# Superoxide signalling required for multicellular development of *Dictyostelium*

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## Summary

**Reactive oxygen species are known to have a signalling role in many organisms. In bacteria and yeast various response systems have evolved to combat oxidative stress which are triggered by reactive oxygen species. Mammals and plants are known to actively generate reactive oxygen species such as superoxide during signalling responses to a variety of extracellular factors. We report here the generation of superoxide as a signalling molecule in early development of *Dictyostelium discoideum*. *Dictyostelium* grows as single amoebae but, on starvation, the single cells aggregate to form a multicellular organism. Superoxide is generated in response to a secreted factor during the transition to the multicellular phase of development. Scavenging superoxide, either pharmacologically or by overexpressing the enzyme superoxide dismutase, inhibits the formation of the aggregate. This report of the use of superoxide as a signalling molecule in a lower eukaryote as it switches to a multicellular phase suggests that this signalling mechanism arose early in the evolution of multicellular organisms, perhaps as a necessary consequence of the need to diversify the number and type of signalling pathways available to facilitate intercellular communication.**

Key words: Superoxide, Cell signalling, *Dictyostelium discoideum*, Superoxide dismutase

## Introduction

Eukaryotic organisms employ sophisticated signalling networks to organise cellular functions. These consist of proteins with specialised enzyme activities and binding affinities, as well as smaller freely diffusible ions and molecules that can specifically modulate protein function. The development of multicellularity brought with it a requirement for more complicated signalling systems to enable communication between different cell types in the organism at different times during its own development. Thus multicellular eukaryotes contain the components of networks found in unicellular eukaryotes, but duplicated and modified, and in addition they have evolved further signalling mechanisms not found in their simpler counterparts.

Recent studies of mammals and some plants have established that reactive oxygen species (ROS) such as hydrogen peroxide and the superoxide anion are used by certain cells in the regulation of their function, playing an integral role in certain signalling networks (Finkel, 1998; Thannickal and Fanburg, 2000). In mammalian cells, ROS are produced in response to a variety of extracellular signals [an exhaustive list is provided by Thannickal and Fanburg (Thannickal and Fanburg, 2000)], and are essential for the functioning of many signals, for example for the mitogenic function of PDGF (Sundaresan et al., 1995) and for NGF-induced neuronal differentiation (Suzukawa et al., 2000). In plants, ROS are produced in response to wounding and infection, leading to the establishment of systemic immunity (Alvarez et al., 1998) via specific gene induction (Orozco-Cardenas et al., 2001). In both mammals and plants ROS are produced by NADPH oxidase enzymes that appear to have evolved specifically to generate superoxide from NADPH and molecular oxygen (Torres et al., 1998; Pei et al., 2000). The superoxide thus generated has a short half-life, mostly reacting with itself in a dismutation reaction catalysed by superoxide dismutase to give oxygen and hydrogen peroxide, which is a more stable molecule. Both superoxide and peroxide are able to affect protein function by reacting with sulphur-containing groups, for example, the catalytic cysteine residues of protein tyrosine phosphatases (Lee et al., 1998; Meng et al., 2002) and cysteine-rich regions of many transcription factors, including AP-1 (Puri et al., 1995) and p53 (Rainwater et al., 1995). Superoxide also inactivates enzymes such as aconitase that require an Fe/S centre for their activity and also the protein serine/threonine phosphatase calcineurin that requires a Fe/Zn centre (Wang et al., 1996; Namgaladze et al., 2002).

Unicellular eukaryotes and bacteria also have signalling intermediates that respond to ROS; in fact the best-understood ROS-responsive transcription factors in terms of molecular mechanism are from these systems. In *E. coli* the OxyR protein is directly oxidised by hydrogen peroxide to form an intramolecular disulphide, activating the transcriptional response to peroxide stress (Zheng et al., 1998); and the SoxR protein is activated by oxidation of an Fe/S centre by superoxide (Hidalgo et al., 1997). The Yap1 transcription factor of *Saccharomyces cerevisiae* (a homologue of mammalian AP-1) is also activated by peroxide, in this case by preventing its export from the nucleus (Delaunay et al., 2000). However, these organisms are not known to actively produce ROS as a signalling function, and they tellingly lack close

homologues of the NADPH oxidases of multicellular eukaryotes. It appears the apparatus required by all organisms to sense potentially dangerous excess free radicals were utilised at some stage of evolution as part of a signalling system in which ROS are produced endogenously in response to a stimulus.

*Dictyostelium discoideum* is a social amoeba, and as such straddles the boundary between unicellular and multicellular life. It lives in the soil as individual amoebae feeding on bacteria, but when food runs out it aggregates to form a multicellular organism of up to a million or so cells. This body of cells can travel to a suitable situation where it forms a fruiting body which consists of a ball of spores supported on a slender stalk. The spores can be dispersed to more clement surroundings where they germinate, each releasing a fresh amoeba. Despite obvious major differences between the development of multicellularity in *Dictyostelium* and other more conventional multicellular organisms, the regulatory mechanisms involved display marked similarities. For example SH2 domains and the STAT family of transcription factors (which are absent in unicellular fungi such as budding yeast) are present and play important roles in development (Kawata et al., 1997).

We were interested to discover whether the active use of ROS in cellular regulation that appears to be characteristic of multicellular eukaryotes also occurs in *Dictyostelium*. A burst of production of peroxide species during the early stages of development is evident in catalase-deficient strains (Fisher et al., 1991). This could be indicative of prior generation of superoxide; we sought to investigate this directly. We established an assay for superoxide production in *Dictyostelium* and found it to be produced in significant amounts during early development. Production could be stimulated by a factor in conditioned medium from developing cells. Reduction of superoxide levels by pharmacological or genetic means lead to an inhibition of aggregation. These data show that superoxide signalling plays an essential role in the transition from the single to multicellular phases of *Dictyostelium*.

## Materials and Methods

### Cell growth and development

Cells were grown either in HL-5 axenic medium or on SM agar in association with *Klebsiella aerogenes* as described previously (Sussman, 1987). SOD-OE cells were grown axenically and the medium was supplemented with 20-100 μg/ml G418. Development was initiated in exponentially growing cells by centrifuging and washing three times in potassium phosphate buffer (20 mM, pH 6.2). Cells were resuspended at the appropriate density and were then either shaken in suspension at 110 rpm, plated in 24-well tissue culture plates, placed as droplets on the surface of potassium phosphate buffer 1% agar plates, or placed on Millipore filters. All development took place at 22°C in the dark.

### Assay for superoxide production

XTT (2,3-bis-[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide) was purchased from Molecular Probes. Stock solutions (10 mM) were prepared in potassium phosphate buffer when required, or stored at –20°C. For the assay, XTT was added to developing cells at a concentration of 500 μM, and incubated for the indicated time. Reaction with superoxide causes the pale yellow XTT to form its bright orange-coloured formazan, the accumulation of which can be measured by monitoring its absorbance at 470 nm (Able et al., 1998; Ukeda et al., 1997). Aliquots of cells were quickly spun at full speed in a microfuge, and the absorption of the supernatant measured in a spectrophotometer. XTT reduction in the absence of cells was always determined as a control and subtracted from the values seen in the presence of cells, before plotting. When assays were performed in the presence of conditioned medium, control experiments were carried out to determine the degree of XTT reduction which was due to the conditioned medium alone. In general the levels were small (a typical OD reading would be 0.095 with the majority of the reduction occurring within the first minute). These values were subtracted from the reduction seen in the presence of cells.

### Vital staining of cells with rhodamine 123

Rhodamine 123 has been shown to be a marker of actively respiring mitochondria in *Dictyostelium* (Matsuyama and Maeda, 1995). Exponentially growing Ax2 cells were washed, resuspended in phosphate buffer at a density of $2\times10^6$ cells/ml. Duplicate samples were treated for 10 minutes with varying concentrations of rotenone or DMSO carrier and then for a further 10 minutes with 10 μg/ml rhodamine 123 dissolved in phosphate buffer. The cells were washed briefly three times in phosphate buffer and observed by fluorescence microscopy to confirm rhodamine accumulation in distinct subcellular organelles as expected for mitochondria. The degree of rhodamine sequestration by actively respiring mitochondria was determined as the degree of fluorescence, determined using a spectrofluorimeter (excitation 507 nm, emission 529 nm) as described by Altenberg et al. (Altenberg et al., 1994). The fluorescence of cells labelled with rhodamine 123 in the absence of rotenone was taken to be 100% and cells treated in the absence of rhodamine 123 as 0%. The degree of fluorescence in the presence of rotenone is expressed relative to these.

### Cell survival experiments

Cell survival in the presence of superoxide scavengers was determined essentially as described by Deering et al. (Deering et al., 1996). Exponentially growing Ax2 cells were diluted 1:12 in potassium phosphate buffer to inhibit cell growth and scavengers added at a range of concentrations. Following 18 hours of incubation at 22°C and 180 rpm, cells were serially diluted in phosphate buffer and 300 cells plated on two 15 cm SM agar plates in association with *Klebsiella aerogenes*. 18 hours of drug treatment was chosen as it is somewhat longer than the aggregation assays and so should represent the survival within the aggregation assay. Plates were incubated at 22°C and colonies counted daily from 3 days. The survival in the presence of the drug is expressed as a percentage of that seen in its absence.

### Preparation of conditioned medium

Exponentially growing cells were washed three times and resuspended in potassium phosphate buffer at a density of $2\times10^7$/ml and developed in shaking suspension for 6 hours. The cells were pelletted and the supernatant filtered through a 0.2 μm syringe filter unit. Medium was used in experiments immediately.

### Generation of cells overexpressing superoxide dismutase

A full-length cDNA was kindly provided by the Japanese cDNA sequencing consortium. It was amplified by polymerase chain reaction using the primers GGGAGATCTGAACAAAAATTATTATCAGA-AGAAGATTTAAATAGGTTACCTACAAAAG and CTCCTCGAG-TTATTGAGAGAACAATGACACC, in order to incorporate a 5′ *Bgl*II restriction site and c-*myc* tag, and a 3′ *Xho*I restriction site. The PCR product was purified, digested with *Bgl*II and *Xho*I, and then ligated into the pAct15-Gal plasmid (Harwood and Drury, 1990), from which

the *lacZ* sequence had been excised using the same endonucleases. Ax2 cells were transformed with this plasmid (Pang et al., 1999), selecting with 10 μg/ml G418. Initial transformants were then further selected by growing sequentially in 20, 50 and 100 μg/ml G418.

### Preparation of RNA and detection of mRNA species

Total cellular RNA was prepared using the Catrimox-14™ method (Insall et al., 1996). 25 μg samples were separated by electrophoresis in a 1% formaldehyde gel and transferred to a nylon membrane. cDNAs of genes to be probed were randomly labelled with [α-$^{32}$P]dCTP (Prime-It® RmT Random Primer Labelling Kit; Stratagene) and hybridised to the membrane, washed and detected according to the method of Huang et al. (Huang and Pears, 1999).

### Western blot

Exponentially growing Ax2 cells and cells overexpressing SodA were lysed directly into hot SDS sample buffer and resolved by 12% SDS-PAGE. After transfer to PVDF paper the blot was probed with polyclonal anti-sera raised against full-length human Cu/Zn superoxide dismutase (FL-154; Santa Cruz Biotechnology). The blot was then stripped and reprobed sequentially with monoclonal antibody specific for the human c-myc tag (9E10; Santa Cruz) and then polyclonal antisera against actin (C-11; Santa Cruz) to confirm equal protein loading.

## Results

A previous investigation had provided evidence for the generation of peroxide species during early *Dictyostelium* development, initiated by starvation (Fisher et al., 1991). These authors concluded that mitochondrial respiration was responsible for this phenomenon, probably via the initial production of superoxide radicals. In order to directly measure production of superoxide, we utilised an assay based on the reduction of the tetrazolium dye, XTT (Able et al., 1998). The reaction of this dye with superoxide results in the production of an orange, water-soluble formazan, the accumulation of which can be easily measured using a spectrophotometer (Ukeda et al., 1997). *Dictyostelium* cells developing under buffer were found to reduce XTT at an approximately constant rate for at least 24 hours after being removed from nutrient medium (Fig. 1A). At least part of this reduction is mediated by superoxide anions as it can be inhibited by up to ~70% by addition of bovine erythrocyte Cu/Zn superoxide dismutase (SOD) to the medium (Fig. 1B); half-maximal inhibition occurs at approximately 1 unit/ml SOD. The remaining XTT reduction could be due to superoxide-independent reactions, or superoxide-dependent reduction at sites inaccessible to extracellular SOD, most notably inside the cell. A comparison of the effect of SOD and the low molecular mass superoxide scavenger tiron (4,5 dihydroxy-1,3-benzene-disulphonic acid) indicated that the further XTT reduction is due to superoxide generation at sites not accessible to SOD (data not shown). In addition, inhibition of intracellular SOD by the chelating agent sodium diethyldithiocarbamate (DEDTC) stimulates XTT reduction (Fig. 1C) further supporting the idea that superoxide generated within the cell can be detected using this assay. A similar assay, widely used to detect superoxide, uses cytochrome c in place of XTT (Babior et al., 1973). Starving *Dictyostelium* cells were also found to rapidly reduce this molecule in a SOD-inhibitable manner, confirming the generation of superoxide (data not shown). However, levels of reduced cytochrome c soon declined, presumably either because of re-oxidation or proteolysis and so XTT reduction was used as the preferred assay.

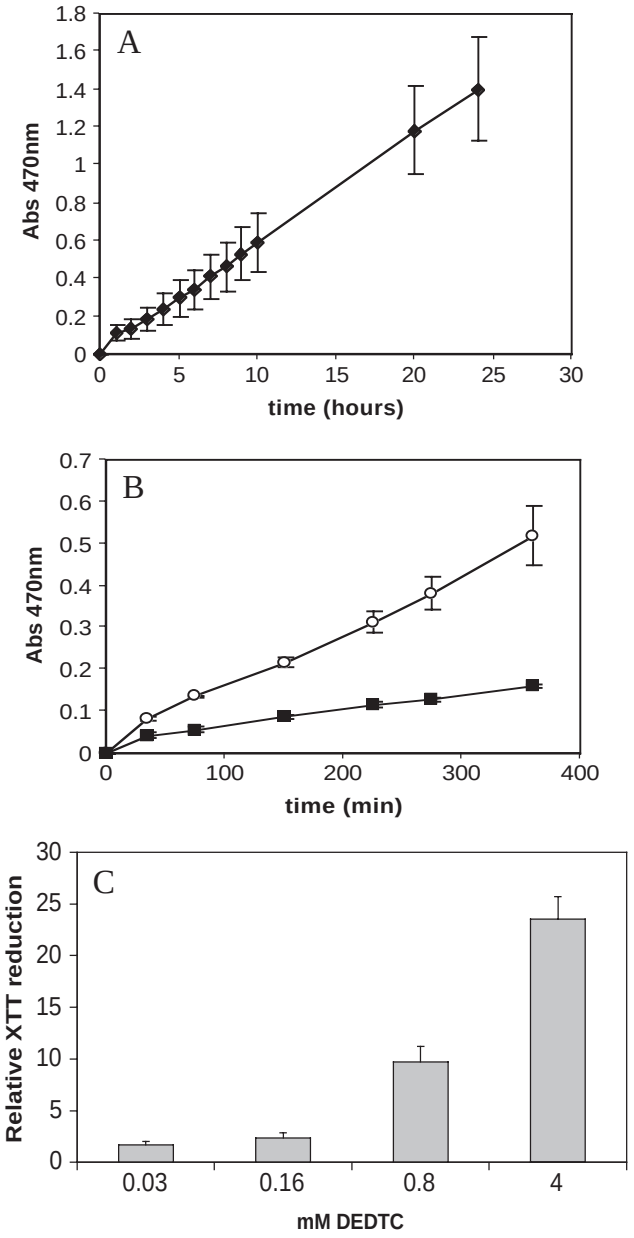


**Fig. 1.** Generation of superoxide during early development. (A) Ax2 cells were starved after plating onto plastic dishes under buffer at a density of $1.4\times10^6$ cells/cm$^2$, and superoxide generation was measured using the XTT assay. The increase in optical density results from the accumulation of the brightly coloured product of the reaction of XTT and superoxide. XTT reduction in the absence of cells was subtracted from each time point. Data are the means ± s.d. of five experiments. (B) Ax2 cells were starved in shaking suspension at a density of $2\times10^7$ cells/ml in the presence (■) and absence (○) of 100 units/ml Cu/Zn SOD. Data are the means ± s.d. of three experiments. (C) Ax2 cells were developed exactly as for A but with the addition of various concentrations of sodium diethyldithiocarbamate (DEDTC). The absorbance was measured after 7 hours. The data are expressed relative to the control lacking DEDTC, and are the means ± s.e.m. of three experiments.

Clearly developing *Dictyostelium* cells generate significant amounts of superoxide anions, but in contrast to the clear peak of peroxide production in catalase-deficient strains observed by Fisher and co-workers, superoxide appears to be generated at a constant rate (Fisher et al., 1991). Fisher et al. concluded that the mitochondrial respiratory chain was a major direct source of ROS. In order to assess the contribution of this source to the superoxide measured using the XTT assay, the effect of the NADH dehydrogenase inhibitor rotenone [which was used to disrupt mitochondrial function by Fisher et al. (Fisher et al., 1991)] was measured. A short-term 10 minute assay was used in order to avoid indirect longer-term effects caused by perturbation of mitochondrial respiration on general cell function. The maximal inhibition of XTT reduction caused by rotenone was less than 20% (Fig. 2). Rotenone was shown to be effective in disrupting mitochondrial function as it reduced the amount of rhodamine 123 sequestered by cells. Rhodamine 123 is a fluorescent dye that accumulates in actively respiring mitochondria (Johnson et al., 1980). Untreated Ax2 cells were found to sequester rhodamine 123 whereas pretreatment of cells for 10 minutes with even the lowest concentration of rotenone used, reduced the rhodamine 123 associated with cells to less than 25% of control levels (Fig. 2B). Generation of superoxide at the plasma membrane is also supported by the proportionately large inhibition of XTT reduction by extracellular SOD (Fig. 1B). Given the short half-life of superoxide and its low membrane permeability (reviewed by Korshunov and Imlay, 2002) it is more likely that the superoxide measured in this assay is generated at the plasma membrane.

Mammals and plants contain superoxide-generating NADPH oxidase enzymes in the plasma membrane that are capable of responding to extracellular factors (Lambeth et al., 2000; Pei et al., 2000). We wished to determine if a similar phenomenon was occurring in *Dictyostelium*. Aggregating *Dictyostelium* cells are known to emit, and respond to, pulses of cAMP that interacts with a cell surface receptor to trigger various responses, including chemotaxis. The addition of a pulse of cAMP, however, had no effect on XTT reduction in a short-term assay, suggesting that this does not stimulate superoxide production (data not shown). A mutant strain lacking the major adenylyl cyclase active during aggregation, ACA (Pitt et al., 1992) caused a similar level of XTT reduction to the parental strain, confirming that extracellular cAMP does not play a role in stimulating superoxide production (data not shown).

In order to determine whether any other factors secreted during aggregation are stimulatory, we prepared conditioned medium from Ax2 cells starved in shaking suspension for 6 hours by centrifugation and filtering of the supernatant. Addition of this conditioned medium to starved cells in the presence of 0.5 mM XTT gave a two-fold stimulation of XTT reduction compared with fresh non-conditioned medium (Fig. 3). Prior heating of the conditioned medium to 80°C destroyed this activity, decreasing this stimulation by about 75%. Thus a heat-labile factor produced by developing cells is able to act in an autocrine manner to increase superoxide generation.

Given this inducible generation of ROS similar to that seen in multicellular eukaryotes, we tested whether superoxide might have specific functional roles during development. Fig. 4A shows the effect of five superoxide scavengers – SOD, tiron and the three tetrazolium salts MTT, NBT and XTT (the last-named at double the concentration used in the superoxide assay) – on aggregation after 12 hours of starvation in their presence. The four low molecular mass scavengers completely prevented aggregation in a dose-dependent manner, while SOD had no discernible effect at any concentration tested. This could be either because the effects of the lower molecular weight molecules were independent of superoxide scavenging, or because of the inability of the relatively large, polar SOD molecule to reach a critical site (for example inside the cell).

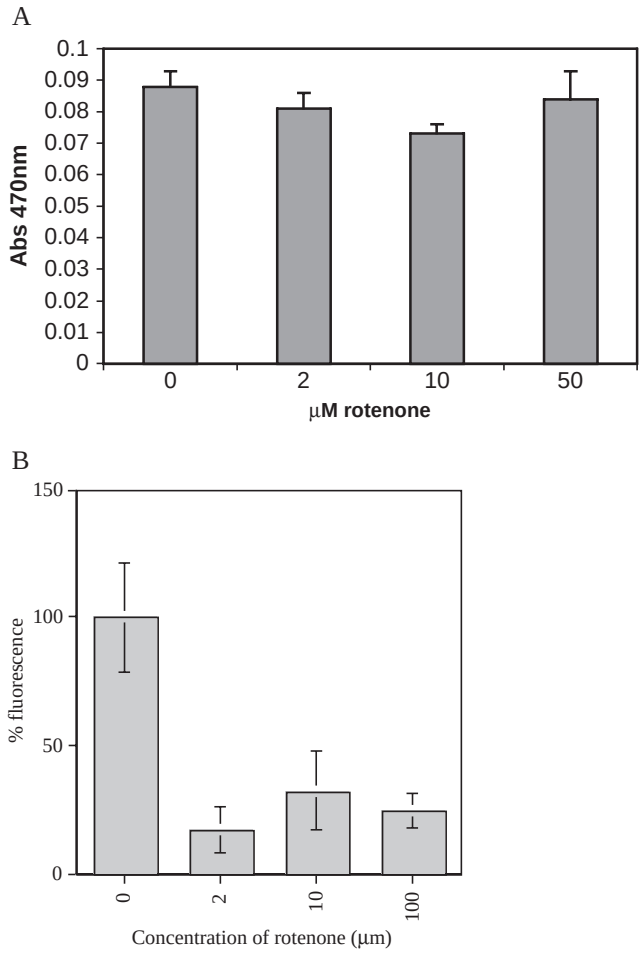


**Fig. 2.** Contribution of mitochondria to superoxide generation. (A) Ax2 cells were starved in shaking suspension for 6 hours at a density of $2\times10^7$ cells/ml, then resuspended at $10^8$ cells/ml, and 0.5 mM XTT and the indicated concentration (μM) of rotenone added. After 10 minutes the absorbance at 470 nm was measured. The data are the means ± s.e.m. of three experiments. (B) Exponentially growing Ax2 cells were washed and resuspended in phosphate buffer at a density of $2\times10^6$ cells/ml. Duplicate samples were treated with rotenone or DMSO carrier for 10 minutes and then for a further 10 minutes with 10 μg/ml rhodamine 123. Cells were washed briefly three times in phosphate buffer and the degree of rhodamine sequestration determined as the relative fluorescence measured using a spectrofluorimeter. One experiment typical of three is shown. Fluorescence microscopy confirmed that rhodamine 123 accumulated in punctate, filamentous structures as expected for mitochondria in cells not treated with rotenone (Matsuyama and Maeda, 1995), but no such staining was detectable in cells treated with 2 μM rotenone (data not shown).

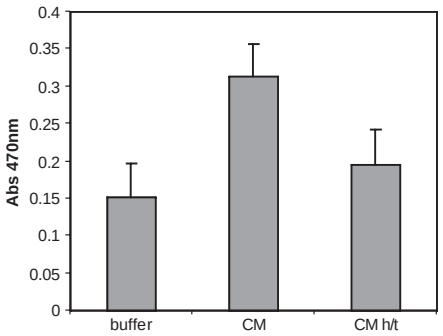


**Fig. 3.** Stimulation of superoxide production by conditioned medium. Ax2 cells were starved in shaking suspension, and after 6 hours were resuspended at $10^8$ cells/ml in fresh buffer, in conditioned medium (CM), or heat-treated conditioned medium (CM h/t), all containing 0.5 mM XTT. After 10 minutes the absorbance at 470 nm was measured. XTT reduction in the presence of the relevant medium (buffer or CM) and the absence of cells was subtracted. The data are the means ± s.e.m. of three experiments.

The dose-dependent inhibition of XTT reduction by MTT, NBT and tiron (Fig. 4B), confirm that these molecules do indeed scavenge superoxide. The large percentage of maximal inhibition compared to maximal inhibition seen by SOD (Fig. 1B) suggests that these molecules are capable of reaching superoxide at sites inaccessible to the enzyme.

The failure to aggregate could be because the drugs were toxic to *Dictyostelium*. However Ax2 cells incubated for 18 hours in the presence of the superoxide scavengers did not show a significant loss of cell viability (Fig. 4C). If anything, the cell number increased in the presence of the tetrazolium salts. One explanation for this may be that the scavengers render the cells insensitive to a starvation-induced withdrawal from the cell cycle. Further evidence that the scavengers were not preventing aggregation purely at the level of toxicity is the density dependence of the effect (Fig. 4D). When the cells were plated for aggregation at high densities, aggregates did form, proving cells to be capable of aggregation at these concentrations of scavenger. Higher concentrations of the drug were required to inhibit aggregation at higher cell densities. These results are consistent with the scavengers lowering the production of, or response to, an extracellular signal that can be restored at high cell density where the level of signal is increased, rather than a general toxic effect. When aggregation occurred, streaming of cells was visible suggesting that the cells were capable of chemotactic movement at these concentrations of drug. It is possible that the rate of movement is reduced but in that case one would expect aggregation at normal cell densities to simply be delayed, not completely inhibited.

From these results we concluded that intracellular superoxide is likely to have some essential function either in the attainment of aggregation competence or in aggregation itself, although it remained possible that the effects on cells observed might be independent of superoxide scavenging.

In order to resolve this doubt, we developed a strategy to overexpress the *Dictyostelium* cytosolic SOD enzyme by stable transformation of amoebae with a construct containing the cDNA encoding the SodA protein (Garcia et al., 2000). The cDNA was courtesy of the Japanese cDNA sequencing consortium (Morio et al., 1998), and expression of the SodA protein, tagged with a c-myc epitope at its N terminus, was driven by the strong actin15 promoter. Overexpression should decrease the level of superoxide in the cell without the potential side-effects of the chemical scavengers. Transformants were selected by resistance to G418 as the transforming plasmid carried the resistance gene, and expression of the myc-tagged protein was confirmed by western blot (Fig. 5A). Polyclonal anti-sera raised against human SOD interacted with a protein precisely co-migrating with the myc- tagged protein. A doublet is detected in the SOD-OE cells with the upper band co-migrating with the anti-myc reactive band. The smaller band seems likely to be SOD protein from which the myc tag has been cleaved as it co-migrates with the band which reacts with the anti-SOD antisera in Ax2 cells. This lower band (slightly smaller than the human one detected in the control Jurkat cell lysate, as expected from the predicted molecular masses) is detectable at lower levels in Ax2 cells. This is consistent with the antisera cross-reacting with the *Dictyostelium* SOD which is overexpressed in the SOD-OE cells.

Increasing the concentration of G418 in a pool of transformed cells has been shown to increase expression of transgenes associated with the neomycin resistance gene (Simon et al., 1989). Selection of cells transformed with the SOD-OE construct with increasing concentrations of G418 progressively decreased the amount of superoxide detected in developing cells with the XTT assay (Fig. 5B). This confirms that XTT reduction is measuring superoxide production and that overexpression of SOD reduces the levels of superoxide. Cells selected at high concentrations of G418 (50 or 100 μg/ml) showed no reduction in growth rate compared to parental Ax2 cells and a transformation control, suggesting that SOD overexpression is not generally toxic to the cells (Fig. 6A). The SOD-OE cells appeared healthy when viewed under the microscope (data not shown). However, the cells do show an aggregation-minus (agg–) phenotype on agar plates after growth on a lawn of *Klebsiella aerogenes* (data not shown). The agg– phenotype was confirmed by developing the SOD-OE cells both on filters (data not shown) and buffered agar (Fig. 6B) where SOD-OE cells failed to form aggregates at cell densities at which control cells could. The SOD-OE cells could form aggregates when plated at high cell densities, again showing that the cells were viable and capable of movement. The higher the concentration of G418 used for selection and therefore the lower the levels of superoxide produced, the higher the cell density required to see aggregates forming on buffered agar. Again, these results are consistent with superoxide acting in a signalling role, rather than that the overexpression has a general toxic effect on the cells. This provides further strong support for the idea that intracellular superoxide plays an essential role in early *Dictyostelium* development.

The course of *Dictyostelium* development is marked by successive changes in gene expression, with the induction of certain genes being necessary for the progression through each stage. In order to gain insight into the developmental defect of the SOD-overexpressing cells, the time-course of expression of a number of developmentally regulated genes was examined by northern blot (Fig. 7). While some genes, for example *pkaC*, which encodes the PKA catalytic subunit, show a normal

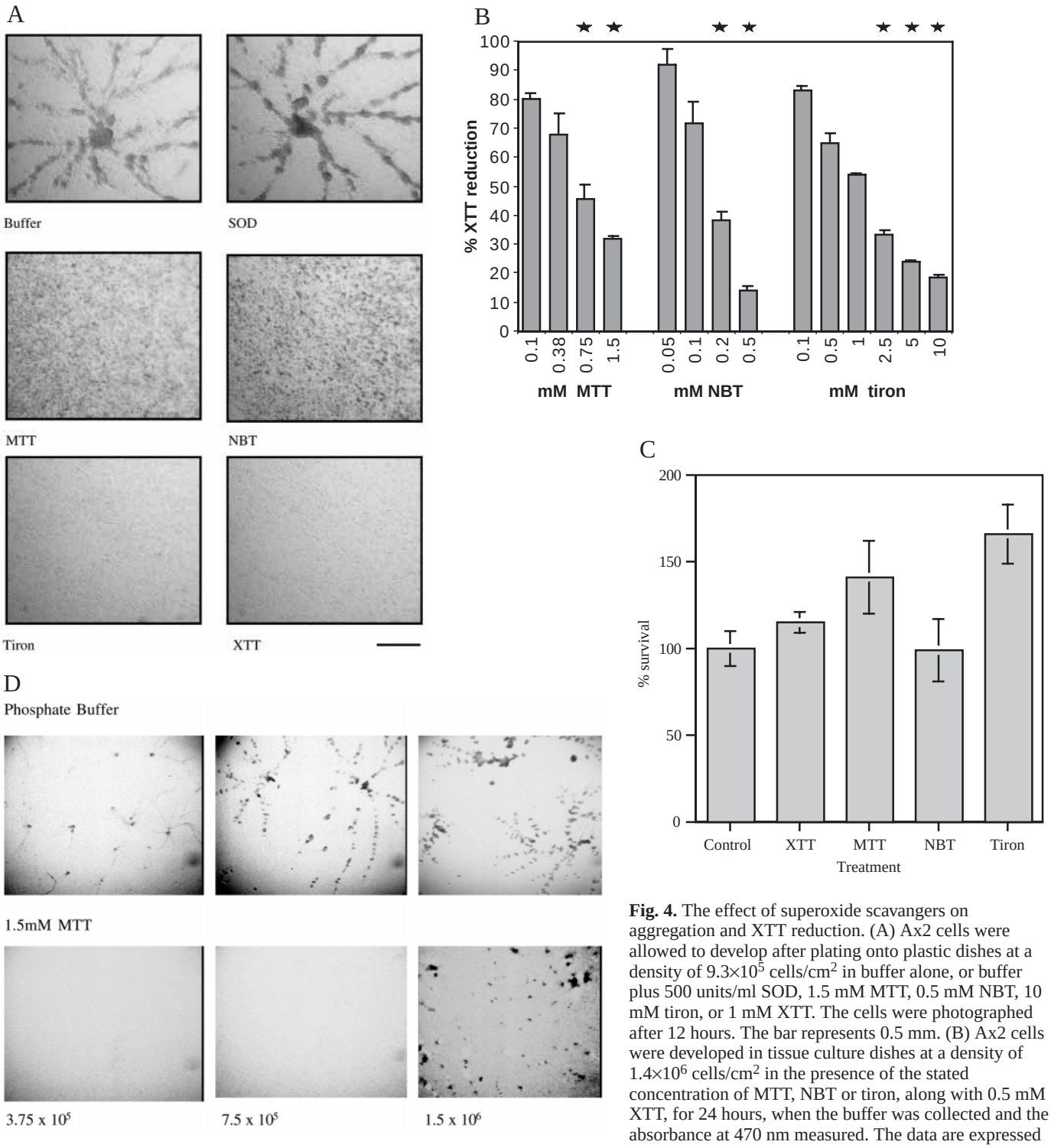


**Fig. 4.** The effect of superoxide scavangers on aggregation and XTT reduction. (A) Ax2 cells were allowed to develop after plating onto plastic dishes at a density of $9.3\times10^5$ cells/cm$^2$ in buffer alone, or buffer plus 500 units/ml SOD, 1.5 mM MTT, 0.5 mM NBT, 10 mM tiron, or 1 mM XTT. The cells were photographed after 12 hours. The bar represents 0.5 mm. (B) Ax2 cells were developed in tissue culture dishes at a density of $1.4\times10^6$ cells/cm$^2$ in the presence of the stated concentration of MTT, NBT or tiron, along with 0.5 mM XTT, for 24 hours, when the buffer was collected and the absorbance at 470 nm measured. The data are expressed as percentage reductions in absorbance relative to a control from cells developed in the presence of 0.5 mM XTT alone, and are the means ± s.e.m. of three experiments. Asterisks indicate concentrations of scavengers that were sufficient to inhibit aggregation at this cell density. (C) Exponentially growing Ax2 cells were diluted 1:12 in phosphate buffer and superoxide scavenger added (XTT 1 mM, MTT 1.5 mM, NBT 0.2 mM, tiron 20 mM). These concentrations of scavenger all inhibited aggregation. The cultures were incubated for 18 hours at 22°C, 180 rpm before serial dilution in phosphate buffer and 300 cells of each were plated over 2× 15-cm SM plates, in association with *Klebsiella aerogenes*. Cell survival was determined by counting the number of clonal *Dictyostelium* colonies appearing on the bacterial lawn. Data are the means ± s.e.m. of triplicate plates within one experiment. One experiment representative of 3 independent experiments is shown. (D) Exponentially growing Ax2 cells were harvested and plated to aggregate under buffer as described in legend to Fig. 4A, but at three different cell densities, as indicated below. 1.5 mM MTT was sufficient to inhibit aggregation at the two lower cell densities, but not at the highest cell density. Similar density dependence was observed for the inhibition of aggregation in the presence of XTT, NBT and tiron (data not shown).

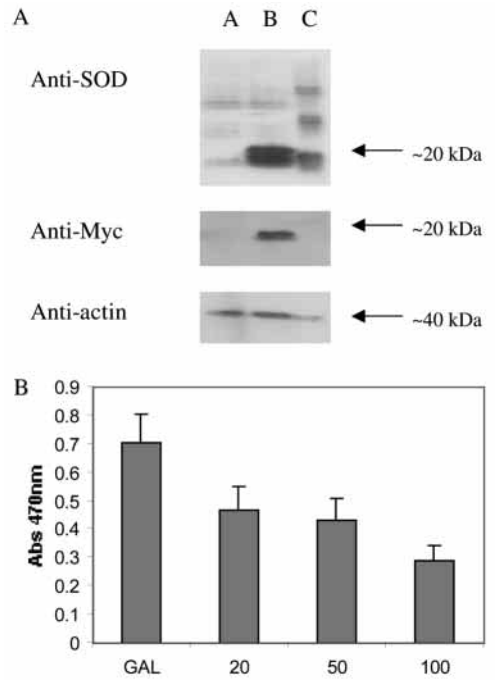


**Fig. 5.** Verification of cells overexpressing SodA. (A) Exponentially growing parental Ax2 (A) and SOD-OE (B) cells (grown in 20 μg/ml G418) were lysed in SDS sample buffer and resolved by 12% SDS-PAGE, along with cell lysate from human Jurkat T cells (C) as a positive control, before being transferred to a PVDF membrane. The membrane was probed with polyclonal antisera raised against SOD (FL-154). A band of around 20 kDa, the expected size for SOD, was detected in both Ax2 and Jurkat cells. Increased immunoreactivity was seen for a doublet in the SOD-OE cells. The blot was then stripped and reprobed with antibody specific for the human c-myc epitope which had been inserted at the N terminus of the exogenous SodA protein, using the 9E10 antibody. This reacted with a single band, only in the SOD-OE cells, co-migrating with the upper of the two bands from the anti-SOD blot. It is likely that the lower band of the doublet seen with the anti-SOD antibody (co-migrating with the band seen in Ax2 cells) is SOD protein in which the N-terminal myc tag has been cleaved. The blot was then reprobed using the C-11 anti-actin antibody as a loading control. (B) 24-hour XTT assays were performed on the following cells aggregating on plastic as described in Fig. 1. GAL indicates Ax2 cells expressing β-galactosidase, after selection at 100 μg/ml G418; 20, 50, 100 indicate Ax2 cells overexpressing SodA, (SOD-OE) after selection at 20, 50 or 100 μg/ml G418, respectively. Data are the means ± s.e.m. of three experiments.

increase in expression upon starvation, a set of genes showed greatly decreased expression in the SOD-overexpressing cells. This set included the genes encoding ACA, cAR1 (the major early cAMP receptor), PDE (the extracellular cAMP phosphodiesterase), and discoidin I. The last-named gene shows the greatest disparity between the SOD-overexpressor and its parent, because it is very strongly expressed in parental strains during early development, increasing in the first few hours from its lower growth-phase level. Strikingly discoidin I mRNA was barely detectable in the SOD-overexpressor during growth; at this stage the level of its expression is controlled by the secreted protein prestarvation factor (PSF), as are the levels of cAR1 and PDE (Rathi et al., 1991; Rathi et al., 1992). The increase in discoidin expression subsequent to starvation is predominantly in response to a second secreted protein, conditioned medium factor (CMF) (Gomer et al., 1991); the later increases in PDE and cAR1 expression are induced by cAMP. These gene expression data raised the possibility that the SOD-overexpressing strain is defective in the production of, or response to, one of these factors secreted in early development.

Pulsing the cells with extracellular cAMP, which rescues the defect of many aggregation-deficient strains such as those lacking cAR1 (Saxe et al., 1991), had no effect on the aggregation of the SOD-OE cells (data not shown). However, development of the SOD-OE cells in conditioned medium prepared from starving AX2 cells resulted in the formation of aggregates (Fig. 8). It had been found that this conditioned medium was able to stimulate superoxide generation (Fig. 3); this stimulation was markedly reduced by heat treatment, and again heating medium to 80°C for 20 minutes decreased its ability to rescue development as aggregates were not formed in its presence. Conditioned medium prepared from SOD-OE cells starved for 6 hours was also able to rescue the aggregation defect of freshly starved SOD-OE cells. This suggests that overexpression of SOD, and thus decreased superoxide levels, may inhibit the production or secretion of some factor or reduce the response to it, such that a higher concentration, accumulated over 6 hours, is required. The heat lability of the factor is inconsistent with its being identical to CMF, and accordingly purified recombinant CMF has no effect on superoxide production (data not shown). PSF is heat labile, as is the little studied differentiation stimulating factor (DSF) reported by Klein et al. (Klein et al., 1976). Neither of these factors has been purified, so their involvement in the phenomena describe here remain to be tested.

## Discussion

Multicellular organisms have evolved sophisticated systems by which their cells regulate their respective functions co-ordinately. *Dictyostelium*, as a social amoeba, represents an unusual mode of multicellularity by which previously independent cells group together to form a greater organism, but requires a similar degree of communication between different cells as do multicellular plants and animals. The present study provides evidence that *Dictyostelium* utilises superoxide as a signalling intermediate at the onset of its development from unicellular to multicellular existence. We found that, using a tetrazolium-based assay, superoxide production could be measured, occurring predominantly outside the cells. As the presence of cells was necessary for superoxide to be detected, and because of this anion's short half-life and poor membrane permeability, it is thus likely that most of the superoxide measured is generated at the plasma membrane. (An extracellular source associated with the surface of cells is also a possibility, but this would require a continuous supply of reducing equivalents.) The assay was however also capable of detecting superoxide produced within cells, as demonstrated by the increased signal upon inhibition of cytosolic superoxide dismutase with diethyldithiocarbamate. Mitochondria are not a likely source for the majority of the superoxide detected in this assay as rotenone has little effect on its generation. The greatest effect is seen at 10 μM and this is significant when assessed using Student's *t*-test. The

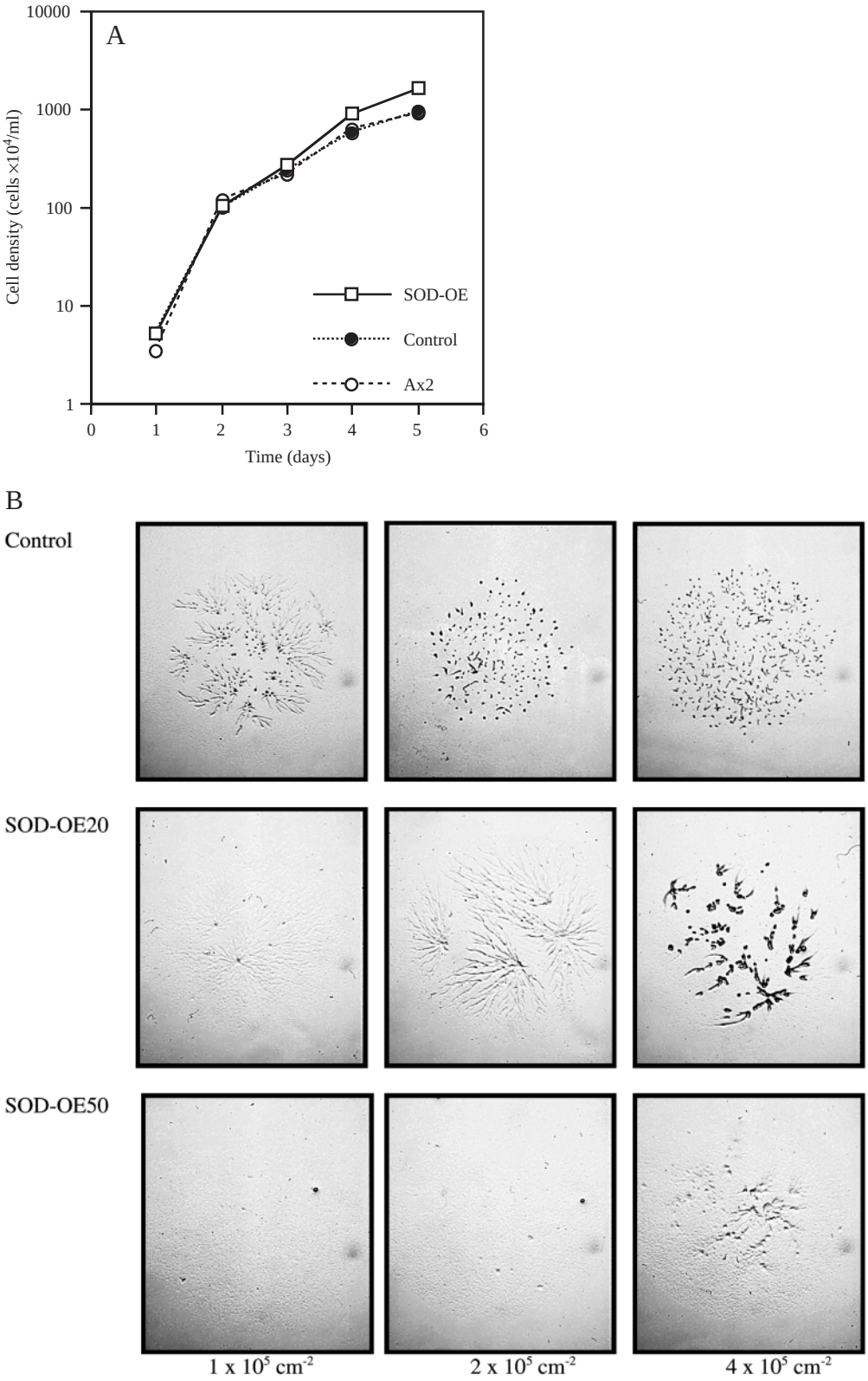


**Fig. 6.** Phenotype of cells overexpressing SodA. (A) Exponentially growing cells (Ax2, control cells expressing β-galactosidase and SOD-OE cells, the latter two grown in 50 μg/ml G418) were diluted to a density of $3\times10^5$/ml in fresh HL5 medium in triplicate. The cultures were incubated at 22°C and 180 rpm and aliquots were taken in duplicate every day to determine cell density. One experiment typical of three independent experiments is shown. (B) Control cells (expressing β-gal from the actin 15 promoter) grown in 100 μg/ml G418 and SOD-OE cells grown in 20 and 50 μg/ml G418 were harvested, washed in phosphate buffer and plated on phosphate-buffered agar at the cell densities indicated. The plates were incubated at 22°C in the dark and photographs were taken after 12 hours. No aggregates subsequently formed on the two plates of SOD-OE 50 cells at the two lowest cell densities and no aggregates were seen at any of these cell densities for SOD-OE cells grown in 100 μg/ml G418 (data not shown).

inhibition of superoxide generation at higher concentrations of rotenone is not, however, significant, raising the possibility that these higher concentrations also stimulate superoxide production, perhaps from an alternative source. Rotenone did inhibit accumulation of rhodamine 123 (which sequesters in actively respiring mitochondria) in the cells, consistent with it entering the cells and disrupting mitochondrial function. When added to starving Ax2 cells, rotenone led to a delay in aggregation, but did not inhibit aggregation, even at 100 μM where a delay of around 5 hours was apparent (data not shown).

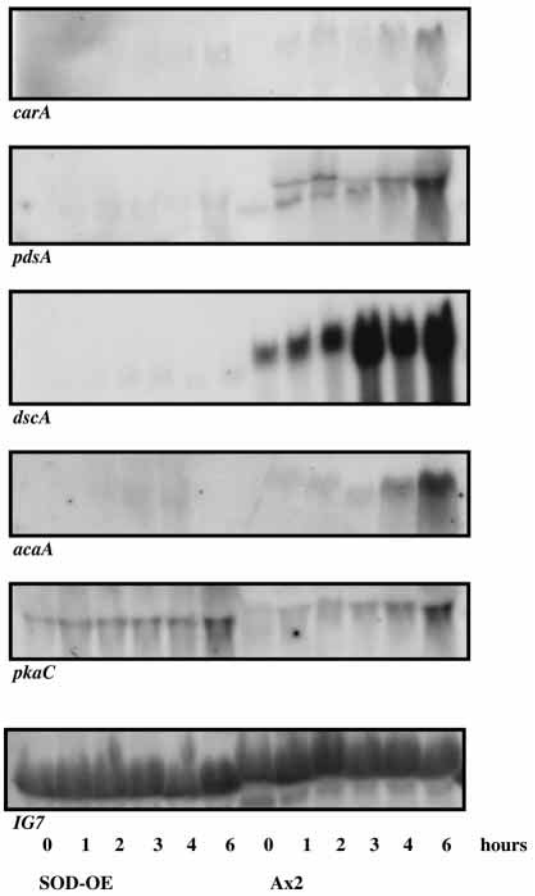


**Fig. 7.** Regulation of gene expression during early development of cells overexpressing superoxide dismutase. SOD-OE and Ax2 cells were separately developed in shaking suspension at $2\times10^7$ cells/ml and aliquots taken for RNA extraction at the indicated times after starvation. The RNA was blotted onto a nylon membrane, and probed with $^{32}$P-labelled fragments of the indicated genes. carA, cAMP receptor cAR1; pdsA, extracellular phosphodiesterase; dscA, discoidin 1; acaA, aggregation stage adenylyl cyclase A; pkaC, the catalytic subunit of cAMP dependent protein kinase; IG7, loading control.

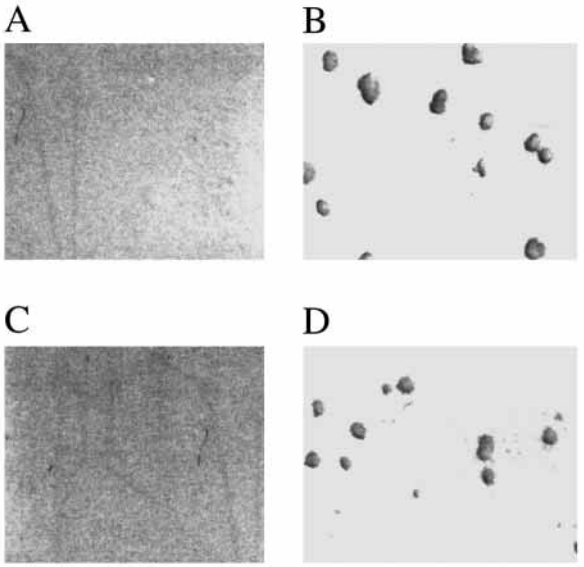


**Fig. 8.** Rescue of aggregation defect of SOD-OE cells by conditioned medium. SOD-OE cells were developed in tissue culture dishes at a density of $5.7\times10^5$ cells/cm$^2$ under (A) buffer, (B) conditioned medium from AX2 cells, (C) heat-treated conditioned medium, (D) conditioned medium from SOD-OE cells.

As the superoxide scavengers inhibit rather than delay aggregation this difference in phenotype of drug-treated cells again is consistent with the scavengers depleting superoxide from a non-mitochondrial source.

An excellent candidate for a source of superoxide anions within the plasma membrane would be an NADPH oxidase enzyme similar to those found in animals and plants (Henderson and Chappell, 1996; Lambeth et al., 2000; Torres et al., 1998). While certain of these enzymes appear to be constitutively active at a low level, the activities of others are markedly stimulated by specific stimuli from outside the cell (Suh et al., 1999; Pei et al., 2000; Banfi et al., 2001). We found that superoxide generation by *Dictyostelium* cells could be rapidly stimulated by a heat-labile factor produced by developing cells. This stimulation occurred within 10 minutes, and thus probably reflects an increase in activity of an existing enzyme rather than the synthesis of new protein. The identity of this factor remains uncertain. The described properties of the protein factors PSF and DSF (which may be identical) make them candidates; however neither has been fully characterised and so their involvement has not been tested. CMF, which is heat-stable, does not stimulate superoxide generation; nor does cAMP phosphodiesterase (G.B. and C.P., unpublished), another factor secreted by developing cells, which is heat-labile and essential for development (Wu et al., 1995).

The best candidate for an enzyme to generate superoxide is a homologue of human NADPH oxidases. Searches of databases of *Dictyostelium* genomic and cDNA sequences (Kreppel and Kimmel, 2002; Morio et al., 1998) have revealed at least three homologues of the NADPH oxidase flavocytochrome subunit, at least one of which is expressed most strongly during the first few hours of development (G.B. and C.P., unpublished). [The sequence of this gene is accessible in GenBank, accession no. AF123275; for a dendrogram displaying the relationship of its sequence with various metazoan homologues see Cheng et al. (Cheng et al., 2001).] Membrane preparations from freshly starved amoebae contain an NADPH oxidase activity that is inhibited by the flavoprotein inhibitor, diphenyliodonium (G.B. and C.P., unpublished), consistent with such an enzyme being a source of superoxide in *Dictyostelium*.

Whatever the source, we found that superoxide is necessary for normal *Dictyostelium* development to occur. Three different tetrazolium compounds, and the phenolic compound tiron, were all found to decrease the amount of detectable superoxide in a dose-dependent manner, and above threshold concentrations to inhibit the aggregation of amoebae that is the first visible stage of development. These concentrations of scavengers did not lead to any reduction in cell viability so the failure to aggregate is unlikely to be due to general toxicity. Also if the cells were plated at high cell densities, then aggregation did occur indicating that the cells were still capable of cell movement and cellular responses in the presence of the drugs. These results are

consistent with the scavengers leading to a reduction in production of, or response to, an extracellular signal which can be overcome at high cell densities where higher concentrations of signal are available, rather than a general toxic effect. Superoxide dismutase added to the medium decreased the amount of superoxide detected, but had no effect on aggregation. Thus either the pharmacological reagents reach sites inaccessible to SOD or their effect on development is unrelated to their ability to scavenge superoxide. As tiron is structurally distinct from the related tetrazolium compounds it seems unlikely that all four would have a common second target. However when superoxide dismutase levels in the cytoplasm were increased by the expression of the native Cu/Zn SOD gene under the control of a strong constitutive promoter, aggregation was inhibited. The clear implication of these results is that intracellular, but not extracellular, superoxide has a necessary role in development.

The possible molecular causes for this defective phenotype were investigated by examining the expression of a number of genes differentially regulated in the first hours of development. Comparison of the SOD-OE strain with its normally developing parent revealed clear differences in expression in a number of these genes. The most strongly affected was the discoidin I mRNA (Blusch et al., 1995). In SOD-OE cells, discoidin I mRNA is barely detectable in growing cells, and not induced at all after starvation. The mRNAs encoding PDE and cAR1 are also markedly underexpressed in these cells. The expression of these three genes is induced during late growth and early development by the secreted protein PSF, noted above as a candidate for the factor present in conditioned medium that stimulates superoxide generation. In the light of this potential connection, we tested the effect of developing SOD-OE cells in conditioned medium. In these conditions, the level of detectable superoxide was restored to near wild-type levels, and aggregates were formed. Conditioned medium from 6-hour-starved SOD-OE cells was also able to rescue the aggregation of freshly starved SOD-OE cells; heat-treated medium could not. This indicates that the developmental defect of these cells results from a deficient response to a factor that promotes the attainment of aggregation competence or from an inability to produce this factor.

The phenotype of cells overexpressing cytosolic SOD has a number of similarities with mutants lacking the dual-specificity protein kinase YakA. This mutant also fails to express early developmental genes, and does not aggregate (Souza et al., 1998) when grown in association with bacteria on an agar plate. YakA is thought to function as a key downstream regulator of heterotrimeric G-proteins; and mutations in the G-protein β and α4 subunits share a small plaque phenotype with *yaka–* (van Es et al., 2001) and SOD-OE cells (G.B. and C.P., unpublished).

This study has demonstrated that a superoxide-dependent signal is necessary for the initiation of development in *Dictyostelium*. Previous studies have pointed to the important roles ROS can play in developmental processes in animals, for example in the *Drosophila* eye (Morey et al., 2001), and in mammalian glial and neuronal cells (Smith et al., 2000; Suzukawa et al., 2000) and also in plants in the formation of cotton fibres (Potikha et al., 1999). Our observations extend the range of organisms that utilise such redox-mediated signals to the protozoa. Inhibition of ROS-dependent signalling events effect the transition from the unicellular to multicellular phase of *Dictyostelium*, suggesting the evolution of the use of superoxide signalling in response to extracellular factors arose with multicellularity. The amenability of *Dictyostelium* to genetic study and the imminent completion of the sequencing of its genome should provide ample opportunity for further study of the mechanisms involved in such signalling.

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