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Sulfation of a cell surface glycoprotein correlates with the developmental program during embryogenesis of *Volvox carteri*

(differentiation/pattern formation/sexual inducer)

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ABSTRACT **A sulfated cell surface glycoprotein with an apparent molecular weight of 185,000 is synthesized in the multicellular organism *Volvox* only during the limited period of embryogenesis. The lifetime of sulfate residues on this glycoprotein is very short (half-life about 20 min). Production of this sulfated glycoprotein sharply decreases to a minimum shortly before the onset of the differentiating cell cleavage—e.g., in asexual development, before the 32-cell embryo divides. It is demonstrated that the sulfated glycoprotein behaves in many respects as would the hypothetical cell surface component postulated by a recently published model [Sumper, M. (1979) *FEBS Lett.* 107, 241–246], which proposes an explanation for the cell-counting mechanism and the spatial control of differentiation that is operative in *Volvox* embryogenesis.**

The colonial green flagellates of the genus *Volvox* present an interesting model for studying the control of cellular differentiation (1–3). With only two different types of cell, *Volvox* is one of the most primitive multicellular organisms. Asexual colonies of *V. carteri* differentiate into 2000–4000 somatic cells with no potential for further division and only 8–16 reproductive cells (gonidia). The somatic cells are always the same in structure and function, but the reproductive cells may differ in number, position, and function, depending on whether the individual is asexual, male, or female. The development of all types of spheroids—asexual, male, and female—is through successive divisions of the asexual reproductive cells (gonidia).

In the developing asexual embryo differentiation into somatic and reproductive cells is seen at the division from 32 to 64 cells (4–6). At this stage, 16 out of the 32 cells undergo unequal cleavage, forming a small somatic and a large reproductive initial. The 16 large reproductive initials stop further cleavage, while the remaining embryonic cells continue cell cleavage and finally differentiate into somatic cells.

Under the influence of a sexual inducer, this developmental program is modified (7, 8): In female strains, the differentiating cleavage is shifted to the 64-cell stage. At the division to the 128-cell embryo, 32 (or a few more) cells divide unequally, forming 32 (or a few more) egg initial cells. In a male strain, the differentiating cleavage is even more delayed under the influence of the sexual inducer. Differentiation into sperm-producing cells (androgonidia) and somatic cells occurs at the final division, which in male embryos is usually encountered at the 128- or 256-cell stage. Obviously, some sort of cell-counting mechanism is operative during embryogenesis, telling a cell that the embryo is in the 2-, 4-, 8-, ...2^n-cell stage. Besides this cell counting problem, the embryogenesis of *Volvox* presents the problem of pattern formation in fascinating simplicity. The spatial arrangement of the reproductive initials within the embryo is exactly controlled, resulting in a highly regular positioning.

We recently have shown that a model that is essentially based on a single assumption is able not only to explain the counting mechanism but also to predict correctly the spatial arrangement of the reproductive cells within the developing embryo (9). The only assumption made by our model is the existence of a limiting amount of a specific cell surface component that mediates cell-to-cell contacts. Such surface components have been detected in a number of different multicellular organisms (10, 11). The pool of such a surface component would be out-titrated by contact formation at a sharply defined stage of division, because the number of cell contacts increases exponentially during embryogenesis. If, for instance, this event occurs at the stage of the 16-cell embryo, then the subsequent cleavage divides the embryonic cells into two subclasses: 16 cells being equipped with this specific cell surface component and another 16 cells lacking it. If the absence of this cell surface component signals the unequal cell cleavage, a number of characteristic features of *Volvox* embryogenesis can be correctly predicted by the model.

A set of experiments supporting this model has recently been published (12, 13). In particular, a sulfated 185-kilodalton (kDal) membrane component was detected that was shown to be a candidate for the postulated cell surface component. In this paper we provide further evidence for an embryonic control function of this sulfated cell surface component.

MATERIALS AND METHODS

Growth of *V. carteri*. *V. carteri* f. *nagariensis* female strain HK 10 and male strain 69-1 b from the Culture Collection of Algae at the University of Texas at Austin were a gift from L. Jaenicke (Cologne). The organism was grown in *Volvox* medium (14) as in ref. 8. Illumination at 10,000 lux on a 16-hr light/8-hr dark cycle at 27°C resulted in a synchronously growing culture. The "sterile" female strain 70–36 was a gift from R. Starr (University of Texas, Austin).

Pulse Labeling Experiments. A clonal culture of *Volvox* was obtained by inoculating one spheroid from a synchronously growing culture into a test tube with 10 ml of medium. After 4 days, shortly before release of daughter colonies, 3 out of the 16 colonies were transferred into a 1000-ml Fernbach flask containing 800 ml of *Volvox* medium. The medium was magnetically stirred at 100 rpm by using a Teflon-coated spinbar with a pivot ring. The medium was aerated by a gentle stream of air injected through a Pasteur pipette. Growth was continued for two further generations, resulting in a *Volvox* suspension containing 15 spheroids per ml. The subsequent gonidial cleavage

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Abbreviations: kDal, kilodalton; SSG 185, sulfated surface glycoprotein, 185 kDal.

period of these colonies occurs in a highly synchronous manner, >90% of the gonidia being in the same stage of division. This culture was used for all pulse labeling experiments. Fifty milliliters was poured over a 40-μm-screen cloth. The trapped colonies were washed four times with a sulfate-free medium ($MgSO_4$ was replaced by $MgCl_2$) and were finally resuspended in 500 μl. After addition of 20 μCi (1 Ci = 3.7×10^{10} becquerels) of radioactive sulfate (specific activity 35 mCi/μmol), the suspension was magnetically stirred under illumination (15,000 lux) in a water bath at 27°C for 30 min.

Spheroids containing sexual embryos were obtained by adding the sexual inducer (8) to the culture medium 15 hr prior to the onset of the last gonidial cleavage period.

Preparation of a Crude Membrane Fraction. *Volvox* spheroids were disintegrated by ultrasonic treatment (30 sec total). The lysate (500 μl) was layered over a 3-ml cushion of 20% sucrose in *Volvox* medium. Centrifugation was at 100,000 × *g* for 3 hr. The green pellet was dissolved in 400 μl of $NaDodSO_4$ sample buffer (15) and heated for 60 sec at 90°C. Aliquots were applied to 6% $NaDodSO_4$/polyacrylamide gels. Fluorography was performed according to ref. 16.

Carbohydrate Analysis. ^{14}C-Labeled neutral sugars were identified by gas chromatography of the alditol acetates. Derivatization was performed as in ref. 17. The alditol acetates were separated on a glass column (914 × 2 mm) packed with 3% SP-2340 on 100/120 Supelcoport (Supelco) run with a temperature program of 180–230°C at 2°C/min. The outlet end of the column was fitted with a 1:10 stream splitter; the radioactivity of 0.9 of the sample was measured as $^{14}CO_2$ in a Packard model 894 gas proportional counter.

RESULTS

V. carteri produces a sulfated membrane component only during the limited period of gonidial cleavage (embryogenesis); synthesis of this compound is shut off or at least strongly reduced at all other stages of the life cycle. The apparent molecular weight of this component was found to be 185,000 (13).

Partially purified sulfated 185-kDal component was found to be sensitive to protease treatment. Digestion by subtilisin or Pronase reduces the apparent molecular weight to about 150,000. The resulting sulfated core material is resistant even to prolonged protease treatment. The 150-kDal core material was further analyzed for the presence of sugars. Incorporation of $^{14}CO_2$ into *Volvox* embryos enabled us to isolate ^{14}C-labeled 185-kDal component. After protease treatment, the remaining 150-kDal core material was hydrolyzed in 2 M HCl for 4 hr at 100°C. After borohydride reduction and acetylation, the hydrolysis products were analyzed by gas chromatography for the presence of neutral sugars. As shown by the gas chromatogram in Fig. 1, radioactivity was mainly detected in the sugars arabinose and galactose, and minor incorporation into xylose, mannose, and glucose was observed. Essentially the same result was obtained by sugar analysis of unlabeled 150-kDal core material obtained by large-scale purification (18). Therefore, the sulfated 185-kDal component is a glycoprotein.

In order to examine the localization of the 185-kDal glycoprotein, intact *Volvox* colonies were treated with subtilisin, which effectively dissociates the organism into single cells. The viability of the reproductive cells is not affected by this treatment, because cell cleavage and development continue after removal of the protease by washing. Thus subtilisin treatment appears to be a mild procedure for digesting only surface-associated components. In the experiment of Fig. 2, *Volvox* colonies containing 4-cell embryos were pulse labeled with $^{35}SO_4^{2-}$ for 30 min. Then the colonies were dissociated by subtilisin and the resulting suspension of somatic cells and embryos

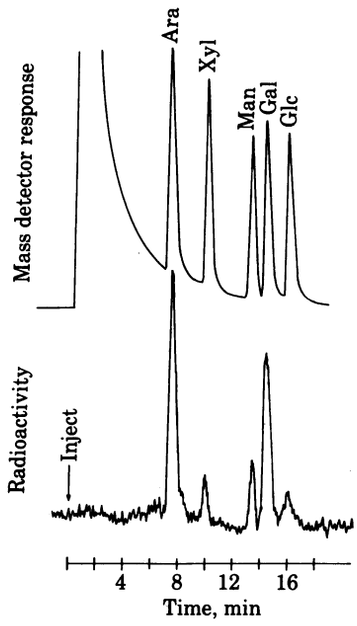

FIG. 1. Gas chromatography of ^{14}C-labeled alditol acetates derived from ^{14}C-labeled 185-kDal sulfated component. *Volvox* embryos were incubated in the presence of $H^{14}CO_3^-$ (70/μCi/ml) for 1 hr. The 185-kDal component was eluted from $NaDodSO_4$ / polyacrylamide gels and digested with subtilisin. The resulting 150-kDal derivative (see Fig. 2) was further purified by $NaDodSO_4$/polyacrylamide electrophoresis. Carrier sugars were added, then after acid hydrolysis (4 hr, 2 M HCl) and derivatization, gas chromatography was performed.

was centrifuged at low speed. The cell-free supernatant and the cell pellet were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography. As shown by the fluorogram of Fig. 2, lane D, the 185-kDal glycoprotein was nearly quantitatively recovered (as its 150-kDal derivative) in the supernatant fraction and nearly no 185-kDal (or 150-kDal derivative) glycoprotein remained associated with the cellular fraction (Fig. 2, lane C). Therefore, the sulfated 185-kDal component is a cell surface-associated glycoprotein and will be denoted in the following as SSG 185 (sulfated surface glycoprotein, 185 kDal).

Pulse-chase labeling experiments revealed an unusually short lifetime of the SSG 185 molecule. It follows from the data given in Fig. 3 that the *in vivo* half-life of SSG 185 is less than 30 min. When the sulfate concentration of the *Volvox* culture medium was raised from its normal value of 0.1 mM to 10 mM at the beginning of the chase period, the lifetime of the sulfate residues of SSG 185 increased substantially. Likewise, the ad-

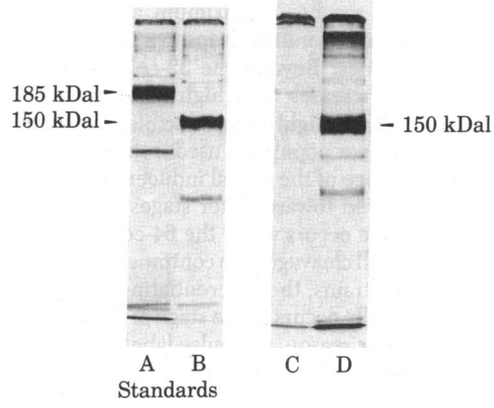

FIG. 2. The 185-kDal glycoprotein is a cell surface component. *Volvox* spheroids containing 4-cell embryos were pulse labeled with $^{35}SO_4^{2-}$ for 30 min. Lane A, analysis of the isolated total membrane fraction by $NaDodSO_4$/6% polyacrylamide gel electrophoresis and fluorography. Lane B, as lane A, after treatment of the membrane fraction with subtilisin at 50 μg/ml for 30 min. Lanes C and D, the pulse-labeled *Volvox* spheroids were dissociated into single cells by subtilisin treatment (350 μg/ml, 30 min at 28°C). After centrifugation (3000 × *g* for 2 min) the cellular fraction (lane C) and the cell-free supernatant (lane D) were analyzed on a $NaDodSO_4$/6% polyacrylamide gel by fluorography.

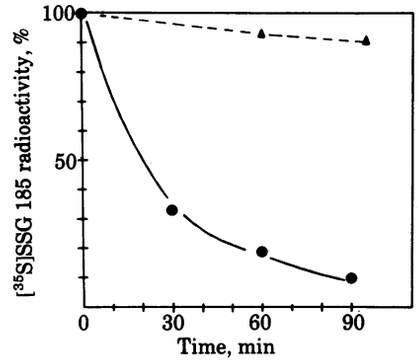

FIG. 3. SSG 185 *in vivo* is very short lived. *Volvox* spheroids containing asexual 4-cell embryos were pulse labeled with $^{35}SO_4^{2-}$ for 30 min. Zero time marks the beginning of the chase period in fresh sulfate-containing *Volvox* medium. After the indicated chase periods, the radioactivity of SSG 185 was analyzed after isolation by NaDodSO$_4$/polyacrylamide gel electrophoresis. ●, Chase in the standard *Volvox* medium; ▲, chase in the presence of 10 mM sulfate.

dition of 10 mM *p*-nitrophenylsulfate to the *Volvox* culture medium inhibited the decay of SSG 185. These characteristics are indicative of the action of a sulfatase-like enzyme on SSG 185.

Short pulse labeling experiments over the whole period of embryogenesis in sexually induced male embryos revealed that SSG 185 behaves as would the hypothetical surface component of our model in one main respect: its net production sharply decreases to a very low level immediately before the last cell cleavage—i.e., the differentiating one. This suggests that a non-sulfated precursor molecule of SSG 185 becomes exhausted at this stage of development (13).

In order to provide further evidence on this point, the same type of pulse labeling experiments were carried out with asexual female embryos as well as with sexually induced female embryos. Asexual female embryos undergo the differentiating cleavage when the 32-cell stage divides. The fluorogram shown in Fig. 4 summarizes the results of pulse labeling experiments performed over the whole period of embryogenesis in a highly synchronously developing culture of asexual female embryos. Net $^{35}SO_4^{2-}$ incorporation into SSG 185 increases during early embryogenesis and reaches a maximum at the 4-cell stage. Thereafter the net incorporation drops to a minimum when the 16-cell embryo divides. Beyond the 32-cell stage the net incorporation again increases to a high level. This pattern of $^{35}SO_4^{2-}$ incorporation is highly reproducible, provided the development of the *Volvox* population used is strictly synchronized.

Under the influence of the sexual inducer the differentiating cell cleavage is shifted towards later stages: in female strains, the unequal cleavage occurs when the 64-cell embryo divides. Thereafter, equal cell cleavages are continued to produce 2000–4000 cells. In male strains, the differentiating cell division is the last one, which usually occurs at the stage of the 128-cell or 256-cell embryo. For this reason, the pulse labeling patterns of sexually induced male and female embryos were compared. The results obtained are shown in Fig. 5. In male embryos net incorporation into SSG 185 reaches a maximum level during early embryogenesis and finally drops to a very low level towards the end of embryogenesis—i.e., at the time of the differentiating division. In sharp contrast, in sexually induced female embryos, a distinct minimal incorporation is observed at the 32-cell and 64-cell stages; beyond these stages a high level of incorporation is restored until the end of embryonic divisions.

Another observation should be stressed in this context: It is not only the $^{35}SO_4^{2-}$ incorporation into SSG 185 that follows a defined pattern, but it is also the apparent molecular weight of

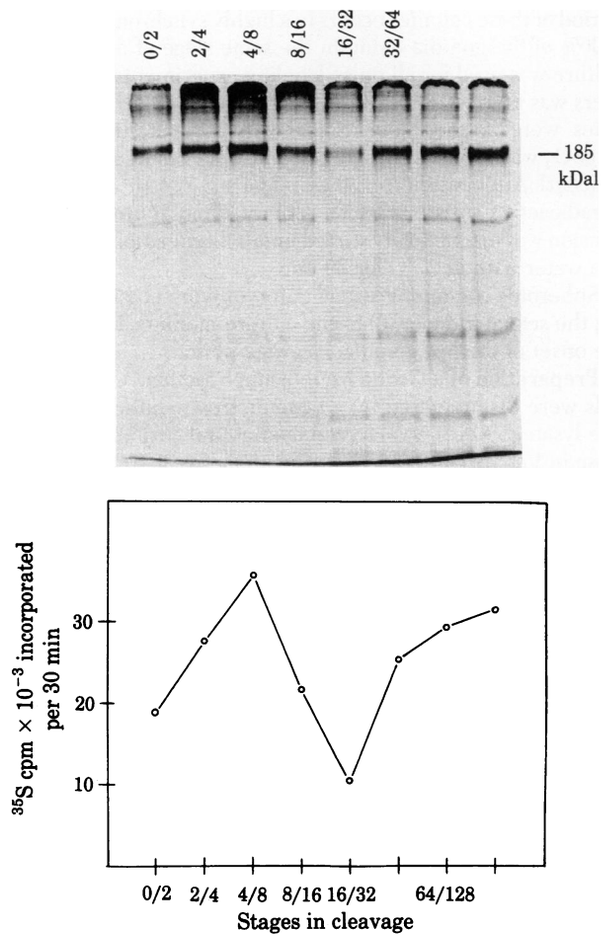

FIG. 4. $^{35}SO_4^{2-}$ incorporation into SSG 185 during embryogenesis of asexual spheroids (female strain HK 10). After onset of gonidial cleavage a pulse labeling experiment was performed at each stage of division, i.e., every 60 min. (*Upper*) Total membrane fractions from spheroids at the indicated cleavage stages were applied to NaDodSO$_4$/6% polyacrylamide gels and visualized by fluorography. (*Lower*) SSG 185 bands were cut out of the polyacrylamide gel and the incorporated radioactivity was measured in a scintillation counter.

SSG 185 that shifts in a defined way. During early embryogenesis the apparent molecular weight of SSG 185 decreases slightly, reaching a constant value at or immediately before the time of the differentiating cell cleavage (Fig. 5). This shift in the apparent molecular weight is more clearly demonstrated by comparing the SSG 185 produced during the 2-cell stage with that of the 654-cell stage in adjacent lanes of a slab gel (Fig. 6). This observation indicates that an additional and as yet unknown modification of SSG 185 may be of importance during early embryogenesis.

Another line of evidence indicates an important role of two additional sulfated surface components in embryogenesis. Besides SSG 185 another sulfated surface component with an apparent molecular weight of 140,000 was found to be synthesized in asexual female spheroids only during embryogenesis (13). Under the influence of the sexual inducer this 140-kDal sulfated surface component is no longer synthesized and a new high molecular weight sulfated component appears during early embryogenesis of female spheroids (indicated by the arrow in Fig. 7). The rate of synthesis of this component sharply decreases immediately before the differentiating cell cleavage and remains very low during the later stages of embryogenesis (Fig. 5). This observation suggests a functional correlation of this molecule with SSG 185. Remarkably, in a *Volvox* mutant strain

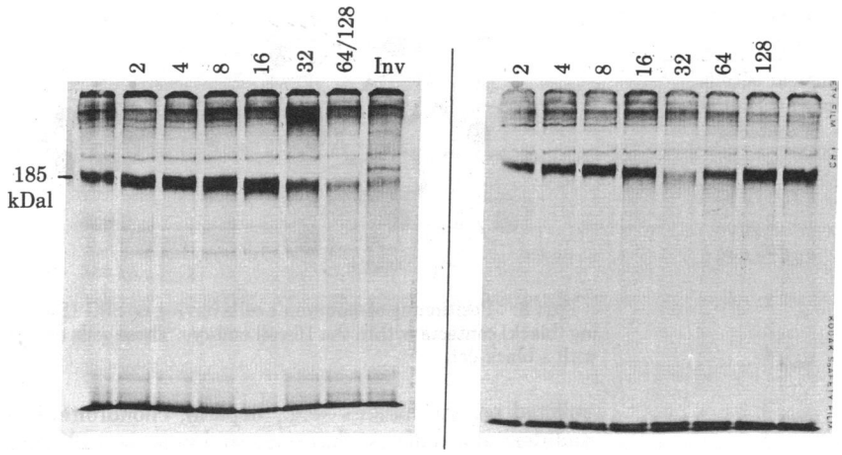

FIG. 5. $^{35}SO_4^{2-}$ incorporation into SSG 185 during embryogenesis of sexually induced spheroids. Stages of cleavage are indicated at the top. Inv, inversion stage. (*Left*) Sexually induced male embryos; (*Right*) Sexually induced female embryos. Pulse labeling was carried out as in Fig. 4. Total membrane fractions were applied to NaDodSO$_4$/6% polyacrylamide gels and visualized by fluorography.

70-36 the pattern of sulfated components shows a corresponding change. Strain 70-36 was derived from a spontaneous female mutant of the HK 10 strain; this spontaneous female mutant forms eggs without having inducer added. In strain 70-36, however, the cells that would have become eggs are no longer capable of doing so, but rather develop as asexual reproductive cells. The pattern and timing of embryonic development remains that of a female, with unequal cleavages at the division of the 64-celled stage. In this mutant strain, the presence of inducer does not result in the synthesis of this high molecular weight component (indicated by the arrow in Fig. 7). This suggests a function for this component in the maturation of egg cells.

DISCUSSION

A great deal of work has pointed to the cell surface as an important site in the control of developmental processes. Experimental work attempting to more clearly characterize the nature of interactions involved is being pursued mainly in animal systems. The experimental advantages offered by the *Volvox* system, as, for instance, synchronous development of large embryo populations, availability of developmental mutants (19, 20) and simplicity of cellular organization make it particularly useful for studying the importance of cell surface components.

The experimental results described in this paper demonstrate that SSG 185 has at least three of the properties postulated for the hypothetical cell surface component involved in the cell-counting mechanism according to our model: (*i*) SSG 185 is located on the cell surface, (*ii*) its production is essentially limited to the short period of embryogenesis, and (*iii*) its net production reaches a minimum exactly one stage of division before the differentiating cleavage, indicating consumption of a precursor molecule at this time. However, one crucial property postulated by the model remains to be established for SSG 185: its capacity to be trapped within the cell-to-cell contact area. This point can be checked experimentally by using antibodies against SSG 185 for immunofluorescence studies.

The observed correlation between SSG 185 production rate and developmental stage suggests but does not prove that SSG 185 synthesis is part of the determinative process. Alternatively, the observed correlation could be a consequence of the determinative process. However, because out-titration of SSG 185 precedes the onset of the differentiating cell cleavage, this latter alternative is less probable.

The scheme of Table 1 demonstrates how SSG 185 could be involved in the control of differentiation. The assumptions are made that (*i*) SSG 185 is engaged in cell-to-cell contact formation and (*ii*) SSG 185 or its (desulfated) degradation product suppresses unequal cell cleavage (or, equivalently, induces somatic

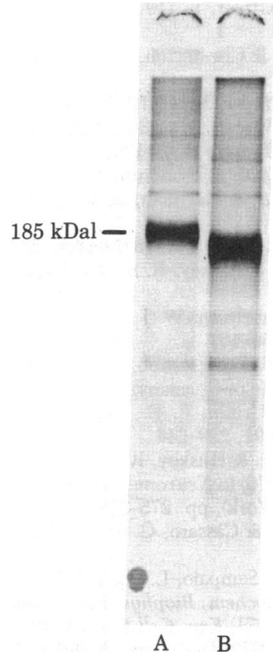

FIG. 6. Comparison of SSG 185 synthesized by 2-cell embryos (lane A) and 64-cell embryos (HK 10, asexual female) (lane B) on a NaDodSO$_4$/5% polyacrylamide gel.

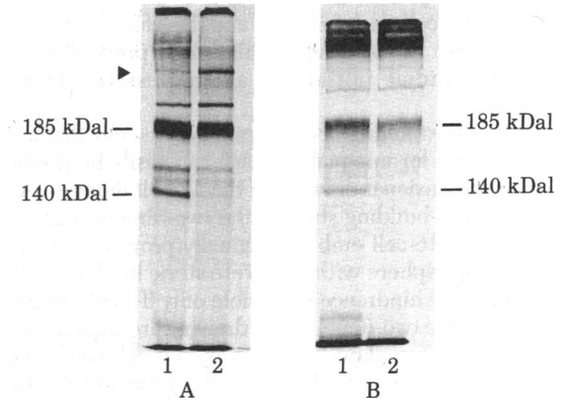

FIG. 7. Effect of the sexual inducer (8) on the synthesis of sulfated membrane components in the female strain HK 10 (*A*) and in the "sterile" female strain 70-36 (*B*). Lanes 1; asexual *Volvox* spheroids containing 2-cell embryos were pulse labeled with $^{35}SO_4^{2-}$ for 30 min. The membrane fraction was analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography. Lanes 2; as lanes 1, but in the presence of the sexual inducer.

Table 1. Cell differentiation in the developing embryo with respect to a given type of cell-to-cell contact

Embryo stage	Type and number of cell-to-cell contacts	Number of cells equipped with this set of contacts
2-cell	$(SSG)_1$	2
4-cell	$(SSG)_2$	2
	$(SSG)_1$	2
8-cell	$(SSG)_3$	2
	$(SSG)_2$	2
	$(SSG)_1$	4
16-cell	$(SSG)_3$ 0	2
	$(SSG)_2$ 0	2
	$(SSG)_1$ 0	4
	0 → unequal cleavage	8

0, no SSG 185 in contact area.

cell development). The first cleavage produces two embryonic cells having a contact in common which traps a given amount of SSG 185 out of the total pool. The second cleavage creates two more cell-to-cell contacts, resulting in a 4-cell embryo in which 2 cells maintain 2 contacts [denoted as $(SSG)_2$] and 2 other cells maintain only one cell-to-cell contact [denoted as $(SSG)_1$]. The number of cell-to-cell contacts formed increases exponentially during embryogenesis. Therefore the pool of SSG 185 or its precursor must be exhausted at a shraply defined stage of division: if, for instance, this happens at the stage of the 8-cell embryo, then in the subsequent cleavage those 8 daughter cells with only a single cell-to-cell contact become completely cleared of SSG 185 on their cell surface. Because the differentiating cleavage is no longer suppressed in this subclass of cells, 8 gonidial initials are formed at the division of the 16-cell embryo. During embryogenesis of *Volvox* the differentiating cleavage is seen only at one stage of division. This means that the suppression of unequal cleavage must be reestablished in all successive stages of division. The pulse labeling experiments of Fig. 4 indeed demonstrate that SSG 185 production is again initiated after the unequal cleavage. In terms of our model, this could be achieved if a 0-contact (a contact lacking SSG) signals reinitiation of SSG 185 synthesis. At the time of the unequal cleavage, all embryonic cells are equipped with a 0-contact (but only half of these cells lack SSG 185) and therefore are triggered to reinitiate SSG 185 synthesis. This would prevent the production of gonidial initials at all successive stages of embryogenesis.

The spatial positioning of those embryonic cells that are no longer repressed for unequal cleavage can easily be predicted from a two-dimensional drawing of the 16-cell embryo. As revealed by model-building studies, the two-dimensional representation of the 16-cell embryo originally proposed cannot be transformed to a sphere without severe steric hindrance. Folding without steric hindrance is possible only if 4 cells of the 16-cell embryo in the two-dimensional drawing are shifted towards the periphery as was done in the corrected cell configuration of Fig. 8. It is clearly seen that the cells of the anterior half of the embryo should undergo unequal cleavage, resulting in 8 gonidial initials which are arranged in two rectangles turned by 45° against each other. This is exactly the observed positioning of gonidia in those *Volvox* embryos undergoing differentiating cleavage already at the 16-cell stage (which occurs only under less than optimal growth conditions).

In recent years a great amount of work primarily with animal

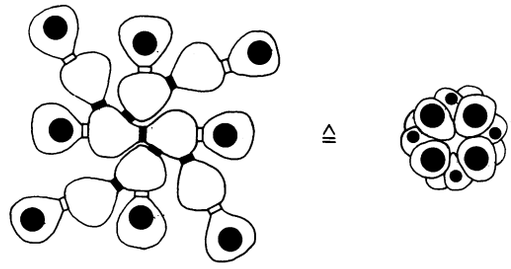

FIG. 8. Positioning of embryonic cells having no SSG 185-containing (black) contacts within the 16-cell embryo. These cells are labeled with a black dot.

sulfated polysaccharides—e.g., heparin, chondroitin sulfates, and dermatan sulfates—has suggested important physiological functions for these substances (21). Dietrich *et al.* (22, 23) proposed a possible role of sulfated mucopolysaccharides in cell recognition and adhesiveness in animal cells. Sulfated polysaccharides were also implicated in embryogenesis of sea urchins; it is assumed that synthesis of sulfated polysaccharides is an indispensable step for postgastrular development (24–26). Studies on the embryogenesis of the brown alga *Fucus* led to the conclusion that the sulfation of polysaccharides (fucan) is a modification required for its localization into a specific region of the cell wall (27, 28).

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