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Sulfate Suicide Selection of *Dictyostelium discoideum* Mutants Defective in Protein Glycosylation

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The assembly and processing of glycoprotein-linked oligosaccharides in *Dictyostelium discoideum* has been shown to generate a wide array of glycan structures which undergo dramatic developmental regulation. As late steps in processing of these oligosaccharides involve sulfation, a sulfate suicide selection procedure was developed to select for temperature-sensitive glycoprotein-processing mutants. Of 673 clones derived from the survivors of suicide selection, 99 were classified by replica-plating fluorography as temperature sensitive for sulfate transport or incorporation. Of these, 74 were unable to complete the developmental program to the fruiting body stage at the restrictive temperature, 29 being blocked in some aspect of aggregation and 45 being blocked at some postaggregation stage. Quantitative metabolic labeling experiments with representative clones showed that they incorporated wild-type levels of [^{35}S]methionine but reduced levels of sulfate at the restrictive temperature. The specific incorporation patterns in the mutants suggest that distinct oligosaccharide-processing steps are involved in different developmental events.

The cellular slime mold, *Dictyostelium discoideum*, is a useful model system for investigating the possible functions of asparagine-linked oligosaccharides (N-linked OLS). The life cycle of this organism is well defined and consists of distinct stages of growth and development. When nutrients are present, the vegetative amoebae grow and divide. Development is initiated upon exhaustion of the food supply. After a few hours of starvation, amoebae stream chemotactically toward aggregation centers. These centers are the source of the chemotactic agent, cyclic AMP. During this period, cells become mutually cohesive and form mounds. Cellular differentiation and sorting occur as the cells in mounds progress through a series of morphogenetic movements. The end product of development is a fruiting body consisting of two major cell types, spore and stalk, which are spatially, functionally, and biochemically distinct (reviewed in references 19 and 20).

The processes of assembly and maturation of N-linked OLS in *D. discoideum* generate a wide array of distinct structures (9, 13, 14) which are also subject to dramatic developmental regulation (13, 14, 15), suggesting that OLS have developmentally important functions. Indeed, evidence from a number of studies indicates that the N-linked OLS of this organism are involved in cell-cell recognition and cohesion, cell sorting, maintenance of spatial patterns between cell types, glycoprotein stability, and intracellular protein sorting (reviewed in reference 10). To explore the relationships between N-linked OLS structures and functions, we undertook the selection and biochemical analysis of mutants defective in N-linked OLS assembly or processing. To enrich for these mutants, a suicide selection procedure (1, 11, 22) was used.

Suicide selection is based on the fact that cells with normal metabolic machinery are able to incorporate radioactive precursors into cellular macromolecules. When these labeled cells are stored frozen, radiation damage accumulates and the cells are killed. However, mutant cells which are unable to transport or incorporate the radioactive precursor survive the selection procedure and can be recovered by thawing and plating the frozen cultures (1). This strategy has been successfully employed by using tritiated mannose and fucose to select glycoprotein-processing mutants of both yeasts and Chinese hamster ovary cells (1, 11, 12; R. M. Baker, W. A. O'Brien III, C. Hirschberg, L. A. Spencer, T. Awerbuch, and T. O. Weisman, J. Cell Biol. **83**:453a, 1979). Transport mutants have also been selected from Chinese hamster fibroblasts by using tritiated 2-deoxy-D-glucose (22).

We extrapolated from these studies to establish a selection based on sulfate suicide. In metabolic labeling studies, there is relatively little sulfation of polysaccharide material and no detectable direct sulfation of amino acid residues. The vast majority of sulfation is of protein-linked OLS (9, 13, 14, 23). The N-linked OLS-processing pathway that is operative during the early development (through the mound stage) of *D. discoideum* is shown in Fig. 1 (9, 13, 14). Since OLS become sulfated during very late steps of processing, survivors of selection should include mutants with defects in specific sulfate transferases or in events required to generate the sulfate acceptor OLS. Finally, since the loss of some OLS could be lethal, we directed selection toward the isolation of temperature-sensitive mutants.

In this report, we describe the sulfate suicide selection procedure and present data from phenotypic screening studies which demonstrate that the majority of mutants identified as defective in sulfate incorporation are also defective in development. Quantitative, developmentally timed metabolic labeling studies indicate that the sulfate incorporation mutants are, indeed, defective in N-linked OLS assembly.

MATERIALS AND METHODS

Culture conditions. *D. discoideum* AX3, an axenic derivative of NC4, is the parent of all mutants isolated in this study. Amoebae were grown at either the permissive (22°C) or nonpermissive (27°C) temperature in shaken suspension (120 rpm on a New Brunswick G10 Gyrotory shaker) in TGC medium (this is identical to HL5 medium (25) except that Trypticase was substituted for thiotone peptone and the medium was supplemented with 0.45 g of cysteine and 0.4 g of glycine per 650 ml). To induce differentiation in suspension, cells were harvested during exponential growth (2 ×

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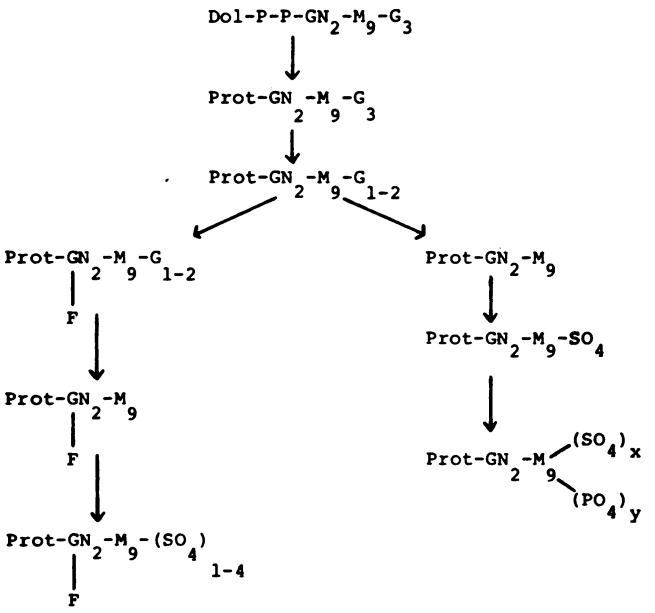

FIG. 1. N-linked OLS processing pathway operative during early development of *D. discoideum*. Abbreviations: Dol-P-P, dolichol pyrophosphate; GN, *N*-acetylglucosamine; M, mannose; G, glucose; F, fucose; Prot, protein.

10^6 to 5×10^6/ml) by centrifugation at 500 g for 5 min, washed twice in KPMC buffer (M/60 potassium phosphate, pH 6.1, 2 mM $MgCl_2$), resuspended in KPMC at 10^7/ml, and shaken at 120 rpm at either 22 or 27°C.

Mutagenesis. Cells were mutagenized by a modification of the procedure described by Barclay and Henderson (2, 3). Log-phase amoebae growing in suspension were harvested from TGC, washed twice with KPMC, and suspended in KPMC at 10^7/ml. *N*-propyl-*N'*-nitro-*N*-nitrosoguanidine was added to a final concentration of 200 μg/ml. The suspension was shaken at 120 rpm for 2 h at 22°C, and the cells were harvested and washed three times with KPMC and then suspended in TGC at 5×10^6/ml.

Immediately after mutagenesis, cell survival (plating efficiency compared with an untreated control) was 42%. The mutagenized culture was split into six subcultures, all of which were allowed to recover from mutagenesis for 36 h (4 wild-type generation times) prior to suicide selection.

Sulfate suicide selection. From each subculture of mutagenized cells, 10^8 cells were harvested, washed twice in KPMC pre-equilibrated to 27°C, and suspended in 0.5 ml of 27°C KPMC. These suspensions were dispersed onto Nuclepore filters (10^8 cells per 47-mm-diameter filter with 0.2-μm pores) resting on SM nutrient agar (24) plates. Cells on filters were incubated for 1 h at 27°C to allow expression of temperature-sensitive phenotypes. The filters were then transferred to fresh, otherwise empty petri plates containing 1.0 mCi of $[^{35}S]O_4$ in 50 μl of KPMC. Cells were labeled for 4 h at 27°C. At the end of the labeling period, cells were harvested by washing each filter once with 1.0 ml of KPMC and twice with 0.5 ml of KPMC. Cells harvested from each filter were washed three times with 30 ml of KPMC to remove all extracellular label. To reduce soluble intracellular label, cells were suspended at 5×10^6/ml in KPMC containing 2 mM $MgSO_4$ and shaken (120 rpm) for 3 h at 22°C. This chase removed about 90% of the intracellular acid-soluble label (data not shown). Chased cells were washed thoroughly with KPMC, prepared for frozen storage (see below) and kept at −70°C for 4 days. After 4 days, the frozen cultures were thawed (see below) and the cells were harvested and suspended in TGC at 10^6/ml. These cultures were shaken for 2 wild-type generation times at 22°C to allow recovery and then washed, plated, and labeled for a second round of selection. After four such selection cycles, surviving cells were harvested and plated at 22°C on SM agar with *Klebsiella aerogenes* to permit eventual formation of fruiting bodies (24). Spores were then harvested and stored in Bonner salts solution (4). With the exception of the $[^{35}S]O_4$ labeling step, control amoebae were taken through all of the procedures described above.

Freezing and thawing of amoebae. Cells to be stored frozen were harvested, washed once with KPMC, and suspended at 5×10^6/ml in freezing medium (65% TGC, 25% horse serum, 10% dimethyl sulfoxide) prechilled to 5°C. Samples (1 ml) of this cell suspension were pipetted into 1-dram, glass, screw-cap vials, which were placed into a styrofoam container for storage at −70°C. The styrofoam container was constructed so that individual vials were completely encased by 2 cm of styrofoam. To recover cell cultures from frozen storage, vials were thawed in air at 22°C. This freeze-thaw procedure allowed recovery of 90% of unselected AX3 cells after 4 days at −70°C (Fig. 2).

Determination of suicide efficiency. To determine the percentage of cells which survived each day at −70°C, one vial of frozen cells was thawed, and a sample of cells was serially diluted with KPMC and plated on SM agar plates with *K. aerogenes*. The formation of plaques in the bacterial lawn was scored daily for 4 days after appearance of the initial plaque (about 3 days after plating). The number of plaques scored was corrected for a plating efficiency of 70% in the wild type.

Control experiments showed that sulfate-labeled cells

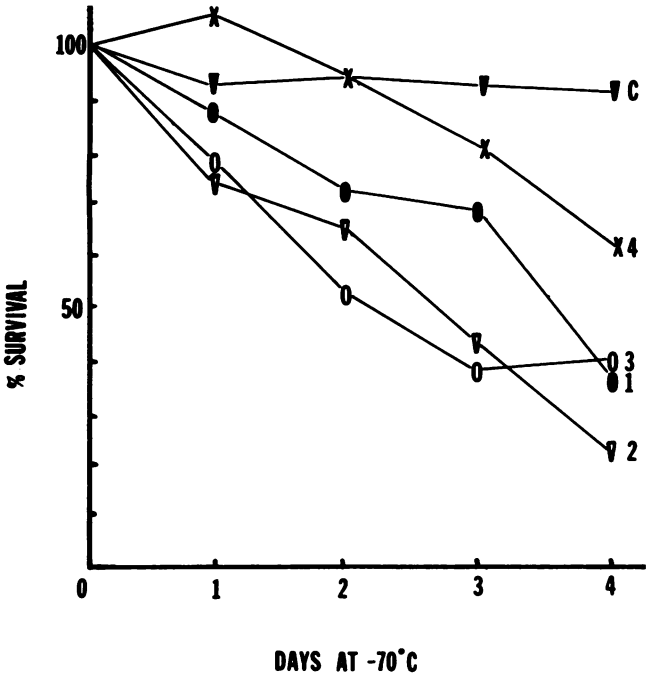

FIG. 2. Suicide efficiency. The percentage of cells which survived each day at −70°C was determined during each of the four selection cycles. Each day, one vial of frozen cells was thawed and a sample of cells was serially diluted and plated to determine the number of viable cells (see Materials and Methods). C, Control.

were progressively killed during frozen storage (Fig. 2). To test the basis for this cell death, populations of labeled and unlabeled cells were mixed prior to freezing and storage for 4 days at −70°C. The results showed that unlabeled cells were not killed by labeled cells, as the number of cells which survived was increased by the number of unlabeled cells in the population. This confirmed that cell death was due to suicide and not radiation damage from neighboring cells.

TCA precipitation. To determine the amount of incorporated $[^{35}S]O_4$ per cell, known numbers of labeled cells were pipetted onto hardened Whatman no. 50 filter paper disks (1-cm diameter) that had been pre-equilibrated in 0.5 mM $MgSO_4$. Disks with cells were dried for 15 min in a 55°C oven and then swirled successively for 10-min periods in ice cold 10% trichloroacetic acid (TCA), 10% acetic acid, and 95% ethanol. Disks were dried in a 55°C oven for 15 min prior to scintillation counting. The number of counts recovered by TCA precipitation was 97% of that obtained by Folch extraction of glycoproteins as described by Ivatt et al. (14) (data not shown).

Replica-plating fluorography. Selection survivors were screened for reduced sulfate incorporation by a modification of the replica-plating fluorography technique of Hirschberg et al. (11). Blunt-end toothpicks were used to stab clones onto lawns of autoclaved *Escherichia coli* B. A frozen paste of *E. coli* B, obtained from Grain Processing Corp., was used to make a 20-mg/ml suspension of bacteria in KPMC, which was then autoclaved. To prepare each lawn, bacteria from 25 ml of the autoclaved suspension were pelleted (2 min at 500 × *g*), suspended in 2.5 ml of KPMC, and transferred onto a 100-mm-diameter KPMC agar plate. The plate was swirled to obtain a uniform lawn, and excess fluid was allowed to evaporate. Identical clones were stabbed onto each of two plates. These plates were incubated at either 22 or 27°C for 3 days to allow clones to generate 1- to 2-mm-diameter plaques. After 3 days, each plate was overlaid by a moist, hardened Whatman no. 50 filter paper that had been pre-equilibrated in 5 mM $MgSO_4$. Filters were weighted down by two layers of 3-mm solid glass beads, and the plates were returned to 22 or 27°C for 3 days to allow cells to attach to the filters. The filters were then removed, rinsed gently with KPMC to remove any adhering bacteria, and placed cell side up for 4 h in petri dishes containing 0.20 mCi of $[^{35}S]O_4$ in 1.25 ml of KPMC. After labeling, the filters were washed gently with KPMC, covered with ice cold 20% TCA for 15 min, washed twice for 30 min in KPMC, and dried with acetone under a stream of N_2. Filter papers were stained with Coomassie brilliant blue G, destained, and processed for fluorography as described by Baker et al. (1). Colonies which had incorporated $[^{35}S]O_4$ were visualized by exposure to preflashed Kodak X-Omat film at −70°C for 2 days (18). Sulfate incorporation mutants were identified by comparing the intensity of the Coomassie stain to that of the fluorogram.

Screening of growth and developmental phenotypes. Clones identified as defective in sulfate incorporation were screened for temperature-sensitive growth and developmental phenotypes. Clones were stabbed onto 2% agar KPMC plates covered with lawns of autoclaved bacteria. These plates were incubated at 22 or 27°C, and clones were subsequently scored for growth and development. Scoring was done daily for up to 1 week after wild-type AX3 clones had reached the fruiting body stage of development.

Metabolic labeling studies. Clones of interest were analyzed for sulfate incorporation in two ways. For a 4-h continuous label, two samples of 10^8 cells each were harvested from 22°C shaking cultures of early-log-phase (2×10^6 to 3×10^6/ml) mutant clones. Cell samples from each clone were suspended in 500 μl of either 22 or 27°C KPMC, dispersed onto Nuclepore filters resting on 47-mm SM agar plates, and incubated at either 22 or 27°C for 2 h. Filters were then transferred to otherwise empty petri plates containing 1.0 mCi of $[^{35}S]O_4$ in 50 μl of KPMC. Cells were labeled at 22 or 27°C for 4 h and then harvested as described above. Harvested cell suspensions were immediately washed three times with 30 ml of KPMC and then chased for 3 h at 22°C with 2 mM $MgSO_4$ as described above. Samples of the chased cell suspensions were TCA precipitated onto filter paper disks and counted in a scintillation counter.

Clones of interest were also pulse-labeled in a microassay to determine developmental time courses of $[^{35}S]O_4$ incorporation at both 22 and 27°C. Shaking cultures of early-log-phase mutant clones at 22°C were split into halves. One half was returned to shaken suspension at 22°C, and the other was brought to 27°C in a 27°C water bath and then placed in a 27°C shaking incubator. Following incubation of the cul-

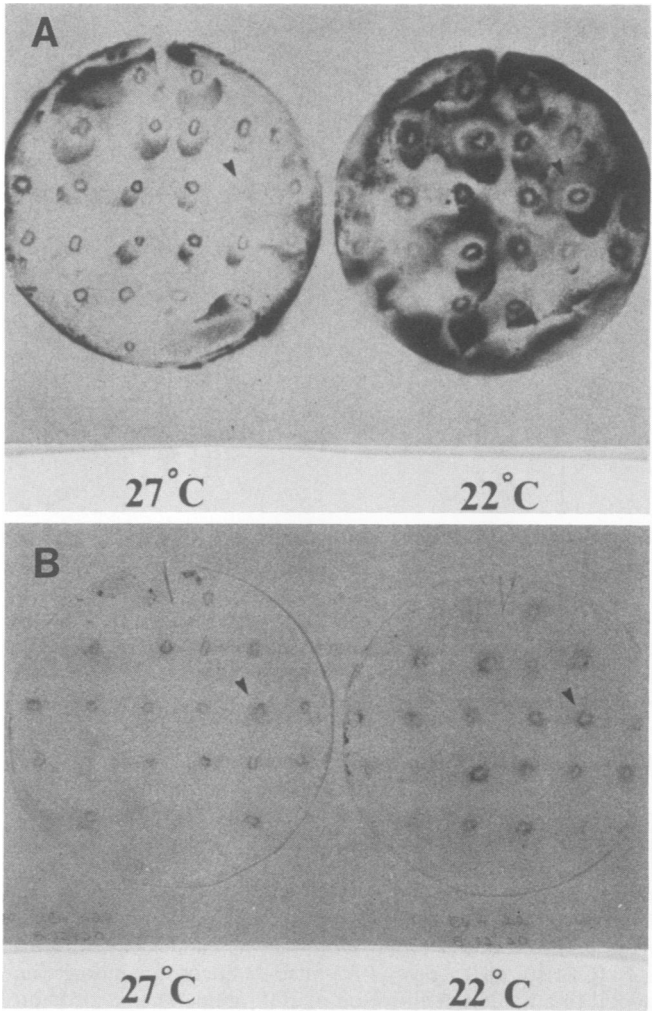

FIG. 3. Replica-plating fluorography. Clones growing on lawns of autoclaved bacteria were transferred to filter papers. The papers were incubated in a solution of $[^{35}S]O_4$, fixed, stained with Coomassie brilliant blue G, and subjected to fluorography as described in Materials and Methods. A is a fluorogram of replica-plated clones that were labeled with $[^{35}S]O_4$ at 27 and 22°C. The corresponding Coomassie-stained filter papers are shown in B.

TABLE 1. Frequency of mutant classes[a]

Origin	No. of clones	Gro^{ts}	Agg^{ts}	Morph^{ts}
Unselected control	500	0	0	5
Potential OLS mutants	99	1	29	45

[a] Mutants classified as Gro^{ts} or Agg^{ts} were temperature sensitive for growth or aggregation, respectively. Mutants classified as Morph^{ts} were blocked in development at a stage between aggregation and the formation of a fruiting body at 27°C.

tures at these temperatures for 2 h, 10^8 cells from each culture were harvested, washed twice in either 22 or 27°C KPMC, and then suspended in 10 ml of 22 or 27°C KPMC. These cell suspensions were shaken for 10 h (120 rpm) at either 22 or 27°C. At several time points during this incubation, a sample of cells from each cell suspension was diluted to 10^5/ml with KPMC, and 100 μl of each diluted cell suspension was transferred to a flat-bottom microtiter plate (Falcon no. 3912) well containing 0.1 mCi of $[^{35}S]O_4$ in a 5-μl droplet of KPMC. The microtiter plates were shaken at 120 rpm for 1 h at either 22 or 27°C. After the labeling period, 20-μl samples from each well were TCA precipitated onto filters as described above.

Clones showing reduced sulfate incorporation at 27°C were analyzed for incorporation of [^{35}S]methionine by a 4-h, continuous-labeling assay with 0.05 mCi of [^{35}S]methionine replacing the 1.0 mCi of $[^{35}S]O_4$ per plate. These cells were not chased after labeling. TCA precipitations were performed on filter paper disks (hardened Whatman no. 50) that had been pre-equilibrated in 10 mM methionine.

Materials. Desiccated horse serum was from Difco Laboratories, as were the organic components of TGC. Dimethyl sulfoxide was analytical grade. *N*-propyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from Sigma, and *E. coli* B was obtained as a frozen cell paste from Grain Processing Corp. $H_2[^{35}S]O_4$ was from ICN Pharmaceuticals Inc. and had a specific activity of 43 Ci/mg. The amount of $H_2[^{35}S]O_4$ used was always well within the buffering capacity of each assay. L-[^{35}S]methionine was from New England Nuclear Corp. or Du Pont Co. and had a specific activity of 1,109 Ci/mmol.

RESULTS

Baker et al. (1) have described the key requirements for tritium suicide selection for glycoprotein-processing mutants in eucaryotic cells. We met all but one of those requirements by the methods described above. Ideally, labeling conditions should generate about 1 cpm of acid-insoluble radioactivity per cell. The maximum amount of TCA-insoluble $[^{35}S]O_4$ observed in *D. discoideum* AX3 under the selection procedures described in Materials and Methods was 0.35 cpm per cell. To compensate for this, amoebae were taken through four rounds of selection to allow cumulative enrichment for mutants.

The sulfate suicide selection was designed to isolate mutants which would be temperature sensitive for sulfate incorporation. Therefore, the mutagenized amoebae were grown at 22°C, the permissive temperature, but labeled at 27°C, the nonpermissive temperature. After the fourth selection cycle, survivors were plated at 22°C to permit growth, development, and sporulation. Amoebae from these spores were used to bias against unconditional growth or developmental defects.

During each of the four selection cycles, the percentage of cells which survived each day at −70°C was determined (Fig. 2). Control, unlabeled cells (C) were not killed by the freeze-thaw conditions, but by day 4 of rounds 1 to 3 of selection, 55 to 75% of the labeled cells were nonviable. By round 4 of selection, a higher proportion of the labeled cells survived. There is precedent for this phenomenon of preferential enrichment for selection survivors during the later rounds of selection in ^{3}H suicide selection (1, 11). After labeling cells with $[^{35}S]O_4$ during selection cycles 1 and 4, samples of cells were TCA precipitated to determine the amount of TCA-insoluble label per cell. Whereas 0.350 cpm of $[^{35}S]O_4$ was incorporated per cell during round 1 of selection, this value decreased to 0.249 cpm per cell during round 4 of selection. The increase in percent survival of cells during selection cycle 4 and the decrease in the amount of acid-insoluble label per cell between rounds 1 and 4 of selection indicated that the procedure had selected for amoebae having a reduced ability to incorporate sulfate at the nonpermissive temperature.

Survivors of selection were screened for reduced $[^{35}S]O_4$ incorporation by a modified replica-plating fluorography technique as described in Materials and Methods. Figure 3A is an example of a fluorogram of replica-plated clones labeled with $[^{35}S]O_4$ at 27 and 22°C. The corresponding Coomassie-stained filter papers are shown in Fig. 3B. Clones which were faint on the fluorogram at 27°C as compared with 22°C and which were detected by Coomassie staining at both temperatures (such as the clone marked by the arrow) were classified as potential temperature-sensitive sulfate incorporation mutants.

The replica-plating fluorography technique was useful as a survey screen, and clones screened repetitively were reproducibly scored as mutant or wild type. However, the technique was not a good quantitative measure for several reasons. First, the fluorograms in Fig. 3 show a particularly dramatic example of differential sulfate incorporation at 22 and 27°C. More quantitative tests, presented below, showed that none of our mutants were fully defective in sulfation, as the figure suggests. This may be due to several factors. (i) Pre-equilibration of the filters with nonradioactive sulfate to reduce background may have altered intracellular pools, and (ii), as shown by the Coomassie-stained control filters, cells grew better on the autoclaved bacterial lawn at 22°C, and the increased number of cells enhanced the apparent differences in labeling at 22 and 27°C, even for nonmutant clones. When the wild type was tested in a more quantitative assay, the actual incorporation per cell at 27°C was only slightly below that at 22°C, as shown below. The other difficulty in the use of this test for *D. discoideum* was due to its development and plaque morphology. Amoebae in the center of plaques were starved and had entered late developmental stages which no longer incorporated sulfate (13, 14). Only growing cells and cells in early stages of development at the edge of the plaques incorporated sulfate, generating the characteristic ring patterns.

Despite these limitations, replica-plating fluorography served as a useful screen for large numbers of clones which could then be more quantitatively analyzed. By this test, 99 of 673 clones of selection survivors appeared to be defective in sulfate transport or incorporation. These 99 clones were replica plated at 22 and 27°C to score for temperature-sensitive growth and development phenotypes. A comparison of mutant numbers and classes for selected and unselected populations is shown in Table 1. Of the 99 mutants, 75% were blocked at a stage of development prior to fruiting body formation. In contrast, the value for developmental

TABLE 2. $[^{35}S]O_4$ and [^{35}S]methionine incorporation[a]

Mutant clone	$[^{35}S]O_4$ cpm incorporated per cell			[^{35}S]methionine cpm incorporated per cell		
	22°C	27°C	27°C/22°C (%)	22°C	27°C	27°C/22°C (%)
Wild type	0.160	0.148	92.5	0.502	0.512	102
HT2	0.157	0.108	68.7	0.490	0.490	98.2
HT4	0.148	0.0864	58.4	0.489	0.479	98.0
HT7	0.135	0.0749	55.5	0.530	0.525	99.1
HT11	0.149	0.116	77.8	0.509	0.611	120
HT12	0.168	0.0912	54.3	0.547	0.552	101

[a] Suicide selection survivors were analyzed for $[^{35}S]O_4$ and [^{35}S]methionine incorporation by a 4-h continuous-label assay. Cells were labeled at either 22 or 27°C, harvested, subjected to a cold chase in the case of sulfate labeling, and TCA precipitated to determine counts per minute per cell as described in Materials and Methods.

defects in a control population of mutagenized but unselected amoebae was 1%.

Several mutant clones identified by fluorography were quantitatively analyzed for $[^{35}S]O_4$ incorporation by a 4-h continuous label with a chase (Table 2), which essentially duplicated the suicide selection procedure. Incorporation of sulfate by wild-type AX3 cells was similar at 22 and 27°C. However, all mutant clones tested showed reduced incorporation of sulfate at 27°C compared with 22°C. The mutants showed no temperature sensitivity in protein synthesis as monitored by [^{35}S]methionine incorporation into TCA-insoluble material (Table 2).

Since sulfation was reduced less than 50% in assays which were identical to the conditions of suicide selection, we took a random selection of clones which showed such temperature sensitivity for sulfation and subjected them to the complete suicide selection procedure, except that cultures were labeled at both 22 and 27°C prior to storage at −70°C. Samples of the cultures were thawed at daily intervals and tested for viability. The wild type produced a suicide curve identical to that labeled round 2 in Fig. 2, independent of the temperature at which they were initially labeled. The mutants showed a similar curve when labeled at 22°C, but when they had been labeled at 27°C they showed survival greater than that of round 4 (Fig. 2). For example, when cells of clones HT7 and HT12 were labeled with sulfate at 22°C, only 22 and 19% of the cells, respectively, survived the 4 days of frozen storage. When these clones were labeled at 27°C, 69 and 79% of the cells survived the 4 days at −70°C. Therefore, partial defects are sufficient to allow survival during sulfate suicide, just as has been reported for tritium suicide.

Since sulfate incorporation is developmentally regulated in *D. discoideum* (13, 14), mutant clones were microassayed by 1-h metabolic pulse-labeling at a series of early developmental points. In this microassay, small samples of cells at 10^7/ml were starved in buffer at 22 or 27°C and then pulse-labeled at different developmental times with $[^{35}S]O_4$ for 1 h prior to TCA precipitation. Wild-type AX3 cells under these assay conditions had a developmental sulfation profile identical to that during normal development on filters (14) and virtually identical sulfate incorporation patterns and levels at the two temperatures (Fig. 4A). On the other hand, although the mutant clones tested showed sulfate incorporation curves similar to that of the wild type at 22°C, they all showed reduced levels of incorporation at 27°C (Fig. 4B to F). In addition, with the exception of clone HT7 (Fig. 4D), all of the mutant clones tested show altered patterns of sulfate incorporation at 27°C. This experiment was repeated at least three times with each clone, and in each case it produced curve shapes and differences between 22 and 27°C identical to those shown.

The first data points in Fig. 4 also show the vegetative levels of sulfation. Though sulfation increased during early development, this change did not occur during the first hour of starvation, as cells labeled for 0 to 10 min of starvation incorporated the same amount of sulfate as cells labeled during the 50- to 60-min time period (data not shown). In Fig. 4, the point at 0 h is, in fact, a zero-time blank value, and the first real data point is total counts incorporated during the first 60 min of labeling. There were some small differences between 22 and 27°C values for the mutants. Thus, HT4 and HT7 appear to have low vegetative sulfation at 27°C relative to the wild type. These mutants grew normally at 27°C, suggesting that the defective element is not required for growth but for the step in development which is blocked.

All of the clones tested were isolated from different subcultures of mutagenized cells and therefore arose independently. In addition to altered sulfation, the clones all exhibited temperature-sensitive development phenotypes. Whereas they developed normally to the fruiting body stage at 22°C, at 27°C clones HT2 and HT4 were blocked at a flat, aggregateless stage; clone HT7 formed very small, loose aggregates which did not develop further; clone HT11 was able to stream at 27°C but could not form completed aggregates, and clone HT12 was blocked after the mound stage of development.

DISCUSSION

It is well established that protein glycosylations play essential roles in the life cycle of *D. discoideum*. For example, tunicamycin, which inhibits assembly of the precursor of N-linked OLS, blocks both growth and development (17, 21, 26). Since some glycosylation mutations could, therefore, be lethal, we endeavored to obtain conditional mutants. Further, mutations in very early steps of OLS assembly should mimic tunicamycin treatment of the cells, and it is not possible from such studies to identify functions of specific OLS species. We therefore selected for mutants blocked in relatively late steps in the processing of OLS.

The processing pathways summarized in Fig. 1 indicate that a number of distinct mutations could lead to a reduction in glycoprotein sulfation. To date, none of our mutants appear to be completely devoid of sulfation. Mutations causing partial reduction in precursor incorporation are common among suicide selection survivors (1, 11, 12, 22), and there are several possible explanations as to why this was the case with our mutants. It is possible that the sulfate suicide mutants were partially defective in a single step common to all sulfation processes (such as sulfate transport or adenosine 3′-phosphate 5′-phosphosulfate synthesis). There is precedent for mutations of this type in that Deutscher et al. (5) have described a Chinese hamster ovary cell glycosylation mutant deficient in the translocation of

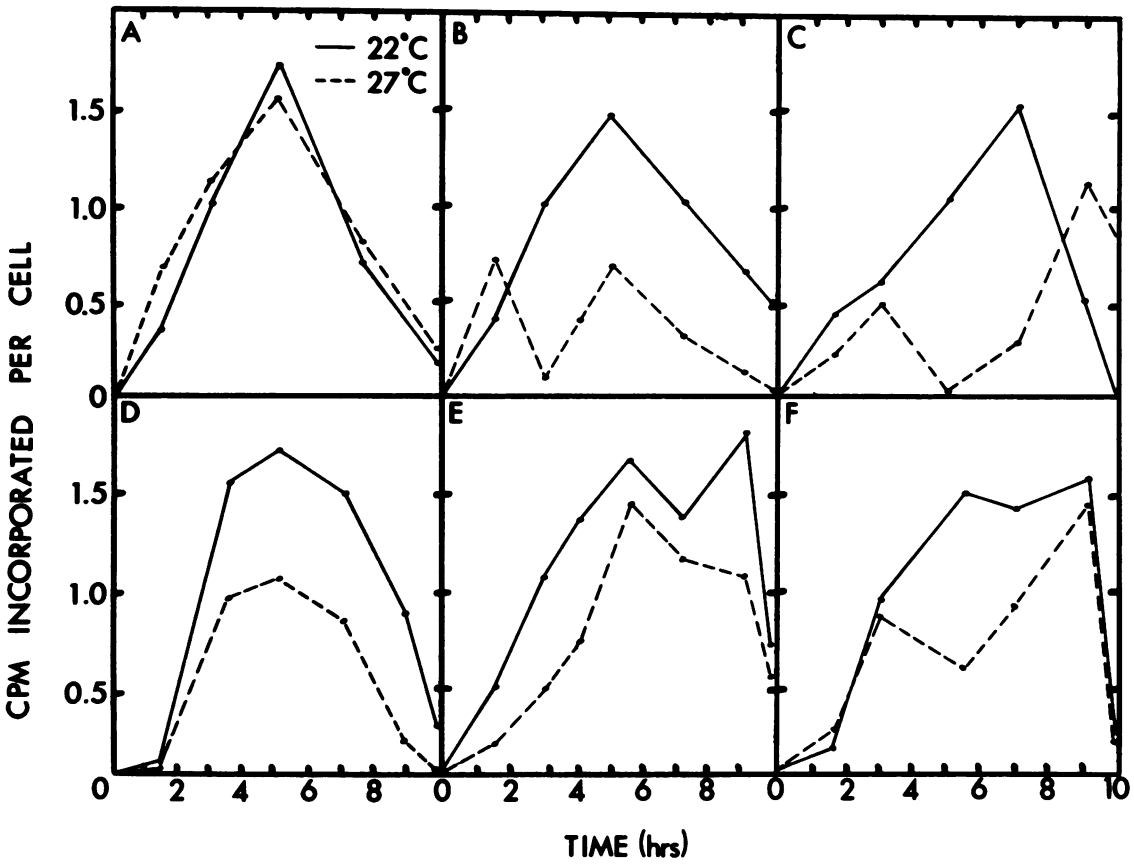

FIG. 4. Sulfate incorporation microassay. Mutant clones were assayed at both 22 and 27°C for their developmental time courses of sulfate incorporation (see Materials and Methods). Data points were plotted at the time corresponding to the end of each labeling period; for example, the data point for the 2- to 3-h pulse-label was plotted at 3 h. The 0-h value of 0 cpm per cell was obtained by adding cells to radiolabel as for all other time points and immediately pipetting a sample of cells from that mixture onto a filter paper for TCA precipitation. This 0-h value was considered to be background and was subtracted from all subsequent data points. A to F show sulfate incorporation curves for (A) wild-type AX3 cells, (B) HT2, (C) HT4, (D) HT7, (E) HT11, and (F) HT12.

CMP-sialic acid across the Golgi apparatus membrane. It is also possible that the mutants were fully defective in one specific sulfation event (such as a single sulfate transferase) or that they were defective in the biosynthesis of the OLS substrate(s) recognized by a particular sulfate transferase. For example, nonconditional *D. discoideum* mutants were isolated by their failure to produce a sulfated OLS which is a common antigenic determinant of lysosomal enzymes synthesized during growth and early development (16). When labeled with sulfate during growth, these mutants all showed reduced incorporation, but only one appeared to be completely devoid of sulfation. All but one (which was thought to contain a secondary mutation) were capable of both growth and development, but at significantly reduced rates compared with the wild-type parent. Since lysosomal enzymes produced late in development are no longer sulfated in wild-type strains, sulfation may not be an essential process for at least minimally required function of these proteins, though it does enhance their stability (6–8). Sulfate incorporation during development in these mutants has not yet been reported.

The sulfated lysosomal oligosaccharides appear to be confined to the branch of sulfated and phosphorylated glycans (9) and do not include the fucosylated structures which, under our conditions, represent the most abundant species. Suicide selection would be expected to obtain mutants in that pathway as well, and this would lead to a partial decrease in sulfation. Finally, since any mutation which lowers the rate of protein synthesis could indirectly affect the rate of protein sulfation, we chose for study only those mutants which show wild-type levels of [^{35}S]methionine incorporation at both the permissive and restrictive temperatures.

Because sulfate incorporation increases over the first 4 h of development in *D. discoideum* (13, 14), it is possible that by screening for reduced sulfate incorporation we selected for developmental program mutants. However, we feel that this is not the case. First, all of the mutants tested at 27°C showed changes in sulfate incorporation (Fig. 4). This indicated that the mutants underwent biochemical differentiation as well as changes in morphogenesis. Second, 24 of the 99 clones identified by replica-plating fluorography as defective in sulfate incorporation showed at least some morphological aspects of aggregation, and only 5 were completely flat at 27°C. Another 45 of the 99 were able to aggregate normally at the nonpermissive temperature and were only blocked in later development. Finally, as mutations which block development presumably block protein synthesis at some stage, working with methionine-positive clones may help bias against mutants defective in developmental program initiation.

From analyses of their phenotypic properties, in contrast

to amoebae treated with tunicamycin, the majority of the mutants isolated by suicide selection were able to grow and complete at least part of the developmental program. However, the fact that 75% of the sulfation-defective mutants were temperature sensitive for growth, some aspect of development, or both confirms that glycoprotein-linked OLS are functionally important throughout the life cycle of *D. discoideum*. These observations also led to our current working hypothesis that some of the later steps in OLS processing are not required for growth, but that the early processing reactions (for example, assembly of the lipid-linked precursor and its transfer to proteins) are required. The early processing steps occur at all stages of the life cycle. However, whereas the later processing steps are known to occur during growth, they are particularly enhanced during early development (13, 14). This hypothesis predicts that mutants blocked in both growth and development are defective in an early processing step, whereas those which grow normally but are unable to initiate or complete development at the nonpermissive temperature are defective in a later step of OLS processing.

Analyses of the suicide selection mutants presented in this study demonstrated that these clones all showed reduced sulfate incorporation at the nonpermissive temperature. Although clones HT4, HT7, and HT12 showed similar levels of $[^{35}S]O_4$ incorporation at 22 and 27°C under the conditions of the 4-h continuous-label assay, results from the pulse-label microassay (Fig. 4C, D, and F) showed that these clones exhibited different developmental patterns of sulfate incorporation at 27°C. It is intriguing that clones HT2 and HT4 showed similar incorporation curves at the restrictive temperature (Fig. 4B and C), as these clones were both blocked at the early aggregation stage of development at this temperature. Both clones exhibited dips in their incorporation patterns at 27°C at about the same time in the developmental program relative to their 22°C curves. Clones HT2 and HT4 were derived from different subcultures of mutagenized cells; hence, it is unlikely that they are siblings. Further, since we used relatively gentle mutagenesis (42% survival), and temperature sensitivity was displayed for both sulfation and the biological phenotype, it is unlikely that these properties are due to independent mutations.

It is anticipated that the study of different mutants having the same temperature-sensitive development phenotype will eventually lead to the identification of N-linked OLS which have functional roles at that stage of development. We are currently in the process of studying the synthesis of specific OLS at 22 and 27°C by the clones presented in this study as well as by additional clones isolated by suicide selection. Results from these assays should enable us to pinpoint the biochemical defect in each mutant. We will then be able to begin classifying mutant development phenotypes with categories of biochemical defects.

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