Intracellular curvature-generating proteins in cell-to-cell fusion

Jean-Philippe RICHARD*1,2, Evgenia LEIKINA*1, Ralf LANGEN†, William Mike HENNE‡3, Margarita POPOVA*, Tamas BALLA§, Harvey T. McMAHON‡, Michael M. KOZLOV∥ and Leonid V. CHERNOMORDIK*4

*Section on Membrane Biology, Laboratory of Cellular and Molecular Biophysics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, 10 Center Dr., Bethesda, MD 20892-1855, U.S.A., †Zilkha Neurogenetic Institute and Department of Biochemistry, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, U.S.A., ‡MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, U.K., §Section on Molecular Signal Transduction, Program for Developmental Neuroscience, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, U.S.A., and ∥Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, 69978 Tel Aviv, Israel

INTRODUCTION

Cell-to-cell fusion plays an important role in normal physiology and in different pathological conditions. Early fusion stages mediated by specialized proteins and yielding fusion pores are followed by a pore expansion stage that is dependent on cell metabolism and yet unidentified machinery. Because of a similarity of membrane bending in the fusion pore rim and in highly curved intracellular membrane compartments, in the present study we explored whether changes in the activity of the proteins that generate these compartments affect cell fusion initiated by protein fusogens of influenza virus and baculovirus. We raised the intracellular concentration of curvature-generating proteins in cells by either expressing or microinjecting the ENTH (epsin N-terminal homology) domain of epsin or by expressing the GRAF1 (GTPase regulator associated with focal adhesion kinase 1) BAR (Bin/amphiphysin/Rvs) domain or the FCH2 (FCH domain-only protein 2) F-BAR domain. Each of these treatments promoted syncytium formation. Cell fusion extents were also influenced by treatments targeting the function of another curvature-generating protein, dynamin. Cell-membrane-permeant inhibitors of dynamin GTPase blocked expansion of fusion pores and dominant-negative mutants of dynamin influenced the syncytium formation extents. We also report that syncytium formation is inhibited by reagents lowering the content and accessibility of PtdIns(4,5)P2, an important regulator of intracellular membrane remodelling. Our findings indicate that fusion pore expansion at late stages of cell-to-cell fusion is mediated, directly or indirectly, by intracellular membrane-shaping proteins.

Cell-to-cell fusion plays an important role in normal physiology and in different pathological conditions. Early fusion stages mediated by specialized proteins and yielding fusion pores are followed by a pore expansion stage that is dependent on cell metabolism and yet unidentified machinery. Because of a similarity of membrane bending in the fusion pore rim and in highly curved intracellular membrane compartments, in the present study we explored whether changes in the activity of the proteins that generate these compartments affect cell fusion initiated by protein fusogens of influenza virus and baculovirus. We raised the intracellular concentration of curvature-generating proteins in cells by either expressing or microinjecting the ENTH (epsin N-terminal homology) domain of epsin or by expressing the GRAF1 (GTPase regulator associated with focal adhesion kinase 1) BAR (Bin/amphiphysin/Rvs) domain or the FCH2 (FCH domain-only protein 2) F-BAR domain. Each of these treatments promoted syncytium formation. Cell fusion extents were also influenced by treatments targeting the function of another curvature-generating protein, dynamin. Cell-membrane-permeant inhibitors of dynamin GTPase blocked expansion of fusion pores and dominant-negative mutants of dynamin influenced the syncytium formation extents. We also report that syncytium formation is inhibited by reagents lowering the content and accessibility of PtdIns(4,5)P2, an important regulator of intracellular membrane remodelling. Our findings indicate that fusion pore expansion at late stages of cell-to-cell fusion is mediated, directly or indirectly, by intracellular membrane-shaping proteins.

INTRODUCTION

Cells fuse in developmental processes, such as fertilization, muscle and bone formation, and in pathological processes, including viral infections and carcinogenesis [1–5]. Early stages of membrane fusion driven by specialized protein fusogens [2,6–8] culminate in the opening of nanometre-sized fusion pores that connect the volumes of the two cells [9–11]. The mechanisms that drive subsequent expansion of the fusion pore(s) to a micrometre-sized lumen that allows complete coalescence of cytoplasmic volumes are poorly understood. In our recent studies, to uncouple later fusion stages from earlier ones, we explored syncytium formation initiated by well-characterized viral envelope proteins: influenza virus HA (haemagglutinin) [12] and baculovirus gp64 [13]. Both HA- and gp64-mediated fusion processes are triggered by treating HA- or gp64-expressing cells with a low pH medium [12,13]. Several studies have proposed that the expansion of fusion pores in cell fusion is driven by the cytoskeleton [16–18]. However, modifications of the microtubule cytoskeleton have no effect on syncytium formation initiated by viral fusogens [12], and depolymerization of actin cytoskeleton promotes rather than inhibits syncytium formation, suggesting that actin structures restrict rather than drive fusion pore expansion [12,13,19].

As long as the fusion pore grows within the tight contact zone, the membrane bilayer at the edge of the pore remains strongly curved and, hence, accumulates the elastic energy of bending (Figure 1, inset). In the course of fusion pore expansion, the length of the pore edge grows and thus the elastic energy increases. The degree of membrane bending at the pore rim is similar to that of intracellular membrane structures such as membrane tubules and endocytic vesicles [20,21], all characterized by the curvature radii of a few tens of nanometres. Therefore our search for protein machinery that can power syncytium formation has focused on the cytosolic proteins involved in the cell-controlled bending of intracellular membranes. CGPs (curvature-generating proteins), such as dynamin [22–24], ENTH (epsin N-terminal

Abbreviations used: BAR, Bin/amphiphysin/Rvs; cELISA, cell surface ELISA; CGP, curvature-generating protein; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; ENTH, epsin N-terminal homology; FCHO2, FCH domain-only protein 2; GFP, green fluorescent protein; GRAF1, GTPase regulator associated with focal adhesion kinase 1; HA, haemagglutinin; MitMAB, tetradecyl trimethylammonium bromide; MOI, multiplicity of infection; PH, pleckstrin homology; PLCδ1PH, PH domain of phospholipase Cδ1.

1 These authors contributed equally to this work
2 Present address: Hugo W. Moser Research Institute at Kennedy Krieger, Department of Neurology, Johns Hopkins School of Medicine, Baltimore, MD 21205, U.S.A.
3 Present address: Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca, NY 14853, U.S.A.
4 To whom correspondence should be addressed (email chernoml@mail.nih.gov).
homology) domain proteins (e.g. epsin 1 [25,26]) and BAR (Bin/amphiphysin/Rvs) domain proteins such as GRAF1 (GTPase regulator associated with focal adhesion kinase 1) [27–29] and F-BAR proteins including FCHo2 (FCH domain-only protein 2) [25,30] are recruited to membranes, often by protein interaction with PtdIns(4,5)\( _2 \) content in the plasma membrane and to compete for PtdIns(4,5)\( _2 \) binding with endogenous PtdIns(4,5)\( _2 \)-binding proteins, we expressed PLC\( _{1PH} \) (PH domain of phospholipase C\( \alpha \)) constructs and in control experiments, its inactive mutant (R40L) construct, which does not bind to the plasma membrane [39]. The recombinant ENTH domain of epsin1 was expressed and purified as described previously [25]. Wild-type baculovirus AcNPV was purchased from Invitrogen. Baculoviruses encoding several dominant-negative dynamin mutants of human neuronal dynamin-1 (S61D [40], I690K [41], K44A [42] and S45N [43,44]) were provided by Dr S. Schmid (Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, U.S.A.). PKH26 was purchased from Sigma.

Figure 1 Overexpression of the F-BAR domain of FCHo2 and the N-BAR domain of GRAF1 in HAb2 cells does not affect HA-mediated early fusion stages (lipid mixing, A), but promotes syncytium formation (B).

Cell culture and preparation

HAb2 cells (NIH 3T3 cells stably expressing HA) were cultured as exponentially growing subconfluent monolayers on 90 mm plates in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with Glutamax (Invitrogen) and 10% (v/v) fetal bovine serum. Exponentially growing cells were dissociated with trypsin (Invitrogen). The cells (2.5 × 10⁵) were plated and cultured overnight on 30 mm plates. In some experiments, human erythrocyte ghosts were labelled with fluorescent lipid PKH26 (Sigma) as described previously [45]. Spodoptera frugiperda (Si9) cells and Si9\(^{\text{F9}}\) cells, i.e. stably transfected Si9 cells expressing a protein fusogen of baculovirus OpMNPV gp64 [14,46], provided by Dr Gary Blissard (Cornell University, Ithaca, NY, U.S.A.), were grown and, in some experiments, labelled with 1-\( \alpha \)-phosphatidylethanolamine-N- (lissamine rhodamine B sulfonyl) (egg) (Rho-PE, Avanti Polar Lipids) as described previously [13,47].

EXPERIMENTAL

Reagents and plasmids

Dynasore [35] was first supplied by Dr T. Kirchhausen (Harvard University, Boston, MA, U.S.A.) and then purchased from Sigma. Another dynamin inhibitor, MitMAB (tetradecyl trimethylammonium bromide) [36], was also purchased from Sigma. Dynole-34-2 and its inactive derivative Dynole-31-2, used as a negative control [37], were purchased from Ascent Scientific. Dynasore [35], MitMAB [36] and Dynoles [37] were applied as described previously. Polysphosphoinositide-binding peptide PB10, a rhodamine B-tagged ten-residue peptide derived from the PtdIns(4,5)\( _2 \)-binding region in segment 2 of gelsolin [38] was purchased from EMD Millipore and was used as suggested by the manufacturer. Alexa Fluor\( ^{\text{TM}} \) 594-conjugated 10000 Da dextran was purchased from Invitrogen.

We also used pEgfp [enhanced GFP (green fluorescent protein)] plasmids containing either the rat ENTH domain of epsin (amino acids 1–164) or its mutant L6E, the F-BAR domain of mouse FCHo2 (amino acids 1–272) or the BAR and PH (pleckstrin homology) domains of GRAF1 (amino acids 1–382). To evaluate PtdIns(4,5)\( _2 \) content in the plasma membrane and to compete for PtdIns(4,5)\( _2 \) binding with endogenous PtdIns(4,5)\( _2 \)-binding proteins, we expressed PLC\( _{1PH} \) (PH domain of phospholipase C\( \alpha \))-GFP construct or, in control experiments, its inactive mutant (R40L) construct, which does not bind to the plasma membrane [39]. The recombinant ENTH domain of epsin1 was expressed and purified as described previously [25]. Wild-type baculovirus AcNPV was purchased from Invitrogen. Baculoviruses encoding several dominant-negative dynamin mutants of human neuronal dynamin-1 (S61D [40], I690K [41], K44A [42] and S45N [43,44]) were provided by Dr S. Schmid (Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, U.S.A.). PKH26 was purchased from Sigma.

In the present study we explored whether the efficiency of the late stages of cell–cell fusion initiated by influenza HA and baculovirus gp64 depends on the activity of intracellular CGPs. Analysis of possible mechanisms of such dependence and identification of specific CGPs involved in biologically relevant cell fusion processes will be examined in future work. We modified the activity of the BAR, F-BAR and ENTH domains of several proteins by either transfecting the cells to express these protein active domains or by microinjecting the domains into cells. To minimize indirect effects, we used constructs lacking protein domains which are responsible for interactions with other proteins but not required for membrane shaping. We found that the GRAF1 BAR domain, the FCHo2 F-BAR domain and the epsin ENTH domain promote cell fusion. Late stages of fusion were affected by PtdIns(4,5)\( _2 \)-targeting reagents and by modifying the functional activity of dynamin either by expressing different dynamin mutants or by blocking dynamin GTPase activity with cell-membrane-permeant inhibitors. The finding that changes in the concentration and/or activity of diverse proteins involved in shaping intracellular membranes and in the membrane concentration of accessible PtdIns(4,5)\( _2 \), an important regulator of membrane shaping, affect late fusion stages substantiates the hypothesis that CGPs drive fusion pore expansion in cell-to-cell fusion.
Intracellular curvature-generating proteins in cell fusion

Transfection

Transfections were performed using Lipofectamine\textsuperscript{TM} 2000 (Invitrogen) using the protocol suggested by the manufacturer. Fusion was triggered 24 h after transfection.

Infection with baculovirus constructs

To drive high levels of mutant dynamin expression in Sf9 cells, we infected the cells with different recombinant baculoviruses or with wild-type baculovirus at an MOI (multiplicity of infection) of 1–10 plaque-forming units per cell. We have adjusted the MOI used for each recombinant baculovirus and for wild-type baculovirus to achieve the same level of expression of baculovirus gp64 at the cell surface, as verified 2 days post-infection by Western blotting of cell-surface gp64 isolated using surface biotinylation and streptavidin beads [48] and by a cELISA (cell surface ELISA) using antibody AcV1 (Santa Cruz Biotechnology) [48]. The levels of expression of exogenous dynamin were similar between different constructs and much higher than the level of endogenous expression of dynamin in Sf9 cells, as verified using Western blotting with the anti-dynamin antibody Hudy 1 (Upstate Biotechnology). We have also confirmed that the cells infected with all of the recombinant baculoviruses used were expressing similar amounts of different dynamin mutants, which were much higher than the amounts of endogenous dynamin observed for non-infected cells.

Microinjection

The microinjection experiments were performed on a Zeiss Axiovert135 with a FemtoJet and InjectMan NI2 (Eppendorf). The microinjected solution was composed of 1 vol. of Alexa Fluor\textsuperscript{®} 594-tagged 10000 Da dextran (5 mg/ml in double-distilled water) (Invitrogen), 1 vol. of the protein of interest, 2 vol. of double-distilled water and 1 vol. of microinjection buffer (250 mM Hepes, pH 7.2. 500 mM KCl and 25 mM Na\textsubscript{2}HPO\textsubscript{4}). In each microinjection experiment, we injected 20 out of 30–40 cells per field of view with either fluorescent dextran alone or fluorescent dextran mixed with the protein of interest (the average fraction of injected cells per field was 50–70%) and five fields per condition (total of 100 injected cells).

Application of dynamin inhibitors and PtdIns(4,5)P\textsubscript{2}-targeting reagent

The GTPase activity of dynamin in HAb2 and Sf9\textsuperscript{biD} cells was inhibited by treating the cells with dynasore, Dynole-34-2 and MitMAB. The reagents were applied either before (for 30 min) or immediately after low pH application (for 20 min). Dynole-34-2 and its inactive analogue Dynole-31-2 were both applied to Sf9\textsuperscript{biD} cells for 20 min immediately after a 1 min application of pH 4.9 medium. PBP10, butan-1-ol and i-butanol were both applied to Sf9\textsuperscript{biD} cells for 15 min immediately after a 1 min application of pH 4.9 medium.

Fusion assays

In all of the results presented, the syncytium index for each condition was determined as the number of nuclei in a multinucleated cell divided by the total number of nuclei in the field [12]. As verified in Supplementary Figure S1 at (http://www.BiochemJ.org/bj/440/bj4400185add.htm), an inhibitor (Dynole-34-2) that lowered the percentage of nuclei in multinucleated Sf9\textsuperscript{biD} cells also shifted the distribution of the number of nuclei per cell towards cells with lower number of nuclei.

Fusion between HAB2 cells was assayed as described previously [12]. In brief, HAB2 cells were pre-treated with trypsin (5 \mu g/ml, 5 min and 37\textdegree C) to cleave HA into the fusion-competent conformation. Then the cells were washed once with PBS with Ca\textsuperscript{2+}/Mg\textsuperscript{2+} and once with pH 4.9 medium, and then exposed for 5 min to pH 4.9 medium at 37\textdegree C, in order to trigger the fusogenic conformational change in HA. Cells were washed two more times with PBS and incubated at 37\textdegree C for 2 h in DMEM supplemented with serum. Cells were scored as syncytia if they had multiple nuclei in a single cell volume or if the connections between fusing cells appeared to be large enough to allow the nucleus passage. In some experiments, in order to evaluate the efficiency of early fusion stages, the extents of lipid mixing between HAB2 cells and PKH26-labelled erythrocytes were measured as the ratio of dye redistributed from bound erythrocytes to the total number of bound erythrocytes [45].

Fusion between Sf9\textsuperscript{biD} cells and between baculovirus-infected Sf9 cells was assayed as described previously [13]. In brief, fusogenic activity of gp64 was triggered at 22\textdegree C by a 5 min application of Grace medium with pH adjusted to 4.9. At 30 min after re-neutralization, the percentage of Sf9 cells or Sf9\textsuperscript{biD} cells in syncytia (the ratio of nuclei within syncytia to the total number of cell nuclei in the same field) was scored using light microscopy [47]. Lipid mixing between either Sf9 cells or Sf9\textsuperscript{biD} cells was quantified as Rho-PE transfer from labelled to unlabelled cells [13].

In the microinjection experiments, 15–30 min after the microinjection we determined the number of surviving Alexa Fluor\textsuperscript{®} 594-labelled mononucleated cells with nuclei labelled with Hoechst. The fusion was then induced by a 5 min application of pH 4.9 medium at 37\textdegree C. At 2 h after inducing fusion, we scored the efficiency of syncytium formation (Pf, percentage of fused injected cells) by determining the percentage of Alexa Fluor\textsuperscript{®} 594-labelled mononucleated cells that remained mononucleated (and thus unfused cells, P\textsubscript{unf}). Since the trauma of injection may influence the fusion rate of injected cells compared with non-injected cells, cell fusion efficiency for the cells injected with protein of interest, which was determined using the expression P\textsubscript{f} = 100 – P\textsubscript{unf}, was compared with that for the cells injected with dextran only.

In the experiments aimed at exploring the effects of the expression of the PLC\textsubscript{C1}PH–GFP construct, we focused on transfected Sf9\textsuperscript{biD} cells. We quantified the efficiency of syncytium formation by dividing the number of nuclei in GFP-labelled (green) multinucleated cells in the field of view by the total number of nuclei in GFP-labelled cells (both mono- and multi-nucleated cells). The efficiency was then normalized to that observed in a parallel experiment on the cells transfected with the inactive mutant R40L that does not bind PtdIns(4,5)P\textsubscript{2} in the plasma membrane [39].

Each experiment of the present study was repeated at least three times and all functional dependencies reported were observed in each independent experiment. Statistical analysis was carried out using SigmaPlot for Windows, version 11.0.

RESULTS

The F-BAR domain of FCHO2 and the BAR domain of GRAF1 promote syncytium formation

To explore whether CGP domains of FCHO2 and GRAF1 affect HA-initiated syncytium formation, we transfected HAB2 cells with constructs containing either the F-BAR domain of FCHO2 [30,49] or the BAR domain and PH domain of GRAF1 [27,28]. Early stages of HA-mediated fusion were unaffected by the
injected with only 10 kDa dextran were significantly different (paired Student’s t test, $P < 0.001$).

Low pH medium inducing fusion was applied 15–30 min after microinjection.

The ENTH domain of epsin promotes syncytium formation

The ENTH domain of epsin is also known to induce membrane curvature. We used two experimental approaches, transfection and microinjection, to test the effects of this domain on HA-initiated syncytium formation. In two out of three experiments, we found the extent of HA-mediated syncytium formation to be somewhat higher for HAb2 cells transfected to express the ENTH domain of epsin1 linked to EGFP than for the mock-transfected cells (Figure 2A). However, in one of the experiments we observed no difference in the syncytium formation for transfected and mock-transfected HAb2 cells (Figure 2A). In one of the experiments we observed no difference in the syncytium formation for transfected and mock-transfected cells. Expression of an ENTH domain of the epsin1 mutant L6E, which is still able to bind to membranes but is defective in membrane bending [25], did not affect syncytium formation.

In another approach, we microinjected 19 μM of the recombinant ENTH domain of epsin1 and observed a statistically significant promotion of syncytium formation compared with mock-injected cells (46 ± 7.8% compared with 30.3 ± 7.5%, values are means ± S.E.M., $n = 6$, $P < 0.001$ in a paired Student’s t test). Although some promotion of cell fusion was also observed in the three experiments where we injected 9.5 or 38 μM ENTH domain, the differences between normalized extents of syncytium formation were not statistically significant (Figure 2B).

A somewhat weaker promotion of cell fusion at 38 compared with 19 μM of the ENTH domain may reflect the toxicity of the injected protein. Note that, in contrast with early fusion stages, syncytium formation strongly depends on metabolic activity of the fusing cells [12,13].

Figure 2 Effects of the ENTH domain of epsin on HA-initiated syncytium formation

(A) Cells were transfected with an EGFP plasmid containing the wild-type ENTH domain of epsin (2) or the mutant L6E (3). (1) Control with mock-transfected cells. (B) Cells were microinjected with Alexa Fluor® 594-conjugated dextran and microinjection buffer without (1) or with (2, 3 and 4) the ENTH domain of epsin at different concentrations (9.5 μM, 19 μM and 38 μM respectively). Low pH medium inducing fusion was applied 15–30 min after microinjection. Not-normalized fusion extents for the cells injected with 19 μM ENTH domain and the cells injected with only 10 kDa dextran were significantly different (paired Student’s t test, $P < 0.001$, $n = 6$). For (A) and (B), the final extents of fusion were measured 2 h after the end of low pH application and normalized to those in the control experiments. Results are means ± S.E.M. ($n$⩾3).

transfection, as shown by the same efficiency of lipid mixing between PKH26-labelled RBCs and either control or transfected HAb2 cells (Figure 1A). However, expression of these CGP domains increased the efficiency of HA-initiated syncytium formation (Figure 1B), indicating that CGPs promote the late stages of cell-to-cell fusion.

Dynamin and the late stages of fusion events

The GTPase dynamin, a key player in budding and scission of intracellular vesicles, is one of the most abundant cytosolic CGPs [22,50]. We explored a possible involvement of this protein in the syncytium formation mechanism using three inhibitors of dynamin GTPase activity and by expression of dynamin mutants. Dynasore, a cell-membrane-permeant inhibitor of dynamin GTPase activity [35], inhibited both gp64-initiated syncytium formation by Sf9Bp1D cells and HA-initiated syncytium formation by HAb2 cells (Figure 3). Figure 4 shows the inhibition of syncytium formation by Sf9Bp1D cells when the low pH application was followed by the application of another cell-permeant dynamin inhibitor Dynole-34-2 that targets an allosteric site at the GTPase domain. Dynole-34-2 lowered both the percentage of nuclei in multinucleate cells (Figure 4) and the sizes of the syncytia (assayed as the distribution of the numbers of nuclei per cell; Supplementary Figure S2 at http://www.BiochemJ.org/bj/440/bj4400185add.htm). No inhibition was observed in the presence of Dynole-31-2, an inactive analogue of Dynole-34-2 [37].

Another cell-membrane-permeant inhibitor of dynamin GTPase, MitMAB, that acts by targeting dynamin interactions with anionic phospholipids [36], also significantly inhibited gp64- or HA-initiated syncytium formation (Figure 5). MitMAB is a reversible dynamin inhibitor [36], and washing Sf9Bp1D cells to remove MitMAB restored the ability of cells to form syncytia (Figure 5A). Importantly, Dynole-34-2 (Figure 4), MitMAB (Figure 5) and dynasore (results not shown) inhibited syncytium formation when added after the end of the low pH pulse. Taking into account that by this time early fusion stages that yield nascent fusion pores had taken place [14,15,45], these findings suggested that the inhibition of the GTPase activity of dynamin blocked the late stages of syncytium formation. Indeed, we found that neither Dynole-34-2 (Figure 4) nor dynasore (results not shown) inhibited lipid mixing in a gp64-mediated fusion.

To explore further the involvement of dynamin in syncytium formation, we infected Sf9 cells with baculoviruses encoding different dominant-negative human dynamin-1 mutants. In contrast with Sf9Bp1D cells that constitutively express the protein fusogen gp64, Sf9 cells expressed gp64 as a result of baculovirus infection. We adjusted the concentrations of all of the baculovirus constructs used to achieve similar levels of gp64 expression (detected by a cELISA, Supplementary Figure S2; and by Western
Dynole-34-2 (bars 2 and 4) or its inactive derivative Dynole-31-2 (bars 3 and 5) was applied to Sf9\(^{\text{Op1D}}\) cells (final concentration 20 \(\mu\)M, bars 2 and 3 or 30 \(\mu\)M, bars 4 and 5) immediately after a 1 min application of pH 4.9 medium. (1) Control with no reagents applied. Final extents of lipid mixing and syncytium formation (black and grey bars respectively) were measured 20 min later. Results are means + S.E.M. (\(n \geq 3\)).

**DISCUSSION**

In the present study, we have explored the late stages of cell–cell fusion in which the nascent fusion pore(s) generated by viral fusogens baculovirus gp64 and influenza HA expand(s) to form

Figure 4 Dynole-34-2, an inhibitor of dynamin GTPase activity, inhibits gp64-initiated syncytium formation, but does not inhibit lipid mixing

Dynole-34-2 (bars 2 and 4) or its inactive derivative Dynole-31-2 (bars 3 and 5) was applied to Sf9\(^{\text{Op1D}}\) cells (final concentration 20 \(\mu\)M, bars 2 and 3 or 30 \(\mu\)M, bars 4 and 5) immediately after a 1 min application of pH 4.9 medium. (1) Control with no reagents applied. Final extents of lipid mixing and syncytium formation (black and grey bars respectively) were measured 20 min later. Results are means + S.E.M. (\(n \geq 3\)).
Fusion pores that develop with a tight contact between cell membranes have a strongly bent rim accumulating the elastic energy of membrane bending. Thus the pore expansion is an energy-consuming process. Earlier studies indicated that the enlargement of micron-scale pores in syncytium formation does not proceed spontaneously but rather involves the cell machinery [12,13,53]. Neither release of membrane lateral tension nor disruption of the force generating intracellular systems such as the actin cytoskeleton and microtubular structures inhibits fusion pore expansion in syncytium formation [12,13,19,54]. We hypothesized that large fusion pores grow because the rim of the pore accumulates proteins that relax its bending energy. The shape of a fusion pore developing in a cell–cell contact zone with an inter-membrane distance of ∼20 nm is similar to a half cylinder with a ∼20 nm diameter [13]. Since the comparable curvature radii are characteristic for highly curved membrane compartments such as endocytic vesicles, we proposed that the late stages of fusion pore growth involve CGPs that drive intracellular membrane shaping. To test this hypothesis, in the present study we explored whether syncytium formation can be promoted by raising the intracellular concentrations of membrane-binding and -bending domains from three CGPs (GRAF1, FCHo2 and epsin) in cells by either overexpressing these proteins or by microinjecting them. Indeed, these diverse CGPs (with BAR, F-BAR and ENTH domains) promoted the late stages of HA-initiated cell–cell fusion. However, only short to form complete circles or helices and thus shaped as open dimers, the building blocks of larger oligomers, are rigid elongated structures with a curved shape. Curvature generated by dynamin can result in different membrane shapes. Long helical oligomers constrain the membrane into tubular shapes. Oligomers that are too short to form helices can generate a wider variety of shapes which include tubular and spherical shapes. Blocking of the GTPase activity of dynamin by dynasore [35], MiTMAB [36] and Dynole-34-2 [37] inhibited syncytium formation initiated by either HA or gp64. Dynamin inhibitors did not inhibit lipid mixing and blocked syncytium formation even when applied after a low pH pulse application, that is, at the time when both HA [56] and, especially, gp64 [14] have already formed initial fusion pores. Thus dynamin plays its role downstream of the opening of fusion pores. Our findings suggest that the recently reported inhibition of HIV env-mediated cell–cell fusion by dynasore [57] also reflects dynamin-dependence of late, rather than early, fusion stages.

Dominant-negative dynamin mutants also affected cell-to-cell fusion. Similar to the experiments using inhibitors, syncytium formation was inhibited by stabilization of the GTP-bound form of dynamin by overexpressing a GTPase-defective S61D mutant [40]. Syncytium formation was also inhibited by the assembly-defective I690K mutant of dynamin, which is impaired in membrane surface affinity [41]. In contrast, K44A and S45N, two mutants of dynamin defective in GTP binding [42–44], promoted syncytium formation. This promotion may indicate that GTP-free dynamins are more likely than GTP–dynamins to form short arc-like oligomers that facilitate fusion pore expansion. However, this hypothesis remains to be tested. Importantly, since impaired GTP binding for these mutants inhibits GTP hydrolysis, the promotion of syncytium formation argues against the hypothesis that fusion pore expansion is somehow driven by the energy derived from the GTP hydrolysis by dynamin.

The finding that diverse dynamin-targeting reagents have notable effects on the efficiency of syncytium formation suggests the involvement of dynamin in cell–cell fusion. However, although the ability of dynamin to shape membranes is well documented, we cannot at the present time offer a specific mechanism by which curvature generation by dynamin promotes fusion pore expansion. In one scenario, dynamin oligomers, too short to form complete circles or helices and thus shaped as open...
arcs bind to the curved membrane of a nascent fusion pore rim. Owing to their ability to generate membrane curvature similar to that of the pore edge, binding of these arc-like oligomers drives expansion of the pore edge by bending additional portions of the initially flat membrane of the intracellular contact into the bent shape of the pore rim. Stabilization of the GTP-bound form of dynamin may promote the formation of the long helical structures and therefore the membrane tubules. As mentioned above, short oligomers can be accommodated both in tubules and in the fusion pore rim, and thus competition between the tubules and pore rim for the short-arc dynamins is expected to deplete the pool of short oligomers in the rim and inhibit pore expansion. Further research will either further develop this mechanism to explain the specific effects of different dynamin mutants or bring about alternative mechanisms that may couple membrane shaping by dynamin with fusion pore expansion at late stages of cell-to-cell fusion. Importantly, since dynamin both directly and via interactions with other proteins regulates many different processes in cell physiology, the role of dynamin in syncytium formation might be complex and indirect.

The activity of many intracellular CGPs is regulated by polyphosphoinositides. The present study is the first demonstration that PtdIns(4,5)P₂ content controls syncytium formation. Inhibition of the late stages of cell fusion by lowering the concentration of accessible PtdIns(4,5)P₂ in the plasma membrane is consistent with the hypothesis that CGPs drive the expansion of fusion pores. However, PtdIns(4,5)P₂ can also affect late fusion stages by regulating PtdIns(4,5)P₂-binding proteins other than CGPs.

To conclude, the late stages of cell-to-cell fusion initiated by well-characterized viral fusogens depend on the functional activity of intracellular CGPs, including dynamin and representatives of the BAR, F-BAR and ENTH protein families. However, a number of important questions remain open. To start with, the mechanism underlying this dependence is yet to be clarified. CGPs can directly facilitate fusion pore expansion by accumulating at the pore edge and lowering its energy. In principle, CGPs can also promote vesiculation of the membrane junction at the edge of the fusion pores [33]. However, at least in the case of gp64-initiated fusion of SF9 cells, fusion pores appear to grow by the displacement of membrane material towards the periphery of the contact zone rather than by vesiculation [13]. The analysis of the role of CGPs in syncytium formation also has to be extended to biologically important examples of cell fusion such as fusion between myoblasts and between macrophages, and then to the identification of specific CGPs involved in these processes. Better understanding of the mechanism and cell machinery responsible for driving fusion pore expansion in cell-to-cell fusion will bring about new ways of controlling fusion in development and in pathological conditions. The dependence of fusion pore expansion on cell machinery can also be of importance for understanding why transient nanotubular connections between plasma membranes of some cells [58,59] do not expand to yield multinucleated cells.

The present study emphasizes an interesting overlap between proteins controlling the late stages of cell-to-cell fusion and proteins that drive the oppositely directed process of membrane remodelling, the fission of one cell membrane into two. Dynamin and curvature-generating domains of GRAF1, FCHo2 and epsin that we found to influence syncytium formation are essential components of different endocytic pathways [23–25,27,28,30] that culminate in membrane fission. Proteins of the dynamin family have been also implicated in mitochondrial fusion [60,61] and fusion of ER membranes [62,63]. Dynamin is involved in an as yet unclear mechanism in HIV fusion with the endosomal membrane, as suggested by a fusion inhibition using the dynamin GTPase inhibitor dynasore [64]. Furthermore, dynasore promotes the release of luminal and membrane biosynthetic cargoes from individual post-Golgi vesicles, suggesting that dynamin redirects fusion pore evolution from expansion to premature closure in ‘kiss-and-run’ exocytosis [65]. A recent study confirms that dynamin regulates the rapidity of fusion pore expansion in exocytosis and suggests that dynamin assembly restricts fusion pore expansion until GTP-hydrolysis-stimulated disassembly [66]. Another component of the endocytic machinery, the BAR domain protein amphiphysin, has also been reported to restrict dilation of fusion pores [67]. Note that, in terms of membrane curvature and positioning of proteins, the closing of the exocytotic fusion pore by CGPs (dynamin [65] and amphiphysin [67]) located outside of the pore lumen is topologically similar to fusion pore expansion in syncytium formation by the proteins located inside the lumen of the pore. Further elucidation of the overlap between the protein players involved in the processes that unite and divide biological membranes is important for finding shared mechanistic principles underlying fusion and fission [68]. Although our results from the present study demonstrate that different CGPs can control the expansion of fusion pores, we still do not know which of the diverse intracellular CGPs control these stages in biologically important cell-to-cell fusion processes. Our finding that increases in concentration of either of the several CGP domains promote transition from early fusion intermediates to syncytium formation suggests a redundancy of their membrane-bending function.

**AUTHOR CONTRIBUTION**

Jean-Philippe Richard, Evgenia Leikina, Michael Kozlov and Leonid Chernomordik conceived and planned the experiments. Jean-Philippe Richard, Evgenia Leikina and Margarita Popova carried out the experiments and analysed the data. Ralf Langen, Tamas Balla, Harvey McMahon and William Mike HNne advised on specific aspects of the experimental strategies and provided constructs and proteins. Leonid Chernomordik, Jean-Philippe Richard and Michael Kozlov wrote the paper.

**ACKNOWLEDGEMENTS**

We are indebted to Dr S. Schmid (The Scripps Research Institute) and Dr T. Kirchhausen for dynamin constructs and dynasore respectively. We thank Dr K. Melikov and Dr J. Zimmerberg for very helpful discussions prior to submission.

**FUNDING**

The research was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health and an National Institute of Allergy and Infectious Diseases, National Institutes of Health Intramural Biodisover Research grant (both to L.V.C.) and by the National Institutes of Health [grant number 2 R01 GM063915 (to R.L.)].

**REFERENCES**

Intracellular curvature-generating proteins in cell fusion


Received 11 July 2011/18 August 2011; accepted 7 September 2011
Published as BJ Immediate Publication 7 September 2011, doi:10.1042/BJ20111243


© The Authors Journal compilation © 2011 Biochemical Society
SUPPLEMENTARY ONLINE DATA

Intracellular curvature-generating proteins in cell-to-cell fusion

Jean-Philippe RICHARD*1,2, Evgenia LEIKINA*1, Ralf LANGEN†, William Mike HENNE‡3, Margarita POPOVA*, Tamas BALLA§, Harvey T. MCMAHON‡, Michael M. KOZLOV∥ and Leonid V. CHERNOMORDIK*4

*Section on Membrane Biology, Laboratory of Cellular and Molecular Biophysics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, 10 Center Dr., Bethesda, MD 20892-1855, U.S.A., †Zilkha Neurogenetic Institute and Department of Biochemistry, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, U.S.A., ‡MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, U.K., §Section on Molecular Signal Transduction, Program for Developmental Neuroscience, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, U.S.A., and ∥Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, 69978 Tel Aviv, Israel

Figure S1 Dynole-34-2, an inhibitor of dynamin GTPase activity, decreases the sizes of syncytia formed by Sf9Op1D cells

Dynole-34-2 (black bars) or its inactive derivative Dynole-31-2 (open bars) was applied to Sf9Op1D cells (final concentrations of 30 μM) immediately after a 1 min application of pH 4.9 medium. Grey bars indicate control with no reagents applied. Syncytium formation was evaluated 20 min later by counting the numbers of cells containing different numbers of nuclei (from one to seven).

Received 11 July 2011/18 August 2011; accepted 7 September 2011
Published as BJ Immediate Publication 7 September 2011, doi:10.1042/BJ20111243

Figure S2 Cell-surface density of baculovirus gp64 in the experiments with Sf9 cells infected with either wild-type baculovirus (BV) or baculovirus constructs expressing wild-type dynamin (w.t.) or different dynamin mutants (K44A, I690K, S45N and S61D)
The surface expression of gp64 was evaluated 2 days post-infection by cELISA. Results are means + S.E.M.