot analysis. Consistent with significantly increased protein expression, enzyme activity of Trr was approximately eightfold higher in Trr^{OE} cells than the parental KAx3 cells (Fig. 2).

Trr is essential for growth and development

We examined axenic growth and development of MBTRA cells. When cultured in the presence of Tc, MBTRA cells showed a similar growth rate to MB cells transformed with MB35 and empty MB38 vectors. In contrast, MBTRA cells which pre-cultured in the absence of Tc grew very slowly (Fig. 3A). This result suggests that thioredoxin reductase may be required for normal growth in *D. discoideum*.

Inhibition of Trr expression during vegetative growth phase caused more severe defects in early development (Fig. 3B). MB and MBTRA cells, which were cultured either with or without Tc, were harvested at early exponential growth phase, washed with KK_2 buffer, and then plated on black filter (Fig. 3B). MB cells underwent normal development indistinguishable from KAx3 cells; tight aggregates were observed by 12 h, slugs by 16 h and fruiting bodies by 24 h. On the contrary, MBTRA cells cultured without Tc failed to aggregate, probably due to the severe growth defect. Moreover, MBTRA cells even cultured with Tc were delayed in developmental progression; tight aggregates were observed by 16 h and small maturating fruiting bodies by 36 h (Fig. 3B). It might be caused by a decrease in Trr level after starvation; the removal of Tc by washing with Tc-free KK_2 buffer induced the expression of *trrA* antisense RNA (data not shown). These results suggest that Trr may be essential for normal development as well as growth in *Dictyostelium*.

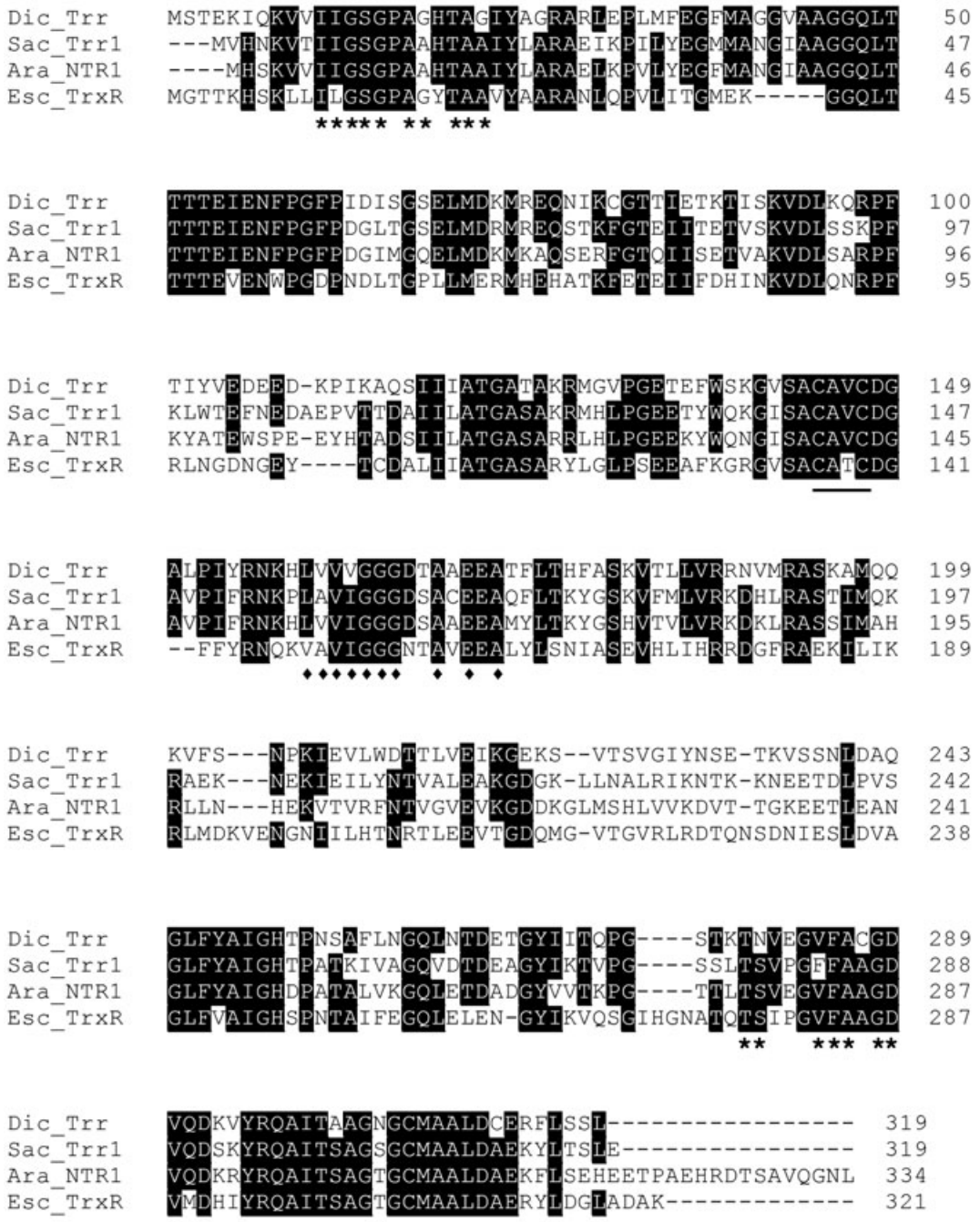

Fig. 1. Multiple sequence alignment of thioredoxin reductase. The deduced amino acid sequence of the *D. discoideum trrA* was aligned with those of thioredoxin reductases from other organisms. Identical amino acid residues are indicated with black backgrounds. The redox active site is underlined. The FAD-binding domain is indicated by asterisks (*) and the NADPH-binding domain by diamonds (◆). The accession numbers are: *D. discoideum*, Dic_Trr (XP_641039); *S. cerevisiae*, Sac_Trr1 (P29509); *A. thaliana*, Ara_NTR1 (Q39243); and *E. coli*, Esc_TrxR (P09625).

Trr^{OE} cells exhibit a defect in phagocytosis

When *Dictyostelium* grows on a bacterial lawn, a plaque is formed from a single cell as the bacteria are eaten. Interestingly, Trr^{OE} cells produced very tiny plaques on a bacterial lawn compared with KAx3 cells (Fig. 4A). The plaque diameter of Trr^{OE} cells did not exceed 30% of that of KAx3 cells. However, the growth rate of Trr^{OE} cells in axenic culture was comparable to that of KAx3 cells (data not shown). To determine whether the poor growth on bacterial lawn is correlated with a defect in phagocytosis, we examined the uptake rate of fluorescein-labelled *Staphylococ-*

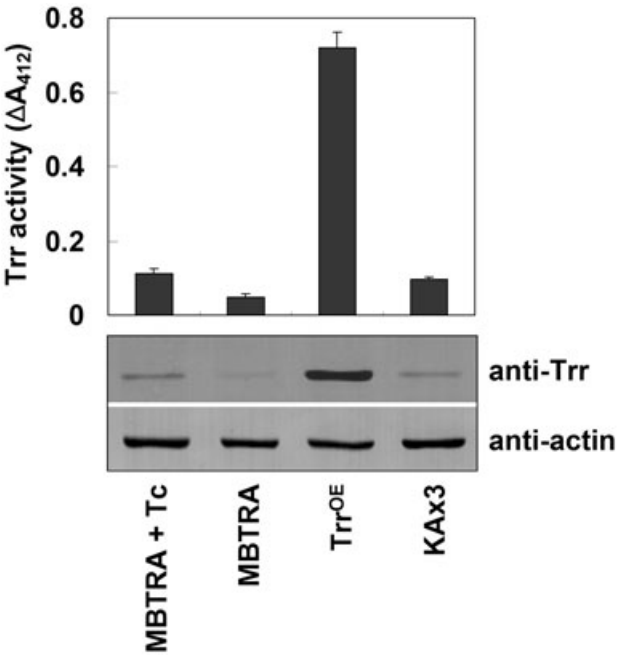

Fig. 2. Enzyme activity and protein level of thioredoxin reductase in KAx3, Trr^{OE} and MBTRA cells. Crude extracts were prepared from KAx3, Trr^{OE} and MBTRA cells. Especially, MBTRA cells were cultured in the presence (MBTRA + Tc) or absence (MBTRA) of Tc to induce the expression of *trrA* antisense RNA. Trr activity was measured spectrophotometrically at 412 nm using the insulin reduction assay (see *Experimental procedures*) (top). The values represent the average (±standard deviation) of three independent experiments. Expression level of Trr in mutants was examined by Western blot analysis (bottom). Twenty micrograms of protein from crude extract was electrotransferred to nitrocellulose membrane, and the membrane was immunoblotted with anti-Trr antibody. Actin is shown as a loading control.

cus aureus. As shown in Fig. 4B, the rate of bacterial uptake in Trr^{OE} cells decreased by 40% compared with that of KAx3 cells. These data suggest that the severe growth defect of Trr^{OE} cells on a bacterial lawn results at least in part from the impairment of phagocytosis.

Trr^{OE} cells display a slugger phenotype

To examine the developmental phenotype, Trr^{OE} cells were developed on non-nutrient agar plate under dark condition. Trr^{OE} cells aggregated and formed slugs normally. However, Trr^{OE} slugs showed a tendency to migrate rather than to enter into culmination. Under dark condition, the slugs of KAx3 cells formed fruiting bodies within 30 h, but those of Trr^{OE} cells continued to migrate more than 40 h and hence left longer slime trails (Fig. 5). Although Trr^{OE} slugs migrated for a long period, they showed normal phototaxis (data not shown).

As slugger mutants have been reported to be hypersensitive to the inhibitory effect of ammonia (Gee *et al*., 1994; Singleton *et al*., 1998; Nelson *et al*., 2000), we examined whether Trr^{OE} cells also possess this property. The cells were deposited on black filters resting on the support pads soaked with KK_2 buffer containing NH_4Cl. With increasing the concentration of NH_4Cl, Trr^{OE} cells were more sensitive than the parental cells (Fig. 6). Trr^{OE} cells formed aggregates and culminants of smaller size than KAx3 cells at 20 mM NH_4Cl. They failed to even aggregate at 50 mM NH_4Cl, whereas KAx3 cells could aggregate. Taken together, these findings indicate that overexpression of Trr leads to the inhibition of culmination and the slugger phenotype.

Vacuolar H$^+$-ATPase activity decreases in Trr^{OE} cells

Ammonia plays a role in the decision between slug migration and culmination (Schindler and Sussman, 1977; Gross, 1994). It was demonstrated that unprotonated ammonia prevents culmination by neutralization of intracellular acidic compartments (Davies *et al*., 1993). Moreover, mutants defective in acidification of intracellular compartments are abnormally sensitive to ammonia and display a slugger phenotype (Davies *et al*., 1996). Thus, in order to determine whether the delayed culmination shown in Trr^{OE} cells is caused by defective acidification of intracellular compartments, we examined intracellular acidic compartments in Trr^{OE} cells by quinacrine vital staining. Quinacrine is a weakly basic fluorescent dye that can accumulate in low pH compartments within the cell, such as the acidic vacuole (Roberts *et al*., 1991). As shown in Fig. 7, quinacrine staining in the parental cells revealed the strong fluorescence of the large acidic compartments. In contrast, the fluorescence in Trr^{OE} cells was very weak, indicating more alkaline vacuolar pH.

It has been well established that the acidic pH of intracellular compartments is generated by V-ATPase, a family of ATP-dependent proton pumps (Padh *et al*., 1989a; Nolta *et al*., 1991; Nelson and Harvey, 1999). As Trr^{OE} cells were defective in acidification of intracellular compartments, we examined V-ATPase activity in Trr^{OE} cells. Table 1 shows 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl)-sensitive V-ATPase activity in the membrane fractions of KAx3 and Trr^{OE} cells. Trr^{OE} cells exhibited a 50% decrease in V-ATPase activity compared with the KAx3 cells. Moreover, the V-ATPase activity in the membrane fractions of KAx3 cells decreased up to approximately 30% after treatment with NADPH, Trr and Trx1 (data not shown). Altogether, these data suggest that the overexpression of Trr cause inhibition of V-ATPase activity, leading to an increase in the pH of acidic compartments, finally resulting in inhibition of culmination.

Mobility shift of VatA depending on redox state

Previous studies have reported that the activity of V-ATPase is regulated by its oxidation and reduction

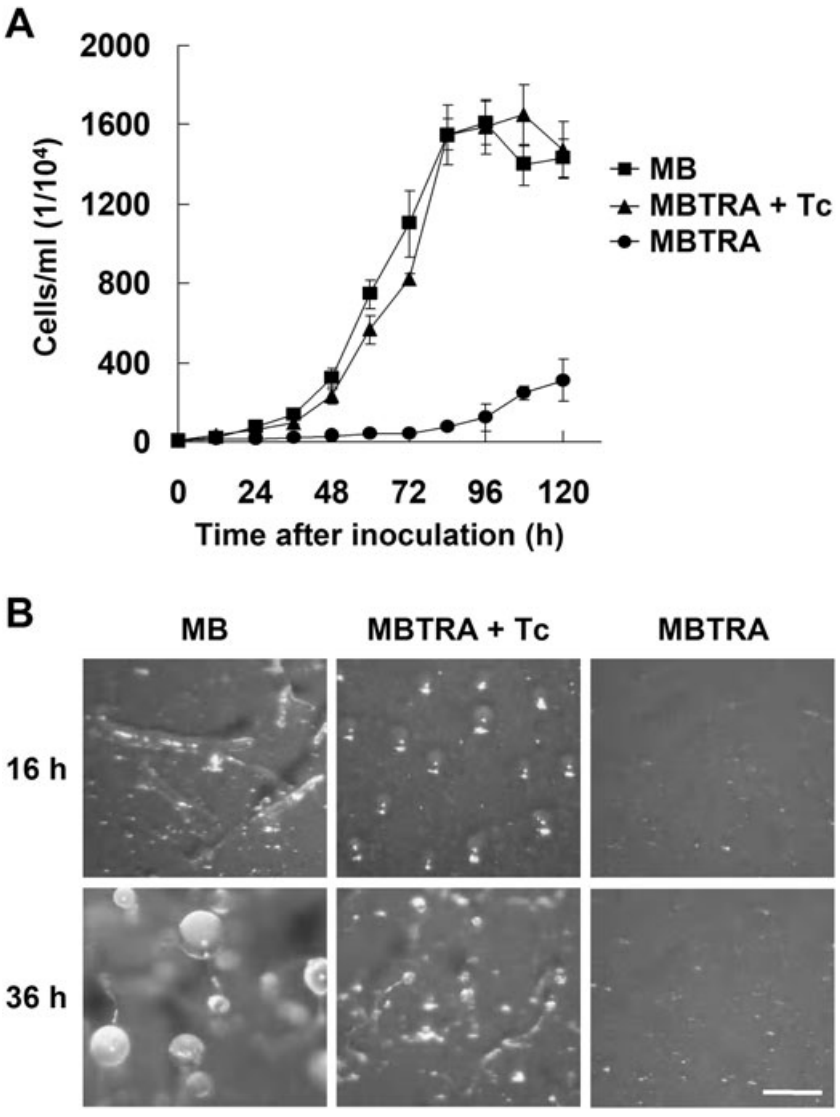

Fig. 3. Growth and development of MB and MBTRA cells.
A. Cells were grown in shaking cultures of HL5 medium supplemented without (MB and MBTRA) or with (MBTRA + Tc) 10 μg ml^{-1} Tc, and cell densities were determined using a haemacytometer at the indicated times. Each point represents the average (±standard deviation) of three independent experiments.
B. Cells were cultured as described above, washed with KK_2 buffer twice and plated for development on black filters. Cells were photographed at the indicated times after development. MBTRA cells pre-cultured in the absence of Tc failed to aggregate. Even when Tc was removed prior to starvation, MBTRA cells were delayed in developmental progression. Scale bar, 0.5 mm. MB, KAx3 cells transformed with MB35 and an empty MB38 vectors; MBTRA + Tc, MBTRA cells pre-cultured in the presence of 10 μg ml^{-1} Tc; MBTRA, MBTRA cells pre-cultured in the absence of Tc to induce the expression of *trrA* antisense RNA.

(Feng and Forgac, 1992a; Oluwatosin and Kane, 1997; Forgac, 1999). Especially, disulphide bond formation in the catalytic subunit of V-ATPase, subunit A, has been proposed to play an important role in regulation of V-ATPase activity (Feng and Forgac, 1994; Taiz *et al*., 1994). Therefore, we were interested in whether the thioredoxin system can directly reduce subunit A of V-ATPase and cloned the *vatA* gene encoding the subunit A (VatA) from *D. discoideum*. The nucleotide sequence of *vatA* consists of an open reading frame of

Table 1. NBD-Cl-sensitive V-ATPase activity in KAx3 and Trr^{OE} cells.

	ATPase activity (nmol P_i per minute per mg protein)		
	NBD-Cl		
	–	+	NBD-Cl-sensitive V-ATPase activity
KAx3	367 ± 6	166 ± 4	201 ± 5
Trr^{OE}	270 ± 8	164 ± 7	106 ± 1

Cells were grown axenically to stationary phase, and then the membrane fractions were isolated (see *Experimental procedures*). The isolated membranes were pre-treated for 10 min without or with 25 μM NBD-Cl, followed by assay of V-ATPase activity. Inorganic phosphorus (P_i) liberated from ATP was quantified spectrophotometrically by measuring the absorbance at 750 nm. NBD-Cl-sensitive V-ATPase activity was determined from subtraction of the NBD-Cl background value. Values represent the average ± standard deviation of three independent experiments.

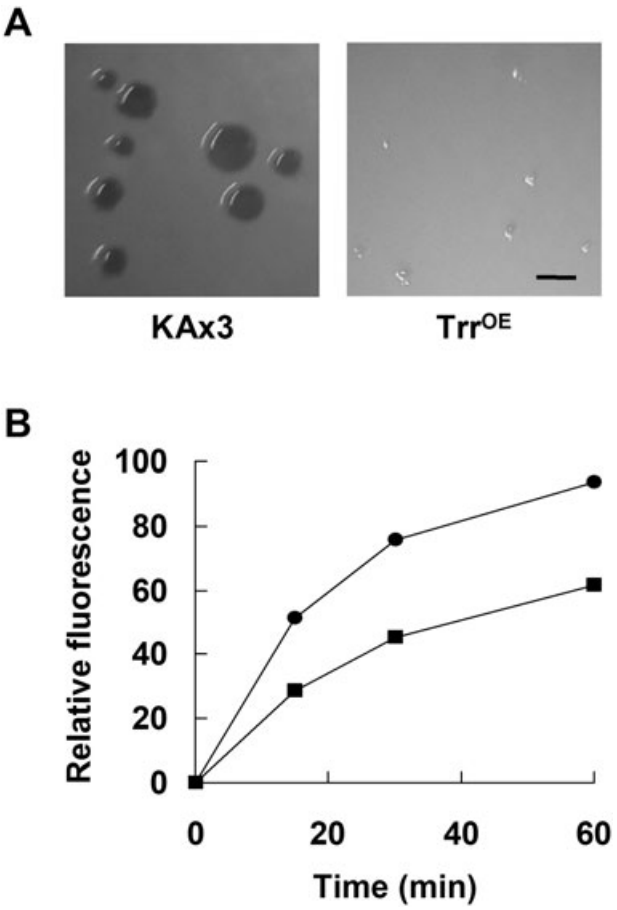

Fig. 4. Growth on bacterial lawns and the uptake rates of bacteria of KAx3 and Trr^{OE} cells.
A. KAx3 and Trr^{OE} cells were plated on SM agar plates with *K. aerogenes* at a density of 100 *D. discoideum* cells per plate. Plaques were photographed after 5 days of incubation. Trr^{OE} cells formed very tiny plaques compared with KAx3 cells. Scale bar, 5 mm.
B. KAx3 (circles) and Trr^{OE} (squares) cells were incubated with fluorescein-labelled *S. aureus* under shaking conditions. Samples were withdrawn at the indicated times and incubated with 2 mg ml^{-1} trypan blue solution to quench fluorescence of non-internalized bacteria. After resuspension in KK_2 buffer, fluorescence was measured at 485 nm for excitation and 535 nm for emission. Each point represents the average of duplicate determinations from a single experiment. Similar results were obtained in three independent experiments.

1857 bp that encodes a polypeptide of 618 amino acids with a calculated molecular mass of 68.2 kDa. *Dictyostelium* VatA shows high sequence similarity to subunit A from other organisms and contains three conserved cysteine residues: Cys-255, Cys-278 and Cys-533 (Fig. 8A).

Recombinant VatA was overexpressed in *E. coli* BL21 and purified by Ni^{2+}-affinity chromatography. To test the electrophoretic mobility, the homogeneously purified VatA was incubated with various oxidizing or reducing agents, and then analysed on SDS-PAGE (Fig. 8B). The VatA treated with reducing agents (Fig. 8B, lanes 1–4) migrated as a single monomeric band on SDS-PAGE. In contrast, the VatA treated with a non-thiol electron donor (Fig. 8B, lane 5) or oxidizing agents (Fig. 8B, lanes 6 and 7) migrated as aggregate forms, which barely entered the running gel. When the reducing agent was removed via desalting, VatA was also observed as the aggregate forms (Fig. 8B, lane 8), which could be reversed by the addition of dithiothreitol (DTT) (data not shown). Therefore, these observations suggest that VatA can form reversibly disulphide bonds *in vitro*, and its redox state can be judged from the change in electrophoretic mobility on SDS-PAGE. A similar electrophoretic pattern has been reported in several proteins: the oxidized proteins migrate as multimers or aggregates on SDS-PAGE under non-reducing condition, caused by forming inter- or intramolecular disulphide bonds (Zheng *et al*., 1992; Bottazzi *et al*., 1997; Landino *et al*., 2004).

The thioredoxin system can reduce VatA

We examined whether VatA can be reduced by the thioredoxin system using non-reducing SDS-PAGE. Recombinant Trr and Trx1 were overexpressed in *E. coli* and purified to homogeneity (see *Experimental procedures*). To obtain oxidized VatA, the purified VatA was incubated with H_2O_2 and desalted just prior to reaction. As expected, the oxidized VatA was detected as aggregate forms on SDS-PAGE under non-reducing condition (Fig. 9A, lane 1). Interestingly, when the oxidized VatA was incubated with the thioredoxin system (NADPH, Trr and Trx1), the band of aggregates disappeared and the monomeric band was detected (Fig. 9A, lane 2). Without Trr (Fig. 9A, lane 4) or Trx1 (data not shown), the monomeric band of VatA was not detected. As the concentration of Trx1 or Trr increased, band intensity of the aggregate form decreased, and that of monomeric form increased (Fig. 9B).

We also confirmed the reduction of VatA by the thioredoxin system via a redox cascade. In this cascade, Trr utilizes NADPH to reduce Trx1, in turn reduces oxidized VatA as a substrate. It occurs continuously until all of VatA is reduced or NADPH is oxidized. The oxidation of NADPH was monitored by a decrease in absorbance at 340 nm. As shown in Fig. 9C, the addition of thioredoxin system to oxidized VatA resulted in the oxidation of NADPH. In contrast, NADPH was barely oxidized when thioredoxin or VatA was omitted from the reaction mixture. Taken together, these results indicate that the thioredoxin system can reduce VatA *in vitro*.

Discussion

The thioredoxin system is involved in the redox regulation of DNA synthesis (Koc *et al*., 2006), gene transcription

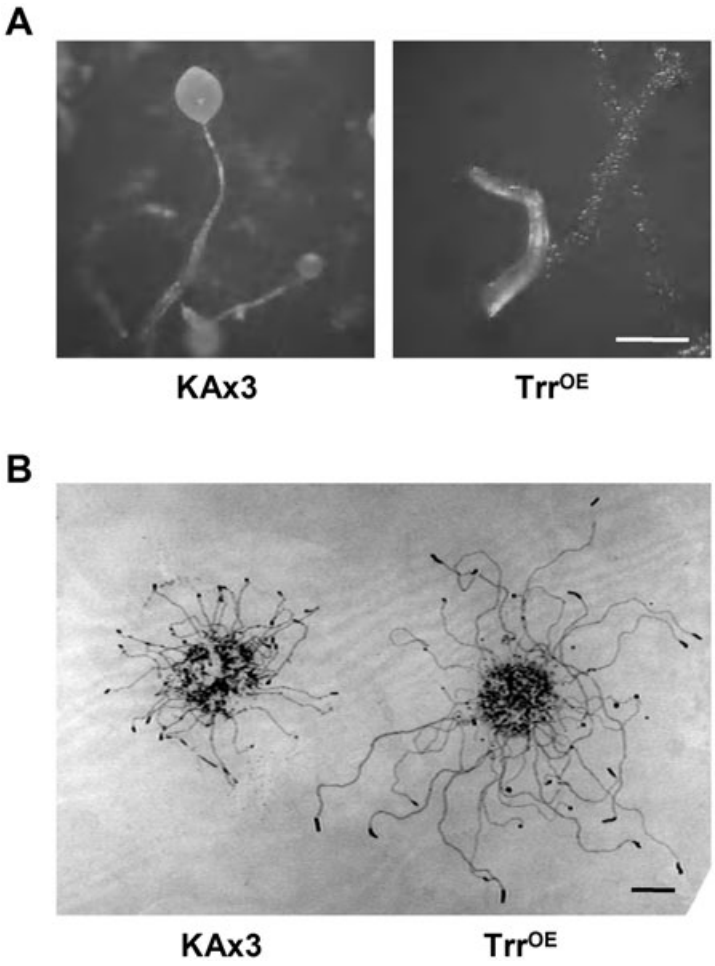

Fig. 5. Developmental phenotypes of KAx3 and Trr^{OE} cells.
A. *Dictyostelium* cells were deposited on KK_2-buffered agar plates under dark condition and photographed after 40 h. Whereas KAx3 cells formed fruiting bodies, Trr^{OE} cells remained in the slug phase. Scale bar, 0.5 mm.
B. After 40 h of development, the resultant slugs, spores and slime trails were transferred to a transparency film, followed by staining with Coomassie Blue. Scale bar, 5 mm.

(Hirota *et al*., 1999; Seemann and Hainaut, 2005) and apoptosis (Saitoh *et al*., 1998; Lindner *et al*., 2002). Also, it has been well established as a component of an efficient antioxidant system in the cell (Spector *et al*., 1988; Chae *et al*., 1994; Garrido and Grant, 2002). Due to these important functions, thioredoxin reductase has been reported to be essential for survival of *Cryptococcus neoformans* (Missall and Lodge, 2005), *S. aureus* (Uziel *et al*., 2004) and *Plasmodium falciparum* (Krnajski *et al*., 2002). As it was unsuccessful to disrupt *trrA* in *D. discoideum* despite of many attempts, we generated the mutant strain in which the expression of Trr was inhibited conditionally. As expected, the Trr-underexpressing cells showed severely poor growth. A possible explanation for their poor growth is that Trr is required for antioxidant defence and maintenance of cellular redox homeostasis. This is supported by the results that transcription of *trrA* was highly induced by hydrogen peroxide and Trr-underexpressing cells were very sensitive to hydrogen peroxide (data not shown).

The phenotype analysis of Trr^{OE} cells proposes that Trr may regulate the entry into culmination via modulation of V-ATPase activity. Trr^{OE} cells showed a slugger phenotype. Trr^{OE} slugs continued to migrate rather than entered into culmination in the dark. Also, they were abnormally sensitive to ammonia like other slugger mutants. Interestingly, Trr^{OE} cells appeared to lack highly acidic compartments compared with the parental cells. Consistent with this finding, V-ATPase activity decreased in Trr^{OE} cells. Previous studies have demonstrated that an increase in the pH of acidic compartments inhibits culmination (Davies *et al*., 1993; 1996; Gross, 1994). The cells transformed with antisense construct of V-ATPase subunit B or mutants defective in acidification of intracellular acidic compartments have a prolonged migrating slug phase (Davies *et al*., 1996). In addition, it was reported that ammonia accumulates in intracellular acidic compartments, where it becomes protonated and raises pH of the compartments, thereby leading to the inhibition of culmination (Davies *et al*., 1993). Thus, the ammonia hypersensitivity of Trr^{OE} cells might be caused by a defect in acidification of intracellular compartments. Taken together, our results suggest that the overexpression of Trr results in the inactivation of V-ATPase in

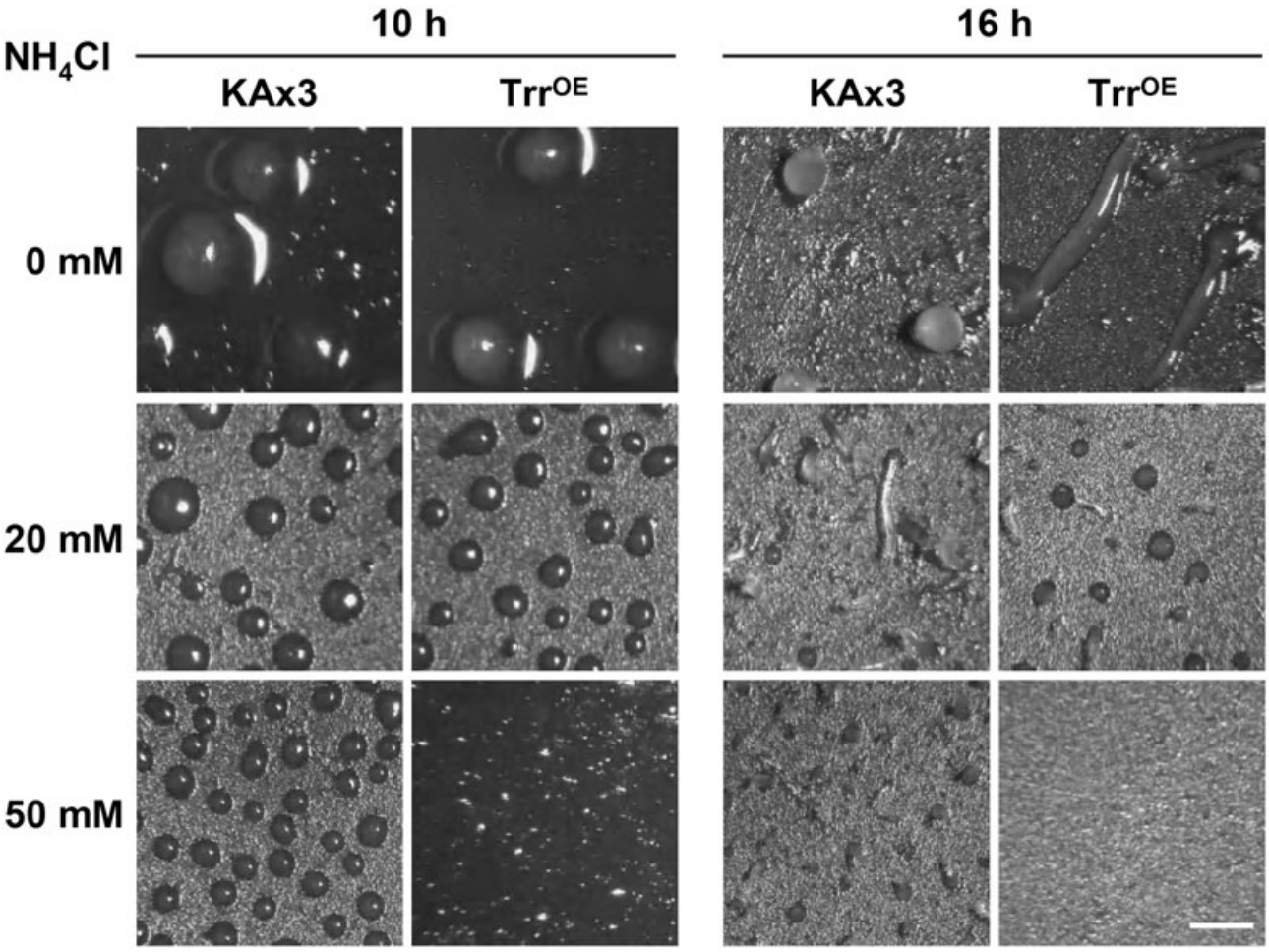

Fig. 6. The effect of ammonia on development of KAx3 and Trr^{OE} cells. Black filters (Millipore) were placed on the filter pads soaked with KK_2 buffer containing the indicated concentrations of ammonium chloride. KAx3 and Trr^{OE} cells were spotted on black filters at a density of 2.5×10^6 cells cm^{-2} and monitored over the course of development. Photographs were taken at 10 h and 16 h after plating. Scale bar, 0.5 mm.

D. discoideum and thus the elevation of pH of intracellular acidic compartments, finally leading to delayed culmination.

Trr-overexpressing cells showed a defective phagocytosis, which is also correlated with V-ATPase. Regulation of phagosomal pH, which has been known as a crucial process in phagosomal maturation (Rupper *et al*., 2001), is accompanied by the recruitment of V-ATPase (Padh *et al*., 1989a). Thus, the decline in phagocytosis capacity of Trr^{OE} cells may be caused by the decrease of V-ATPase activity.

It has been reported that V-ATPase is sensitive to the redox state, and in particular subunit A is responsible for the sensitivity of V-ATPase to sulphydryl reagents in several organisms (Feng and Forgac, 1992b; Dschida and Bowman, 1995). Analysis of the electrophoretic mobility indicated that VatA displays a different mobility depending on its redox state and is reduced by the thioredoxin system. Interestingly, oxidized VatA were observed as high-molecular-weight aggregates on SDS-PAGE under non-reducing condition. The aggregation may be attributed to conformational change by disulphide bonds. Based on the biochemical studies, we first suggest that the thioredoxin system may be a natural reductant of VatA in *D. discoideum.*

The phenotype analysis in Trr^{OE} cells and VatA reduction assay *in vitro* suggest that the thioredoxin system may regulate the activity of V-ATPase via the reduction of VatA in *D. discoideum*. Although the detailed molecular mechanism of the inactivation of V-ATPase by the thioredoxin system remains unknown, the existence of three conserved cysteine residues in VatA may provide a clue. Previous studies have demonstrated that three highly conserved cysteine residues in subunit A of V-ATPase are critical for the regulation of V-ATPase activity. Feng and Forgac (1994) reported that the V-ATPase of bovine clathrin-coated vesicles can be reversibly inhibited by the formation of intramolecular disulphide bond between Cys-

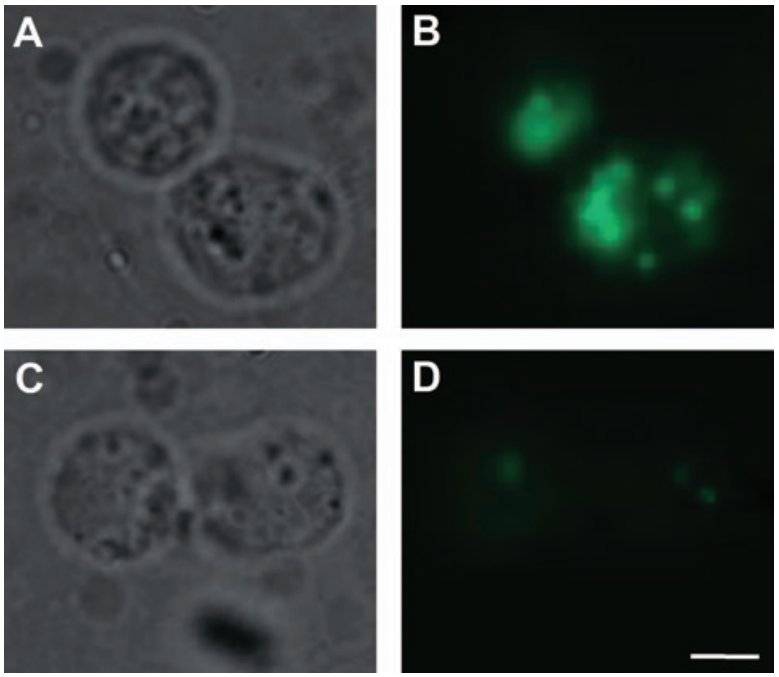

Fig. 7. Quinacrine staining of acidic compartments in KAx3 and Trr^{OE} cells. KAx3 (A and B) and Trr^{OE} cell (C and D), which were cultured axenically to late log phase, were incubated in KK_2 buffer containing 200 μM quinacrine for 5 min to label the acidic compartments. Cells were observed with a fluorescence microscope. (A) and (C) are bright-field images of (B) and (D) respectively. Trr^{OE} cells exhibited weak and diffuse fluorescence. Scale bar, 5 μm.

254 and Cys-532 in subunit A. Although our result is seemingly contradictory with their report, it may be explained by the existence of another disulphide bond which is required for activation of V-ATPase. Moreover, it has been proposed a model that V-ATPase might be active when a disulphide bond between Cys-532 and another Cys in subunit A is formed (Feng and Forgac, 1994; Nishi and Forgac, 2002). Therefore, we suggest the possibility that subunit A in active V-ATPase might be in the disulphide bonded form, which can be reduced by the thioredoxin system. Further site-directed mutagenesis and X-ray crystallographic studies will be required to elucidate the sites and roles of disulphide bonds in subunit A.

The redox status in the cell is a major regulator of a broad range of physiological and cellular functions. Especially, it has been reported that the differentiation of eukaryotic microorganisms can be controlled by reactive oxygen species or antioxidant enzymes (Hansberg *et al*., 1993; Lara-Ortíz *et al*., 2003; Aguirre *et al*., 2005). In *D. discoideum*, the overexpression of superoxide dismutase inhibits the formation of aggregates (Bloomfield and Pears, 2003). In addition, we have recently reported that glutathione (GSH) is essential for development of *Dictyostelium* (Kim *et al*., 2005) and an increase in GSH levels leads to the inhibition of culmination (Choi *et al*., 2006). Various target proteins of *D. discoideum* Trx1 were identified using yeast two-hybrid screening (Brodegger *et al*., 2004), but little is known about the physiological significance of these interactions. Herein, we suggest that Trr plays a role in the redox control of culmination and VatA is a new potential target protein controlled by the thioredoxin system in *D. discoideum*. These studies will provide insights into the redox regulation in development.

Experimental procedures

Cell culture and development

Dictyostelium discoideum axenic strain KAx3 and its derivatives were grown axenically in HL5 media (Cocucci and Sussman, 1970) at 22°C. *Dictyostelium* cells also were plated on SM agar plates (Sussman, 1987) with *Klebsiella aerogenes* and incubated at 22°C for 3–4 days until *Dictyostelium* plaques appeared on the bacterial lawns. To obtain single clones of *Dictyostelium*, 20–100 cells were plated on SM agar plates. For development, exponentially growing cells were washed twice, resuspended in KK_2 buffer (16.5 mM KH_2PO_4 and 3.8 mM K_2HPO_4, pH 6.2), and placed on KK_2-buffered agar plates at a density of 2.5×10^6 cells cm^{-2}. The pH of KK_2 buffer containing 20 mM or 50 mM NH_4Cl was adjusted to 6.2 with KOH. For dark condition, the plates were wrapped with aluminum foil.

Cloning and strain construction

The full-length cDNA of *trrA* was isolated by polymerase chain reaction based on the sequence from *Dictyostelium* genome database. To overexpress Trr in *D. discoideum*, the full-length *trrA* was subcloned into BglII and XhoI sites of EXP4(+) vector. The construct was introduced into KAx3 cells by electroporation (Pang *et al*., 1999), and the transformed cells were selected with 20 μg ml^{-1} G418.

To conditionally underexpress Trr in *D. discoideum*, Tc-regulated expression system (Blaauw *et al*., 2000) was

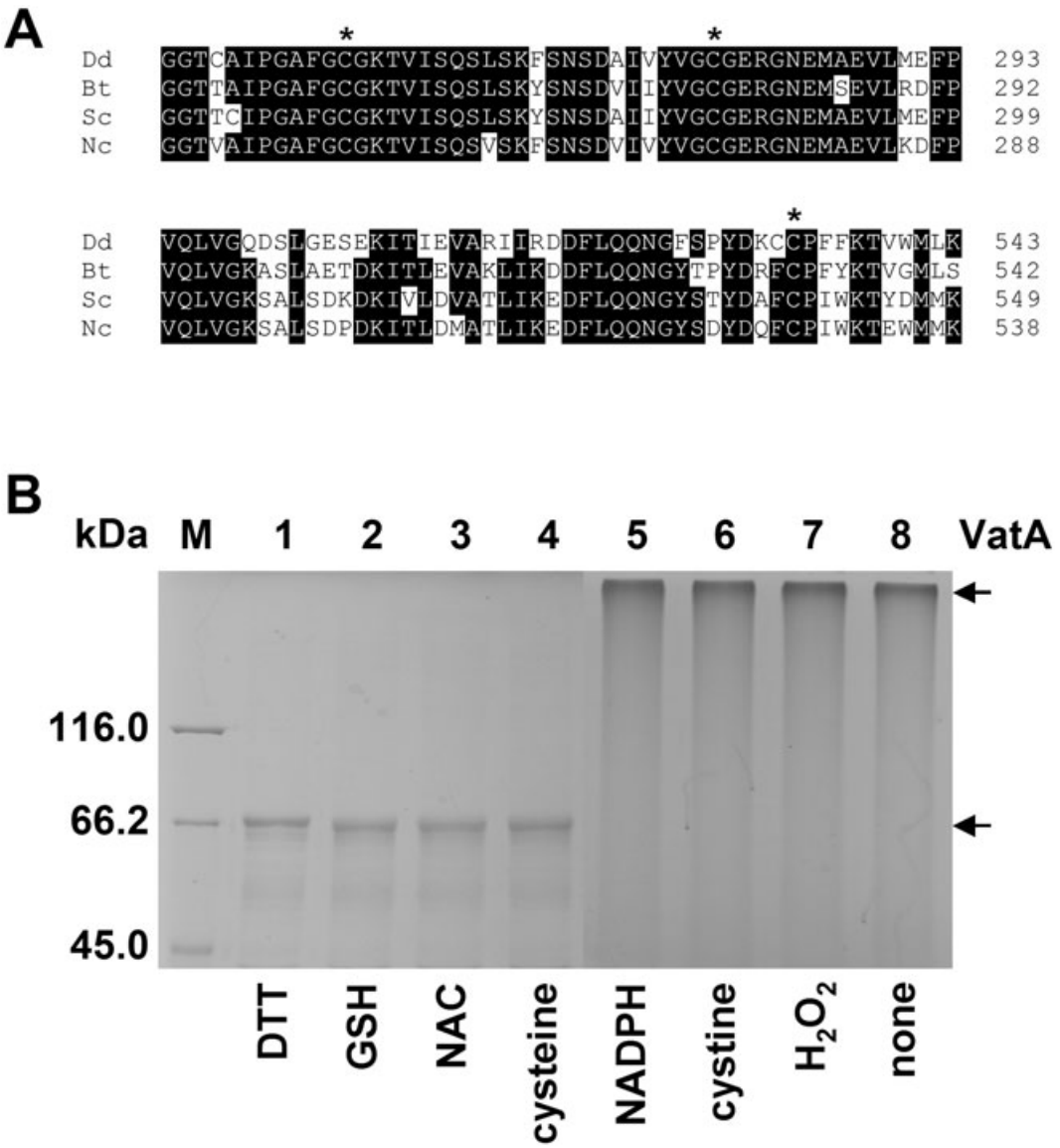

Fig. 8. Conserved cysteine residues in subunit A of V-ATPase and redox-dependent electrophoretic mobility of VatA.
A. Multiple sequence alignment was performed for the amino acid sequences of subunit A of V-ATPase. Identical amino acid residues are indicated with black backgrounds, and three conserved cysteine residues are indicated by asterisks (*). Dd, *D. discoideum* VatA (Accession No. XP_637351); Bt, *Bos taurus* VatA (P31404); Sc, *S. cerevisiae* VmaA (P17255); Nc, *Neurospora crassa* Vma-1 (P11592).
B. The purified VatA was desalted (lane 8) and treated with various reducing or oxidizing agents: 5 mM DTT, GSH, *N*-acetylcysteine (NAC), L-cysteine, NADPH, cystine or H_2O_2. After the incubation at room temperature for 15 min, proteins were resolved on 7% SDS-PAGE. The upper and lower arrows indicate aggregated and monomeric VatA respectively. M, molecular weight markers.

applied. Two vectors MB35 and MB38 were kindly provided by P.J. van Haastert. MB35 vector encoding a Tc-controlled transcription activator was introduced into KAx3 cells, and the resulting transformants were named MB35 cells (Choi *et al.*, 2006). The full-length *trrA* was inserted in the antisense orientation into BglII site linked to the Tc-responsive promoter in MB38 vector. The resulting construct was introduced into MB35 cells. The transformed cells were selected in HL5 medium supplemented with 20 μg ml^{-1} G418, 10 μg ml^{-1} blasticidin S and 10 μg ml^{-1} Tc, and maintained at a low density. Prior to experiments, they were pre-cultured in the absence of Tc for 5 days to induce the expression of *trrA* antisense RNA. As a control, MB cells were generated by introducing an empty MB38 vector into MB35 cells.

General methods

Crude extract of *D. discoideum* was prepared by sonication in phosphate-buffered saline (PBS, pH 7.4) and centrifugation at 12 000 r.p.m. for 10 min. Protein concentration was determined using the *DC* Protein Assay Kit (Bio-Rad). SDS-PAGE was performed according to the method of Laemmli (1970). After electrophoresis, the gel was stained with Coomassie

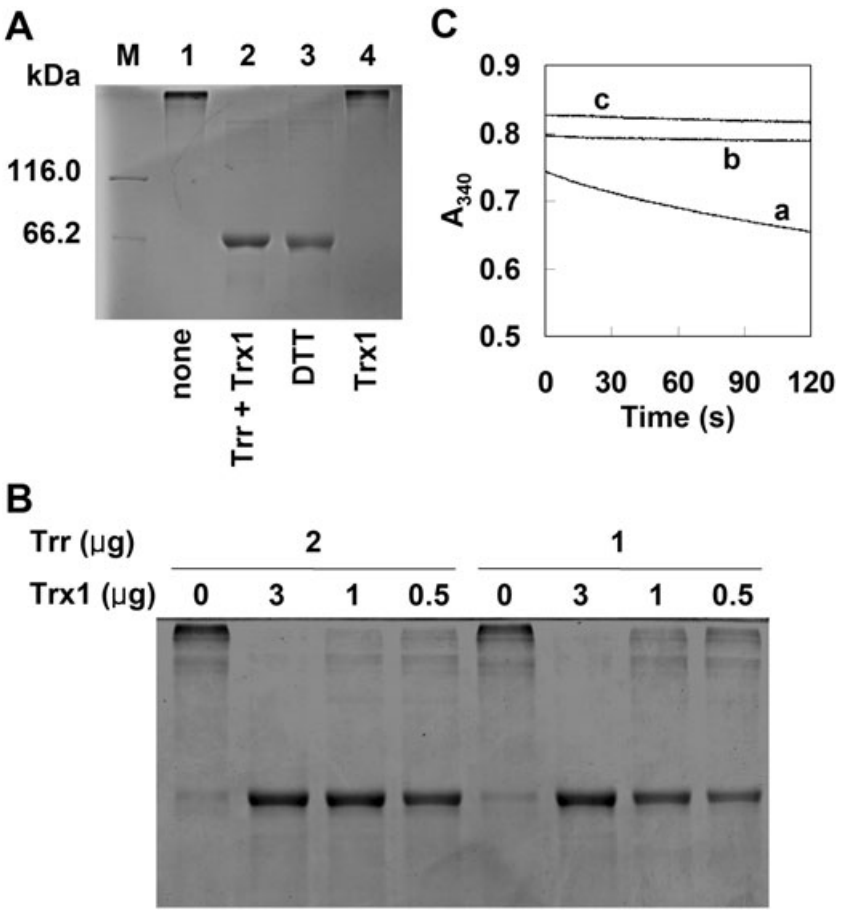

Fig. 9. Reduction of VatA by the thioredoxin system. All three recombinant proteins were purified to homogeneity. To prepare oxidized VatA, the purified protein was treated with 5 mM H_2O_2 for 15 min and desalted using PD-10 column.
A. Eight micrograms of the oxidized VatA was incubated in 50 mM potassium phosphate, pH 7.0, containing DTT or the thioredoxin system for 15 min and resolved on 7% SDS-PAGE. Each lane shows VatA incubated with the following: lane 1, none; lane 2, 100 μM NADPH, 2 μg of Trr and 3 μg of Trx1; lane 3, 5 mM DTT; lane 4, 100 μM NADPH and 3 μg of Trx1. M, molecular weight markers.
B. Eight micrograms of the oxidized VatA was incubated with various concentrations of the thioredoxin system as described above. It was resolved on 7% SDS-PAGE under non-reducing condition.
C. Reduction of VatA by the thioredoxin system was determined by monitoring the decrease in absorbance at 340 nm. The reaction mixture contained 100 μM NADPH, 50 nM Trr, 1 μM Trx1 and 8 μM oxidized VatA in 0.1 M potassium phosphate buffer, pH 7.0 (a). The mixture lacking VatA (b) or Trx1 (c) was also monitored.

Blue R-250. Western blot analysis (Towbin *et al*., 1979) was performed using mouse anti-Trr antiserum or rabbit anti-actin (Sigma). The signals were visualized using a colorimetric detection (Lee *et al*., 2005).

Thioredoxin reductase assay

Trr activity was determined as previously described for mammalian Trr (Holmgren and Björnstedt, 1995) with a slight modification: *D. discoideum* crude extracts (100 μg of protein) were incubated with 0.2 mM EDTA, 1 mg ml^{-1} insulin, 200 μM NADPH and 5 μg of recombinant Trx1 in 0.1 M potassium phosphate, pH 7.0, for 10 min at 37°C. Reaction was terminated by the addition of 6 M guanidine-HCl in 0.2 M Tris-HCl, pH 8.0, containing 0.4 mg ml^{-1} 5,5′-dithio-bis(2-nitrobenzoic acid). As a reference, the crude extracts were incubated with the reaction mixture without Trx1. Absorbance was measured at 412 nm, and the reference value was subtracted from each sample.

Phagocytosis assay

The uptake rate of bacteria was measured using fluorescein-labelled *S. aureus* (Molecular Probes). Around 1×10^6 *Dictyostelium* cells were incubated with the fluorescent bacteria at a final concentration of 100 bacteria per amoeba. At each time point, cells were harvested and then resuspended in 2 mg ml^{-1} trypan blue solution to quench the fluorescence of non-internalized bacteria. The cells were incubated for 1 min and then washed twice with KK_2 buffer. The fluorescence of internalized bacteria was measured with an EnVision multi-label plate reader (Perkin-Elmer).

Isolation of vacuolar membranes and V-ATPase activity assay

Stationary cells were pelleted, resuspended in ice-cold homogenization buffer (100 mM sucrose in 5 mM sodium-glycinate, pH 8.5), and lysed through two nucleopore filters (pore size, 5 μm). Cell debris and nuclei were removed by centrifugation at 1000 *g* for 10 min. The post-nuclear supernatant was centrifuged at 100 000 *g* for 1 h. The resultant membrane pellet was resuspended in homogenization buffer.

Vacuolar H^+-ATPase activity in the isolated vacuolar membrane fraction was assayed spectrophotometrically by the release of inorganic phosphorus (P_i) from ATP as previously described (Padh *et al*., 1989b) with some modifications: the reaction mixture consisted of 5 mM $MgCl_2$, 5 mM NaN_3, 0.1 mM Na_3VO_4 and 50 mM MES buffer, pH adjusted to 7.0 with Tris base. Thirty micrograms of the membrane proteins were added and pre-incubated at 30°C for 5 min without or with 25 μM NBD-Cl, a V-ATPase inhibitor. The reaction was

initiated by the addition of 5 mM ATP. After 10 min incubation, liberated P_i was determined according to the method of Fiske and Subbarow (1925). Raw data were converted to nmol P_i per mg protein per minute.

Quinacrine staining of acidic compartments

To examine intracellular acidic compartments, the acidic pH probe quinacrine (Sigma) was used (Roberts *et al*., 1991). *Dictyostelium* cells were grown axenically to late log phase and incubated in KK_2 buffer containing 200 μM quinacrine for 5 min. The cells were harvested by centrifugation, washed once with KK_2 buffer and resuspended in the same buffer. Fluorescence images were obtained immediately using an Axiolab microscope (Carl Zeiss).

Recombinant protein purification

Escherichia coli BL21 (DE3) pLysS was used to overproduce various recombinant proteins. The full-length cDNAs encoding Trr and Trx1 were subcloned downstream of the GST sequences in pGEX-4T-1 respectively. Each protein was purified by affinity chromatography using Glutathione-Sepharose 4B column (GE Healthcare) according to the manufacturer's instructions. The GST-tags were removed by thrombin.

The full-length *vatA* was subcloned into NdeI and XhoI sites of pET-15b, and the N-terminal His_6-tagged VatA protein was purified using His·Bind column (Novagen) under denaturing condition. To obtain soluble protein, the denatured VatA was diluted by 100-fold directly into refolding buffer (0.4 M L-arginine, 5 mM GSH and 0.5 mM oxidized glutathione in 0.1 M Tris-HCl, pH 7.5) (Mishig-Ochiriin *et al*., 2005). Then the protein was desalted using a PD-10 column (GE Healthcare) and subsequently analysed on SDS-PAGE.

Reduction of VatA by the thioredoxin system

To obtain oxidized VatA, the purified VatA was incubated with 5 mM H_2O_2 for 15 min and then desalted. Eight micrograms of the oxidized VatA was reacted with 3 μg of Trx1, 2 μg of Trr and 100 μM NADPH in 50 mM Tris-HCl, pH 7.0, for 15 min at room temperature. The proteins were resolved on 7% SDS-PAGE under non-reducing condition.

The reduction of the oxidized VatA by the thioredoxin system was measured by monitoring a decrease in absorbance at 340 nm in the reaction coupled with NADPH oxidation. The reaction was performed at room temperature in 500 μl of 0.1 M potassium phosphate, pH 7.0, containing 100 μM NADPH, 1 μM Trx1, 50 nM Trr and 8 μM VatA.

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