Review Letter-Hypothesis

Do hydrophobic sequences cleaved from cellular polypeptides induce membrane fusion reactions in vivo?

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The concept that a direct interaction between Ca^{2+} and phospholipids is a major factor in membrane fusion reactions is questioned. Attention is drawn to a number of findings on associations between fusion and the proteolysis of membrane proteins. It is proposed that hydrophobic polypeptides, which are functionally comparable to the fusogenic proteins of certain viruses but which are produced in cells by the endogenous proteolysis of membrane and cellular proteins, may induce membrane fusion reactions in vivo.

Membrane fusion *Cell fusion* *Calcium* *Membrane protein* *Proteolysis* *Hydrophobic polypeptide*

1. INTRODUCTION

Membrane fusion is a biological phenomenon of major importance because it is an essential feature of innumerable cellular functions, including cell fusion which is a special case that occurs in a comparatively limited number of situations. Of these, fertilization, the fusion of myoblasts in the development of skeletal muscle, and cell fusion induced by Sendai virus have probably been studied the most extensively. Also, cell fusion induced by poly(ethylene glycol) has recently achieved a special importance as a laboratory tool for the production of monoclonal antibodies, despite the fact that the mechanism of cell fusion involved is not fully understood.

This article reviews some recent studies on fusion reactions in cells and in model systems. It also develops a new proposal for the mechanism of cellular membrane fusion reactions.

2. MEMBRANE PROTEINS

2.1. *Aggregation of integral proteins*

It has been established from model experiments that membranes can be fused by electrical [1] or chemical [2] stimuli but, apart from virus-induced membrane fusion, unequivocal evidence is lacking on the molecular nature of the physiological/pathological stimuli that initiate membrane fusion in living cells. Although the central event of membrane fusion is the interaction and fusion of the phospholipid components of two closely apposed membranes, the minimum area of contact between the two bilayers that is necessary for fusion to develop is also unknown. It is quite probably very small, perhaps about 500 Å in radius [3], but a sufficient area of free lipid bilayer may not be available in membranes in vivo unless a change in molecular organisation is induced as an early event in the fusion process.

In [4] it was proposed that the movement of intramembranous particles and the lateral mobility of integral proteins is of importance in biomem-

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brane fusion reactions because of the movement of intramembranous particles seen in preparations of erythrocyte membranes treated with Sendai virus [5]. It was thought that fusion might then proceed by interdigitation in the two membranes of aggregated membrane proteins, and the areas of free lipid bilayer arising from protein aggregation were not considered to be of any significance. Shortly afterwards it was suggested that fusion may occur in the areas of membrane that are free from integral proteins [6]. This is consistent with the ability of lipid vesicles to fuse readily under appropriate conditions, and also with many [7–11] but not all [12] observations on the fusion of biomembranes. It is noteworthy, however, that clearing of intramembranous particles from the sites of membrane fusion was not found when rapid freezing techniques were applied to exocytosis in rat mast cells [13]. Protein clearing, or at least clearing of the extent that has been observed, may therefore be an artefact and this point remains to be resolved by further experimental work.

Some interesting comparative findings have recently been reported on intramembranous particles in mouse L cells and fusion-deficient sublines treated with poly(ethylene glycol) (PEG) [14]. With 50% PEG, 90% of the parental L cells were fused and cold-induced particle aggregation was extensive. By contrast, identical treatment of fusion-deficient cell lines showed neither extensive fusion nor particle aggregation, but higher concentrations of PEG caused extensive fusion and particle aggregation was then seen. Fusion and aggregation were, however, not invariably related since no measurable aggregation was seen with low levels of fusion in fusion-deficient cells.

2.2. *Proteolysis*

Ca^{2+} is important both in cell fusion in particular and in membrane fusion in general. For example, fertilization requires Ca^{2+} as does the fusion of myoblasts, and many exocytosis reactions are Ca^{2+}-dependent [4].

Is Ca^{2+} involved in the putative lateral displacements of membrane proteins at the sites of fusion? The influx of Ca^{2+} into cells that is observed in secretion might induce a contraction of filamentous proteins, attached to intramembranous particles, which drags the integral proteins away and creates bald patches in the plasma membrane. Should a similar process occur in the membrane of a secretory vesicle, membrane fusion in exocytosis could result when a random collision brings protein-free patches on the two membranes into apposition [3]. The integral proteins of the human erythrocyte membrane are normally unable to move because some of them are attached, via ankyrin, to the underlying membrane skeleton of spectrin and actin; a similar situation may apply in other cells that contain structures which are comparable to the spectrin-actin proteins of the erythrocyte [15,16]. In these cases, lateral movement of the integral proteins and randomly generated protein-free patches for fusion would probably result from the endogenous proteolysis of key proteins in the network such as ankyrin (band 2.1). Proteolysis could also release integral membrane proteins that are attached to contractile networks.

In this connection, we have observed that human erythrocytes will fuse on treatment with exogenous proteolytic enzymes, e.g., subtilisin, and that the intramembranous particles of the treated cells were free to move on cooling to 0°C, but a very high concentration of enzyme (1 mg/ml) was used in this work [17]. However, we also observed that human erythrocytes induced to fuse by treatment with oleoyl glycerol showed a loss of bands 2.1, 2.2 and 3, and increases in band 2.3, 4.3 and 4.5 [18]. In addition, changes in the behaviour of the intramembranous particles were seen. It was therefore suggested that endogenous proteolysis of membrane proteins, activated by the perturbing fusogen, results in an increased freedom of movement of membrane proteins that may lead to fusion. Our more recent work on human erythrocytes fused by chlorpromazine has indicated that a Ca^{2+}-insensitive component of this fusion reaction is associated with degradation of ankyrin (band 2.1) to bands 2.3–2.6 and to smaller polypeptides by a serine proteinase [19]. The Ca^{2+}-stimulated component of fusion induced by chlorpromazine is related to the degradation of band 3 to band 4.5 by a Ca^{2+}-activated cysteine proteinase. Proteolysis of ankyrin appeared to be sufficient to permit fusion and the intramembranous particles were freed from their attachment to the membrane skeleton both in the presence and absence of Ca^{2+} in the fusogen-treated cells, but fusion occurred more rapidly when band 3 was

also degraded in the presence of Ca^{2+}

Unlike the fusion of human erythrocytes by chlorpromazine and oleoylglycerol, the fusion of rat red blood cells by benzyl alcohol and the associated degradation of their membrane proteins is prevented by agents that chelate Ca^{2+} and by *N*-ethylmaleimide [20]. This indicates that the Ca^{2+}-activated cysteine proteinase is more important in these cells, and it is interesting that the content of calpastatin, an endogenous inhibitor of the Ca^{2+}-activated, cysteine proteinase known as calpain I is extremely small in rat erythrocytes, while in human erythrocytes there is much more inhibitor than enzyme [21]. A recent report has shown that the fusogenic action of PEG on mouse erythrocytes is similarly related to the activity of a Ca^{2+}-activated cysteine proteinase in both normal red blood cells and with malaria-infected cells [22]. A Ca^{2+}-sensitive, cysteine proteinase is also involved in the breakdown of microtubule-associated proteins, α-spectrin and globin that parallels the release of microvesicles from the plasma membrane by fusion in chicken erythrocytes treated with ionophore A23187 and Ca^{2+} [23]. In addition Ca^{2+}-activated neutral proteinase activity has been reported to appear concomitantly with the fusion of rat myoblasts into myotubes, while other proteases such as cathepsin D and plasminogen activator did not show any change in their activities [24]. Interestingly, the fusion of rat myoblasts requires the activity of a neutral metalloendoprotease at the time of fusion, although the relationship between the requirement for Ca^{2+} in fusion and the activity of the enzyme is at present unknown [25]. Furthermore, recent data indicate that synaptic transmission involves a metalloendoprotease in the pre-synaptic nerve terminal, and that proteolysis may be an important step in the exocytosis of neurotransmitters [26].

Protease treatments enhance cell fusion induced by PEG [27] and by high pH/high Ca^{2+} [28], but not all chemically-induced cell fusion seems to depend on a proteolytic breakdown of the membrane skeleton. Thus, by contrast with the actions of PEG on the fusion of mouse erythrocytes, the fusion of human erythrocytes by PEG has been reported not to involve proteolysis of membrane proteins [29,30]. In human erythrocytes fusion may be a consequence of ultrastructural changes in the membrane skeleton that are induced by the dehydrating action of concentrated solutions of PEG [31–33]. It has, however, been suggested that PEG itself merely induces cell aggregation and that, when it is purified and small contaminant molecules are removed by recrystallisation, PEG does not cause cell fusion [34]. This proposal was made on the basis of the behaviour towards erythrocytes of commercial PEG-6000 from a particular commercial source (Wako Pure Chemical Industries Ltd). We have found that erythrocytes treated with recrystallized PEG (Wako) for 15 min, instead of 1 min as in [34], are extensively fused [35]. We also found that recrystallizing commercial preparations of PEG from 4 other sources was without effect on their fusogenic properties, and none of these preparations was able to fuse erythrocytes in 1 min. Unpurified PEG (Wako) thus appears to contain impurities that enhance its fusogenic action in short incubation periods. It also seems that PEG is itself able to fuse cells but that, depending on the specific experimental conditions and the preparation of PEG employed, the extent of cell fusion obtained in some applications may depend on the enhancement of fusion by contaminating molecules [36]. In view of the fact that concentrations of 35–50% of PEG are required to induce cell fusion, small molecules such as oleoyl glycerol, chlorpromazine and benzyl alcohol would nevertheless seem to be more appropriate models than PEG for the unknown chemical mediators of biomembrane fusion reactions in vivo, regardless of the undoubted value of PEG as a laboratory tool.

3. MEMBRANE LIPIDS

3.1. *Calcium ions*

A simple function of Ca^{2+} in fusion reactions would be to neutralize the surface charges of membranes, contributed by the proteins or by phospholipids, but it has been pointed out that one should not expect Ca^{2+} to act only by screening negative charges on membranes otherwise very high concentrations of internal Mg^{2+} would enhance neutrotransmitter release [37]. Furthermore, two phospholipid membranes that are closer than about 30 Å repel each other with a force that is apparently only weakly dependent on membrane charge, and removal of water from the polar

groups that stabilize the membrane surface is probably more important in allowing membranes to become closely apposed. It has therefore been suggested that Ca^{2+} may act to trigger a phospholipase that removes polar groups, and hence water, from the phospholipid bilayers of membranes [37]. Since lysophosphatidylcholine is fusogenic [38] another possibility is that Ca^{2+} stimulates its production by a phospholipase but there is little evidence for lysophospholipids being mediators of biomembrane fusion other than under experimental conditions.

An interesting recent paper has reported that phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphophate are degraded, and diacylglycerol and phosphatidic acid are formed, when fusion-competent, chick embryonic myoblasts are stimulated to fuse by increasing the concentration of Ca^{2+} in the medium to 1.4 mM [39]. Similar findings were made with phosphatidylinositol itself but other common phospholipids were unaffected [40]. Sr^{2+}, which can replace Ca^{2+} in stimulating myoblast fusion, also stimulated the breakdown of all 3 inositol phospholipids, whereas Mg^{2+} inhibited both the Ca^{2+}-stimulated fusion and breakdown of the phospholipids. As the breakdown of phosphatidylinositol 4,5-bisphosphate may be a primary event in Ca^{2+}-gating in membranes, and since Ca^{2+} is now known to enter myoblasts before fusion [41], it was suggested that the changes in myoblast phospholipids are part of a gating process for extracellular Ca^{2+}.

Since 1974 Papahadjopoulos and his colleagues have developed a general theory for the role of Ca^{2+} in membrane fusion reactions on the basis of an extensive series of investigations on the behaviour of phospholipids as a model system for the investigation of biomembrane fusion reactions. In particular, observations made on the mechanism of the Ca^{2+}-induced fusion of phosphatidylserine vesicles were taken to indicate that a phase change from a fluid to a solid state is a key factor in fusion induced by Ca^{2+} [42]. They proposed that the crucial event in fusion responsible for triggering most, and perhaps all membrane fusion phenomena, is a Ca^{2+}-induced separation of acidic phospholipids such as phosphatidylserine into rigid crystalline domains, with fusion occurring at domain boundaries between adjacent membranes [43]. This was put forward as a single mechanism to explain extracellular and intracellular fusion reactions, and they have subsequently shown that Mg^{2+} [44], the protein synexin [45], phosphate ions [46] and polyamines [47] each decrease the concentration of Ca^{2+} required for the fusion of vesicles containing phosphatidylserine mixed with other phospholipids.

However, apart from the fact that relatively small quantities of phosphatidylserine are present in the majority of biomembranes, it does not follow that the requirement for Ca^{2+} in fusion occurring in vivo simply reflects an involvement of Ca^{2+} in the fusion of acidic phospholipid bilayers, particularly as under some conditions phospholipid vesicles fuse in the absence of Ca^{2+} or in the presence of chelating agents. For example, it has been reported that fusion is induced in 1 min by 50% PEG when unilamellar vesicles of egg phosphatidylcholine and even of bovine phosphatidylserine, are treated in aqueous buffer containing 0.2 M EDTA; in this work fusion was monitored by electron microscopy and by turbidity measurements [48]. With a mixture of egg phosphatidylcholine, egg phosphatidic acid and cholesterol (molar ratios of 7:2:1), large vesicles were observed after 3 h with 30% PEG in the absence of added Ca^{2+} [32]. Changes consistent with vesicle fusion have also been noted in studies on the calorimetric behaviour of mixed phospholipids in the presence of PEG and 5 mM EDTA [49], and alkyl bromide-induced fusion of both egg phosphatidylcholine and phosphatidylcholine/phosphatidic acid vesicles shows no dependence on the concentration of Ca^{2+} over a range of 0.5 to 10 mM assayed either by light-scattering or by microscopy [50]. There are, furthermore, a number of reports on the fusion of phospholipid vesicles occurring on treatment with peptides and proteins either in the absence of Ca^{2+} or in the presence of EGTA [51–56].

Erythrocytes fuse in the presence of EGTA when they are treated with oleoylglycerol or chlorpromazine as mentioned [18,19], or with tetracaine [57], or Sendai virus [58,59] and it has been suggested that the entry of Ca^{2+} into Sendai virus-treated erythrocytes is a consequence and not a cause of the fusion event [60]. Even some fusion of chick myoblasts (but not rat myoblasts) occurs in high-density cultures in 1.75 mM EGTA [61]. Additionally, the toxic effects of PEG are decreased,

and the yield of hybrid cells is increased, when PEG is used in a Ca^{2+}-free medium and the cells then maintained in Ca^{2+}-free medium for at least 15 min [62]. In the light of these data and those on the fusion of liposomes without Ca^{2+} it seems unlikely that a direct interaction of Ca^{2+} with acidic phospholipids is a major factor in membrane fusion in vivo. Ca^{2+} may well be more involved in other aspects of biomembrane fusion reactions, possibly involving membrane proteins as discussed above.

3.2. *Non-bilayer phases*

What happens to the structures of the lipid bilayers when two closely apposed biomembranes fuse? An intermediate structure of lower stability than a bilayer seems likely to occur as a transient intermediate. One of the first proposals for a transient non-bilayer phase in membrane fusion reactions was the suggestion that globular micelles might be involved [63]. This was based on the known effects of lysolecithin on the structures of phospholipid membranes and its ability to fuse erythrocytes. Other workers have proposed that a semi-micelle configuration might be formed [64], and the participation of inverted micelles in fusion induced by alamethicin [51] and by membrane mobility agents [65] has been suggested.

In early studies, a hexagonal phase was also observed, by electron microscopy, when phospholipids were treated with fusogenic lipids, e.g., oleic acid, but not when corresponding non-fusogenic lipids were used, e.g., stearic acid [66]. In the last 5 years a large number of papers devoted to NMR studies of membranes and model systems treated with chemical fusogens and Ca^{2+} have been published, in which the authors suggested a common mechanism of action for fusogenic lipids whereby structures, such as long cylinders of the hexagonal H_{II} phase (or inverted micelles, see below) are formed that serve as intermediates in the fusion event [67,68]. No X-ray diffraction data in support of this hypothesis appear to have been published yet, however, and it is relevant to note that it is conceivable that NMR spectra attributed to a hexagonal phase may actually correspond to a minor change in the organisation of bilayer phospholipids. Indeed, a theoretical study has shown that it is possible to generate spectra, considered to be typical of the hexagonal H_{II} phase, simply by changing the conformation of the phospholipid head group of phosphatidylethanolamine while retaining the molecules in the bilayer [69]. On a separate point, even if the hexagonal phase is a fusion intermediate in centrifuged, packed preparations of erythrocyte ghosts [68], the possibility remains that it is not involved when fusion occurs between individual pairs of biomembranes in vivo.

Small particles (60–120 Å diameter) have been seen on the fracture faces in related freeze-fracture studies of lipid systems that exhibit the NMR spectra attributed to the hexagonal phase, and it has been suggested that the particles represent inverted micelles lying within the phospholipid bilayers [70]. It has also been proposed that factors, which promote the H_{II} phase in multilamellar systems, cause fusion in unilamellar vesicles via inverted micelles [71,72] but other workers consider that the lipidic particles seen in these experiments are artefacts [73].

Thus, although most investigators would probably agree that a non-bilayer structure of some kind probably occurs as an intermediate in fusion, there is no consensus on its nature even in model systems let alone in vivo. Also, it is not impossible that in various membranes, fusing under differing conditions, different intermediates may be involved.

4. LIPID–PROTEIN INTERACTIONS

4.1. *Fusogenic viral polypeptides*

Membrane fusion induced by certain enveloped viruses is the only case of naturally-occurring biomembrane fusion in which the molecular initiators of the process are known. Thus with paramyxoviruses, e.g., Sendai virus particles (which fuse with the plasma membranes of cells and induce cell fusion), and with viruses such as the Semliki Forest virus and influenza virus (which fuse with the membranes of endocytic vacuoles at low pH) the fusogenic agents are glycoproteins. The F glycoprotein of Sendai virus has a hydrophobic sequence at its amino end that is thought to interact with the target membrane [74]. Despite its highly hydrophobic nature, the N-terminal segment is readily attacked by proteolytic enzymes [75]: this indicates that the segment is exposed to the surrounding aqueous medium. At pH

9, the optimum for fusion by Sendai virus, a conformational change occurs that may allow the hydrophobic sequence to interact with the target membrane [76]. The haemagglutinin glycoprotein HA of influenza virus similarly undergoes a conformational change at low pH to expose a hydrophobic amino-terminal segment [77].

How these hydrophobic peptide sequences actually perturb membranes to induce fusion is unknown but further support for the importance of the aggregation of intramembranous particles in fusion induced by Sendai virus has come from a recent finding that the inclusion of high concentrations of anti-spectrin antibody within erythrocyte ghosts simultaneously inhibits clustering of the particles and fusion [78]. In addition to its fusogenic glycoprotein, the virus may therefore need to possess proteolytic activity towards the membrane skeleton or cell cytoskeleton to which the integral membrane proteins are normally attached. Hence it is interesting that fusion of Ehrlich ascites-tumour cells by Sendai virus is inhibited by proteolytic inhibitors [79]. Recently, also, proteolytic activity has been found in Sendai virus that degrades band 3 in human erythrocytes; several inhibitors of proteolytic enzymes inhibited the activity of the enzyme and the fusion of erythrocytes by the virus. It was therefore suggested that the viral protease may remove membrane glycoproteins to expose the phospholipids to the viral hydrophobic glycoprotein [80].

4.2. *Hypothesis*

Do hydrophobic peptides or proteins initiate any of the innumerable biomembrane fusion reactions that occur, without the aid of viruses, in the normal behaviour of mammalian cells? In [81] authors remarked that fusogenic viruses may have acquired the mechanism by which they induce fusion from a pre-existing cellular mechanism and, in view of the associations between fusion and proteolysis discussed above, I have recently suggested that endogenous cellular proteinases may release hydrophobic fragments (derived from integral proteins at the cytoplasmic surface or from membrane skeleton proteins) that perturb the lipid bilayers of cells and thus induce fusion [82]. Cell fusion induced in this way would be complementary to that caused by penetration into the lipid bilayer from outside cells of the fusogenic glycoprotein of Sendai virus. The production in situ of fusogenic peptides or proteins by endogenous proteinases, which are activated in vivo by appropriate stimuli, might however be a general mechanism for the fusion of biomembranes in a wide variety of situations, including endocytosis and exocytosis. It may be significant in this connection that, when Sendai virus is grown in chick embryos it contains the fusogenic F_1 glycoprotein, but that when grown in bovine kidney (MDBK) cells it contains the precursor F_0 that is inactive in fusion. Thus, activation is dependent on cleavage of a viral protein by a cellular enzyme [83].

The literature contains a number of reports on the fusogenic behaviour of (non-viral) peptides and proteins. For example, two amphipathic polypeptides, melittin [55,56] and alamethicin [51], which are structurally related and contain lipophilic amino acids in their N-terminal regions, fuse phospholipid vesicles. Proteins that have been reported to fuse phospholipid vesicles include tobacco mosaic virus coat protein [54], concanavalin A [52] and, rather surprisingly, serum albumin [53]. It is conceivable, however, that relatively non-polar sections of soluble proteins may induce membrane fusion, particularly since sequences of some 20 hydrophobic amino acid residues can occur in the folded interior of globular proteins. Similarly, when a peripheral membrane protein is degraded by proteolysis, hydrophobic segments can be released some of which could be fusogenic. Spectrin, for example, contains hydrophobic regions which are thought to occur in α-helical domains that are linked by protease-sensitive loops. Ankyrin also contains a substantial hydrophobic domain, and it is relevant to the possible general applicability of the present hypothesis that cells other than erythrocytes have structures that are comparable to the spectrin-actin skeleton of the erythrocyte membrane [15,16]. It is also interesting that myelin basic protein will fuse erythrocytes in the presence of Ca^{2+} but that a mixture of sulphatides and myelin basic protein is not fusogenic [84]; i.e., the dissociation of molecules that normally co-exist in membranes can yield components that are membrane-disruptive and fusogenic.

What is known about the possible importance of the products of proteolysis in biomembrane fusion occurring in vivo? Many inflammatory cells, in-

cluding mast cells and neutrophils, can be activated by the addition of exogenous proteases, and the secretory activities of a number of them are inhibited by inhibitors of serine proteases. Platelet protein band 2 is cleaved into two fragments concurrently with platelet aggregation, and it has been proposed that on stimulation a Ca^{2+}-dependent proteolytic activity appears on the platelet surface and that proteolysis of band 2 occurs on platelet aggregation [85]. Another secretory membrane system of interest in the present context is the exocytosis of the cortical granules of egg cells that accompanies fertilization. A limited proteolysis of surface proteins has been observed following fertilization of sea urchin eggs and several proteins are present in greatly reduced quantities in the plasma membrane after fertilization [86]. Authors in [87] have suggested that the release of protein is related to the rise in intracellular Ca^{2+} that induces the exocytosis reaction, and they have speculated on whether the released polypeptides have a functional role. It is not known how an increase in cytoplasmic Ca^{2+} promotes exocytosis. It is interesting however, in relation to the inhibition by *N*-ethylmaleimide of the Ca^{2+}-stimulated fusion of erythrocytes and the associated proteolysis discussed above, that this thiol agent inhibits the exocytosis of egg cortical granules and also protects the granules from a spontaneous breakdown that occurs on incubation at 20°C [88].

Certain polypeptide hormones have been shown to stimulate the fusion of intracellular vesicles with the plasma membrane to increase the number of transporters for water (vasopressin on toad bladder cells) and for glucose (insulin on fat cells) [89]. However, should fusogenic peptides be involved in either of these two systems they are unlikely to be derived from the hormones themselves, despite their relatively high contents of non-polar amino acids, because the action of vasopressin is reproduced by dibutyryl cyclic AMP and it is probably unnecessary for insulin to enter cells to stimulate glucose transport. Functional fusogenic peptides or polypeptides may nevertheless be generated from non-membranous proteins in some circumstances. For instance, the importance of hydrophobic amino acid residues in the signal sequence of secreted proteins is well established, but relatively little is known about the fate of such sequences after their release by a signal peptidase [90]. If signal peptides are not further degraded but accumulate within the endoplasmic reticulum they may perhaps participate in one or more of the membrane fusion reactions that mediate the pathways followed by enzymes and proteins destined for secretion.

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