SULPHATION–DESULPHATION OF A MEMBRANE COMPONENT PROPOSED TO BE INVOLVED IN CONTROL OF DIFFERENTIATION IN *VOLVOX CARTERI*

Manfred SUMPER and Stephan WENZL

Institut für Biochemie, Genetik und Mikrobiologie, Lehrstuhl Biochemie I, Universität Regensburg, Universitätsstr. 31, 8400 Regensburg, FRG

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1. Introduction

In the developing asexual embryo of *Volvox carteri* the differentiation into somatic and reproductive cells is seen at the division from 32–64 cells. At this stage, 16 cells of the 32-celled embryo undergo unequal cleavage, forming 16 large reproductive cells [1,2]. In sexual embryos developing under the influence of an inducer the differentiating cleavage is delayed in the male strain up to the final division, which usually occurs at the 128- or 256-celled stage [2].

We have proposed a model that explains the counting mechanism which tells a cell that the embryo is in the 2-, 4-, 8-, 16-, 32- or 64-celled stage [3]. This model is able to predict correctly the very regular spatial arrangement of the reproductive cells (gonidia) within the developing *Volvox* embryo. A necessary assumption in the model is the existence of a membrane component which mediates cell- to- cell contacts. The pool of this membrane component would be exhausted at a sharply defined stage of division since the number of cell contacts increases exponentially during cell division. This event was assumed to signal differentiation.

A first set of experiments supporting this model appeared in [4]. We have studied the developing embryo for components meeting the following criteria:

1. The component in question should be a membrane-bound (glyco-)protein.
2. Its cellular level should correlate with the developmental program.
3. Its production should be (reversibly) inhibited by chemicals known to (reversibly) disturb the mechanism of gonidial differentiation.

Here we report the existence of a sulphated membrane glycoprotein with an extremely high turnover rate. We postulate that this glycoprotein is involved in the control of differentiation.

2. Materials and methods

2.1. *Growth of* Volvox carteri

Volvox carteri f. *nagariensis* female strain HK 10 and male strain 69-1b were a gift from Professor L. Jaenicke, Cologne. The organism was grown in *Volvox* medium [5] as in [6]. Illumination at 10 000 lux on a 16 h light/8 h dark cycle at 27°C resulted in a synchronously growing culture.

2.2. *Pulse labelling experiments*

A clonal culture of *Volvox* was obtained by inoculating one spheroid from a synchronously growing culture into a test tube with 10 ml medium. After 4 days, shortly before release of daughter colonies, 4 out of the 16 colonies were transferred into a 1000 ml Fernbach flask containing 800 ml *Volvox* medium. The medium was magnetically stirred at 100 rev./min 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 ~20 spheroids/ml. The subsequent gonidial cleavage 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 labelling experiments: 50 ml were poured over a 40 μm screen cloth.

The trapped colonies were washed several times with a sulphate free medium ($MgSO_4$ was replaced by $MgCl_2$) and were finally resuspended in 500 µl. After addition of radioactive sulphate (spec. act. 17 mCi/µmol), the suspension was shaken under illumination (18 000 lux) in a water bath at 27°C.

Spheroids containing sexual embryos were obtained by adding the sexual inducer [6] to the culture medium 15 h prior to the onset of the last gonidial cleavage period.

2.3. *Preparation of a crude membrane fraction*

Volvox spheroids were disintegrated by ultrasonic treatment (30 s 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 h. The green pellet was dissolved in 400 µl SDS sample buffer [7], and heated for 60 s at 90°C. Aliquots (10–20 µl) were applied to 6% SDS–polyacrylamide gels. Fluorography was performed according to [8].

2.4. *Binding of the 185 k component to Con A–Sepharose*

A crude membrane fraction was solubilized in the presence of 1% SDS. Excess SDS was precipitated by addition of 150 mM KCl [9]. The supernatant was diluted 2–5-fold with the following buffer (Con A buffer): 0.1 M Tris–HCl (pH 7.5) containing 0.4% Triton X-100, 0.1 M NaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$. After the addition of Con A–Sepharose, the suspension was incubated at room temperature for 30 min. The Con A–Sepharose was then washed 5 times with 10 vol. Con A buffer. Bound radioactivity was eluted with SDS sample buffer [7]. We could not observe desorption of the 185 k component using α-D-methylglucoside.

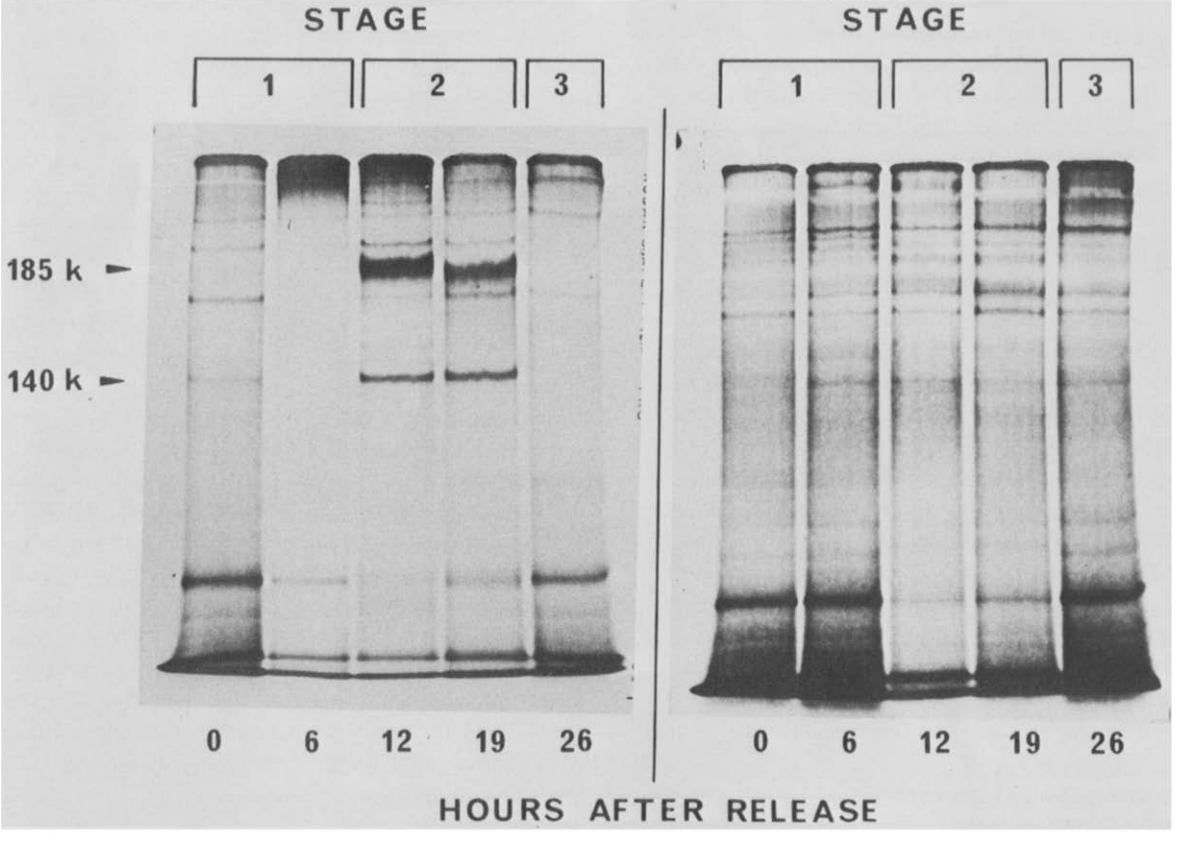

Fig.1. SDS–polyacrylamide electrophoresis of membrane proteins pulse labelled in vivo for 30 min at different developmental stages. *Volvox* spheroids (strain HK 10) were pulse-labelled as in section 2. The total membrane fraction was applied to 6% SDS–polyacrylamide gels and the patterns visualized by fluorography. (Left) Patterns obtained by pulse-labelling with 25 µCi/ml $^{35}SO_4^{2-}$ for 30 min. (Right) Patterns obtained by labelling with 10 µCi/ml $H^{14}CO_3^-$. For definition of stages 1–3 see section 3.

3. Results

The life cycle (48 h) of *Volvox carteri* was divided as proposed [1] into the following:

Stage 1. Release of daughter colonies from the parent colony to beginning of gonidial cleavage. This stage covers the period of gonidial enlargement (12 h).

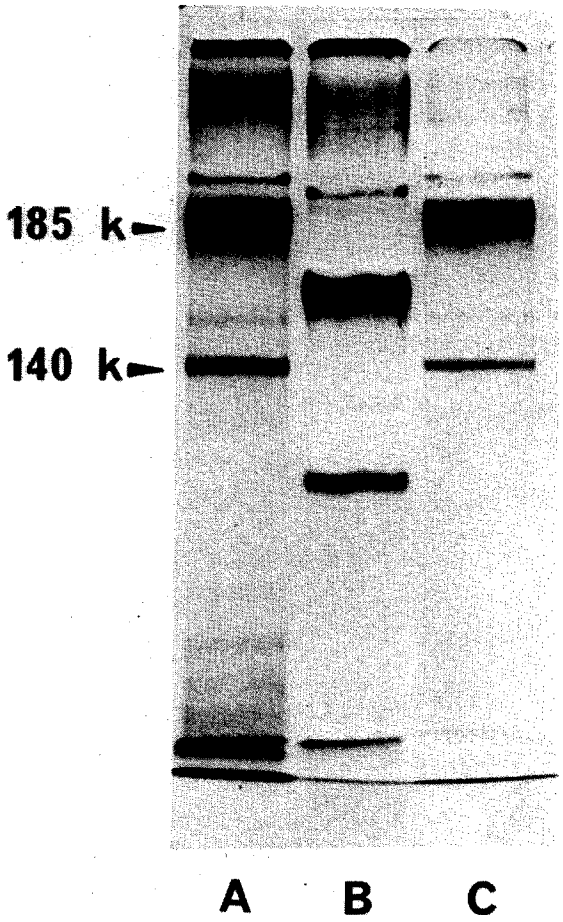

Fig.2. Component 185 k is sensitive to protease treatment (B) and binds to Con A–Sepharose (C). A $^{35}SO_4^{2-}$-labelled crude membrane preparation (100 μl) containing the 185 k component was treated with 2.5 μg subtilisin for 60 min in the presence of 50 μg serum albumin as carrier protein. An aliquot was applied to a 6% SDS–polyacrylamide gel. The labelled substances were visualized by fluorography. (A) Pattern before protease treatment; (B) after protease treatment. The same membrane preparation was solubilized by SDS as in section 2 and incubated with Con A–Sepharose. After extensive washing, lectin-bound components were eluted by SDS and analysed on a 6% SDS–polyacrylamide gel (C).

Stage 2. Beginning of gonidial cleavage to end of inversion.

Stage 3. End of inversion to release of daughter colonies.

A highly synchronously growing *Volvox* population was pulse labelled in order to determine the patterns of membrane proteins synthesized at the different developmental stages. Fig.1 (right) shows fluorograms of SDS–polyacrylamide gels obtained by using $^{14}CO_2$ as the radioactive label. It is obvious that the membrane protein patterns obtained at different developmental stages do not differ very much from each other. In sharp contrast, a quite distinct change is observed during development when the same type of pulse labelling experiment is with $^{35}SO_4^{2-}$ as the radioactive label (fig.1, left). Exactly at the beginning of cell cleavage (stage 2) two highly labelled membrane components appear. Production of these components abruptly stops after the end of stage 2 (cleavage period). The more prominent component of the two has app. mol. wt 185 000 (185 k), as estimated by electrophoresis on 6% SDS–polyacrylamide gels using fatty acid synthase from yeast and RNA polymerase from *Escherichia coli* as standards.

Since the time dependence of the 185 k component is only detected by labelling with $^{35}SO_4^{2-}$, a de novo synthesis is less probable than the sulphation of a pre-existing membrane component.

3.1. *The 185 k component is a sulphated glycoprotein*

To determine the chemical nature of the ^{35}S-label, the 185 k compound was eluted from SDS–polyacrylamide gels and subjected to mild acid hydrolysis (2 N HCl, 30 min at 80°C). The hydrolysate was analyzed by thin-layer ion exchange chromatography on polyethylene imine cellulose plates. All of the incorporated radioactivity comigrated with authentic sulphate, indicating that the 185 k compound is a sulphated molecule. It is also sensitive to protease (subtilisin) treatment and in addition, strongly binds to Con A–Sepharose, indicating a glycoprotein structure. The latter properties are documented in fig.2.

3.2. *The 185 k glycoprotein sulphate residue has a high turnover rate*

Eight-celled embryos of *Volvox* were pulse labelled with $^{35}SO_4^{2-}$ for 20 min. Within a subsequent chase period of only 40 min, the label of the 185 k component is nearly completely lost (fig.3). If *p*-nitrophenylsulphate (10 mM) is added during the chase

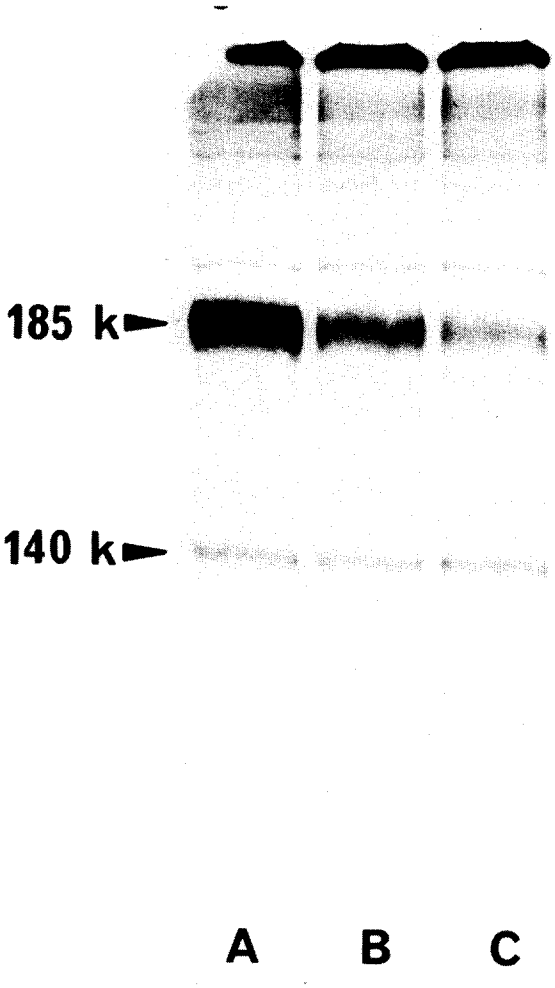

Fig.3. The sulphated 185 k component is very short-lived. 8-celled asexual embryos were pulse-labelled with $^{35}SO_4^{2-}$ (20 μCi/ml) as in section 2 (A). After 20 min, unlabelled sulphate was added to 1 mM final conc. After a chase period of 20 min (B) and 40 min (C), the radioactivity of the 185 k component was analyzed by fluorography of 6% SDS–polyacrylamide gel.

period, degradation of the ^{35}S-label is effectively inhibited. This observation indicates the presence of a sulphatase acting on the sulphated 185 k component.

3.3. *Evidence for control of differentiation by 185 k glycoprotein*

Short pulse labelling experiments were performed over the whole cleavage period (stage 2) to investigate whether the production or degradation of the sulphated 185 k glycoprotein correlates with the differentiating cell division. Sexual embryos of the male strain were used for this type of experiment, since the whole cleavage takes only ~6 h, i.e., about half the time required for asexual embryos. Cleavage occurs every 50–60 min. Therefore, a pulse length of 30 min was selected. As shown in fig.1, exactly at the beginning of cleavage, production of the ^{35}S-labelled 185 k component is initiated. The results of the subsequent labelling are shown in fig.4. The net production reaches a maximum level during the early cleavage period and sharply declines as division proceeds. The minimum level of net $^{35}SO_4^{2-}$-incorporation is reached immediately before the differentiating cleavage, i.e., at the 64- and 128-celled stage (under our growth conditions, a mixture of male spheroids with 128 or 256 cells is produced). At the time of the final division (the differentiating one) a new ^{35}S-labelled membrane component emerges with app. mol. wt ~195 000. Production of this component is strictly limited to this short period (not shown in fig.4). This pattern of $^{35}SO_4^{2-}$-incorporation is highly reproducible. Thus,

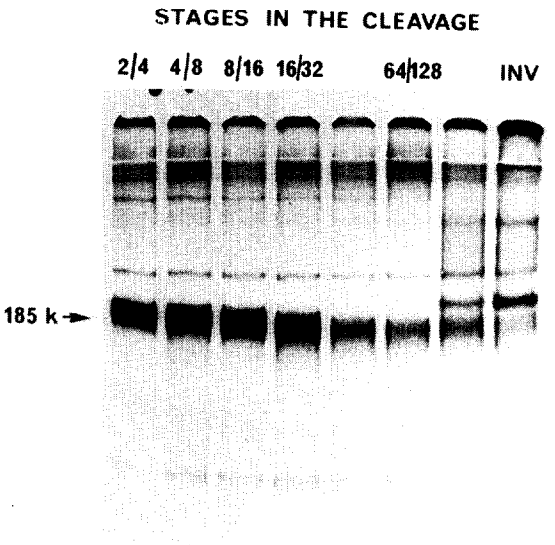

Fig.4. Net $^{35}SO_4^{2-}$-incorporation into the 185 k component during embryogenesis. *Volvox* spheroids (male strain 69-1b, sexually induced) were pulse labelled in vivo for 30 min as in section 2 (25 μCi/ml $^{35}SO_4^{2-}$). Immediately after the initiation of gonidial cleavage a pulse-labelling experiment was performed every 60 min until the end of inversion (INV). The total membrane fractions were applied to 6% SDS–polyacrylamide gels and visualized by fluorography.

the correlation of net $^{35}SO_4^{2-}$-incorporation and cleavage stage suggests a role of this sulphation–desulphation reaction in the control of differentiation. Preliminary experiments with asexual embryos in which the differentiating cleavage is at the 32-celled stage indeed show a corresponding time shift of the minimum of $^{35}SO_4^{2-}$-incorporation into the 185 k glycoprotein.

3.4. *Reversible inhibition of sulphation by borate*

A drastic disturbance of the control of differentiation is achieved by treating embryos with borate during the early cleavage stages [4]. Short treatments at any other developmental stages do not at all affect the developmental program. In fig.5, *Volvox* embryos in the 4-celled stage were pulse-labelled for 20 min in the presence and absence of borate. Another embryo suspension was first treated with borate for 20 min then, after resuspension in borate-free medium, pulse-labelled. The results clearly show that borate completely inhibits the production of sulphated 185 k glycoprotein. After removal of borate the sulphation reaction is re-established within a few minutes. These results also support the idea of a control function of the sulphated 185 k component in differentiation.

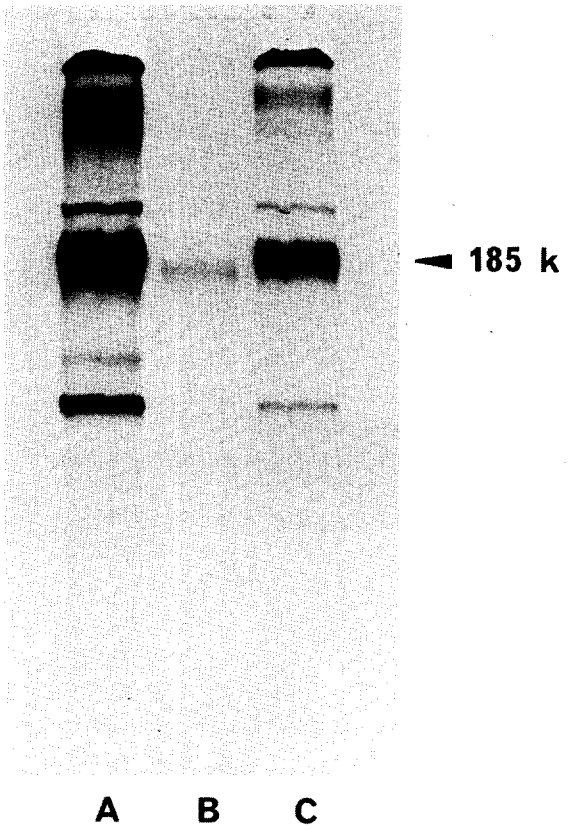

Fig.5. Borate reversibly inhibits formation of 185 k sulphated glycoprotein. Fluorograms of total membrane fractions separated on 6% SDS–polyacrylamide gels: (A) $^{35}SO_4^{2-}$ pulse-labelling of 4-celled embryos (20 min) in the absence of borate; (B) $^{35}SO_4^{2-}$ pulse-labelling (20 min) in the presence of 10 mM borate; (C) 4-celled embryos were first treated for 20 min with 10 mM borate, then washed extensively with borate-free *Volvox* medium and 5 min later, pulse-labelled with $^{35}SO_4^{2-}$ for 20 min.

4. Discussion

How could sulphation of a membrane component and its rapid degradation (desulphation) control differentiation during embryogenesis? In terms of our model, a possible explanation would be as follows:

Let us assume that the desulphated 185 k component directly or indirectly inhibits development to a reproductive cell, while the sulphated molecule does not.

Let us further assume that the desulphation is catalysed by a membrane bound sulphatase which is active on the 185 k substrate only if it is localized in the adjoining membrane of the partner cell (i.e., the enzyme does not degrade substrate localized within the same cell membrane).

Although desulphation and sulphation are operative continuously, in the steady state the enzyme accumulates within the contact areas due to enzyme–substrate complex formation. Contact formation (1,3,7.., etc.) in the developing embryo would consume an exponentially increasing amount of the pre-existing (or linearly growing) pool of the sulphatase. Thus, the pool would become exhausted at a sharply defined stage in the cleavage, producing daughter cells with no sulphatase.

If, for instance, this event occurs at the stage of the 16-celled embryo, then in the subsequent cleavage exactly 16 cells of the 32-celled embryo will lack the sulphatase in their cell-to-cell contact areas. As a consequence, these 16 cells convert all their 185 k glycoprotein to the sulphated form and are therefore no longer repressed to develop into reproductive cells.

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