Yolk granule tethering: a role in cell resealing and identification of several protein components

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Summary

Homotypic fusion among echinoderm egg yolk granules has previously been reconstituted in vitro, and shown to be a rapid, Ca^{2+}-triggered reaction that can produce extremely large (>10 μm diameter) fusion products. We here show that, prior to Ca^{2+}-triggered fusion, yolk granules in vitro, if isolated in an appropriate buffer, became tethered to one another, forming large aggregates of more than 100 granules. Granule washing with mildly chaotropic salt abolished this tethering reaction, and prevented Ca^{2+}-triggered formation of the large fusion products characteristic of tethered granules. Protein factors present in the wash restored tethering activity and these factors could be substantially enriched by anion exchange chromatography. The enriched fraction behaved under native conditions as a high molecular weight (~670 kDa), multisubunit complex of at least seven proteins. Monoclonal antibodies directed against this complex of proteins were capable of immunodepleting tethering activity, confirming the role of the complex in granule tethering. These antibodies selectively stained the surface of yolk granules in the intact egg. We therefore propose a new role for tethering: it can promote the formation of large vesicular fusion products, such as those required for successful resealing. We have, moreover, identified several proteins that may be critical to this tethering mechanism.

Key words: Resealing, Tethering, Fusion, Membranes, Calcium

Introduction

Cell survival in vivo often depends on the capacity for rapid repair or resealing of a plasma membrane disruption. Skeletal (McNeil and Khakee, 1992) and cardiac muscle cells (Clarke et al., 1995) for example, commonly suffer disruptions during normal contractile, force-generating activities. Failure to reseal a plasma membrane disruption results in rapid (seconds to minutes) cell death, probably as a result of excessive entry of Ca^{2+} ions.

The mechanism of resealing large disruptions (>1 μm diameter) has been investigated extensively in the echinoderm egg. Resealing has an absolute requirement for external Ca^{2+} at near physiological concentration (Steinhardt et al., 1994). Ca^{2+} entering through the disruption locally triggers both homotypic (Terasaki et al., 1997) and heterotypic membrane fusion events (Steinhardt et al., 1994). The combined result of these fusion events is the application of a reparative 'patch', derived from internal membrane, across the disruption site. The homotypic fusion events occur between yolk granules, and lead to the formation near to the disruption site of abnormally large intracellular vesicles, termed patch vesicles, that correspond in size to the disruption defect (Terasaki et al., 1997). Homotypic fusion of yolk granules can be reconstituted in vitro (Chestkov et al., 1998; McNeil et al., 2000), where it closely mimics the resealing-based process envisioned to occur in vivo: fusion is triggered by Ca^{2+}, and strikingly, can produce extremely large vesicular products (>10 μm diameter) very rapidly. The heterotypic fusion events induced by a disruption occur between the patch vesicle and the plasma membrane, and are exocytotic in nature. These exocytotic fusion events join the product of homotypic fusion, the patch vesicle, to the native plasma membrane surrounding the disruption site, completing defect repair. A similar mechanism, involving homotypic and heterotypic fusion events, is thought to mediate resealing in mammalian cells (Bansal et al., 2003), but this process has not yet been reconstituted in vitro.

Two of the paradigmatic protein components in the fusion field have been implicated in resealing-based fusion. Botulinum or tetanus toxins, injected into fibroblasts or sea urchin eggs, inhibit resealing, suggesting a requirement for SNAREs (Steinhardt et al., 1994). Injection of function-neutralizing antibodies or competitive peptide fragments of synaptotagmin VII into fibroblasts (Reddy et al., 2001) or squid giant axons (Detrait et al., 2000a; Detrait et al., 2000b) partially inhibit resealing, suggesting a role for this possible Ca^{2+} sensor. However, a recent study casts doubt on the role of synaptotagmin VII (Jaiswal et al., 2004): it showed that, in fibroblasts at least, synaptotagmin VII actually acts as a brake on the resealing-based exocytotic event it was supposed to mediate. Moreover, another recent study (Shen et al., 2004) failed to confirm that the C2A domain of Syt VII inhibits resealing. The C2B domain, however, which does not block lysosomal exocytosis (Martinez et al., 2000), was effective in blocking resealing.

Does resealing-based fusion use any novel protein components, not so far identified in other systems? An answer to this question requires that one go beyond previously used approaches based on reasoning by analogy from other systems.

A biochemical approach is one possible route to the discovery of resealing-specific components.

We previously reconstituted resealing-based yolk granule fusion in vitro, allowing us to identify biochemically protein fusion components and analyze their potential role in resealing. We here show that yolk granules isolated in a non-chaotropic buffer exhibit a potent tethering apparatus and that this tethering apparatus is required for the formation of large patch vesicles in vitro. This suggests a further biological role for tethering: the promotion of large products of fusion suitable for patching large disruptions. Moreover, we describe here methods that allowed us to dissociate tethering activity from isolated yolk granules and to biochemically enrich it. This enriched activity acts as a high molecular weight complex comprised of at least seven protein subunits.

Materials and Methods

Reconstitution of yolk granule fusion in vitro

Gravid starfish *Asterias forbesi* were obtained from the Marine Biological Laboratory (Woods Hole, MA). Excised ovaries were minced using scissors and frequently swirled, in Ca^{2+}-free, ice-cold artificial seawater (Marine Biological Laboratory) and the eggs thus liberated descanted through a meshwork filter. One gravid starfish typically yields between 5-20 ml of wet-packed eggs. Eggs (10-20 wet packed ml) were washed three times (2 minutes, 500 ***g***) in Ca^{2+}-free seawater, and twice in glycine-based IM buffer (pH 6.7, 220 mM potassium glutamate, 500 mM glycine, 10 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, protease inhibitor cocktail, EDTA-free; Roche), homogenized by 15-25 passes through a 40-ml Dounce-type glass tissue grinder (Kontes, NJ). Yolk granules were then purified either on a Percoll gradient, as previously described (McNeil et al., 2000), or by differential centrifugation (Vater and Jackson, 1989). No difference was observed between the behavior of granules isolated on the Percoll gradient and those isolated by differential centrifugation, though the latter procedure is a cheaper and faster method for isolation of large quantities of granules. Cytosol was obtained by taking the supernatant of the first high-speed spin used in the differential isolation approach, followed by a centrifugation step (60 minutes, 50,000 ***g***) for removal of residual organelles and membrane fragments. Fusion was initiated in these isolated granules by the addition of calibrated Ca^{2+} buffers (McNeil et al., 2000), and either observed directly in the light microscope as the formation of vesicles greater in diameter than that of the native granule (~1 μm), or assessed by a semi-quantitative, light scatter-based assay, as previously described (McNeil et al., 2000).

KI stripping of granule tethering activity

Yolk granules were centrifuged (10 minutes, 3200 ***g***) to remove IM buffer and suspended for 30 minutes at room temperature in a KI-based buffer (pH 6.85; 470 mM KI, 5 mM $MgCl_2$, 2 mM EGTA, 10 mM PIPES, protease inhibitor cocktail, EDTA-free) to strip the granules of peripheral protein candidate tethering factors. These 'KI-washed' yolk granules were pelleted by centrifugation and resuspended in IM buffer and saved for use in assays of tethering. The KI buffer harvested as the supernatant in this centrifugation step was saved as the starting point for further purification of protein candidates of the granule tethering apparatus.

Microscopic analysis of granule tethering

KI-washed, tether-incompetent granules were vortexed for 10-15 seconds, and then a 5-μl volume (suspension OD_{405} ~0.5) was dispensed onto a glass slide. This was diluted with 20 μl IM buffer alone (negative control) or with 20 μl IM buffer containing test substances. Slides were immediately examined by transmitted light microscopy for the presence of tethered clusters.

Semi-quantitative analysis of granule tethering

KI-washed yolk granules (100 μl) with an OD_{405} of ~0.350 were mixed with 100 μl IM containing test substances, or with IM alone (negative control), in 96-well, round-bottomed plates. After a 10-minute incubation at room temperature, the plate was centrifuged (3 minutes, 100 ***g***) in order to separate tethered granule aggregates (pellet) from non-tethered granules (supernatant). Finally, 75 μl of the top layer of each well, containing non-tethered granules, was transferred into a new well, and the absorbance of this transferred volume read on a plate reader (Cambridge Technology).

Biochemical enrichment and analysis of the granule-tethering factor

Proteins of interest were salted out of KI wash buffer by the addition of ammonium sulfate (2.4 M final concentration), and the precipitate redissolved in 10 mM sodium phosphate buffer, pH 6.8, preparatory to further separation. Column chromatography (FPLC, Pharmacia) was performed with a strong anion exchanger (HQ, BioRad) and a size exclusion gel (Sephacryl S-300, Amersham). Protein was eluted from the anion exchanger with a linear 1 M NaCl gradient; size exclusion chromatography utilized 10 mM sodium phosphate buffer (pH 6.8). Fractions were analyzed by conventional SDS-PAGE followed by staining with Coomassie Blue; protein amounts were quantified using a modified Lowry assay for all relevant experiments (DC protein assay, BioRad). Prior to all bioassays of tethering activity, protein fractions were desalted in columns (BioRad) into IM.

Granules were lysed by a 1:10 dilution in distilled water, and a supernatant harvested after centrifugation (60 minutes, 50,000 ***g***). This lysate was then desalted into IM buffer prior to assay for tethering activity.

Generation of monoclonal antibodies against tethering-factor subunits

Purified (anion exchange fraction) tethering complex was used as an antigen for the production of monoclonal antibodies. Clones were selected for expansion based on the production of antibodies that stained intact (not detergent-permeabilized) IM-washed granules more strongly than KI-washed granules. For this purpose, flow cytofluorometry was used to evaluate relative fluorescence levels. Western blotting was done using standard laboratory procedures. All monoclonal antibodies were used at 1:1000 dilution and detected using goat anti-mouse antibody conjugated to HRP (Jackson ImmunoResearch Laboratories). Visualization of HRP was accomplished using an ECL kit (Amersham).

Immunostaining of eggs

Eggs were fixed in 3.7% formalin/Ca^{2+}-free seawater and frozen sections (10 μm) cut for immunostaining. Incubations in primary and secondary antibodies were for 60 minutes at room temperature at dilutions of 1:1000 and 1:200 respectively. Confocal images of the immunostained eggs were acquired on a confocal microscope (Zeiss, Germany).

Results

Tethering of yolk granules promotes the formation of large fusion products

In a previous study we observed that echinoderm egg organelles extruded from a microneedle into seawater without

Ca^{2+}, adhere to one another as they leave the tip, forming a strand-like array. In contrast, if Ca^{2+} was present, the organelles fused forming an elongate vesicular structure (Terasaki et al., 1997). A similar phenomenon has been observed in broken echinoderm eggs dating back to the classical experiments of Heilbrunn: breakage in Ca^{2+} elicits a rapid fusion reaction among organelles at the breakage site (termed the surface precipitation reaction), whereas breakage in the absence of Ca^{2+} results in an outflow of organelles that rapidly adhere to one another to form a large extracellular mass (Heilbrunn, 1956). This raised the possibility that aggregation or tethering of egg organelles, in particular the yolk granule, was an important step in the formation of membrane patches for use in the repair of plasma membrane disruptions.

To investigate this possibility further, we developed conditions for isolating yolk granules that retained this tethering capacity. We found that yolk granules isolated in a glycine-based, non-chaotropic buffer (IM) were strongly tethered to one another: they formed clusters consisting of many hundreds of granules (Fig. 1A). Upon Ca^{2+} buffer addition, fusion products of a size proportionate to the tethered sub-population were observed to form rapidly, in less than 2 seconds (the limit of resolution of the video detection method) (Fig. 1B-D). In contrast to previous studies in which the granules were isolated in a KCl-based buffer (Chestkov et al., 1998; McNeil et al., 2000), fusion to form large products did not require that the granules be forced into contact with one another by a centrifugation step prior to Ca^{2+} buffer addition.

As described in previous studies (Chestkov et al., 1998; McNeil et al., 2000), Ca^{2+}-triggered granule fusion is a reaction that is initiated rapidly (second to sub-second time scale) and fusion, measured as a decline in light scatter in those studies, was observed to cease within 1 minute of Ca^{2+} buffer addition. To confirm that in our system, in which granules become spontaneously tethered to one another, is a similar temporal limit to fusion, we continued incubations of granules for over an hour. After the initial rapid decline in light scatter (completed before the plate could be introduced into the plate reader, <3 minutes) induced by Ca^{2+} addition, no further change in light scatter was observed for the next 65 minutes (Fig. 2). Neither addition of further Ca^{2+}, even that sufficient to produce fusion in granules previously exposed only to IM buffer, nor forcing of vesicles into contact with one another by centrifugation, resulted in additional fusion (data not shown), confirming that neither sequestration of added Ca^{2+}, nor fusion induced decreases in vesicle density and therefore contacts, explain the fusion block. Thus, fusion of yolk granules triggered by Ca^{2+} is a self-limiting process, severely constrained temporally: multiple rounds of fusion occurring over a minute-to-hour-long time scale, such as are often recorded in other systems (Merz and Wickner, 2004), are not permitted in the yolk granule system.

To confirm the importance of tethering in the yolk granule fusion response, we washed granules in a mildly chaotropic

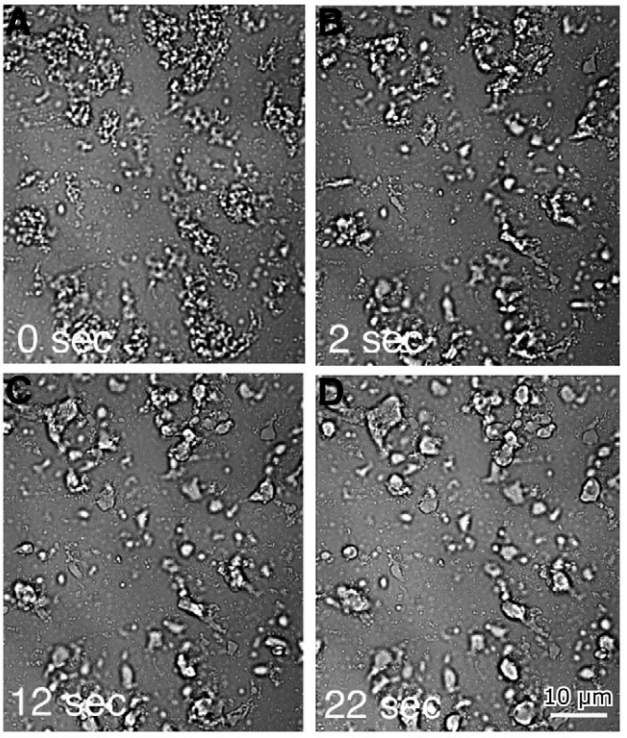


Fig. 1. Tethering and fusion of yolk granules isolated in IM buffer. Tethered yolk granules (A) before (0 second) and (B-D) at various time points after perfusion of Ca^{2+} (300 μM) into a slide coverslip chamber. Prior to Ca^{2+} buffer addition the vesicles are tethered to one another, forming large aggregates. These tend to fuse with one another as soon as Ca^{2+} reaches them, forming a large 'patch' vesicle product whose size reflects that of the tethered aggregate. Bar, 10 μm.

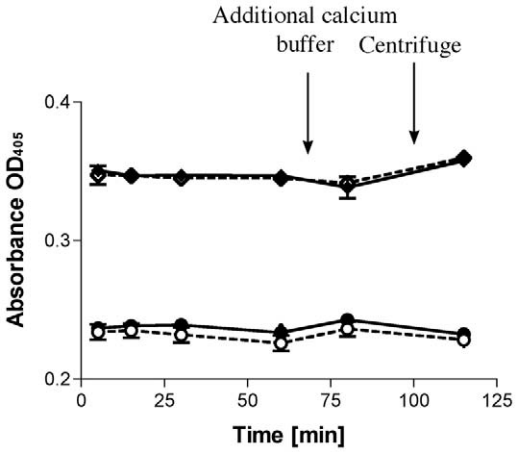
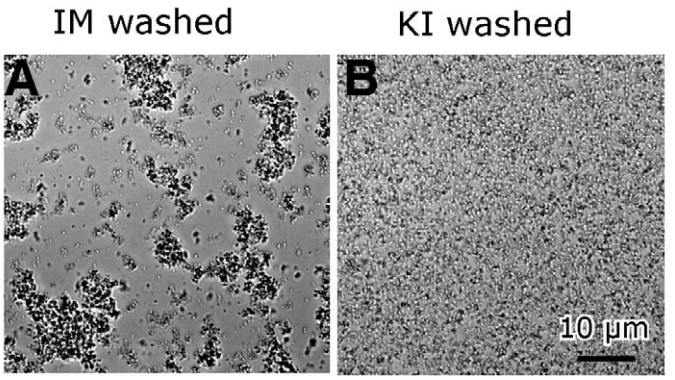


Fig. 2. Long-term time course of Ca^{2+}-triggered granule fusion. Granule light scattering, a semi-quantitative measure of granule fusion, was measured at intervals after the addition of Ca^{2+} buffer to give the following final concentrations: 0.001 μM (IM buffer), ◆; 11.9 μM, ◇; 572 μM, ●; 1151 μM, ○. The decrease in light scatter characteristic of fusion in this system was complete at the two higher Ca^{2+} concentrations at the earliest interval (~3 minutes after Ca^{2+} buffer addition) measurable. No further change in light scattering was recorded over the remaining measurement interval. Additional Ca^{2+} buffer was added at the 65-minute time point, resulting in the following approximate final concentration levels: <0.001 μM (IM buffer), ◆; 572 μM, ◇; 1450 μM, ●; 2680 μM, ○. The granules were pelleted by centrifugation at the 110-minute time point, concentrating them in the well bottom. No change in absorbance, indicative of further fusion, was observed in either case. Error bars indicate s.d. (n=3).



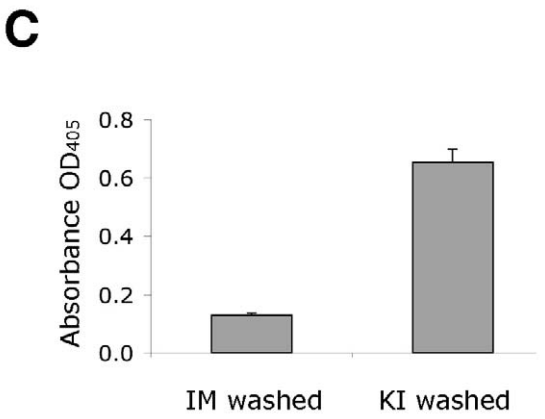


Fig. 3. Washing of yolk granules in the chaotropic salt, KI, inhibits tethering. (A) Granules washed three times post-isolation in IM buffer. (B) Granules washed three times post-isolation in KI buffer. Tethering is no longer evident. (C) Comparison of KI-washed and control, IM-washed granule tethering responses using a novel, rapid, semi-quantitative assay. Granule samples in 96-well plates are centrifuged to pellet the large tethered aggregates. Granules not tethered, which remain suspended in buffer in the upper half of the contents of each well, are then transferred to a fresh well and the absorbance of this transferred measured. Tethering efficiency is an inverse function of measured absorbance. Hence the absorbance of the KI-washed granules (not tethered, see panel B) is higher than that of IM-washed granules (tethered, see panel A). Error bars indicate s.d. (n=6). Bar, 10 μm.

buffer (KI-, NaCl-, KCl-based, in order of decreasing effectiveness). This was found to be capable of completely abolishing tethering: compared to the highly clustered IM-washed granules (Fig. 3A), KI-washed granules were observed to be an approximately monodisperse population (Fig. 3B). This tethering failure in KI-washed granules was further demonstrable in a semi-quantitative assay for tethering (Fig. 3C). Importantly, this KI wash-induced failure to tether had a dramatic effect on the yolk granule fusion response: the size of

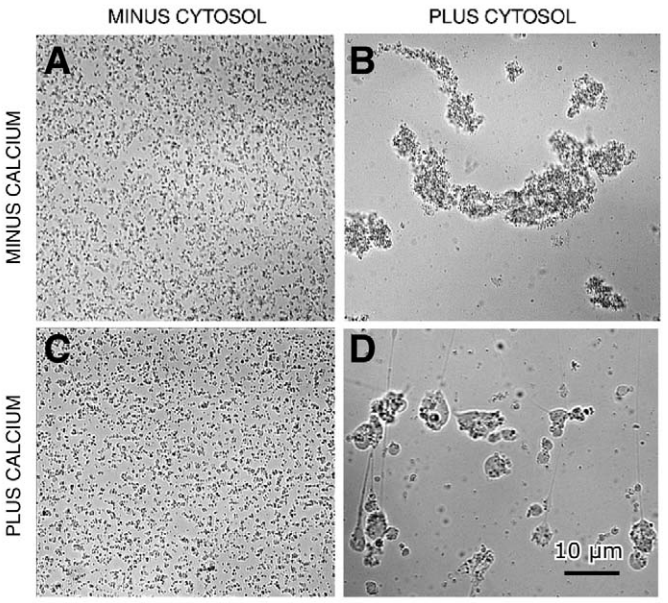


Fig. 4. Restoration of tethering by cytosol. (A) After washing in KI, granules are not capable of the tethering response. (B) Addition of cytosol (100 μg) restores tethering. (C) Ca^{2+} addition (300 μM) to the non-tethered population of granules produces small fusion products only. (D) Granules tethered by prior cytosol addition, by contrast, fuse upon Ca^{2+} addition to form large patch vesicle products. Bar, 10 μm.

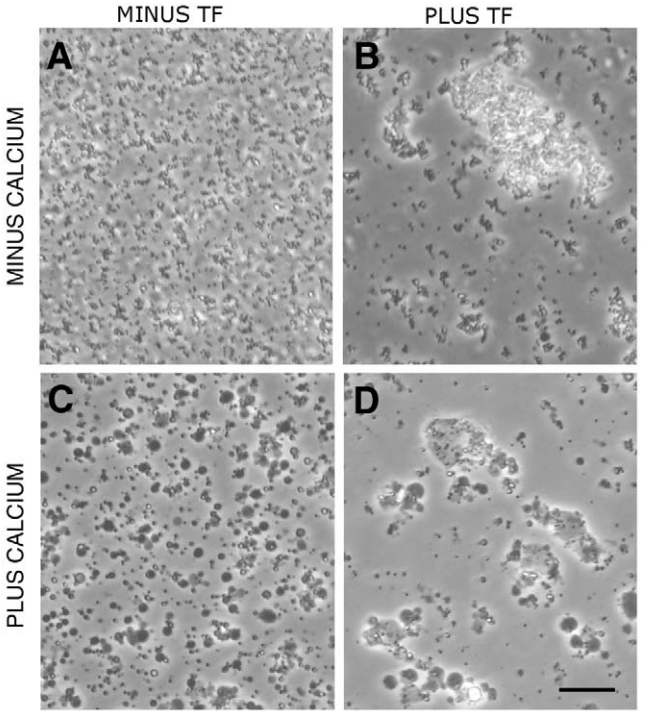


Fig. 5. Restoration of tethering by factors present in KI salt wash. (A) KI-washed vesicles (tether incompetent) received IM buffer only or (B) IM buffer conditioned by the addition of protein factors (40 μg desalted into IM buffer) stripped from granules during incubation in KI. Tethering is restored by these factors. (C) Fusion products produced by Ca^{2+} addition to KI-washed granules. (D) Fusion products produced by Ca^{2+} addition (300 μM) to granules tethered, as in B by the prior addition of tethering factors (TF) from a KI wash of granules. Note the increased size of the resultant fusion products. Bar, 10 μm.

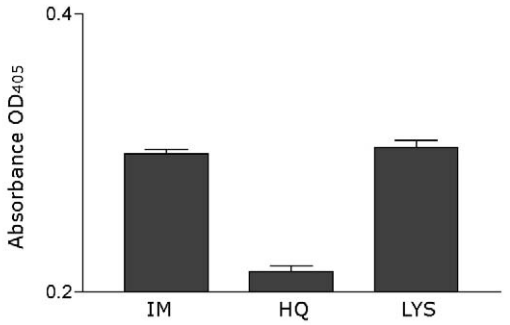


Fig. 6. Lack of tethering activity in a crude yolk granule lysate. Yolk granules were incubated with IM buffer only (IM), with equal amount (100 μg) anion-exchange-purified tethering factor (HQ) or with a crude granule lysate (LYS) and tethering activity measured. The buffer only and lysate samples did not differ significantly from one another, whereas both were significantly different from the tethering factor sample (P<0.005). Error bars indicate s.d. (n=6).

the fusion products elicited by Ca^{2+} addition was dramatically reduced (Fig. 4A,C). Thus, tethering is required for the production of large fusion products.

To further demonstrate this tethering requirement, we attempted to reconstitute tethering in KI-washed granules. We found that cytosol (Fig. 4B,D) and the KI buffer used for vesicle stripping of tethering activity (Fig. 5) could both restore tethering and Ca^{2+}-triggered production of enlarged fusion products. To demonstrate that the tethering activity present in the KI wash buffer was not simply a product of vesicle lysis, we compared the tethering activity of lysed granules with anion-exchange-purified tethering factor (see below). The lysis product had no demonstrable tethering activity (Fig. 6). These results not only confirm the importance of tethering for the production of large fusion products, they suggest that protein components with a tethering function are present in the cytosol and are associated with the granule membrane.

Biochemical isolation of the yolk granule tethering factor

As a starting point for purification of protein 'tethering factors' we used KI wash buffer, as it contains a far simpler mixture of proteins than cytosol. Tethering activity was precipitable with ammonium sulfate, allowing us to efficiently concentrate and desalt tether candidate proteins prior to anion exchange chromatography. Tethering activity was found to elute in a linear salt gradient as a single peak from the anion exchanger resin (Fig. 7A). Enrichment, based on the semi-quantitative assay for tethering, was about fivefold over the simplified starting material (Fig. 7C). The active peak consisted of seven major polypeptide bands, of ~100, 105, 110, 155, 170, 215 and 225 kDa (Fig. 7B).

When the enriched fraction from the anion exchange column was subject to size exclusion chromatography, tethering

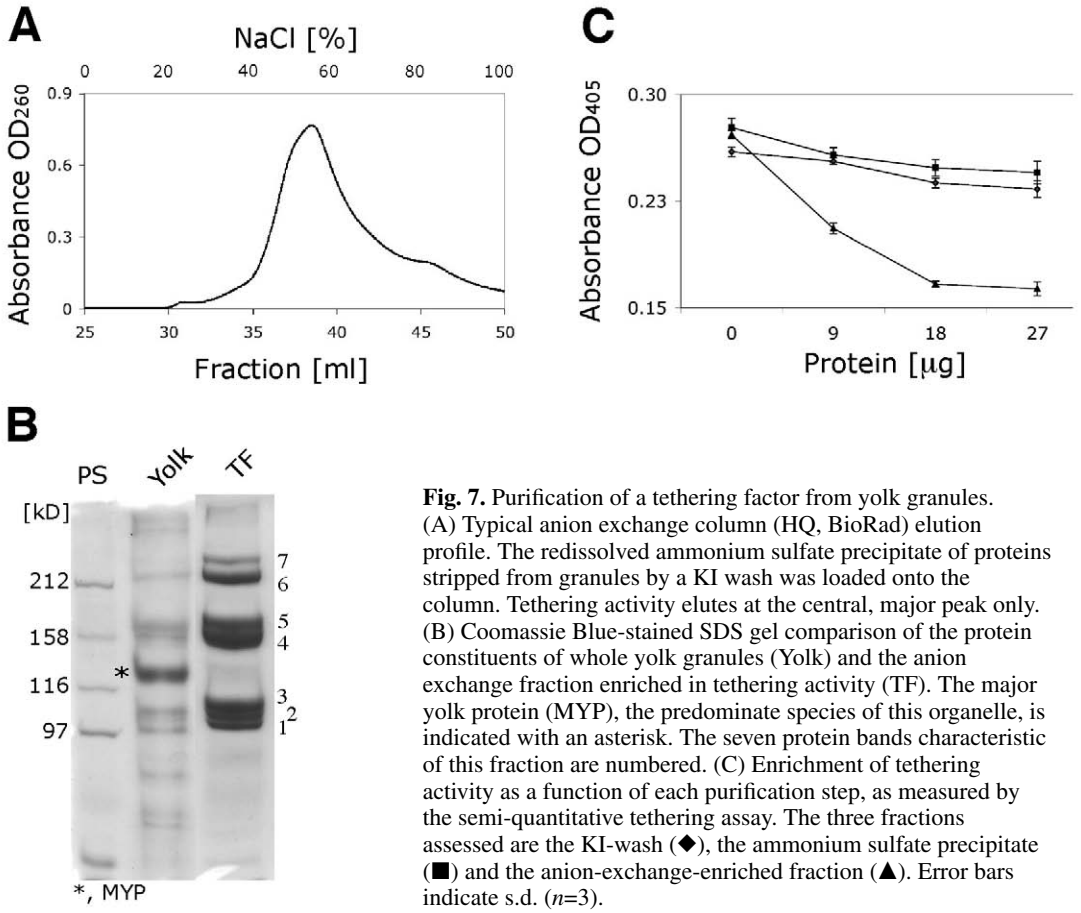


Fig. 7. Purification of a tethering factor from yolk granules. (A) Typical anion exchange column (HQ, BioRad) elution profile. The redissolved ammonium sulfate precipitate of proteins stripped from granules by a KI wash was loaded onto the column. Tethering activity elutes at the central, major peak only. (B) Coomassie Blue-stained SDS gel comparison of the protein constituents of whole yolk granules (Yolk) and the anion exchange fraction enriched in tethering activity (TF). The major yolk protein (MYP), the predominate species of this organelle, is indicated with an asterisk. The seven protein bands characteristic of this fraction are numbered. (C) Enrichment of tethering activity as a function of each purification step, as measured by the semi-quantitative tethering assay. The three fractions assessed are the KI-wash (◆), the ammonium sulfate precipitate (■) and the anion-exchange-enriched fraction (▲). Error bars indicate s.d. (n=3).

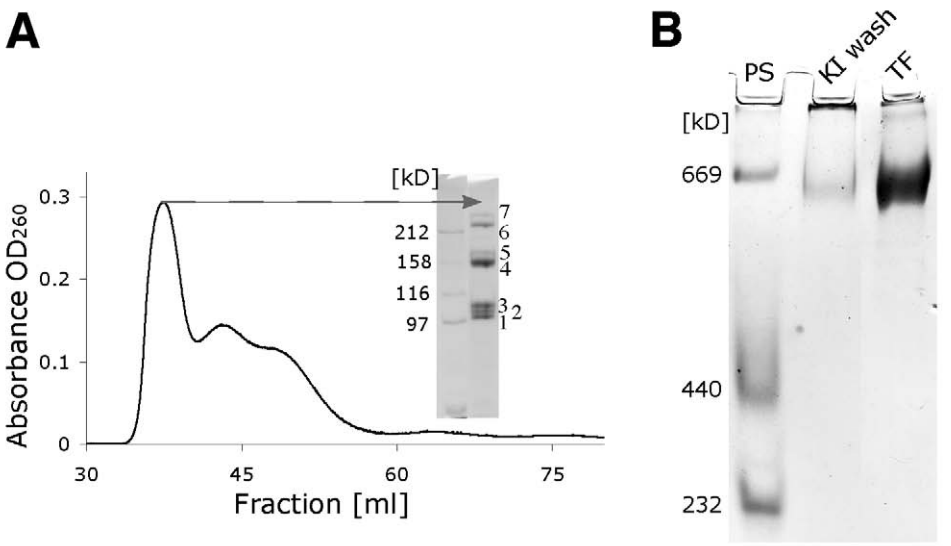


Fig. 8. The tethering factor behaves as a high molecular weight complex. (A) The anion exchange fraction enriched in tethering activity elutes predominantly as a single peak at the void volume of a size exclusion column (Sephacryl S-300). This fraction, and none of the others, has tethering activity. Gel electrophoretic analysis of the peak fraction with tethering activity is also shown in this panel. The complex of proteins purified as described above is present in this fraction. (B) Non-denaturing gel electrophoresis of equal amounts (20 μg) of protein from a KI wash (KI wash) and from the anion-exchange-enriched fraction (TF). A high molecular weight species of ~670 kDa is evident in both samples, but more prominent in the highly active anion exchange fraction. PS, protein standard markers.

activity emerged in a single peak running at the column void volume (~670 kDa) (Fig. 8A). Gel electrophoresis of the material in this peak confirmed that the previously described complex of proteins was present in this peak (Fig. 8A). When subjected to non-denaturing electrophoresis, it ran as single band of ~ 670 kDa (Fig. 8B). We conclude that the yolk granule tethering factor is a high molecular weight, multimeric complex of proteins.

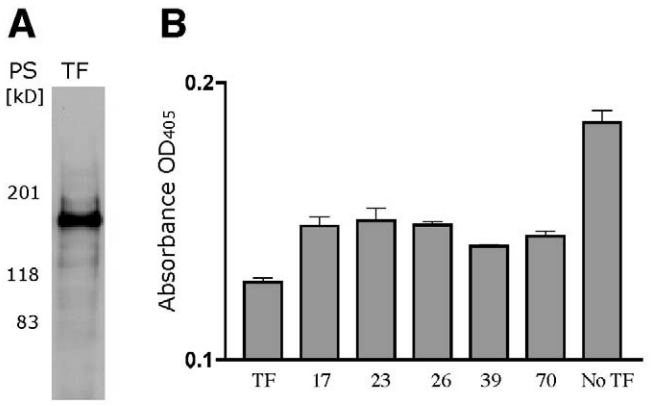


Fig. 9. Monoclonal antibodies raised against the tethering complex react against the 170 kDa subunit and can immunodeplete tethering activity. (A) Western analysis of monoclonal antibody staining of the 170 kDa component of the tethering factor. PS, position of protein standards. (B) Granules were incubated with anion-exchange-purified tethering factor (TF) or with an equivalent amount of tethering factor (15 μg) after it adsorption to protein G beads to which various (numbered columns) monoclonal antibodies had been bound. The activity depleted by protein G beads alone (in the absence of antibodies) has been subtracted from the antibody samples. One sample, a negative control, received IM buffer containing no tethering factor (No TF). Tethering activity was measured in the semi-quantitative assay. Significant immunodepletion (P<0.05) relative to the TF control is indicated for numerous monoclonal antibodies (identified by number). Error bars indicate s.d. (n=3).

Antibody immunodepletion and localization of the tethering factor

Monoclonal antibodies were raised against the tethering factor and selected based on higher affinity staining of IM- compared to KI-washed (i.e. tether-competent compared to tether-incompetent) granules. All clones thus obtained were against the 170-kDa subunit (Fig. 9A). Many of these clones were capable of depleting tethering activity from KI wash buffer, confirming that one subunit of the complex at least, is required for granule tethering (Fig. 9B).

Monoclonal antibodies against the tethering complex labeled a ubiquitous and abundant organelle compartment of ~1 μm diameter in the egg (Fig. 10A), strongly suggesting labeling of yolk granules. That the yolk granules and not other major egg organelles were labeled was confirmed by centrifuging eggs prior to staining, which stratifies the organelle compartments: only the heavy end of the stratified egg, in which the predominant organelle is the yolk granule, were stained with these monoclonal antibodies (data not shown). When the yolk granules were imaged at high resolution, apparent peripheral staining of granules was observed (Fig. 10B). Moreover, staining was observed in the absence of detergent permeabilization of eggs sheared to expose cytoplasmic constituents, and flow cytofluorometry analysis revealed no difference in the intensity of staining between detergent-permeabilized and intact yolk granules (data not shown). These results are all consistent with the antigen having the location expected of a cytosol-facing, peripheral membrane protein. The egg surface, possibly the plasma membrane, was also strongly stained with these monoclonal antibodies (Fig. 10C).

Discussion

Resealing may employ several novel fusion protein components, specialized for this emergency-based cellular reaction (McNeil and Kirchhausen, 2005). Two recently described candidates in mammalian cells, dysferlin (Bansal et al., 2003) and annexin (Lennon et al., 2003), are both Ca^{2+}-activated phospholipid-binding proteins. However, the exact

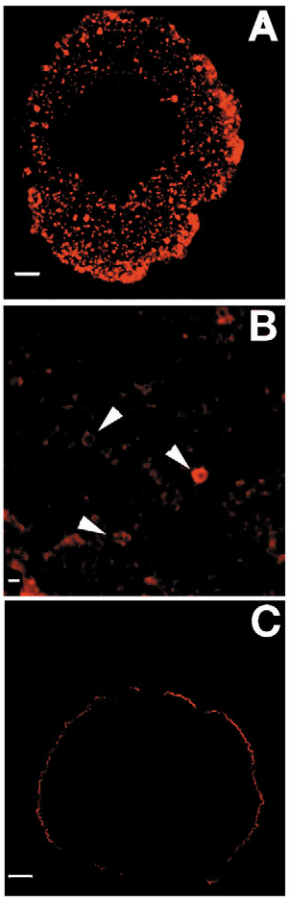


Fig. 10. Immunostaining of starfish eggs with a monoclonal antibody raised against tethering factor. (A) A ubiquitous, ~1 μm diameter, extranuclear compartment is labeled by a monoclonal antibody raised against the purified tethering complex. This pattern is highly consistent with yolk granule labeling. (B) Higher magnification reveals that the intracellular compartment staining is peripheral (arrowheads). This is consistent with labeling of a peripheral protein. (C) At a low-gain setting, intense staining of the plasma membrane is emphasized in this confocal micrograph. Bar, 10 μm (A,C); 1 μm (B).

roles of dysferlin and/or annexin in resealing remain unknown. Do these proteins participate in early events, such as tethering or docking of membranes, and/or as the Ca^{2+} sensor, and/or in the actual fusion or bilayer merger step? An answer to these questions requires that the various steps of a fusion process can be staged in vitro and the effects of manipulation of a protein at each stage observed. This paper reports the results of just such an approach using reconstituted yolk granule fusion as a model system to study an initial step – tethering – in a resealing-based fusion process.

Tethering is proposed to be an early, reversible event in numerous membrane fusion reactions (Whyte and Munro, 2002). Single, extremely large coiled-coil proteins, or multisubunit tethering complexes, are envisioned to bind to and physically span two membranes destined for fusion, restricting their ability to move apart. This forced physical interaction is hypothesized to promote, in turn, activity of downstream components of the fusion machinery, such as the SNAREs. The proximal nature of the tethering step in fusion suggests that tethers may confer specificity. Such a role is consistent with the known sequence diversity of tethers, and with their precise spatial localization to specific organelles (Waters and Hughson, 2000) and strikingly, to localized domains of the plasma membrane (Guo et al., 1999). Here we propose an additional role for membrane tethers: they are required for the production of large membrane fusion products, such as those utilized in a successful resealing response.

We have shown, by reconstituting yolk granule fusion in vitro and analyzing the staging of this reaction, that a Ca^{2+}-independent tethering step precedes Ca^{2+}-triggered granule fusion. Fusion clearly occurs preferentially between the members of a tethered granule population: large tethered clusters from large fusion products. We suggest that tethering in the egg is an essential step in the repair of large plasma membrane disruptions, which, as much work has demonstrated, are 'patched' by proportionately large products of homotypic fusion.

It is conceivable that large fusion products could be produced in the absence of tethering by multiple rounds of fusion occurring sequentially over time. The behavior of yolk granules, as documented here and elsewhere, rules out this possibility. Granule fusion events triggered by Ca^{2+} are constrained to a narrow, second-scale time window. We propose that tethering of one yolk granule to another ensures that a fusion partner is nearby during this narrow time window: tethering thus acts kinetically to promote fusion in this system. The self-limiting nature of the Ca^{2+}-induced fusion response observed in vitro would, in vivo, be of benefit as a means for limiting fusion events to those productive of repair.

This study is the first to identify protein components of resealing-based yolk granule homotypic fusion. From a highly simplified starting material, the approximately 15 major proteins stripped from the granule by a mildly chaotropic salt wash, a fraction enriched in tethering activity was produced by anion exchange chromatography. This fraction, which SDS electrophoresis resolves into seven major protein bands, behaves, under native conditions, as a high molecular weight complex based on analysis by size exclusion chromatography and non-denaturing gel electrophoresis. Immunodepletion of the complex inhibits tethering, confirming its role in this event in vitro. Immunostaining with a monoclonal antibody raised against one of the components of the tethering complex confirm that it associates strongly with the yolk granule, and not other major egg organelles. Yolk granules, and not other known organelles, are required for egg resealing of large disruptions (Terasaki et al., 1997). The yolk granule tether may, in addition to promoting large fusion product formation, also determine the organelle utilized in its formation.

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