Role for YakA, cAMP, and Protein Kinase A in Regulation of Stress Responses of Dictyostelium discoideum Cells

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The Dictyostelium protein kinase YakA is required for the growth-to-development transition. During growth YakA controls the cell cycle, regulating the intervals between cell divisions. When starved for nutrients Dictyostelium cells arrest growth and undergo changes in gene expression, decreasing vegetative mRNAs and inducing the expression of pkaC. YakA is an effector of these changes, being necessary for the decrease of vegetative mRNA expression and the increase of protein kinase A (PKA) activity that will ultimately regulate expression of adenylyl cyclase, cAMP synthesis, and the induction of development. We report a role for this kinase in the response to nitrosative or oxidative stress of Dictyostelium cells. Hydrogen peroxide and sodium nitroprusside arrest the growth of cells and trigger cAMP synthesis and activation of PKA in a manner similar to the well-established response to nutrient starvation. We have found that yakA null cells are hypersensitive to nitrosative/oxidative stress and that a second-site mutation in pkaC suppresses this sensitivity. The response to different stresses has been investigated and YakA, cAMP, and PKA have been identified as components of the pathway that regulate the growth arrest that follows treatment with compounds that generate reactive oxygen species. The effect of different types of stress was evaluated in Dictyostelium and the YakA/PKA pathway was also implicated in the response to heat stress.

INTRODUCTION

A common feature to all living cells is the capacity to use survival mechanisms in response to environmental stresses, the most common stress encountered by many organisms being nutrient depletion. Dictyostelium discoideum responds to starvation by triggering a developmental program where isolated amoebae adopt a multicellular mode of living and differentiate into spores to survive the harsh conditions. In Dictyostelium, the responses observed in the first few hours that follow sensing of nutrient exhaustion include growth cessation, induction of cAMP synthesis and its secretion, and migration of the cells through cAMP gradients that guide the formation of the multicellular organisms (reviewed by Firtel, 1996; Loomis, 1998). A complex change in gene expression is observed upon starvation where growth-related genes are turned off and developmental genes are induced. Protein kinase A (PKA) levels increase severalfold upon starvation and this increase seems to be necessary for the up-regulation of genes related to cAMP synthesis and detection, such as the adenylyl cyclase acaA and the cAMP receptor carA. PKA has also been shown to regulate the cell type specialization that follows this process.

We have determined that several aspects of the starvation response in Dictyostelium are coordinated by the kinase YakA. YakA is necessary for the decrease in vegetative gene expression that occurs when cells are starved and in particular, for the decrease in the mRNA levels for the pufA gene. PufA inhibits translation of the pkaC mRNA, and its down-regulation seems to be essential for the increase in PKA production that will trigger the adenylyl cyclase acaA and the cAMP receptor carA mRNA expression and allow aggregation to proceed (Souza et al., 1999). The growth cessation that accompanies nutrient depletion is also under the control of the YakA pathway. YakA overexpression induces growth arrest and faster development, whereas YakA-deficient strains have a faster cell cycle and do not undergo development (Souza et al., 1998).
YakA belongs to a family of kinases that include Yak1 from yeast, the Dyrk/MNB-related kinases, and several other kinases from mouse, Caenorhabditis elegans, Arabidopsis, humans, and Drosophila. The recurring theme in all studies related to this kinase family is their involvement in the control of the cell cycle. In Dictyostelium YakA inhibits growth when overexpressed and yakA null mutants have a faster cell cycle and a smaller cell size (Souza et al., 1998). Minibrain (MNB) is located at the Down Syndrome critical region (Smith et al., 1997), being expressed in the regions of the brain that are abnormal in individuals with Down Syndrome (Guineri et al., 1996). Strong expression has also been found in epithelial cells that are highly mitotic (Rahmani et al., 1998). In Drosophila MNB is reported to be required for neuroblast proliferation during postembryonic neurogenesis (Tejedor et al., 1995). In yeast Yak1 is induced in conditions that arrest the cell cycle and acts as a growth attenuator in response to stresses and nutrient conditions (Garrett and Brouch, 1989; Garrett et al., 1991). The similarity of the Dyrk/MNB/Yaks with the cdk kinases involved in the regulation of cell division also suggests a role in the control of the cell cycle.

To investigate the role for YakA in the regulation of growth in response to stress we submitted Dictyostelium cells to several environmental challenges and observed a severe deficiency of yakA null cells to survive nitrosative or oxidative stress. The isolation of second site suppressors of this phenotype revealed a role for cAMP and PKA in the growth inhibition observed when cells are treated with compounds that generate oxidative species. Herein, we describe a new role for YakA in the regulation of the growth arrest induced by nitrosative, oxidative, and heat stress in Dictyostelium.

MATERIALS AND METHODS

Cell Strains

All strains are derived from the axenic D. discoideum strain AX4 (Knecht et al., 1986). Mutant strains used were as follows: pkaC null (Mann and Firtel, 1991), acaA null (Pitt et al., 1992), yakA null AK800 (Souza et al., 1998), yakA/pufA null AK804 (Souza et al., 1999), and yakA [pkaC/pkac] (Souza et al., 1999). The original yakA/pkaC null strain isolated in the suppressor screen was named 1-20. The isolation of second site suppressors of this phenotype was carried out as follows. The YakA-null mutant AK800, which harbors a plasmid insertion (IS800) in the sequence that encodes the protein kinase core (Souza et al., 1998), was used as the parental strain for insertional mutagenesis. A REMI-mutagenized population of 70,000 clones divided into 24 pools of 3000–3000 mutants was diluted to 5 × 10^6 cells/ml in HL-5 supplemented with 500 μM SNP. The cells were shaken at 22°C for 10 d, after which time growth was observed in nine of the pools. Cells were diluted and plated in association with K. aerogenes for clone isolation. Nine-sixty clones from each of the nine positive pools were picked into 96-well plates. Duplicates of each plate were prepared. One set of plates was treated with SNP for 1 wk and wells were inspected for cell growth. Ten clones that grew in the presence of SNP were picked for each pool from the untreated plates. These clones were grown, frozen, and cultures were expanded for genomic DNA isolation.

Growth and Stress Conditions for Dictyostelium Cells

All strains were grown in axenic media (HL-5) or on SM agar plates in the presence of Klebsiella aerogenes (Sussman, 1987). Treatments for survival rate scoring and growth curves were performed in fresh axenic cultures kept exponentially growing in HL-5 for 1 wk. For both cases cells were collected at 1–2 × 10^6/ml, diluted to 0.5–1 × 10^6/ml in HL-5, and 500 μM H_2O_2, 500 μM sodium nitroprusside (SNP), or 500 μM spermine N-(2-aminomethyl)-N-(2-hydroxy-2-nitrosohydrzino)-1,2-ethylenediamine (NONOate) was added. Oxyhemoglobin was added to 40 μM 30 min before SNP was added. The osmotic shocks were performed in 300 mM glucose, 20 mM phoshate pH 6.8 for 1 or 2 h with cells diluted to 1 × 10^6/ml. Cells were grown in HL-5 at 27 or 30°C for thermal stress experiments. Cells were counted with the aid of a hemocytometer. Growth curves for mutants were determined in side-by-side tests with nonmutant sibling transformants. Survival rates were determined by counting the cells after the treatments, plating in association with K. aerogenes, and counting the colonies formed.

Transformation

Restriction enzyme-mediated integration (REMI) mutagenesis was carried out using 40 μg of the BamHI-linearized plasmid pBSrI, and the restriction enzyme DpgI, according to Adachi et al. (1994). Confirmation that a mutation in pkaC was responsible for the resistance to SNP treatment observed in the 1-20 strain was done by recapitulation of the resistance phenotype by disruption of the pkaC gene in the yakA null background. Homologous recombination to disrupt pkaC was carried out by electroporation of yakA null cells with 40 μg of p292 digested with EcoRI/NheI. Transformants were selected in HL-5 supplemented with 4 μg/ml blasticidin.

Isolation of Suppressors

The screen for mutations that suppress the yakA-null sensitivity to SNP was carried out as follows. The YakA-null mutant AK800, which harbors a plasmid insertion (IS800) in the sequence that encodes the protein kinase core (Souza et al., 1998), was used as the parental strain for insertional mutagenesis. A REMI-mutagenized population of 70,000 clones divided into 24 pools of 3000–3000 mutants was diluted to 5 × 10^6 cells/ml in HL-5 supplemented with 500 μM SNP. The cells were shaken at 22°C for 10 d, after which time growth was observed in nine of the pools. Cells were diluted and plated in association with K. aerogenes for clone isolation. Ninety-six clones from each of the nine positive pools were picked into 96-well plates. Duplicates of each plate were prepared. One set of plates was treated with SNP for 1 wk and wells were inspected for cell growth. Ten clones that grew in the presence of SNP were picked for each pool from the untreated plates. These clones were grown, frozen, and cultures were expanded for genomic DNA isolation.

DNA and RNA Manipulations

Standard DNA and RNA manipulations were carried out as described previously (Sambrook et al., 1989). Genomic DNA from isolated suppressor mutants was extracted as described previously (Kuspa and Loomis, 1994). Flanking genomic DNA was recovered from the genomic DNA of strain 1-20 by plasmid rescue with the enzyme HindIII to liberate a 5-kb fragment that was cloned as described in Kuspa and Loomis (1994) to generate the plasmid p120HindIII. This plasmid was sequenced and the insertion was identified to disrupt the open reading frame of pkaC between codons 284 and 285. Homologous recombination at the pkaC site by using plasmid p292 was confirmed by digestion of genomic DNA from candidate clones with HindIII or ClaI and hybridization with a HindIII/BamHI pkaC fragment as a probe on Southern blots. The DNA used to prepare the antisense probe was a BclI/HindII fragment of yakA and a HindIII/KpnI fragment of pkaC subcloned in pGEM and digested with HindIII. RNA was extracted using the TRIzol reagent as described by the manufacturer (Invitrogen, Carlsbad, CA). The antisense probe was obtained by in vitro transcription with the T7 RNA polymerase and the Riboprobe System (Promega, Madison, WI). The RNase protection assay was performed using the RPAII Ribonuclease Protection Assay Kit (Ambion, Houston, TX) and analyzed using denaturing conditions according to the manufacturer’s instruction. Control experiments confirmed that all reactions were performed in excess of probe RNA.
Protein Manipulations

Protein extracts were prepared by freezing and thawing frozen cell pellets in 10 mM Tris, pH 7.8, containing 4 μg/ml pepstatin, 4 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The extracts were clarified by centrifugation at 12,000 x g for 10 min, the samples were submitted to SDS-PAGE in 10% polyacrylamide gels and transferred to nitrocellulose filters as described previously (Laemmli, 1970; Harlow and Lane, 1988). Immunological detection of PKA-C was accomplished by incubation of the blots with rabbit anti-PKA-C antibodies (generously provided by M. Veron and F. Traincard, Institut Pasteur, Paris, France). The crude antiserum was diluted 1:1000 in 10 mM Tris, pH 7.8, containing 4 μg/ml pepstatin, 4 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The extracts were clarified by centrifugation at 12,000 x g for 10 min, the samples were submitted to SDS-PAGE in 10% polyacrylamide gels and transferred to nitrocellulose filters as described previously (Laemmli, 1970; Harlow and Lane, 1988). Immunological detection of PKA-C was accomplished by incubation of the blots with rabbit anti-PKA-C antibodies (generously provided by M. Veron and F. Traincard, Institut Pasteur, Paris, France). The crude antiserum was diluted 1:1000 in 10 mM Tris, pH 7.8, containing 4 μg/ml pepstatin, 4 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The extracts were clarified by centrifugation at 12,000 x g for 10 min, the samples were submitted to SDS-PAGE in 10% polyacrylamide gels and transferred to nitrocellulose filters as described previously (Laemmli, 1970; Harlow and Lane, 1988).

Biochemical Analysis

PKA activity measurements were carried out using the SignaTECT PKA Assay System (Promega). Samples were prepared from cells diluted to 1 × 10^6/ml in HL-5 media with or without SNP or H_2O_2. Cell extracts containing 100 μg of protein were prepared according to the manufacturer's instructions at the indicated treatment times and were used in reactions in the presence or absence of 10 mM of the PKA-specific inhibitor PKI, which inhibits the Dictostelium enzyme (Mann et al., 1992) to 10 μM cAMP. PKA activity is defined as the amount (pmol/min/mg protein) of kemptide substrate phosphorylated in the absence of PKI minus the amount phosphorylated in the presence of PKI. Different amounts of protein were used to ensure linearity of the assay.

DNA and Protein Sequence Analyses

Clone 1-20 sequence was compared with the sequences present in the databanks by using the BLAST search program from the National Center of Biotechnology Information and indicated complete identity to the PKA-C amino acid and nucleotide sequences deposited in GenBank under the accession number P28178.

RESULTS

Nitrosoative, Oxidative, Osmotic, or Heat Stress Induces Growth Arrest and Death of Dictyostelium Cells

To investigate the pathways that regulate growth arrest in response to stress, the general stress response of Dictyostelium cells was accessed by submitting this organism to a variety of challenges. Growth curves were determined in the presence of the nitric oxide generators SNP and spermine NONOate, H_2O_2, or at high temperature (30°C). After ~24 h an inhibition of growth was observed in response to these treatments (Figure 1). Growth inhibition induced by SNP was greatly abolished when SNP was added in conjugation with oxyhemoglobin, a scavenger of nitric oxide. H_2O_2 caused 20–30% of the cells to lyse in the initial 12 h (see below) and also led to growth arrest. The ability of the cells to survive the stress and sustain growth after the treatments was measured by their capacity to form plaques on a bacterial lawn. Survival rates of wild-type cells submitted to nitrosoative, oxidative, heat, or osmotic stress are shown in Table 1. SNP caused significant cell death after 24 h of exposure. Shorter incubations did not produce significant death. H_2O_2 led to death after 12 h of incubation. High glucose led to decreased survival after 1 h of incubation. Growth at 30°C also caused a decrease in cell viability.

YakA Is Essential for Survival to Nitrosoative and Oxidative Stresses

We have previously shown that yakA null cells have an impaired response to nutrient starvation and an altered cell cycle. To test whether these growth anomalies would be reflected in an altered sensitivity to stress, yakA null cells were submitted to the same environmental challenges as described above (Table 1). yakA null cells were more sensitive to treatments that generated nitrosoative/oxidative stress compared with wild-type cells, and no differences were observed in response to osmotic shock. Irreversible damage seemed to occur only after 12 h of treatment because no significant death was observed in shorter treatments. If incubations were performed at 10°C no loss of cell viability was observed. Figure 2A shows the growth profile of wild-type AX4 cells (WT) and yakA null cells treated or not with SNP. yakA null cells presented a faster doubling time than WT cells as mentioned above. SNP inhibited growth of cells after 20–24 h of treatment. Wild-type cells arrested cell growth but no extensive cell lysis was observed for ~1 wk in the presence of SNP. yakA null cells also presented the initial growth inhibition (compared with untreated cells) and after 2 d this was followed by extensive cell lysis. SNP caused a growth arrest that persisted as long as the media were not exchanged, but did not induce extensive cell lysis. Removal of the SNP media allowed growth to resume for wild-type cells but not yakA null cells (Figure 2, B and C). If SNP was removed after 12 h of treatment yakA null cells presented some growth recovery after 36 h but later failed to maintain...
pkC Suppresses yakA

To identify components of the pathways that modulate the nitrosative/oxidative stress responses we isolated yakA− second-site suppressors that may mediate death in response to the SNP treatment. Seventy thousand insertional mutants generated by REMI (Kuspa and Loomis, 1992) in the yakA minus background were obtained. Insertional mutant pools were grown in the presence of SNP. DNA from confirmed SNP resistant cultures was isolated, the mutated genes were cloned by plasmid rescue and one of them was identified by sequence analysis as the catalytic subunit of PKA, pkC (Mann and Firtel, 1991). pkC was identified only once among the suppressor genes, indicating that the screen did not reach saturation. To verify the suppression phenotype, a pkC-Bsr construct was reintroduced into the yakA null strain, and insertions in the pkC gene were confirmed by Southern blots.

To confirm a role for PKA-C in the nitrosative/oxidative stress response, the growth rate of cells that either lack or overexpress this kinase were analyzed after SNP treatment. Figure 4 shows the growth profile of wild-type, yakA null, pkC null, yakA/pkaC double null, yaka/pufA double null, and yakA null cells that overexpress pkC under the control of its own promoter (yakA[pkAC/pkAC]) grown in the presence of SNP. Growth rates in the presence of SNP for strains with a disruption on the pkC gene (pkC null or yakA/pkaC double mutants) were higher compared with wild-type cells. During exponential growth wild-type cells double at ~8-h intervals. When treated with SNP for longer than 24 h the doubling time increased to ~100 h. For pkC null strains treated with SNP this rate was 4 times faster, with an average 24-h interval. Strains with higher PKA activity (either pkC overexpressing strains, or pufA null cells), on the other hand, were more sensitive to SNP treatment. Extensive cell lysis was observed immediately after treatment, in a manner similar, but more pronounced, to that observed for yakA null cells.

PKA-C and AcaA Are Involved in Modulation of Nitrosative/Oxidative Stress Responses

Because cAMP metabolism seemed to be involved in the inhibition of growth, mutants for the adenyl cyclase acaA (Pitt et al., 1992) were also investigated. Observation of the growth pattern of cells during 7 d of H2O2 treatment indicated different profiles for each strain. For wild-type cells 20–30% cell lysis was observed in the first 12 h (Figure 5B) and slow growth continued for 5 d (Figure 5A) after which time growth rates accelerated. This is expected because H2O2 is labile. Wild-type cells resumed growth if the H2O2 was removed after 12 or 24 h of treatment (Figure 5C), but no recovery was observed for yakA null cells. For yakA null cells growth was observed for 6–8 h when lysis started (Figure 5B) with no apparent recovery (Figure 5A). For pkC null and acaA null cells, no cell lysis was observed (Figure 5B), growth continued, even although at a slower rate (compared with untreated cells), and recovery started after 1 d in the presence of H2O2. Recovery after H2O2 removal was faster for pkC null cells than for wild-type cells (Figure 5C). Cell viability after SNP, H2O2, and high glucose treatment was also investigated for pkC and acaA null cells (Table 1).

No significant differences were observed when these strains were compared with wild-type cells treated with SNP or H2O2 for 4 or 12 h or with 300 mM glucose. Longer treatments (SNP or H2O2 for 24 h), however, distinguish wild-

**Table 1. Survival of cells submitted to nitrosoative, oxidative, osmotic, and thermal challenges**

Exponentially growing cells were diluted to 1 × 10^6 cells/ml and incubated with 500 μM SNP, 500 μM H2O2, or 300 mM glucose for the indicated times. Cells were grown at 22°C unless otherwise specified. Cells were counted and plated in association with K. aerogenes for colony number scoring. The percentage of survival is relative to the untreated control. The results are significant of 12 independent experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wild-type</th>
<th>yakA−</th>
<th>pkC−</th>
<th>acaA−</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP 4 h</td>
<td>96.8 ± 2.0</td>
<td>95.2 ± 2.6</td>
<td>97.0 ± 3.5</td>
<td>98.1 ± 3.8</td>
</tr>
<tr>
<td>SNP 12 h</td>
<td>93.5 ± 4.2</td>
<td>85.2 ± 6.6</td>
<td>96.0 ± 2.5</td>
<td>96.1 ± 2.0</td>
</tr>
<tr>
<td>SNP 24 h</td>
<td>83.6 ± 3.2</td>
<td>35.6 ± 5.1</td>
<td>95.0 ± 2.0</td>
<td>95.3 ± 1.5</td>
</tr>
<tr>
<td>SNP 24 h at 10°C</td>
<td>96.1 ± 3.4</td>
<td>90.2 ± 2.1</td>
<td>99.8 ± 9.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>H2O2 4 h</td>
<td>98.5 ± 6.2</td>
<td>90.2 ± 3.9</td>
<td>96.2 ± 5.8</td>
<td>96.1 ± 2.0</td>
</tr>
<tr>
<td>H2O2 12 h</td>
<td>87.3 ± 3.0</td>
<td>75.3 ± 4.5</td>
<td>89.3 ± 3.5</td>
<td>90.3 ± 6.5</td>
</tr>
<tr>
<td>H2O2 24 h</td>
<td>67.3 ± 2.0</td>
<td>15.3 ± 2.5</td>
<td>95.3 ± 2.5</td>
<td>93.4 ± 2.5</td>
</tr>
<tr>
<td>Glucose 1 h</td>
<td>78.6 ± 3.2</td>
<td>72.0 ± 2.2</td>
<td>76.3 ± 1.7</td>
<td>79.0 ± 3.2</td>
</tr>
<tr>
<td>Glucose 2 h</td>
<td>56.6 ± 5.3</td>
<td>50.0 ± 3.5</td>
<td>48.3 ± 6.1</td>
<td>53.0 ± 3.6</td>
</tr>
<tr>
<td>30°C 12 h</td>
<td>66.5 ± 5.4</td>
<td>71.6 ± 5.1</td>
<td>70.0 ± 2.4</td>
<td>76.3 ± 3.7</td>
</tr>
<tr>
<td>30°C 24 h</td>
<td>58.4 ± 6.2</td>
<td>85.5 ± 4.0</td>
<td>69.0 ± 3.1</td>
<td>67.2 ± 4.3</td>
</tr>
</tbody>
</table>

n.d., not determined.
type cells from pkaC and acaA null cells. A marked decrease in cell viability was observed for wild-type cells, whereas only around 5% of pkaC null or acaA null cells died under these conditions.

**SNP and H₂O₂ Treatment Induces cAMP Synthesis and PKA Activation**

To test whether SNP and H₂O₂ would induce an increase in PKA activity we performed enzyme activity measurements in extracts from cells treated with these compounds (Figure 6A). Exponentially growing cultures were diluted to 1 × 10⁶ cells/ml in the presence of SNP or H₂O₂, and aliquots were collected after 4, 12, and 24 h for activity assays. No significant differences were observed up to 12 h of treatment. A 60% increase in activity was observed after 24 h of treatment of wild-type cells with SNP, a 44% increase in activity was observed after 12 h of treatment with H₂O₂, no significant increase in activity was observed in treated yakA null cells and no activity was observed in pkaC null cells treated or not. The increase in activity is not apparent when cAMP is added to the assay, an indication that it is not a result of higher amounts of PKA-C protein in the extracts from treated cells (Figure 6B). Activation of PKA seems to be transient because the increased levels of activity do not persist in longer than 24-h incubations with the compounds (our unpublished data).

**Figure 3.** H₂O₂ induces death of yakA null cells. Wild-type cells and yakA null cells were diluted to 5 × 10⁶ cells/ml and H₂O₂ was added to 500 µM. Cells were counted at the indicated times.

**Figure 4.** PKA is involved in the growth inhibition response induced by SNP. (A) Wild-type cells, yakA null cells, pkaC null cells, yakA null overexpressing pkaC (yakA⁻ [pkaC/pkaC]), yakA/pkaC double, and yakA/ pufA double mutants were diluted to 5 × 10⁶ cells/ml and SNP was added to 500 µM. Cells were counted at the indicated times. (B) Same as in A, with Y-axis expanded for better visualization.
To investigate whether PKA activation was a response to activation of the adenylyl cyclase acaA, cAMP levels were measured in wild-type cells, yakA null cells, and pkaC null cells were diluted to $5 \times 10^5$ cells/ml and $H_2O_2$ was added to 500 $\mu$M. Cells were counted at the indicated times. (B) yakA null and pkaC null cells have similar initial responses to SNP treatment. Same as in A, with X- and Y-axes expanded for better visualization of the early response. (C) yakA null cells do not recover from the $H_2O_2$-induced growth arrest. Wild-type, yakA null, and pkaC null cells were diluted to $5 \times 10^5$ cells/ml and $H_2O_2$ was added to 500 $\mu$M. $H_2O_2$-containing media were washed off, and cells were resuspended in $H_2O_2$-free media after 12 or 24 h of incubation. Cells were counted at the indicated times.

**Figure 5.** pkaC and acaA null cells have a less pronounced growth inhibition. (A) Wild-type cells, yakA null cells, and pkaC null cells were diluted to $5 \times 10^5$ cells/ml and $H_2O_2$ was added to 500 $\mu$M. Cells were counted at the indicated times. (B) yakA null and pkaC null cells have similar initial responses to SNP treatment. Same as in A, with X- and Y-axes expanded for better visualization of the early response. (C) yakA null cells do not recover from the $H_2O_2$-induced growth arrest. Wild-type, yakA null, and pkaC null cells were diluted to $5 \times 10^5$ cells/ml and $H_2O_2$ was added to 500 $\mu$M. $H_2O_2$-containing media were washed off, and cells were resuspended in $H_2O_2$-free media after 12 or 24 h of incubation. Cells were counted at the indicated times.

Stress Does Not Induce Changes in yakA or pkaC Expression

We had shown previously that yakA mRNA increased during growth with an increase in cell density (decreased food resources). To determine whether yakA expression was regulated by SNP or $H_2O_2$ RNase protection assays were performed on RNA from cells that were treated with SNP or $H_2O_2$ for 24 h. As a control we also performed this assay with RNA from cells grown in bacteria and collected at low cell densities (44 h from the time of plating) and at high cell densities (clearing plates, 50 h from the time of plating). Increased levels of yakA mRNA in response to an increase in cell density were observed as expected but no increase in response to treatments with either SNP or $H_2O_2$ was observed (Figure 7A). The same assay was also used to determine pkaC mRNA levels in response to these treatments. Figure 7B shows an increase in pkaC mRNA levels in cells during aggregation as expected, but no increase in response to SNP or $H_2O_2$ treatment. PKA-C protein levels were also investigated by analyzing protein extracts on Western blots.
incubated with an antibody against PKA-C. PKA-C protein content was not altered after treatment with SNP or H₂O₂ for 12 and 24 h (Figure 7C).

YakA and PKA Mediate Response to Heat Stress

To determine whether YakA, PKA, and AcaA have roles in the regulation of the heat shock response in Dictyostelium the growth profile and survival rates of wild-type, yakA null, pkaC null, and acaA null cells at 27 and 30°C were determined. yakA null, pkaC null, and acaA null cells are more resistant to heat stress than wild-type cells (Table 1). Incubation of cells during axenic growth at 30°C caused an inhibition of growth of all strains with a seemingly less pronounced effect for the mutants (Figure 8). At 27°C the growth inhibition was more pronounced in the first 72 h of treatment for wild-type, pkaC, and acaA null cells. yakA null cells was less affected by the treatment during the first 96 h of treatment and after this period the mutant ceased to grow.

DISCUSSION

The data indicate that YakA and PKA may integrate the responses to several stresses in Dictyostelium as depicted in Figure 9. Yak1p and PKA have been shown to modulate starvation, oxidative, and thermal stress responses also in yeast (Hartley et al., 1994; Smith et al., 1998). Yak1p has recently been shown to phosphorylate Pop2 upon glucose limitation (Moriya et al., 2001). Pop2, a component of the global transcription factor complex CCR4, is a member of the deadenylase complex as well (Tucker et al., 2001). PufA, a member of the pumilio protein family, which is a target of YakA regulation in Dictyostelium (Souza et al., 1998), has also been implicated in the regulation of deadenylation events (Wreden et al., 1997), and its knockout in the yakA null background renders the cells more sensitive to stress (this work). Our findings indicate that YakA is a general sensor of environmental conditions effecting changes through PKA. This kinase has been shown to be essential for aggregation, prespore and prestalk cell type differentiation, and spore germination (Loomis, 1998). Both the catalytic and regulatory subunits are also present at low levels during growth, but because null mutants for both genes grow normally, a role for this kinase during this phase was not obvious. The exit from the growth phase to development is regulated by the kinase YakA whose mRNA increases with an increase in cell density (Souza et al., 1998). YakA levels are modulated by PSF, a secreted factor that signals food availability (Clarke and Gomer, 1995). Our previous studies indicate

**Figure 7.** yakA mRNA, pkaC mRNA, or PKA-C protein content is not altered by SNP or H₂O₂ treatment. (A) RNase protection assays were performed on RNA samples extracted from wild-type cells grown in the presence of bacteria and collected after 44 h (low cell density) or 50 h (high cell density) or axenically grown wild-type cells treated or not with SNP and H₂O₂ for 24 h. Total RNA was extracted from the cells and assayed using a [³²P]CTP-labeled antisense yakA mRNA. (B) RNase protection assays were performed on RNA samples extracted from wild-type cells starved for 8 h on filters and collected at the aggregation stage or axenically grown wild-type cells treated or not with SNP for 24 h and H₂O₂ for 12 h. Total RNA was extracted from the cells and assayed using a [³²P]CTP-labeled antisense pkaC mRNA. (C) Western blot analysis using PKA-C antibodies were performed on protein extracts of axenically grown wild-type cells treated or not with SNP and H₂O₂ for 12 or 24 h.

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that during growth, increased cell density and decreased food resources increase YakA levels and, in response to this, growth is attenuated. When overt starvation takes place, YakA may reach levels that will inhibit growth phase-specific genes (such as pufA) with the consequent up-regulation of PkaC, production of cAMP by AcaA, and triggering of development. PKA activity increases fourfold in 12 h of starvation, but we have not detected any changes in PKA activity during growth at different cell densities (unpublished data). In fact, several lines of evidence suggest that YakA impinges on the cell cycle through a pathway that is independent of PKA: yakA overexpression in pkaC minus cells induces growth arrest; pkaC overexpressing strains, or pufA minus cells do not show any growth rate deficiencies; pkaC minus strains do not present cell cycle-related phenotypes. In this work we report a new role for PKA and YakA, now in the regulation of growth rates and cell survival in response to nitrosoative/oxidative and heat stress. An increase of cAMP production and PKA activity was observed in cells stimulated with SNP and H$_2$O$_2$. These compounds inhibit growth of wild-type cells and the inhibition is less severe in mutants of pkaC and acaA. yakA null cells, in contrast, are hypersensitive to the treatment. cAMP signaling through the aggregation phase adenylyl cyclase acaA and activation of PKA seem therefore to mediate the growth inhibition response that may protect the cells under stress situations.

Overproduction of nitric oxide has been shown to lead to inhibition of DNA synthesis, damage to mitochondria, loss of cell membrane integrity, apoptosis, changes in the cell cycle, and DNA strand breaks in other systems (Burney et al., 1997). Hydrogen peroxide can lead to the production of more reactive oxygen species that are highly damaging toward cellular constituents, including DNA, lipids, and proteins. Treatment of wild-type cells during growth with SNP, spermine NONOate, or H$_2$O$_2$ leads to some loss of cell viability. The same treatments in yakA null cells lead to extensive killing. The growth curves also indicate death of yakA null cells in nitrosoative/oxidative stress conditions. Approximately after 24 h of treatment cell lysis is evident, whereas in wild-type cells, growth is inhibited but little cell lysis occurs. Observation of the growth pattern during the first hours of stress indicates that yakA null cells attempt to grow during the first hours of treatment and this is followed by cell lysis. This coincides with the observed doubling time for this mutant during exponential growth. Wild-type cells, however, show an immediate inhibition of growth, which may protect them of the deleterious effects of cell division under the stress conditions. SNP treatment at 10°C, a temperature that does not support growth of Dictyostelium cells, does not lead to loss of viability in yakA null cells, indicating that death, as a result of the stress-inflicted damage, may be directly related to their inability to arrest growth.

The discovery that the cAMP pathway might be involved in the nitrosoative/oxidative stress response of Dictyostelium cells came from the isolation of pkaC as a second site suppressor of the death induced by SNP in yakA mutants. It appears that YakA activity is essential for endurance of the stress conditions, unless pkaC is absent. We have previously reported that yakA null cells have very low PKA activity levels during growth and after nutrient starvation (Souza et al., 1998, 1999). In the first 6 h of treatment yakA null cells respond to SNP in a manner similar to that observed in pkaC and acaA null cells. Growth is inhibited but occurs, and no cell lysis is observed. This similar early response might be due to the lack of PKA induction in all these strains. The increase in PKA activity and cAMP levels in wild-type cells seem to coincide with the period that cells responded to the stress with growth arrest. Because pkaC overexpression renders the cells hypersensitive to SNP treatment, inhibition of this kinase activity may follow the initial increase allowing for growth to continue after damage repair. The pathways in Figure 9 summarize our view that changes in growth rates are regulated by PKA-dependent and -independent pathways. The exact contribution of both YakA and PKA to these pathways remains to be established, however, because both the yakA minus strain and the pkaC null strain lack induction of PKA activity in response to SNP, but yakA null cells die and pkaC null cells do not. SNP and H$_2$O$_2$ seem to induce the stress response by direct activation of YakA and PKA, because no increase in message levels for both enzymes was observed in response to these treatments, and neither was any increase in PKA-C protein content observed. It is still possible that the basal PKA activity found in yakA null cells is incompatible with the continuation of the cell cycle under stress conditions or with the maintenance of the arrest status. It is also possible that PKA allows the cells to attempt to begin development and that a PKA-independent/YakA-dependent pathway feeds back to inhibit that from occurring (for example, by stabilizing PufA protein). In the absence of YakA, such responses may or may not be coordinated and PKA promotes death through an inappropriate response.

Figure 9. Pathways proposed to mediate stress responses of Dictyostelium cells. The regulatory relationship between genes and events is described, with arrows representing a positive requirement for a gene or event and bars representing an inhibitory role. See DISCUSSION for details.
The experiments described in this work were designed to investigate the signaling pathways activated in response to lethal concentrations of reactive oxygen species (ROS), but Dictyostelium cells are most probably not routinely exposed to these conditions in the soil. ROS are endogenously produced by normal aerobic metabolism. YakA and PKA may have a role in the modulation of growth rates in the day-to-day variations of metabolic status and the clearance of its by-products. ROS are also produced as signaling molecules that regulate transcription, cell fate, proliferation, and apoptosis. In this respect, it is interesting to note that Dictyostelium cells have been shown to produce nitric oxide and that this compound has been postulated as a signaling molecule in this organism (Tao et al., 1997).

The osmotic stress response in Dictyostelium has been shown to be regulated by cGMP signaling, tyrosine phosphorylation, and a hybrid histidine kinase (Kuwayama et al., 1996; Schuster et al., 1996; Gamper et al., 1999). Our data confirm previous work on the osmotic shock response that indicated that cAMP does not mediate cGMP synthesis induction (Kuwayama and Van Haastert, 1998) because no differences in the osmotic shock responses were found for either yakA, pkaC, or acaA null cells.

The observation that growth is less affected at 27Â°C in mutants that lacked yakA, pkaC, or acaA indicates a role for YakA, PKA, and CAMP in the regulation of thermal tolerance. YakA seems to affect the early response to thermal stress because increased growth at 27Â°C is observed in the first 96 h of treatment. After this period growth deteriorates and yakA null cells are less successful at 27Â°C than wild-type cells. pkaC null and acaA null cells do not show this inhibition with time of the growth rates and seem actually to grow better after extended incubations at 27Â°C. The same is observed for wild-type cells that seem to adapt to the heat shock conditions. The apparent early success (compared with wild-type) of the growth of yakA null cells at 30Â°C for 24 h and at 27Â°C for 96 h may be similar to the early response observed when these cells were treated with SNP and H2O2. When submitted to these stresses yakA null sustained growth for a few hours and then died, whereas wild type sustained growth at reduced rates.

The response to nitrosative/oxidative stress, at least in part, seems to work in a manner similar to the starvation response, which involves growth arrest, induction of cAMP synthesis, and PKA activation by YakA. Overall, Yak proteins seem to have several roles in cell survival, regulating the cell cycle, and eliciting changes at the transcriptional and posttranscriptional levels to maintain cell homeostasis. Our findings indicate a broad function for Yak and PKA proteins in the regulation of growth and the responses to environmental signals.

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REFERENCES


