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Biochemical Approaches to Problems of Cellular Patterning*†

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SYNOPSIS. The expression of intracellular patterning is perhaps nowhere more impressive than in the arrangements of structural elements associated with the cell surface in protozoa. The view is proposed that biochemical studies of protozoan plasma membranes and associated surface structures represent important contributions of potential significance for the understanding of the perpetuation and expression of positional information at the intracellular level. Some recent work dealing with the isolation, identification, and purification of macromolecular proteins in Tetrahymena is presented and discussed. Some integral membrane proteins have been identified by iodination and polyacrylamide gel electrophoresis. Labeling studies suggest heterogeneous turnover rates within the group of presently identified membrane proteins. High molecular weight proteins with some similarity to spectrin have been isolated from Tetrahymena. It is suggested that the ciliate epithelium is one example of membrane-associated, actomyosin-like systems found in a variety of cell types. The epithelium may play a role in the positioning of surface-associated structures and in the control of cell shape.

Index Key Words: Cell surface; membrane proteins; turnover; epithelium; cortical pattern; Tetrahymena.

It is generally recognized that a variety of experimental approaches is required to develop an adequate understanding of cellular patterning. The importance of the biochemical approach, however, is perhaps not as widely appreciated as it should be. There have been significant contributions in the past, and it is clear that the biochemical approach will play an increasingly important role in the future. Of importance in this context are the biochemical studies which have been made in an attempt to identify and localize the molecular species present in the surface regions of protozoa, including the search for cortical DNA in ciliates. Because the arrangement of structural elements in the cell surface of protozoa is a significant manifestation of the general problem of pattern formation at the intracellular level, these studies must be regarded as important beginnings in the chemical approach to the study of cellular patterning. Moreover, investigations at the molecular level of the cell surface of protozoa appear to be on the increase, probably stimulated in part by the rapid advances which have occurred in the general area of membrane biology in recent years. From studies of this kind, it is reasonable to expect that we can achieve a rather complete understanding of the molecular constituents of the cell surface and of the assembly processes by which complex surface structures are formed in protozoa. In addition, it may not be unrealistic to expect that information of this type will at least

* Presented as part of a Symposium, "Cellular Patterning: Substance, Expression, and Transmission" (sponsored by The Society of Protozoologists and co-sponsored by The American Society of Zoologists), held at the 29th Annual Meeting of The Society of Protozoologists, Tulane University, New Orleans, Louisiana, June, 1976.
† This investigation was supported by Research Grant GB-41389, the National Science Foundation.
contain clues to the mystery of the operation of positional information in the cell cortex. Possessed of such faith, we have begun a series of biochemical studies of the cortex of Tetrahymena. The following is a discussion of our recent unpublished work dealing with cortical proteins in Tetrahymena.

Integral Membrane Proteins

Tetrahymena "ghosts" (pellicles) can be isolated both with and without the presence of surface membranes. Pellicles (Fig. 1) which retain the outer and alveolar membranes (Fig. 2) can be prepared conveniently by the nondetergent method of Nozawa & Thompson (8). At least some methods which involve detergents, for example the procedure described recently by Vaudaux (21), yield pellicles without membranes (Fig. 3). The integrity of these pellicles depends entirely upon the persisting epiplasm, which is the fibrogranular layer normally located just beneath the inner surface membrane of the pellicle (Figs. 2, 3, arrows). Following the convention of Singer (16), membrane proteins may be classified as either "integral" or "peripheral." The epiplasmic proteins belong to the latter category, and will be discussed in a subsequent section.

Considerable progress has been made in studying the lipid composition and lipid metabolism of surface membranes in Tetrahymena (2, 6, 9, 18). Using appropriate isolation methods, it should also be possible to isolate and identify the integral membrane proteins of Tetrahymena cilia and pellicles. Unfortunately, isolated pellicles contain several structures other than membranes which contain proteins, e.g. basal bodies and kinetodesmal fibers, whereas only the membranes in this preparation are expected to contain lipids. This means that further fractionation is essential if integral membrane proteins are to be identified and studied. Two approaches may be followed. In the first, the membranes are removed from the isolated pellicles and subsequently purified. In the 2nd, externally located membrane proteins can be bound to markers of various sorts; these proteins can then be purified using the marker as an assay during the purification procedures.

Subbaiah & Thompson (17) used low concentrations of detergent to free membrane from isolated Tetrahymena cilia and then harvested the resulting small vesicles and separated the proteins by polyacrylamide gel electrophoresis. We have applied the same procedure to isolated pellicles. By this method, we have tentatively identified 4 polypeptides as ciliary membrane components and 4 as pellicle membrane components. The results must be regarded as tentative, however, because the method gives incomplete separations (considerable amounts of putative membrane proteins are found in the supernatant fluid and in the non-membranous pellet) and somewhat variable results. Further work with membrane separation methods is needed before integral membrane proteins can be identified with complete confidence. The most promising possibility under discussion at the moment seems to be the isolation of surface membrane by attachment to polylysine-coated surfaces, although effective procedures involving ciliates have not yet been published.

Certain externally disposed membrane proteins can be identified, and also localized in some cases, by binding to externally applied probes of various kinds. This approach is now being employed extensively in membrane research, and important studies using protozoan cells have recently appeared (e.g. 4, 5). Using the lactoperoxidase method of Phillips & Morrison (12), we have iodinated Tetrahymena surface membranes and then looked for iodinated proteins among those separated from isolated cilia and pellicles by SDS polyacrylamide gel electrophoresis. A full report of these studies will appear elsewhere.

Fig. 1. Photomicrograph of Tetrahymena ghosts (isolated pellicles) prepared by the nondetergent method of Nozawa & Thompson (8). × 430.

Figs. 2, 3. [Electronmicrographs of sections through pellicles of Tetrahymena isolated by 2 different methods.] 2. General surface structure of pellicles isolated by the nondetergent method of Nozawa & Thompson (8). Beneath the outer membrane (top of figure) are the 2 membranes of the alveolar sac. The epiplasm (arrow) is the dense layer of material associated with the inner alveolar membrane. × 128,000. 3. Surface structure of pellicles isolated by the detergent method of Vaudaux (21). The integrity of these pellicles is due entirely to the persisting epiplasmic layer (arrow), and no unit membranes are found in these preparations. As with the nondetergent method, cilia are removed during the isolation; however, oral apparatuses, somatic basal bodies, kinetodesmal fibers, and cortical microtubule ribbons remain attached and in their normal positions in both types of preparations. × 100,000.
Fig. 4. Separation of pellicle proteins by polyacrylamide gel electrophoresis in 8% gels, and identification of 125I-labeled species following iodination of whole cells by the lactoperoxidase method. A single peak of radioactivity is found in the region of the gel just below β-tubulin.

(Williams, et al. in preparation). In brief, gels of isolated cilia and isolated pellicles both have 125I localized in a single region just below β-tubulin (Fig. 4). This region, however, may contain more than a single protein. Two-dimensional separations will be required to check this possibility and to determine whether the same iodinated polypeptides are found in both ciliary and pellicular surface membranes. Iodination is only one of several methods developed recently for the identification and localization of surface proteins in ciliates. The usefulness of plant lectins and tagged antibodies, for example, has not been explored adequately as yet.

Following identification of surface proteins, studies of their synthesis, assembly, and turnover can be carried out. Such studies should contribute to our understanding of pattern, if only indirectly, by providing basic information concerning the biogenesis of surface components. We have studied the turnover of iodinated surface proteins and amino acid-labeled integral and peripheral proteins in nongrowing Tetrahymena. Our results confirm the dynamic nature of the molecular components of surface membranes, first recognized in mammalian cells (22). We have found that the rate of change in specific activities of prelabeled membrane proteins in chase experiments in nongrowing cells is nearly as great as in growing cells. Using double labeling, we have also examined the turnover rates of all presently identified surface proteins in Tetrahymena to see whether extensive differences exist among them. We have found, like in the results reported earlier with rat liver membrane proteins (3, 10), that all proteins do not have similar turnover rates.

Caution must be exercised, however, in interpreting this result. As in many previous studies, the changes in membrane protein specific activities were followed in pulse-chase experiments with labeled amino acids. In this procedure the rate of synthesis and degradation ("turnover") is measured in the population of proteins obtained when membranes are isolated. It is not necessarily true, however, as is sometimes concluded, that this accurately reflects the rate at which proteins "turn over" in membranes, i.e. enter and exit the membrane itself. Neither is the issue clarified by the tendency to use the word "turnover" for both processes. In this context, it might be helpful to restrict "turnover" to its original meaning in biochemistry, i.e. the synthesis and degradation of molecules. The term "exchange" might then be applied to the replacement of molecules within membranes (Fig. 5). Using this convention, for the moment at least, it can be pointed out that turnover rates estimated using proteins which have been isolated from membranes reflect actual exchange rates only if certain conditions are met. For example, the 2 clearly are not equivalent if membrane proteins recycle in membranes to any extent (Fig. 5). Because much has yet to be learned about membrane protein metabolism, including whether or not they recycle in membranes, it is perhaps premature to suggest that uniform turnover rates indicate copolymerization, or membrane flow, whereas heterogeneous rates mean independent replacement in membrane structure. Although this remains a real possibility, we cannot be sure that it is the correct interpretation until more is known about the basic me-
whether any of the major bands formed in polyacrylamide gel
oral apparatuses. Gels of such preparations contain 6 major
desmal fibers, somatic microtubules (including basal bodies), and
bands, designated A-F in a recent paper by Vaudaux (21). In
electrophoresis of protein material extracted from this layer was called "spectrin" (7). Subsequently, epiplasmic-like layers and spectrin-like proteins associated with surface membranes have been demonstrated in a variety of other cell types (11).

We have carried out a series of experiments designed to see whether any of the major bands formed in polyacrylamide gel electrophoresis of Tetrahymena pellicles can be identified with any of the individual structures present, and in particular whether the ciliate epiplasm contains spectrin-like proteins (Williams & Vaudaux, in preparation). Pellicles isolated by the detergent method of Vaudaux contain epiplasm without membrane, kinetodesmal fibers, somatic microtubules and oral apparatuses. Gels of such preparations contain 6 major bands, designated A-F in a recent paper by Vaudaux (21). In the same study, it was shown that bands D and E are found only in the oral apparatus, and that band F is tubulin. The latter runs as a pair under appropriate conditions, as is expected of tubulin. The 3 remaining bands, A through C, are high molecular weight proteins found associated exclusively with the somatic cortex. These bands can be seen in Figs. 4 and 6, because they are also present in gels of pellicles obtained by the nondetergent method used in these preparations. Except for microtubules and oral structures then, the major structures remaining which might contain these 3 largest major proteins are the epiplasm and the kinetodesmal fibers.

To determine which structures contain proteins A-C, we extracted pellicles in various ways, examined both soluble and insoluble fractions by gel electrophoresis, and then analyzed the insoluble fraction by electron microscopy. Using this approach, we found that proteins A and C were extracted from the membrane-less pellicles at both high and low ionic strength, and that protein B was relatively insoluble under both conditions. It was shown with the aid of electron microscopy that kinetodesmal fibers had been solubilized. The epiplasm, though not completely gone, had disintegrated, and only thin fragments containing the rings normally present around the basal bodies and paraxialal sacs could be found. From this result we concluded that protein B is not found in kinetodesmal fibers, and assumed that it might account for part of the epiplasm, perhaps being concentrated in the area of the rings.

We then performed extractions on pellicles with membranes, in the hope that the membranes would aid in the microscopic studies by holding the remaining structures together. With this material, we found that only band C was selectively removed at low ionic strength (Fig. 6, arrow), and that kinetodesmal fibers persisted. This, together with the previous information, suggested that protein A is most likely the major protein of the kinetodesmal fibers. The selective solubilization of protein C permitted us to determine its source by looking for the major structure(s) missing from pellicles dialyzed against buffer at low ionic strength. It was clearly evident in sectioned material that the epiplasmic layer, normally seen just below the inner alveolar membrane as a dense layer (Fig. 2), was missing. All the results suggest that the epiplasm may be composed of 2 high molecular weight proteins (B and C), and that the largest protein (A) may be the major kinetodesmal fiber protein.

The molecular weights of the 2 epiplasmic proteins from strain GL are estimated at 165,000 and 145,000 daltons (21). It remains to be seen to what extent these proteins resemble spectrin. The molecular weights are certainly not the same, with molecular weights of 250,000 and 220,000 being reported for the 2 spectrin polypeptides (19). Nevertheless, the presence of 2 high molecular weight proteins in the submembranous layer of ciliates and the red cell represents at least some basic similarity.

Painter et al. (11) have suggested that there may be a large family of myosin-like proteins in cells and that subsurface layers in a variety of cell types consist of actin associated with one or more myosin-like proteins. Major support for this idea comes from the isolation of actin from red cell ghosts (19) and from the discussion that antibodies directed against smooth muscle myosin cross-react with erythrocyte spectrin (11). Especially convincing is the additional demonstration by the same authors that the submembranous layer in human fibroblasts contains a major protein which is antigenically identical with human smooth muscle myosin. In view of this information, it may be proper to regard the ciliate epiplasm as but one example of the membrane-associated, actomyosin-like

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Fig. 6. Extraction of an epiplasmic protein from pellicles by
 treatment at low ionic strength. The major protein bands obtained
from control pellicles (nondetergent method of isolation) are evi-
dent in the gel on the left. The gel on the right shows the bands
remaining after dialyzing pellicles overnight against Tris-EDTA
(for details, see text). Protein C, the 3rd major band from the
top of the gel, is differentially extracted from the pellicles (ar-
rows). Electron-microscopic examination of the extracted pellicles
indicates that most of the epiplasm, and none of the other struc-
tures, is removed by this procedure.
systems found in cells. We are presently testing this by further studies of proteins B and C and by attempting to demonstrate actin in Tetrahymena pellicles. We have learned so far that there are "isozymes" of the epiplastic proteins in Tetrahymena; the molecular weights of proteins B and C vary according to species (including syngens).

It has been suggested that the ciliate epiplasms may serve to prevent water loss (20) and to insulate the cytoplasm from physical and chemical changes in the environment (14). Another possibility, suggested by studies with mammalian cells, is that the epiplasms may play a role in the positioning of structures associated with the cell surface. Studies of the lateral mobility of lectin receptors and other proteins in mammalian species (including syngens) have led a number of workers to suggest that the spatial distribution of these membrane components may be under the control of the actomyosin-like layer associated with the plasma membrane (1, 11, 15, 23). If, as suggested above, the ciliate epiplasms are analogous to the epiplastic structure, it may be that epiplasms have a similar function in ciliates. This might include the spatial regulation of individual surface proteins, although the trilaminar construction of the ciliate cortex creates difficulties for simple versions of this hypothesis. A related function might be to control the positioning of larger structures, such as basal bodies and paraxonal sacs. Finally, the epiplasms may be important in the control of cell shape.

Further biochemical studies will be of importance in clarifying these and related possibilities. A detailed analysis of the chemical makeup and metabolism of ciliate surface structures, especially in combination with form, pattern, and assembly mutants, should go far toward establishing a molecular biology of the ciliate cortex capable of dealing directly with problems of positional information in these complex cellular organisms.

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