Proteomic and Microarray Analyses of the Dictyostelium Zak1–GSK-3 Signaling Pathway Reveal a Role in Early Development

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GskA, the Dictyostelium GSK-3 orthologue, is modified and activated by the dual-specificity tyrosine kinase Zak1, and the two kinases form part of a signaling pathway that responds to extracellular cyclic AMP. We identify potential cellular effectors for the two kinases by analyzing the corresponding null mutants. There are proteins and mRNAs that are altered in abundance in only one or the other of the two mutants, indicating that each kinase has some unique functions. However, proteomic and microarray analyses identified a number of proteins and genes, respectively, that are similarly misregulated in both mutant strains. The positive correlation between the array data and the proteomic data is consistent with the Zak1-GskA signaling pathway’s functioning by directly or indirectly regulating gene expression. The discoidin 1 genes are positively regulated by the pathway, while the abundance of the H5 protein is negatively regulated. Two of the targets, H5 and discoidin 1, are well-characterized markers for early development, indicating that the Zak1-GskA pathway plays a role in development earlier than previously observed.

GSK-3 is a multifunctional serine/threonine protein kinase that regulates a large number of key eukaryotic cellular processes, including intermediate metabolism, cytoskeleton maintenance, and development (12). A significant question is how GSK-3 can be active in a broad range of signal pathways yet retain pathway specificity. For example, in the same cell, insulin can control glycogen synthesis and Wnt can control β-catenin protein levels, both in a GSK-3-dependent manner but without crossover between the two pathways (10). This specificity appears to arise through a number of mechanisms. Phosphorylation by GSK-3 generally requires prior phosphorylation of a substrate by another kinase at a priming site four residues toward the C terminus from the phosphoacceptor site. As a consequence, in most instances, GSK-3 operates in combination with other kinases. Substrate specificity can be determined by the identity and activation profile of the priming kinase, such as in the case of CRMP-2 and CRMP-4 (8). Both are substrates for GSK-3 but can be phosphorylated by GSK-3 at different times, as phosphorylation is dependent on different priming kinases which are not coordinately regulated. In addition, GSK-3 and its priming kinases may be brought into close contact with its substrate through the action of scaffold proteins, such as Axin, a protein which brings together the kinases CK1 and GSK-3 with their substrate β-catenin. Wnt signaling can disrupt this protein complex to block β-catenin phosphorylation in a manner that is distinct from the regulation via serine phosphorylation at the N terminus of GSK-3 seen in response to signals such as insulin (24).

A third, but less understood, regulatory mechanism is tyrosine phosphorylation at residue 216 (in GSK-3β). This phosphorylation event is not required for kinase activity but may influence substrate interaction with the active site (9). Although in most cases, phospho-Tyr216 does not change during GSK-3 regulation, there are a number of situations where it does regulate GSK-3 function (4, 17, 29). A well-characterized example of phosphotyrosine regulation of GSK-3 during the multicellular development of Dictyostelium has been observed (22).

Dictyostelium amoebae exist in a unicellular state while feeding on bacteria, but starvation triggers the formation of a multicellular organism. The aggregation of single cells into a mound is coordinated by the pulsatile release of cyclic AMP (cAMP). cAMP acts as a chemoattractant and as a regulator of the developmental gene expression program, acting through high-affinity cAMP receptors cAR1 and cAR3. In the mound, extracellular cAMP rises to millimolar levels, stimulating the low-affinity cAMP receptors cAR2 and cAR4. The process eventually leads to the generation of a terminal structure consisting of a ball of spores supported on a stalk composed of vacuolated cells (34).

Deletion of the gene encoding GskA, the Dictyostelium homologue of GSK-3, leads to ectopic expression of a marker of stalk cell differentiation, ecmB, suggesting that GskA forms part of the repressive signaling pathway that prevents premature stalk cell differentiation (18, 30). Both ecmB expression...
and stalk cell formation are repressed by extracellular cAMP, whereas the formation of the spore precursor cells (prespore cells) requires cAMP and the loss of GskA or cAR3 can disrupt these effects during the multicellular stages of development (18, 27, 30). A dual-specificity kinase, Zak1 (22), acts downstream of cAR3 and directly phosphorylates GskA on Tyr214 (the equivalent of Tyr216 in the mammalian GSK-3β). The activation profile of Zak1 coincides with that of GskA, and the peak of GskA activation normally seen around the mound stage of development is lost in a zak1− strain. The balance between Zak1 kinase activity and an unidentified phosphatase activity has been proposed to cause the differential activation of GskA in different cell types, thus regulating the expression of cell type-specific markers (21).

Despite the importance of GskA, only one substrate for this kinase has been identified. The transcription factor STATa is phosphorylated by GskA, and this event leads to its enhanced nuclear export (15). In order to establish the prevalence of the Zak1-GskA regulatory mode, we set out to identify other cellular effectors of the two kinases by searching for changes in the proteomes and the transcriptomes of cells bearing null mutations in the genes encoding Zak1 or GskA. We identify a number of features that are coordinately altered in the two mutant strains, providing potential targets in a unified Zak1-GskA pathway. Some targets identified are genes or proteins whose expression is regulated during the early stages of development, suggesting that the Zak1-GskA pathway has a role at a much earlier stage than previously thought.

MATERIALS AND METHODS

Cell culture and development. Axenic Dictyostelium Ax2 cells were grown at 22°C in HL5 medium (40). The Ax2gskA-null and statA-null cells have been described previously (25, 30). For development, exponentially growing cells were resuspended in KK2 (16.5 mM KH₂PO₄, 3.8 mM K₂HPO₄) at 2 × 10⁷ cells/ml and shaken at 120 rpm and 22°C for 5 h, being pulsed with 5 nM cAMP every 5 min.

Construction of the zak1− null strain in an Ax2 background. The gene encoding Zak1 was disrupted by an in vitro transposition technique (1) using an artificial transposon carrying a blasticidin S resistance cassette for selection in Dictyostelium. The integration position of the transposon was determined by sequencing, and the transposon disrupts the coding sequence corresponding to the second, or DL kinase domain of Zak1 after amino acid 574. The original zak1− strain also contains a disruption corresponding to this second kinase domain, predicted to be a tyrosine kinase. Transcription could not be detected by real-time (RT)-PCR, but we cannot rule out the possibility that a truncated Zak1 containing a functional N-terminal serine/threonine kinase domain is expressed. Four independent clones showed identical phenotypes, so one was chosen for further analysis and called zak1−Δ₅₇⁴ to distinguish it from the original zak1− strain. When developed on nutrient-free filters, most aggregates arrested at the tip-mound stage. A few aggregates developed further into slugs, and a small strain. When developed on nutrient-free filters, most aggregates arrested at the tipped-mound stage. A few aggregates developed further into slugs, and a small

Two-dimensional gel electrophoresis. Aliquots containing 100 μg of soluble whole-cell extracts were acetone precipitated and resubmitted in 125 μl of sample buffer (5 mM urea, 2 mM thio尿, 4% [wt/vol] 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 4% [wt/vol] NСD-256 (dimethylbenzylammonium phosphate sulfate), 1% [wt/vol] TBP (tritutylphosphine), 1% [wt/vol] dihydrothiouracil, 10 mM benzimidamine, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and a trace of bromophenol blue). The samples were loaded onto non-linear immobilized pH gradient strips (pH range, 3 to 11; Amersham), and isoelectric focusing was performed on a MULTIPHOR II apparatus (Amersham) as recommended by the manufacturer. Equilibration of isoelectric focusing strips was performed for 10 min in equilibration buffer (4 M urea, 2 mM thio尿, 2% [wt/vol] dihydrothiouracil, 2% [vol/vol] sodium dodecyl sulfate [SDS], 0.05 M Tris [pH 6.8], 30% [vol/vol] glycerol, and a trace of bromophenol blue). The second dimension was performed by standard gel electrophoresis on NuPAGE bis-Tris 4 to 12% ZOOM gels (Invitrogen). The gels were subsequently stained using colloidal blue (Invitrogen). Gels were analyzed using ImageMaster 2.0 GE DGE Platinum software (Amersham).

Protein identification. Protein mass fingerprinting data were obtained by matrix-assisted laser desorption ionization-tandem time of flight (tandem mass spectrometry [MS-MS]) analysis performed at the University of Dundee “Fingerprints” Proteomics Facility using an Applied Biosystems (AB) 4700 proteomic analyzer. Excised protein spots from two-dimensional electrophoresis analysis were destained and in-gel digested by trypsin (Roche; modified sequencing grade) as previously described (38). One-tenth of each digested sample was then applied to a 192-well matrix-assisted laser desorption ionization sample plate (AB), allowed to air dry, and then supplemented with 0.5 μl of a 5-mg/ml solution of 4-cyano-4-hydroxy-trans-cinnamic acid matrix (Sigma) plus 10 mM ammonium dihydrogen phosphate in 50% (vol/vol) acetone in 0.1% (vol/vol) trifluoroacetic acid, mixed and allowed to dry prior to analysis. The mass spectrometer was internally calibrated using the AB 4700 proteomic analyzer calibration mix. Using the 4000 series Explorer software (AB), MS spectral data were acquired from the samples and an MS-MS list was automatically generated for further analysis based on the top five most intense ions present (trypsin and major keratin ions were excluded). The MS and MS-MS spectral data obtained were exported from the 4700 proteomic analyzer by using the global proteome server Explorer software (AB). The data were then submitted to a local Mascot search engine for comparison against entries in the NCBI nr and Dictyostelium databases for identification. Methionine oxidation and cysteine carboxymethylation modifications were allowed for with a peptide mass tolerance of 50 ppm and one missed cleavage.

Northern transfer analyses. Total RNA was extracted from approximately 10⁷ cells by using a TRizol RNA extraction kit (Sigma) according to the manufacturer’s protocol. Samples were treated with 2 U/ml total RNA was treated with 2 U/ml DNase I (Promega), which had been developed in shaken suspensions for 5 h with nanomolar pulses of cAMP. Each sample was primed with oligo(dT) and separately labeled with Cy3 and Cy5 by using Superscript III reverse transcriptase (Invitrogen). Each set of labeled mutant total RNA was paired with the control cDNA labeled with the complementary cDNA, which was confirmed by the analysis of microarray data. Therefore, levels of cad4 were used as an internal control.

Genome-wide expression profiling. Total RNA was extracted from three independent samples of control, zak1−Δ₅₇⁴ and gsk4− cells which had been developed in shaken suspensions for 5 h with nanomolar pulses of cAMP. Each sample was primed with oligo(dT) and separately labeled with Cy3 and Cy5 by using Superscript III reverse transcriptase (Invitrogen). Each set of labeled mutant cDNA was paired with the control cDNA labeled with the complementary cDNA, which was confirmed by the analysis of microarray data. Therefore, levels of cad4 were used as an internal control.
Tyrosine phosphorylation events induced by cAMP in gskA− and zak1−/Ax2 strains. We first disrupted the zak1 gene in Ax2 cells in order to allow comparison with an existing gskA− strain that was generated in the Ax2 background (30). The resulting zak1-null mutants in the Ax2 background (zak1−/Ax2 cells) have a phenotype similar to that reported for a different (Ax4) genetic background (22) (see Materials and Methods). The Zak1 and GskA kinases are regulated by millimolar concentrations of extracellular cAMP at the mound stage of development, and consistent with this pattern, the two mutants develop relatively normally until mound formation (ca. 8 to 10 h) (data not shown). We therefore chose to study cells at a nominally earlier stage than the mound stage: by starving cells in shaking suspensions for 5 h. In a further attempt to ensure that comparisons were made when the strains were at similar developmental stages, we chose to drive the developmental program by pulsing with exogenous nanomolar cAMP.

We first analyzed the known components of the signaling system. After 5 h of pulsing with low-level cAMP, cells were exposed to 5 mM cAMP for 1 min, conditions expected to induce tyrosine phosphorylation of STATa (2). For the control strain and both the mutant strains, there was an increase after 1 min in the phosphotyrosine content of a band migrating at the position predicted for STATa (Fig. 1A). The use of an antibody specific for the tyrosine-phosphorylated form of the position predicted for STATa (Fig. 1A). The use of an antibody specific for the tyrosine-phosphorylated form of STATa-null (data not shown). We therefore chose to study cells at a nominally earlier stage than the mound stage: by starving cells in shaking suspensions for 5 h. In a further attempt to ensure that comparisons were made when the strains were at similar developmental stages, we chose to drive the developmental program by pulsing with exogenous nanomolar cAMP.

A tyrosine-phosphorylated band of approximately 52 kDa was missing from gskA− cells, and this is the molecular mass expected for GskA. There was no apparent increase in the level of tyrosine phosphorylation of this protein following treatment with 5 mM cAMP in control cells, presumably because the pulsing-shaking conditions led to the activation of GskA. Zak1 appears not to be the only kinase responsible for the tyrosine phosphorylation of the band we hypothesize to be GskA because it was tyrosine phosphorylated in the zak1−/Ax2 null mutant. Interestingly, a close homologue of Zak1 is present in the genome (16). This other kinase, known as DPYK4, could be responsible for the phosphorylation of GskA during these earlier stages of development.

In Western analysis of zak1−/Ax2 cells, there was no obvious loss of a tyrosine-phosphorylated protein with the molecular mass predicted for Zak1. However, this reflects the low abundance of Zak1 because, upon enrichment with phosphotyrosine-containing protein by immunoprecipitation from nuclear extracts with an anti-phosphotyrosine antibody, a difference between the control and zak1−/Ax2 cells was apparent. One phosphotyrosine-containing band was missing in the zak1−/Ax2 cells (Fig. 1C), and the molecular mass of this band was consistent with its being a tyrosine-phosphorylated form of Zak1. The total level of tyrosine phosphorylation of this nuclear band was also unchanged upon treatment of control and gskA− cells with 5 mM cAMP.

Changes in the proteome induced by loss of Zak1 and GskA function. In order to identify transcriptionally and nontranscriptionally regulated, high- to medium-abundance targets in the Zak1 and GskA signaling pathway, we first performed proteomic analysis. We compared the patterns of features detectable on two-dimensional gels bearing whole-cell extracts from control, zak1−/Ax2, and gskA− cells. Samples were harvested following 5 h of development in shaken suspensions with exposure to pulses of cAMP. Representative pairs of two-dimensional gels are shown in Fig. 2A. The majority of features show equal staining intensities in all three strains, and a number of these constant spots were used for normalization. Several features were reproducibly altered in one or both of the mutant strains (Fig. 2 and Table 1), and their identities were determined by mass spectrometry.
A number of proteins were misregulated in only one or the other of the two mutants, e.g., the metabolic enzyme aldo-keto reductase (alrA) was underrepresented in the gskA− samples and overrepresented in the zak1−Δc2 samples (Fig. 2 and 3A).

(ii) Coordinately regulated proteins. The metabolic enzyme transketolase was down-regulated in the two mutants, as was the product of the DD7-1 gene, which encodes a homologue of the discoidin 1 proteins (Fig. 2 and 3B). In contrast, vegetative protein H5 (the product of the cinB gene) was up-regulated in both zak1−Δc2 and gskA− cells (Fig. 3B). Changes in spot intensities observed on protein gels could be the result of transcriptional or posttranscriptional events. Quantitative real-time PCR showed that the message encoding H5 was always present at higher levels in both mutant strains than in control cells, although there was considerable variation in the level of increase among biological samples. These data are consistent with the idea that the alteration in H5 protein levels is due at least in part to transcriptional changes (Fig. 3C).

Interestingly, although the AlrA protein level was significantly reduced in the gskA− cells, quantitative PCR revealed that the alrA mRNA level was not reduced and, if anything, may have actually been higher in both mutant strains (Fig. 3C). This finding suggests that GskA alone is responsible for a posttranscriptional event that leads to an overall reduction of the amount of AlrA protein in the spot in its absence.

Transcriptional targets regulated by Zak1 and GskA. In order to extend the analysis down to lower abundance limits, and also to investigate regulation at the RNA level, expression profiles of zak1−Δc2 and gskA− cells were compared with those of control cells. The microarray bears PCR products from approximately 8,600 genes, identified from the complete Dictyostelium genome sequence. Only genes for which unique PCR primer sets could be predicted were included. Gene prediction suggests that the genome contains around 12,000 genes. Subsequent detailed analysis has reduced the predicted number by around 1,500 genes by removing those derived from retrotransposons, pseudogenes, and those coding for very small predicted proteins (26). The array therefore likely represents around 80% of the total number of Dictyostelium genes.

The great majority of genes showed no difference between control and mutant strains. However, a number of genes were aberrantly expressed in similar ways in both zak1−Δc2 and statistical significance and are marked with an asterisk. One of the features which showed different intensities on the two-dimensional gels for gskA− cells was DdCAD-1. This is an abundant and relatively well-studied protein from Dictyostelium for which there are a range of tools available (6). The relevant spot was absent in two-dimensional gel analysis of cad1− cells (42), confirming its identity (data not shown). Despite the differences apparent on two-dimensional gels, Northern blot analysis revealed no detectable differences in cad1 mRNA levels in the three strains (data not shown). Consistent with the equal mRNA levels, antisera against DdCAD-1 demonstrated no differences in total DdCAD-1 protein levels when DdCAD-1 from all three strains was extracted into SDS buffer on one-dimensional gels (data not shown). The change in the intensity of the feature corresponding to DdCAD-1 on two-dimensional gels could therefore represent a posttranscriptional modification or could be due to different solubility of DdCAD-1 in the gskA− cells.
A total of 24 of the coordinately altered genes have functions that are known or can be reliably inferred. The genes that were differentially expressed in both mutant strains, whether they were up-regulated or down-regulated, encode proteins with a range of cellular functions (Table 3). Nine of the 24 are involved in metabolism, and six of the others have developmental functions. The microarray data were used to investigate the levels of mRNA encoding each of the proteins identified as being altered in the mutant strains in the proteomic analysis (Fig. 4B). There is, for most of the gene products, qualitative agreement between the microarray data and the proteomic data, but the mutant expression level/control expression level ratio is generally lower with the array data. If we take a standard cutoff unadjusted P value of 0.05 for the array data, then there is significant agreement between the results of the proteomic and array analyses for H5 (cinB), tkt, and DDB0187880 in gskA− and alrA in zak1−. In most other cases, there is qualitative agreement but the P value for the three experiments is greater than 0.05. In only one case is there significant disagreement between the proteomic and the array data. In the zak1− Ax2 strain, DDB0187880 was underexpressed according to the array data but was not seen to change on two-dimensional gels. Apart from this case, the fact that there is a good correlation between the changes in protein and mRNA levels is consistent with transcription being the major target of the Zak1-GskA pathway at this stage in development.

The microarray analysis identified the family of discoidin 1 genes as being coordinately altered in zak1− Ax2 and gskA− cells. We confirmed this array result by Northern transfer, using conditions that detect all three discoidin 1 genes (Fig. 5). As predicted by the microarray analysis, the discoidin 1 genes were underepressed in both the mutant strains during the first 6 h of development.

The expression levels of the genes encoding both H5 (identified in the proteomic analysis) and discoidin 1 are commonly used markers of early development in Dictyostelium. We there-

![FIG. 3. Representative features. (A) Noncoordinate regulation. Cropped images showing a control and two representative gels from mutant strains showing features which are not coordinately regulated in the two mutant strains. AlrA is up-regulated in zak1− Ax2 cells but down-regulated in gskA− cells, and DDB0187880 (DDB01) is down-regulated only in gskA− cells. (B) Coordinate regulation. Cropped images showing a control and two representative gels from mutant strains showing features which are coordinately regulated in the two mutant strains. H5 protein is present at higher levels (Bii) in the mutant strains than in the control strain, while DD7-1 is underrepresented (Bi) in both mutant strains. The feature labeled DD7-1 contains peptide sequences which correspond to this gene and a second, nearly identical, gene (DDB0190881) which lies directly adjacent to DD7-1 on chromosome 1. This seems likely to be the result of a recent gene duplication, and mass spectrometry could not distinguish between the two genes. The protein encoded by these two genes shows high homology to discoidin 1. (C) Quantitative real-time PCR was used to determine the relative levels of mRNA from the alrA and cinB genes under developmental conditions equivalent to those used to isolate the protein for two-dimensional gel analysis. The level in control cells (WT) was defined as 1 and the increase (n-fold) relative to this level in gskA− and zak1− Ax2 cells is shown. The averages of results from three independent experiments are shown with standard errors of the means. The change in alrA expression in zak1− Ax2 cells was shown to be statistically significant (P < 0.05) by a single sample t test (*). All other samples showed the same trend in that features were expressed at higher levels in gskA− and zak1− Ax2 cells than in control cells, although the biological variation among samples is such that the differences are just outside statistical significance (P < 0.08 for the other three).

| Feature(s) | Change in zak1− Ax2 cells | Up-regulation | Down-regulation |
|------------|---------------------------|---------------|-----------------|
| H5 (cinB) | Up-regulation | Aldoketo reductase (alrA) | |
| Coronin (corA) | Down-regulation | Transketolase (tkt), DD7-1b | |
| None | None | DDB0187880, b Dd-CAD1 (cadA) | |

*The identities of features reproducibly over- or underexpressed in zak1− Ax2 and gskA− cells were determined by mass spectrometry. Gene names are shown in parentheses (see dictybase.org).

*Expression profile data from the microarray analysis performed at Baylor College of Medicine (36) are available for the genes encoding all of these proteins (see dictybase.org). The change in expression was greater than a log ratio of 1 during the first 4 h of development. Only tkt does not show such a major change in the expression profile in early development.
fore used existing microarray data (36; see dictybase.org) to examine the expression profiles of all the genes identified by both analyses to see if any of the other potential targets also showed major changes in gene expression during early development (first 4 h). Data were available for all of the targets identified proteomically, and the expression of 8 out of 10 targets was altered by at least twofold during the first 4 h of development (Table 1). Expression profile data were not available for all of the targets identified by the microarray, but among the 21 genes for which data were available, 10 showed at least a twofold change in expression levels during this early stage of development (Table 3). Taken together, these findings support the conclusion that the unified Zak1-GskA pathway plays a role in regulating gene expression during the early stages of development, prior to the mound stage as previously thought.

### Table 2. Numbers of genes misregulated in zak1⁻/⁻ and gskA⁻/⁻ cells after 5 h of development in a shaken suspension

| Change in zak1⁻/⁻ cells | No. of genes with the following change in gskA⁻/⁻ cells: |
|-------------------------|----------------------------------------------------------|
|                         | Up-regulation | Down-regulation | None                  |
| Up-regulation           | 6            | 3              | 41                    |
| Down-regulation         | 3            | 31             | 84                    |
| None                    | 72           | 78             | 8,290                 |

### Table 3. Functional analysis of genes showing coordinate alteration in gene expression in zak1⁻/⁻ and gskA⁻/⁻ cells

| Genes and proteins overexpressed in both zak1⁻/⁻ and gskA⁻/⁻ cells | Cellular process |
|---------------------------------------------------------------|------------------|
| ampA                                                          | Development      |
| DDB0187621                                                     | Metabolism       |
| vatB, vatE, *                                                  | Ion transport    |
| DDB0186611*                                                   | Unknown          |
| pkiF                                                          | Biosynthesis     |
| DDB02017438                                                    | Unknown          |

| Genes and proteins underexpressed in both zak1⁻/⁻ and gskA⁻/⁻ cells | Cellular process |
|---------------------------------------------------------------------|------------------|
| dscA, *                                                            | Development      |
| dscD, pdcA, *                                                      | Metabolism       |
| psp3B, sglA*                                                      | Unknown          |
| pakF                                                               | Signal transduction |
| tpsC, methionine adenosyltransferase regulatory beta subunit, cyclopropane-fatty-acyl-phospholipid synthase | Biosynthesis |
| sodC, *                                                           | Unknown          |
| iunH, CYP508D1, DDB0167345, DDB02011047, DDB0168923, *         | Unknown          |
| DDB0217438                                                      | Unknown          |

* The genes encoding the transcripts suggested to be coordinately misregulated in zak1⁻/⁻ and gskA⁻/⁻ cells have been subdivided into functional categories. Gene names are given wherever possible; otherwise, identifying DDB numbers (see dictybase.org) are given. Expression profile data from the microarray analysis performed at Baylor College of Medicine are available for some of the genes identified by microarray (36; see dictybase.org). For 16 of these 37 genes, no expression profile data are available. Ten of the remaining 21 genes, marked with an asterisk, show a change in expression of more than a log₂ ratio of 1 during the first 4 h of development.
FIG. 5. Northern analysis of discoidin gene expression in zak1\(^{-}\) and gskA\(^{-}\) cells. Control, zak1\(^{-}\) and gskA\(^{-}\) cells were harvested, washed, and plated for development on filters. Cells were harvested at the times shown, and RNA was extracted and subjected to Northern analysis using probes specific for discoidin 1 (dsc) before stripping and reprobing with IG7 as a loading control.

DISCUSSION

Common and distinct targets for GskA and Zak1. The proteomic and the array analyses identified common targets of the two kinases GskA and Zak1, the common targets being defined as mRNAs or protein features which showed qualitatively similar patterns of misregulation in both mutants. It is not possible to determine whether the changes seen were direct or indirect effects of the loss of Zak1 or GskA. The relatively small number of changes identified and the fact that the analysis was carried out with cells prior to overt phenotypic alterations of the mutant strains might mean that this analysis may include direct targets. However, elucidation of the pathway linking Zak1-GskA to a single target would be necessary to demonstrate a direct link. This identification of a number of coregulated proteins and mRNAs significantly strengthens the case for a common pathway. However, both methods also identified targets specific to one or the other kinase. These two observations are entirely reconcilable with published data on the two mutant phenotypes. There are similarities between zak1\(^{-}\) and gskA\(^{-}\) cells, most notably in the loss of stalk cell repression by cAMP, but the developmental phenotypes of the null strains are not identical. Thus, in an Ax2 background zak1\(^{-}\) strains fail to complete development on filters, whereas gskA\(^{-}\) cells culminate. Conversely, gskA\(^{-}\) cells show a slightly increased rate of aggregation while zak1\(^{-}\) cells aggregate normally. The independent effects of GskA suggest either that constitutive GskA activity plays a role in early development or that GskA activation can be regulated by other means. The small increase in GskA activity detected during the development or that GskA activation can be regulated by other means. The common pathway regulates gene expression in early development. H5 (CinB) is an esterase/lipase/thioesterase domain-containing protein used as a marker for growth as the abundance of its mRNA decreases as cells arrest growth and enter development (31). The proteomic analysis showed that the abundance of H5 was higher in both mutants than in controls, and the array and quantitative PCR analysis suggested that this difference was at the level of gene expression. In contrast, the discoidin 1 genes were underexpressed in the two mutants. These observations can be accommodated into the model of a unitary signaling pathway because the discoidin 1 genes, in contrast to the H5 gene, are activated rather than repressed during early development.

The discoidin 1 family is one of the best-characterized gene families, first expressed as cells grow to a high density in axenic medium and then further activated during early development. Discoidin 1 expression is induced in response to two density-sensing factors, conditioned medium factor and prestarvation factor, and is later repressed by pulses of extracellular cAMP during aggregation (5, 41). The promoter of the discoidin 1c gene has been dissected into separate regions that are needed for prestarvation factor-inducible expression and cAMP repression (28, 37).
In summary, the targets H5 and discoidin 1 both indicate that Zak1 and GskA have a role in transcriptional regulation at a much earlier stage in development than was previously thought. The discoidin 1 genes are a particularly attractive entry point for dissecting the pathway further because there is a very considerable body of information on their regulation.

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