PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Report of the Ciba Foundation Discussion Meeting on Protein and Membrane Interaction Held at the Ciba Foundation on 26 May 1982

W. T. Dixon

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Report of the Ciba Foundation discussion meeting on protein and membrane interaction held at the Ciba Foundation on 26 May 1982

By W. T. Dixon

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

The Deputy Director of the Ciba Foundation, Dr Ruth Porter, in her opening remarks, described the main function of the Ciba Foundation as one of encouraging multidisciplinary international cooperation in scientific and medical research. The purpose of this informal half-day discussion meeting was firstly to provide an atmosphere in which participants in the Royal Society Discussion Meeting to be held on the following two days could meet one another, and secondly to hear three lectures loosely grouped around the theme of 'Protein and membrane interaction'.

Professor D. H. Northcote, the organizer of the Royal Society meeting, after welcoming the participants, introduced Dr Gordon Koch of the M.R.C. Molecular Biology Unit, Cambridge. Dr Koch addressed the problem of 'Associations between extracellular glycoproteins and microfilaments in the pseudopodial reactions of motile cells'.

Various morphological specializations of the cell surface are known including pseudopodia, microvilli and pits. Associated with all of these specializations is a local differentiation of the cell surface so that particular surface components become highly enriched in these areas. These local differentiations are related to the function carried out by the morphological specializations, which, in pseudopodial reactions, include locomotion, phagocytosis and cell–cell interactions. It is therefore important to elucidate the basic mechanism that generates the morphological specialization, bearing in mind that the plasma membrane is an intrinsically fluid structure.

In pseudopodia there was a variety of circumstantial evidence to suggest that interactions between the surface and the intracellular microfilaments operate during the function of these organelles.

Koch and co-workers became interested in this problem from the discovery of a cultured cell line, P815, in which the cells possessed a large number of long thin pseudopodia called filopodia. A great advantage of using these cells was that the filopodia could be easily sheared away from the cell body with little or no damage. This allowed the isolation of relatively pure preparations of intact filopodia, which could be subjected to biochemical analysis. In earlier work (Koch & Smith 1978), they had shown that certain surface histocompatibility antigens could form a stable association with the microfilaments from such filopodial preparations. However, they were unable to demonstrate any detectable biological function of such an association.

From a P815 cell suspension culture a variant was isolated that underwent cell spreading when transferred to a substratum. This spreading phenomenon could be readily assayed and served as a laboratory analogue of the pseudopodial reactions. It occurred in two stages whereby the cell would initially produce numerous filopodial extensions, followed by the flow of

membraneous lamellae between them to adopt the fully spread morphology. A crucial stage of this spreading reaction was the formation of contact between the filopodia and substratum, which occurred via a layer of serum proteins adsorbed to the inert substratum. In fibroblasts this spreading factor is known to be fibronectin. However, depletion of fibronectin from serum did not reduce the capacity of serum to support the spreading activity of the P815 cells, indicating that fibronectin was not the major spreading factor in calf serum.

By using either whole cells or isolated filopodia as immunogens, antibodies were developed to isolate the spreading factor from the growth medium, on the rationale that this factor must have the capacity to bind to the filopodia. Such antisera recognize two major Concanavalin A-binding glycoproteins from calf serum, with molecular masses of 85 and 160 kDa. To determine whether these were responsible for cell spreading, Koch and co-workers adopted two approaches. Using anti-filopodia antibodies, they either specifically depleted the growth medium of the glycoproteins or alternatively overlayered the serum coated substratum with the antibody. In both cases the spreading reaction was inhibited. When they attempted to isolate and purify the individual serum factors, named GP85 and GP160, they were only able to purify the former to homogeneity.

To investigate whether GP85 had any stable interaction with the filopodia, two-dimensional glycoprotein 'fingerprint' gels of whole cells, isolated membranes and whole filopodia were compared. They found that the GP85 was quantitatively enriched in the filopodial preparation. Indeed, when anti-GP85 was made immunofluorescent and applied to P815 cells, preferential staining in 'plaque' regions of intercalation and hence local enrichment of filopodia was found.

Koch and co-workers then tested by the 'myosin-affinity technique' whether the GP85 formed a similar association with microfilaments as they had found earlier with the H-2 antigens. Pure preparations of filopodia were treated with detergent to dissolve membranes and combined with synthetic filaments of rabbit muscle myosin to selectively extract actin, the main component of microfilaments. The remainder fraction and the spun-out microfilament fraction were analysed for the retention of the GP85 and GP160. The microfilament fraction was found to be highly enriched with both of the serum glycoproteins.

This suggested a direct physical connection between the two entities, but the problem still remained of identifying the GP85 receptor on the filopodia. A clue to this problem had arisen when cell lines similar to P815 were infected with RNA tumour viruses.These shed large quantities of virus particles rather than filopodia but interestingly were also found to contain high concentrations of GP85. The receptor might therefore be a viral surface component, and subsequent electron-microscope examination of whole cells revealed single dense RNA tumour virus particles at the tips of filopodia. This surface component might be involved in the attachment of the serum spreading factors to the surface of the filopodia and it was probably the envelope glycoprotein of the virus. To test this, a panel of well characterized polyclonal and monoclonal antibodies to the major GP70 viral glycoprotein were used. After surface labelling P815 cells the anti-GP70 antibodies immunoprecipitated a single component with a molecular mass of 85 kDa. In control experiments on cells grown in the absence of calf serum the immunoprecipitable glycoprotein was absent. This suggested that the antiGP70 was pulling down the GP85 serum protein and two-dimensional gels of the 85 kDa glycoprotein supported this identification.

In summary, Koch and co-workers have shown not only an association between one of the major serum spreading factors and the filopodia but also that these serum factors are pre-

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ferentially retained by the microfilaments. In addition, evidence was presented for an association between a viral GP70 antigen and the filopodia, during spreading reactions and that this may be the receptor for the GP85 serum factor.

The second speaker, Dr Graham Warren of E.M.B.L. in Heidelberg, Germany, presented the work that he and his colleagues have performed on the 'Structural and functional dissection of the Golgi complex'. They have used an enveloped-animal virus, Semliki Forest virus, as a model system for plasma membrane proteins that are assembled in the endoplasmic reticulum and pass through the Golgi apparatus on their way to the cell surface.

The virus consists of a central nucleocapsid composed of a single RNA molecule complexed with about 240 capsid proteins and surrounded by a host-derived membrane bilayer. There are about 240 spike proteins in the membrane, each composed of three component glycoprotein molecules, E_1, E_2 and E_3. The first two of these are membrane-spanning proteins, and the third is peripheral. Both E_2 and E_3 are synthesized as a precursor in the endoplasmic reticulum and are later cleaved. These glycoproteins carry both simple and complex oligosaccharide moieties.

The life cycle of this virus has been examined with the use of baby hamster kidney (BHK) cells. It first binds to the plasma membrane before being internalized by coated pits and is ultimately delivered to the lysosomal compartment. Here the low pH causes a rapid and specific fusion of the viral and lysosomal membranes so that the nucleocapsid is expelled into the cell cytoplasm (Helenius *et al.* 1980). This is the infection stage and it is here that the RNA molecule unravels and transcription and translation occur. The three glycoproteins E_1, E_2 and E_3 are synthesized on membrane-bound ribosomes (Garoff *et al.* 1978) and then transported through the Golgi apparatus to the cell surface (Green *et al.* 1981). There they are recruited by underlying nucleocapsids to form new intact virus particles, which then bud off from the plasma membrane.

An advantage of using a viral system is that the virus turns off host cell protein synthesis and creates a system that is devoted to conveying large numbers of just a few types of membrane protein from the endoplasmic reticulum to the cell surface. It is known that approximately 30 000 spikes are synthesized per minute in an infected BHK cell and since it takes at least 30 min for these to move to the surface, there may be as many as a million spikes moving through the cell at any one time.

To examine the route taken by the viral glycoproteins, Warren and co-workers used immunofluorescence techniques. They treated fixed, permeabilized cells with antibodies to the viral glycoproteins and visualized the first antibody with a second antibody conjugated to either rhodamine or fluorescein. To locate the viral proteins in a particular organelle, they carried out double-labelling experiments with fluorescent antibodies specific to the endoplasmic reticulum or Golgi apparatus (Louvard *et al.* 1982) in conjunction with the fluorescent anti-viral antibodies. In this way they were able to follow the viral proteins from one compartment to another as defined by antigenic determinants rather than morphological criteria.

To quantitate viral protein movement, Warren and co-workers used the e.m. technique of thin (0.1 μm) frozen sections, labelled with anti-viral antibody and visualized with protein A conjugated to gold. This gives a static picture; to observe the movement of viral proteins the cells were treated with cycloheximide, which stops synthesis but not intracellular transport. At different times thin frozen sections were prepared and the amount of label over each of the intracellular membranes was determined. For the viral proteins the half-times for leaving the

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endoplasmic reticulum and Golgi apparatus were 10 and 22 min respectively (Green *et al.* 1981).

It is well established that as glycoproteins move through the Golgi stack they acquire the complex oligosaccharides, which make up 40 % of the viral glycoprotein oligosaccharide moieties. The site of this trimming and addition of sugars within the Golgi apparatus is unknown. *Ricinus communis* agglutinin I (r.c.a.) is a lectin specific for the galactose residues of complex oligosaccharides and could be used to detect these oligosaccharides as soon as they were formed within the Golgi stack. Frozen thin sections were prepared and labelled with r.c.a. followed by an anti-lectin antibody and protein A–gold. This labelled about two-thirds of the Golgi cisternae and always left one or two cisternae on the *cis* side of the Golgi stack unlabelled (Griffiths *et al.* 1982*a*).

The labelling patterns with r.c.a. in conjunction with three cytochemical marker enzymes, glucose-6-phosphatase, thiamine pyrophosphatase and acid phosphatase allowed Warren and co-workers to delineate tentatively three distinct Golgi compartments: *cis*, medial and *trans* Golgi compartments. Each was composed of one or two cisternae. Further evidence for the existence of distinct medial cisternae came from experiments with the sodium ionophore monensin, which has been shown to block the intracellular transport of proteins without affecting their synthesis (Tartakoff & Vassalli 1978). The advantage of this ionophore in the viral system is that it causes viral proteins to accumulate in the Golgi compartment before the transport block. Owing to their viral nature these proteins bind nucleocapsids by a process that would normally only occur at the plasma membrane surface. This allowed the direct visualization under the e.m. of two distinct classes of Golgi cisternae, those that were smooth and presumably occurred topologically after the monensin block, and those that Warren calls i.c.b.ms (intracellular capsid-binding membranes), which occurred topologically before the monensin block. These i.c.b.ms all have the cytochemical characteristics attributed to the putative medial Golgi compartment. This demonstrated that even though monensin also caused swelling and distortion of the Golgi cisternae, their cytochemical integrity was mintained (Griffiths *et al.* 1982*b*).

To investigate the precise location of Golgi associated enzyme activities within the stack, Warren and co-workers were able to exploit the fact that, owing to the presence of nucleocapsids, the i.c.b.ms were much denser in sucrose gradients than smooth cisternae. If parallel experiments in the presence or absence of monensin were carried out, the medial Golgi compartment (i.c.b.ms) could be selectively shifted down a sucrose gradient after treatment of infected cells with the ionophore. Of three activities of the Golgi apparatus – fatty acid acylation α-1,2-mannosidase and galactosyltransferase – the first was the only activity that shifted down the gradient after treatment with monensin. This suggested that fatty acid acylation was an activity of the *cis* or medial Golgi compartment. The other two were associated with the *trans* Golgi compartment, which might be expected because they were both involved in the synthesis of complex oligosaccharides, which the labelling with r.c.a. had shown was confined to *trans* cisternae (Quinn *et al.* 1982).

In conclusion Dr Warren stressed the value of raising antibodies to cell membrane organelles. These were prepared by the injection of a relatively crude membrane fraction into a rabbit. The crude antisera raised could be cleaned up by specifically adsorbing out all the undesired antibody activities onto the membrane and secretory proteins usually discarded during the preparation of the original purified membrane fraction.

These antibodies were very specific to one type of membrane organelle; indeed Warren showed evidence for an anti-Golgi antibody that recognized only a single protein of the *trans*

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Golgi compartment (Louvard *et al.* 1982). Using these powerful tools they hoped to study the assembly and disassembly of complex organelles such as the Golgi apparatus during the life cycle of the cell.

The final speaker of the morning was Dr M. Lord of the University of Bradford who moved the discussion onto the subject of plant glycoproteins and 'Protein glycosylation in the castor bean endosperm'. In the castor bean system the primary metabolic event during seed germination is the breakdown of stored triglycerides, which compose 60–70 % of the total dry mass of mature seeds. These triglycerides are broken down to hexoses and serve as the energy source for the developing embryo until such time as it becomes photosynthetically competent. The metabolic pathway of this breakdown involves the conversion of fatty acids to acetyl CoA units, which then enter a glyoxylate cycle within specialized organelles called glyoxysomes. These in turn produce succinate, which enters mitochondria and is converted to oxaloacetate before entering the cytoplasm where it is ultimately converted to sucrose.

The glyoxysome is analogous in plants to the animal peroxisome (or microbody) and shares many common biochemical markers such as β-oxidation, catalase and hydroxyacid oxidase, in addition to its unique glyoxylate cycle enzymes. Its morphology is very similar to a peroxisome and it is believed to be assembled in an analogous way.

The castor bean system is appropriate for studying protein synthesis and organelle assembly for several reasons that have been exploited by Lord and his co-workers. The endosperm of the castor bean is a short-lived tissue that only lasts for about 7 days at 30 °C after water imbibition. There is no net protein synthesis and no cell division. Reserve proteins from protein bodies are broken down to their constituent amino acids from which a range of gluconeogenic enzymes, and the organelles that contain them, are synthesized. These are rapidly synthesized over 3–4 days and vanish as the tissue becomes photosynthetic. The tissue can be easily fractionated and after a differential spin to remove plastid inclusions, Lord and co-workers found that three major organelle fractions could be isolated on sucrose gradients: endoplasmic reticulum, mitochondria and glyoxysomes. The last of these could be isolated in an undamaged state and represented 20–25 % of the total particulate protein.

The problem that Lord and co-workers addressed was to determine the mechanism of assembly of the glyoxysomes in this system. If the mechanism initially proposed for microbody biogenesis occurred, then the various matrix and membrane proteins would be synthesized on membrane-bound polysomes and either co-translationally discharged across the endoplasmic reticulum membrane or inserted into it. These would then collect in a hypothetical region of the endoplasmic reticulum from which vesicles formed the microbodies by budding (de Duve & Baudhuin 1966).

However, Lord and other workers have shown that matrix proteins such as isocitrate lyase or malate synthase appear to be exclusively synthesized on free polysomes (Kindl *et al.* 1980; Zimmerman & Neupert 1980; Roberts & Lord 1981*a*). These proteins are initially released into the cytosol and ultimately accumulate in the glyoxysome. Some support for an endoplasmic reticulum origin of at least some of the microbody membrane comes from experiments on *N*-glycosylated glycoproteins, which they find to be present in the glyoxysomal membrane (Mellor *et al.* 1980; Bergner & Tanner 1981). These are believed to be synthesized at the level of the endoplasmic reticulum and glycosylated in a co-translational manner after membrane insertion or vectorial discharge across the membrane.

As a first step to show that the glycoproteins that they had identified as glyoxysomal did not

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arise from cross-contamination by other organelles, they used marker enzymes to identify membrane fractions. Choline phosphotransferase, fumarase and catalase were taken as marker enzymes for endoplasmic reticulum, mitochondria and glyoxysomes respectively. Electron microscope examination of isolated membrane fractions gave them a further measure of the homogeneity of their fractions. If they fractionated [^{35}S]methionine-labelled endosperm homogenate on sucrose gradients, remixed the isolated membranes with fresh non-radioactive carrier homogenate and recentrifuged, they found that their radioactive membranes all purified again as single peaks.

Analysis by g.l.c. of glycopeptides extracted from KCl-washed endosperm membranes indicated that a variety of sugars characteristic of *N*-linked glycoproteins were present (Mellor *et al.* 1980). Work on lipid intermediates had suggested that castor bean was similar to other systems in its ability to synthesize *N*-linked glycoproteins by a dolichol-mediated pathway. These synthetic pathways were restricted to the endoplasmic reticulum and could be inhibited with tunicamycin.

Obvious qualitative differences between the protein component of the three organelle fractions could be seen on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis, which again indicated that pure fractions had been obtained. After labelling with radioactive sugar precursors, clear differences in the glycoprotein complement of the organelle fractions were found. These glycoproteins were fractionated by Triton-X114, a detergent that has been shown to separate membrane proteins into those which are hydrophobic (integral) or hydrophilic (peripheral) (Bordier 1981). Perhaps more surprisingly, they found identical bands between endoplasmic reticulum and glyoxysomes and more especially between glyoxysomes and mitochondria. The bulk of these glycoproteins appeared in the hydrophobic (integral) Triton-X114 fraction as expected, and when glycosidases were used to probe the topology of the sugar moieties of these glycoproteins, they appeared to be expressed at the luminal face.

Membranes were prepared from endosperm tissue that was pulsed with radioactive *N*-acetyl glucosamine and chased with non-radioactive *N*-acetyl glcosamine for various times, and were extracted with Triton-X114 at each time point and the radioactivity measured. The endoplasmic reticulum became radioactive first and began to chase out, whereas incorporation into glyoxysomes and mitochondria increased over the time course.

From these data Lord and co-workers tentatively concluded that the glycoproteins of the glyoxysome and the membranes in which they are inserted, could have come from endoplasmic reticulum whereas the matrix proteins appeared to enter the organelle at a later stage of development. Finally Dr Lord used the synthesis of *Ricinus communis* agglutinin (r.c.a.) as a clearcut example of a castor bean glycoprotein that is synthesized on membrane-bound polysomes and that has a site of glycosylation exclusively in the endoplasmic reticulum. It is encoded as a single large 60 kDa polypeptide that is first cleaved to remove an *N*-terminal 'signal sequence' before the cleavage into subunits of the ricin (toxin) and r.c.a. molecules (Roberts & Lord 1981*b*).

Professor Northcote then opened up the three papers for general discussion. A lively question period ensued in which some controversial aspects of glycoprotein synthesis and membrane biology were considered. It was hoped that some of these important questions would be raised again at the following two-day meeting.

In summary, these three lectures served as an ideal preface to the Royal Society Discussion

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Meeting and, on behalf of all the participants, Professor Northcote expressed gratitude to Dr R. Porter and the Ciba Foundation for their generous hospitality.

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