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Induction of cell fusion in cultured fibroblasts and epithelial cells by microinjection of EGTA, GTPγS and antifodrin antibodies

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Abstract

$CaCl_2$, EGTA, GTPγS and anti-α-fodrin antibodies were injected into fibroblast-like IMR-33 cells and Madin–Darby bovine kidney (MDBK) epithelial cells cultured both in the presence and absence of cycloheximide and fetal calf serum. EGTA, GTPγS and antifodrin antibody induced fusion of MDBK cells within one hour after injection. The cells formed polykaryons with up to 15 nuclei, reaching an average fusion index of 20%. IMR-33 cells fused at a slower kinetics and only upon injection of GTPγS or antifodrin antibodies. No fusions were seen in serum-deprived, quiescent cells. On the other hand, cycloheximide treatment did not prevent the fusions. The results show that cells can be induced to fuse by using agents that interfere with the regulation of the G-proteins, intracellular calcium level or membrane skeleton. We suggest that the putative fusogens are resident proteins of the plasma membrane which become exposed upon destabilization of the membrane skeleton.

Key words: Membrane skeleton; Intrinsic fusogen; GTP-binding protein; Intracellular calcium level

1. Introduction

Cell fusion is a crucial step in various biological phenomena such as fertilization, formation of myotubes and virus infections. In the fusion process the cells lose their shape and individuality and share their content and membrane structure with neighbouring cells. Despite intensive research, the basic mechanisms of the cell fusion have remained largely unknown.

The membrane skeleton underlying the plasma membrane, together with the cytoskeleton proper is important in the maintenance of the cellular integrety. It provides a link between various integral membrane proteins and the cytoskeleton and is considered to take part in the domain formation and maintenance of the specialized plasma membrane areas in differentiated cells [1–3]. The membrane skeleton consists of fodrin (non-erythroid spectrin), short actin filaments, protein 4.1, ankyrin and adducin as its principal components [2]. Fodrin binds calcium and calmodulin [4–6] which makes it a candidate molecule for various calcium-regulated phenomena. Indeed, fodrin appears to be involved in such dynamic events occurring at the cytoplasmic face of the plasma membrane as signal transduction, receptor capping, and fusion of exocytic vesicles to the plasma membrane [7–9].

In this study we microinjected $CaCl_2$, EGTA, GTPγS, and antibodies to α-fodrin into cultured MDBK cells and IMR-33 cells. We found that microinjection of EGTA, GTPγS and antifodrin antibodies led to numerous cell fusions in MDBK cells. GTPγS and antifodrin antibodies induced fusions of IMR-33 cells. In contrast, $CaCl_2$ had no detectable effect on either type of the cells. The results suggest that the fusion of nucleated mammalian cells is controlled, at least partially, by GTP-binding proteins, intracellular level of free calcium and the integrity of the membrane skeleton.

2. Materials and methods

2.1. Cells

Madin–Darby bovine kidney (MDBK) cells and fibroblast-like gerbil fibroma IMR-33 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). MDBK cells were grown in Eagle's minimal essential medium with Earle's salts (E-MEM; Gibco, Gaithersburg, MD, USA), supplemented with 2 mM glutamine, 10% fetal calf serum (FCS), and antibiotics in a water-saturated atmosphere of 5% CO_2 in air. The cells were grown to confluency on glass cover slips with etched squares marked by numbers and letters (Bellco, Vineland, NJ, USA). IMR-33 cells were cultured in E-MEM supplemented with 2 mM glutamine, non-essential amino acids (Gibco), 10% FCS and antibiotics. For some experiments, quiescent MDBK cells, grown in E-MEM medium without serum for 18 h, were used.

2.2 Chemicals

EGTA (Sigma, St. Louis, MO, USA) was dissolved in the injection buffer (100 mM KCl, 5 mM HEPES, pH 7.4) at 50 mM concentration and pH adjusted to 7.4 using 5 M NaOH solution. $CaCl_2$ (Merck, Darmstadt, Germany) was dissolved in the injection buffer at 10 mM concentration. GTPγS (Sigma) was dissolved in the injection buffer at a concentration 6.7 mg/ml. Cycloheximide (Sigma) was dissolved in ethanol at 40 mM concentration and diluted in the growth media to a final concentration of 0.4 mM. In cycloheximide experiments both injection and subsequent incubations of the cells were performed in the presence of 0.4 mM cycloheximide.

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2.3. Antibodies

A monoclonal antibody cross-reacting with mammalian α-fodrin (101AA6) was obtained from Professor Ismo Virtanen (Department of Anatomy, University of Helsinki, Finland; [10]). It was purified using CM Affi-Gel Blue chromatography gel column (Bio-Rad, Richmond, CA, USA) and concentrated by lyophilization. The purified antibodies were dialyzed against the injection buffer and filtered before injection.

2.4 Microinjection

Microinjections were carried out using a micromanipulator 5170 and a microinjector 5242 from Eppendorf (Hamburg, Germany) installed on an Axiovert 405M inverted microscope with a heating stage (Zeiss, Oberkochen, Germany). Microinjections were performed under nitrogen gas pressure using glass micropipettes either purchased from Eppendorf (Femtotips, the diameter of the aperture 0.5 ± 0.2 μm) or pulled from borosilicate capillaries (Clark Electromedical Instruments, Reading, UK) using Sutter microelectrode puller (Sutter Instruments Co., San Rafael, CA, USA). The applied pressure was adjusted between 300 and 500 hPa and the time of injection was 0.3 s. Injection was targeted to the perinuclear area and a small wave inside the cells was regarded as an indicator for a successful injection. Typically, all the cells within one or two squares of the etched cover slips, i.e. about 300 to 600 cells, were injected within a time period of 20 to 30 min for each experiment. In order to keep the intracellular pH normal the cells were transferred to Eagle's minimal essential medium with Hanks' salts for the injection and returned to the normal growth medium immediately after the injection. The injected cells were monitored by phase contrast microscopy and by photographing at several time points indicated in the figure legends. Each experiment was repeated 2 to 5 times.

2.5. Fusion index

Cell fusion was quantitated from the photographs by counting the number of mononucleate (unfused) and multinucleate (fused) cells and scoring the nuclei present in both unfused and fused cells within the injected area. The fusion index f was calculated according to the following formula: $f = 1 - C/N$, where C is the total number of cells and N is the total number of nuclei [11]. As the spontaneous cell fusion was very low (fusion index 0.2% in uninjected cell cultures), it was not necessary to subtract any background values from the scores of multinucleated cells.

3. Results

Injection of EGTA, GTPγS and antifodrin antibody into MDBK cells led to a formation of polykaryons within one hour (Figs. 1 and 2). Similarly, fusions occurred in IMR-33 cells after injecting antifodrin antibodies and GTPγS, but not with EGTA (Figs. 1 and 3). The MDBK cell fusions, especially those induced by antifodrin antibodies, were unstable and the polykaryons began to die within three hours (Fig. 1). With GTPγS, large and more stable polykaryons with over 15 nuclei were induced. Calculation of the fusion index of GTPγS-injected cells was impossible, however, due to rounding and detachment of the majority of the unfused cells.

In IMR-33 cells antifodrin antibodies and GTPγS induced fusions, but at a slower rate than in epithelial cells (Figs. 1 and 3). The number of fusions after EGTA remained undistinguishable from the control experiments carried out with the injection buffer (Fig. 1B). In contrast to MDBK cells, the fused IMR-33 cells were remarkably stable: after injection of antifodrin antibodies the polykaryons with a fusion index of 25% were still observable after 6–7 h in culture.

Nu fusions were seen in cultures injected with $CaCl_2$ or upon microinjecting antifodrin antibodies into cells cultured in the absence of serum. Cycloheximide did not prevent fusions induced by antifodrin antibodies.

Control experiments were carried out by injecting KCl buffer. Some fusions were occasionally seen, but the fusion index remained low in both types of cells (Fig. 1).

4. Discussion

The results showed that fusions could be induced in both epithelial MDBK cells and fibroblast-like IMR-33 cells by microinjecting into the cells either GTPγS or antifodrin antibodies. EGTA, on the other hand, induced fusions only in MDBK cells.

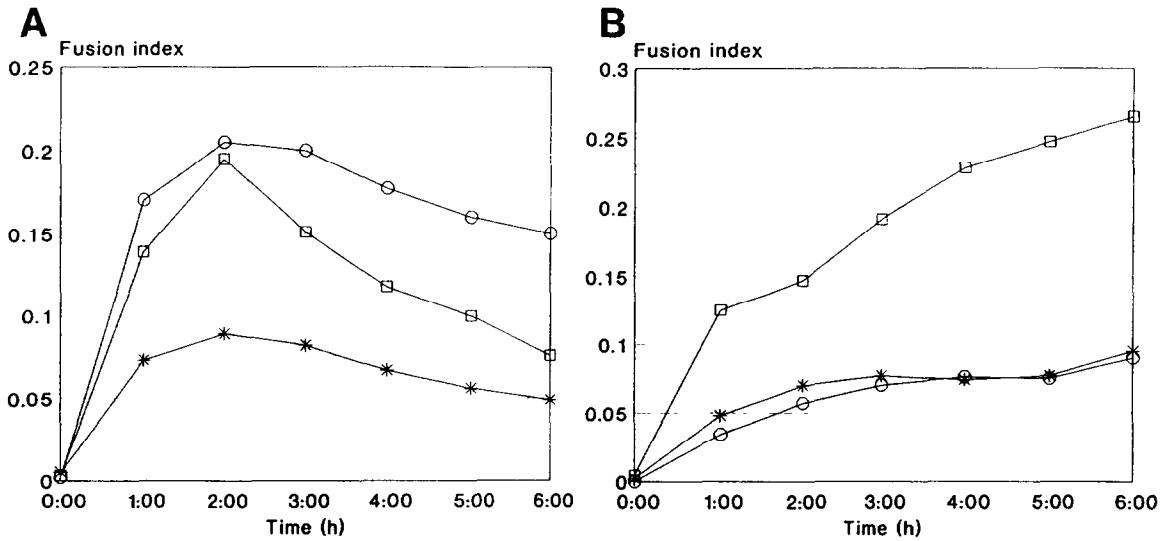

Fig. 1. Fusion index as a function of time after injection into MDBK cells (A) and IMR-33 cells (B) of EGTA (○-○); antifodrin antibody (□-□); or KCl buffer (*-*).

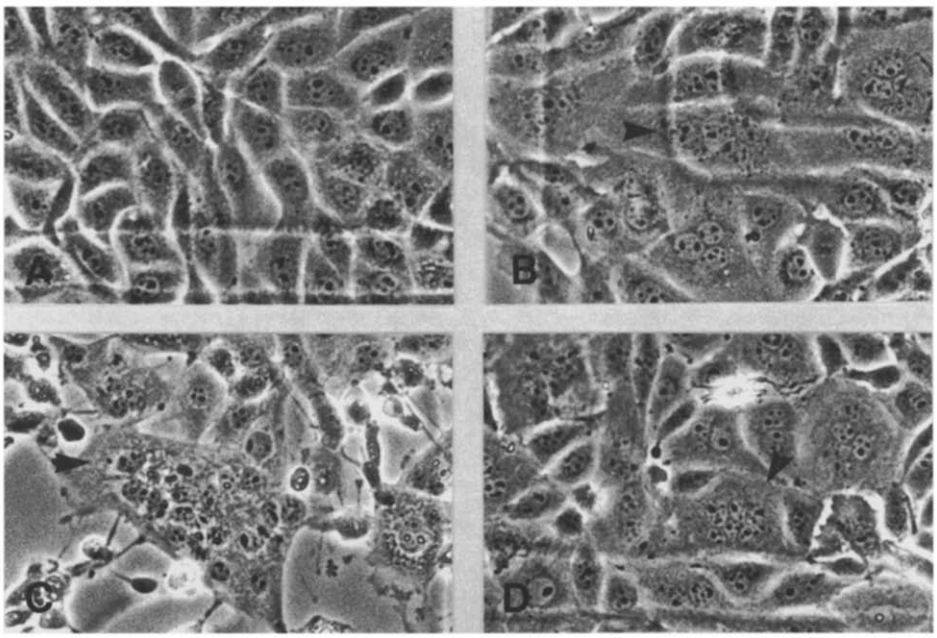

Fig. 2. Phase contrast micrographs of unfixed, uninjected MDBK cells (A), MDBK cells two hours after injection of EGTA (B), GTPγS (C) or antifodrin antibodies (D). A group of fused cells in indicated by arrows.

4.1. Fusion kinetics

The fusion index of MDBK cells reached its maximum within two hours of the injection. In comparison, in Sendai virus-infected MDCK cells the fusion starts in the lateral region subjacent to the tight junctions within 30 min, and multinucelated fused cells can be observed within 60 min after warming the infected cells to the fusion permissive temperature [12]. The most accurate triggering of fusion can be achieved by an electrical pulse which leads to merging of adjacent membranes of neighbouring cells in less than one second [13]. In electrofused fibroblast-like CV-1 cells, bundles of microtubuli begin to extend into the cytoplasmic bridges in a few minutes after initiation of the fusion, resulting in the aggregation of nuclei after 30 min and complete fusion in about 2–3 h [14,15]. Thus, in terms of kinetics, the fusion of

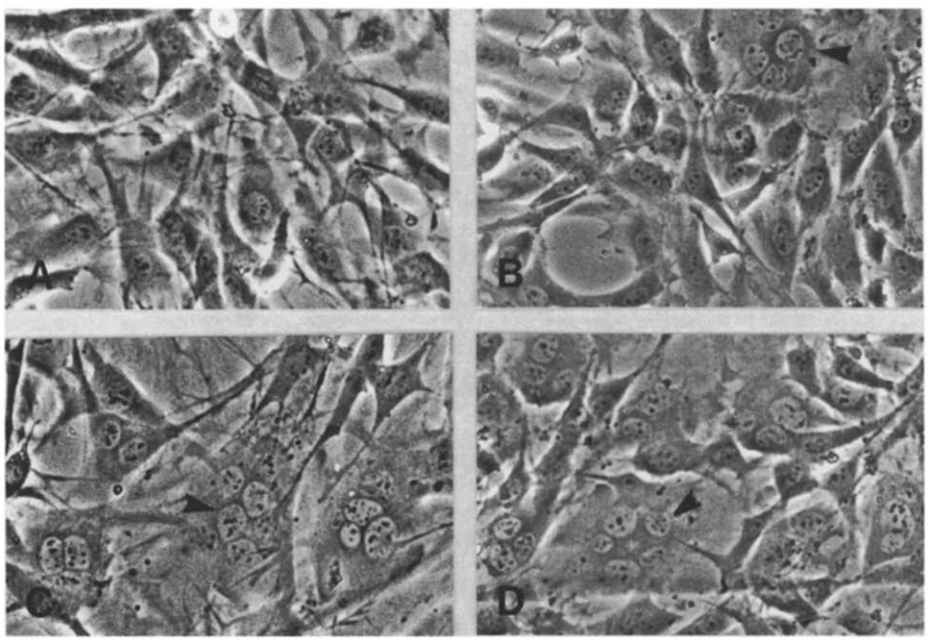

Fig. 3. Phase contrast micrographs of unfixed, uninjected IMR-33 cells (A), IMR-33 cells seven hours after injection of EGTA (B), four hours after injection of GTPγS (C) and six hours after injection of antifodrin antibodies (D). A group of fused cells is indicated by arrows.

MDBK cells resembles that induced by viruses or electrical pulses. This also suggests that critical for the rapid onset of the fusion in epithelial cells is their close proximity and the specialized cell–cell contacts between the apposing cell walls of the fusing cells.

In contrast to MDBK cells, the fusion in IMR-33 cells was much slower. This is most probably due to the lack of specific cell–cell contacts in fibroblasts. Therefore, several steps, including cell migration, attachment, membrane fusion and structural rearrangements of the cells are required for the formation of polykaryons. In this respect, it is interesting that a calcium chelator EGTA induced fusion of MDBK cells, but not IMR-33 cells. We suggest that this difference in the response is due to the presence of calcium-dependent specialized cell–cell adhesion sites in MDBK cells, but not in fibroblasts [16].

4.2. Membrane skeleton in fusion

Characteristic of the MDBK fusion induced by antifodrin and EGTA was its rapid initiation after injection. The target of antifodrin antibodies, α-fodrin, is located along the lateral walls of MDBK cells where it forms the membrane-associated subplasmalemmal skeleton [2]. Therefore it is logical to think that the microinjected antibodies induce fusion by interfering with the membrane skeletons of the adjacent cells.

There are only few observations on the effect of microinjected antifodrin antibodies on cultured cells: Mangeat and Burridge [17] injected antifodrin antibodies both into fibroblasts and epithelial cells and observed intracellular precipitation of fodrin. In contrast to the present results, however, there were no alterations in the cell shape or any cell fusions. This discrepancy is most probably due to the differences in the experimental set up; in the present study we always used confluent cells and injected all the cells within a defined area. On the other hand, microinjection of antifodrin antibodies into *Amoeba proteus* causes drastic changes in the cellular shape and movement [18].

There is indirect evidence supporting the idea that interference with the integrity of the spectrin/fodrin-based membrane skeleton could lead to cell fusion. First, an intact spectrin skeleton restricts the expansion of the diameter of the fusion zone of electrofused erythrocyte ghosts [19]. Secondly, degradation of spectrin-associated proteins, ankyrin and band 3, has been observed in chlorpromazine-induced fusions of erythrocytes [20]. Thus, it seems that the intact membrane skeleton is required to maintain the cellular individuality and to prevent cell fusion.

4.3. G-proteins in fusion

G-proteins, encompassing both small *ras*-related and heterotrimeric GTP-binding proteins, serve important functions in intracellular protein trafficking, endo- and exocytosis. *Ras*-like G-proteins participate in the fusion of secretory vesicles with the plasma membrane [21,22]. Moreover, several small G-proteins of the *rab* family are localized on endosomal and secretory vesicles of mammalian cells [23]. Also heterotrimeric G-proteins seem to be involved in endosome fusion [24]. On the other hand, the role of G-proteins in cell–cell fusion events is unknown. In vitro studies on the fusion between rat pancreactic zymogen granules and plasma membranes by GTPγS provides evidence for the existence of GTP-binding proteins both in the plasma membrane and granule membrane which control fusion [25]. In the present study microinjection of GTPγS induced cell fusions both in MDBK and IMR-33 cells suggesting that activation of G-proteins is important in cell–cell fusion. Since GTPγS activates the G-proteins indiscriminately by locking them in GTP-bound state, it is impossible to say, without further study, which G-proteins are involved in the fusion events.

4.4. Fusion mechanism

No fusions could be obtained by microinjecting antifodrin antibodies into serum-deprived MDBK cells. This indicates that the prerequisites for the cell fusion are (i) an active intracellular signalling machinery, and/or (ii) synthesis of new proteins, and/or (iii) serum-mediated changes in the membrane lipids, and/or (iv) serum-mediated exposure or cleavage of fusogenic proteins. Although liposome fusion can occur in the absence of proteins, the available evidence strongly suggests that proteins play a vital role in membrane fusions in vivo. They are involved e.g. in bringing the fusion membranes to a close apposition and in providing the fusion specificity. However, excluding viral fusion proteins and a recently found protein in sperm cells resembling viral fusion peptides [26], biological fusogens are largely unknown [27,28].

In our experiments fusion was not inhibited by cycloheximide treatment, indicating that protein synthesis is not involved. Thus, it seems that the already existing proteins or peptides are utilized in the fusion. This is in line with the previous observations which indicate that hydrophobic peptide moieties of membrane proteins can serve as fusion peptides. These can be exposed in different ways: through dissociation of an oligomeric protein or through an extensive denaturation of the protein. 'Professional' fusion proteins display these changes under physiological and easily triggered conditions such as low endosomal pH [29,30]. It has also been suggested that proteolysis of integral and skeletal membrane proteins would lead to formation of polypeptides with fusogenic properties. Experimental evidence for this are observations that inhibitors of metalloendoproteases block sperm-egg fusion or fusion of myoblasts [31,32]. The present study shows that fibroblasts and epithelial cells, incapable to fuse with each other in tissues, possess, however, an inherent capability to fuse. This property

can be evoked by activating GTP-binding proteins, lowering intracellular calcium level or affecting the integrity of the membrane skeleton.

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