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Above the fray: Surface remodeling by secreted lysosomal enzymes leads to endocytosis-mediated plasma membrane repair

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Abstract

The study of plasma membrane repair is coming of age. Mirroring human adolescence, the field shows at the same time signs of maturity and significant uncertainty, confusion and skepticism. Here we discuss concepts that emerged from experimental data over the years, some of which are solidly established while others are still subject to different interpretations. The firmly established concepts include the critical requirement for Ca^{2+} in wound repair, and the role of rapid exocytosis of intracellular vesicles. Lysosomes are being increasingly recognized as the major vesicles involved in injury-induced exocytosis in many cell types, as a growing number of laboratories detect markers for these organelles on the cell surface and lysosomal hydrolases in the supernatant of wounded cells. The more recent observation of massive endocytosis following Ca^{2+} -triggered exocytosis initially came as a surprise, but this finding is also being increasingly reported by different groups, shifting the discussion to the mechanisms by which endocytosis promotes repair, and whether it operates or not in parallel with the shedding of membrane blebs. We discuss how the abundant intracellular vesicles that undergo homotypic fusion close to wound sites, previously interpreted as exocytic membrane patches, actually acquire extracellular tracers demonstrating their endocytic origin. We also suggest that an initial, temporary patch that prevents cyto-sol loss until the bilayer is restored might result not from vesicular fusion, but from rapid Ca^{2+} -dependent crosslinking and aggregation of cytosolic proteins. Finally, we propose that cell surface remodeling, orchestrated by the extracellular release of lysosomal hydrolases and perhaps also cytosolic molecules, may represent a key aspect of the plasma membrane repair mechanism that has received little attention so far.

Keywords

Plasma membrane; Repair; Resealing; Lysosome; Hydrolase; Sphingomyelinase; Protease

1. Introduction

Considering the long and distinguished history of the eukaryotic cell biology field, the systematic study of plasma membrane repair is relatively new, having been developed only

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since the 1990s. Based on what we know today about the cellular housekeeping pathways of metazoans, it is surprising that such a fundamental mechanism that ensures cell survival after injury did not receive more attention earlier. The reason for this may have been twofold. First, the frequency by which cells suffer plasma membrane wounds may have been underestimated. Second, when plasma membrane damage was observed, it was assumed that cells would either die, or membrane integrity would be restored by spontaneous lipid bilayer resealing. In recent years great strides were made in our understanding of plasma membrane repair, leading to the realization that it represents an active, energy-dependent process that is orchestrated by specific membrane traffic events. Membrane traffic is an extremely well developed area of cell biological research, so there are now powerful tools available to study how cells reseal after injury. In this review we discuss a few important concepts that are emerging in this new field, and suggest a framework for the integration of observations that may appear, at first glance, disconnected and even contradictory with each other.

2. The great consensus: Ca^{2+} , ATP and the exocytosis of endomembranes are required for plasma membrane repair

Studies performed in the 1950s in sea urchin eggs revealed the essential role of Ca^{2+} for plasma membrane repair. Injured eggs kept in seawater rapidly restored their membrane integrity and remained viable, even developing after fertilization. However, extracting Ca^{2+} from the seawater severely impaired resealing, leading to cytosol loss and cell death [1,2]. This seminal observation launched the field of plasma membrane repair, and highlighted the importance of extracellular Ca^{2+} for maintaining cell viability. This realization proved to be particularly important during conditions that cause plasma membrane injury, such as experiments involving cell motility and microinjection. Recent studies revealed that Ca^{2+} release from the lumen of lysosomes also plays a role in triggering plasma membrane repair, in addition to the influx of extracellular Ca^{2+} [3,4]. The steep Ca^{2+} gradient maintained across the plasma membrane (mM range extracellularly and nM range in the cytosol) allows Ca^{2+} to function as a very effective second messenger that activates cytoplasmic proteins involved in exocytosis and other steps of the wound repair process.

It was only several decades after the original studies with sea urchin eggs that the cellular mechanism responsible for membrane resealing began to emerge. Work from the McNeil and Steinhardt laboratories in the 1990s demonstrated an important concept -that Ca^{2+} entry into wounded cells triggers exocytosis of intracellular vesicles close to the wound site [5,6]. Neurotoxins known to inhibit SNARE-mediated membrane fusion interfered with the ability of cells to reseal after injury, consistent with the involvement of membrane traffic events [7]. These key observations gave rise to two initial hypotheses to explain how exocytosis facilitates plasma membrane repair. The first, known as the “patch” hypothesis, postulated that Ca^{2+} would trigger homotypic fusion of pre-existing intracellular vesicles, forming a large vesicle that would then somehow be flattened and fused to the wound, “patching” the membrane [8]. The second, the tension release hypothesis, postulated that Ca^{2+} -dependent exocytosis in the vicinity of the wound would reduce membrane tension (an event that can be measured experimentally), facilitating spontaneous resealing of the lipid bilayer [9]. Both hypotheses were firmly grounded on the need for endomembrane to enable the repair

machinery, a requirement that was elegantly demonstrated in experiments comparing the ability of nucleated cells and red blood cells to repair plasma membrane wounds [10]. Below we discuss recent evidence that is significantly expanding our understanding of the ATP-dependent [11] membrane traffic events responsible for restoration of plasma membrane integrity.

3. It is time to revisit the “patch” hypothesis - the abundant vesicles next to wound sites originate by endocytosis, not exocytosis

The observation of intracellular vesicles fusing with the plasma membrane a few seconds after wounding [5,6] led to an investigation of the nature of those Ca^{2+} -sensitive exocytic compartments. The major membrane-bound compartments (Golgi, ER, early endo-somes, late endosomes and lysosomes) of CHO cells were tagged with fluorescent markers and imaged by total internal fluorescence microscopy after exposure to a Ca^{2+} ionophore. This technique, which allows a precise visualization of vesicular fusion events at the plasma membrane, identified lysosomes as the only tagged vesicles capable of rapid exocytosis in response to an elevation in cytoplasmic Ca^{2+} [12]. These results were fully consistent with previous experiments showing that conventional lysosomes behave as Ca^{2+} -regulated secretory vesicles when the intracellular free Ca^{2+} concentration rises above 1 μM [13]. Additional studies demonstrated that interfering with the molecular machinery that mediates lysosome exocytosis inhibits plasma membrane repair [4,14]. Fusion of lysosomes with the plasma membrane in response to injury has now been observed in several laboratories with different cell types, including cells exposed to pathogens, wounded mechanically or permeabilized by pore-forming proteins such as bacterial toxins or perforin from cytotoxic lymphocytes (CTL) [14–20]. Given the consistency by which lysosomal exocytosis is observed in wounded cells, it has been increasingly suggested that lysosomes are the main intracellular organelle that originates the “patch” responsible for membrane resealing [18,21–24].

Recent findings, however, suggest a very different interpretation. Light and electron microscopy imaging experiments, performed shortly after plasma membrane wounding, led to a surprising discovery. When Ca^{2+} enters cells through plasma membrane wounds and exocytosis of lysosomes occurs, there is also a rapid and massive formation of endocytic vesicles, which accumulate close to the injury site. These vesicles are clearly originated by invagination of the plasma membrane, because they retain in their lumen BSA-gold added extracellularly before wounding [11,25]. Independent electrophysiological studies confirmed that exocytosis triggered by Ca^{2+} influx is rapidly followed by massive endocytosis [26,27]. Accumulation of newly formed endosomes was also observed in CTL target cells after permeabilization with perforin [19]. A detailed study of the fate of streptolysin O (SLO) pores inserted on the plasma membrane revealed that the toxin is internalized in injury-induced endosomes and directed to lysosomes for degradation [28]. Collectively, these findings call for a re-interpretation of the observations that led to formulation of the “patch” hypothesis of plasma membrane repair. Those earlier experiments [5,8] were not performed in the presence of extracellular endocytic tracers, so the numerous vesicles observed next to wound sites were interpreted as pre-existing intracellular compartments, proposed to be

recruited to the wound site, fuse homotypically in response to Ca^{2+} and then applied to the wound as a repair “patch” [29]. However, the presence of endocytic tracers in the lumen of the abundant vesicles generated after wounding changes this view, indicating that after lysosomal exocytosis there is a massive formation of small endosomes (<100nm) that gradually merge with each other forming larger endosomes [25] that subsequently join the endocytic pathway and deliver their contents to lysosomes for degradation [28] (Fig. 1). Caveolae, ~80 nm plasma membrane invaginations that are abundant in muscle fibers and other cells prone to injury such as endothelial cells [30,31], were recently implicated in this massive endocytic response, and in the removal of plasma membrane lesions [32]. As further discussed below, these findings provide an explanation for the serious muscle pathology associated with mutations in proteins required for caveolae formation [33,34].

4. Repair of large and small wounds - similarities and differences

An interesting ongoing discussion in the field is whether different mechanisms are involved in the repair of large and small lesions. This possibility was initially raised when lipid bilayer tension reduction was proposed to play a more important role in the resealing of small lesions, while exocytic “patching” would repair large wounds [35]. However, this idea has been difficult to test, because of the lack of approaches for accurately assessing wound size. A recent study addressed this issue with an imaginative assay combining quantitative analysis of the influx of a membrane impermeant dye with mathematical modeling. This study concluded that components of the ESCRT III complex, involved in deforming membranes and promoting vesicle budding away from the cytosol, are required for the repair of lesions smaller than 100nm, but not of larger ones [17]. This was an intriguing finding, because the ESCRT-mediated vesicles described so far are quite small, in the 50 nm range [36], while membrane blebbing events seen during wounding and repair are significantly larger [17,20]. As discussed elsewhere [37], disruption of the cortical actin cytoskeleton at the site of Ca^{2+} influx triggers the transient formation of large plasma membrane blebs, which retract back into the cell body within a few seconds, as the cell reseals [38]. Thus, additional studies are needed to clarify whether plasma membrane blebbing and shedding are a phenomenon that occurs in parallel with other wound repair mechanisms, or if it is directly responsible for lesion removal [20,24]. It will be important to determine if there is massive membrane shedding specifically from wounded cells undergoing resealing, if this occurs at a rate compatible with the rapid kinetics of plasma membrane repair, and whether this mechanism only applies to wounds of a certain size range.

When cells permeabilized by SLO toxin pores (in the 30 nm diameter range) or wounded by scraping (which is assumed to generate larger lesions) were examined in parallel, it was found that Ca^{2+} influx, lysosomal exocytosis and massive endocytosis were involved in the repair of both types of lesions [11,25,32]. Strikingly, a differential ability of cells to reseal after SLO permeabilization or scrape wounding was observed when cells were depleted in components of the exocyst, a cytosolic protein complex involved in polarized exocytosis events [39]. The exocyst components Exo70 and Sec8 were needed for the repair of mechanical wounds, but not for resealing of SLO-permeabilized cells [40]. These results suggest an additional level of complexity in the resealing of larger, irregular wounds, which

may require the highly localized, spatially regulated exocytic events coupled to interactions with the actin cytoskeleton that are characteristic of the exocyst complex [41]. This is a very interesting possibility, because it can explain the apparent contradiction regarding involvement of the actin cytoskeleton in the resealing of certain forms of wounds, but not others [24,42].

5. Ca^{2+} -activated proteins implicated in plasma membrane repair: do we understand how they work?

A substantial amount of evidence indicates that cytosolic proteins activated by Ca^{2+} participate in plasma membrane repair. Annexins in particular have been implicated by several independent studies, ranging from quantitative trait locus mapping of genetic modifiers associated with sarcolemma damage [43] to the direct rescue of resealing defects by recombinant annexins [44]. Annexins are soluble cytosolic proteins that when exposed to elevated Ca^{2+} bind tightly to membranes exposing negatively charged phospholipids such as phosphatidylserine (PS), which are abundant on the cytosolic leaflet of the plasma membrane [45]. The 12 different annexin isoforms found in vertebrates share a highly conserved 70 amino acid repeat domain that forms the membrane-binding core, while a variable amino-terminal domain is thought to confer functional specificity [46]. Considerable variation has been observed in the Ca^{2+} concentration required to recruit different annexins to membranes [46], making members of this family attractive candidates for the management of plasma membrane wounds, given that the amount of Ca^{2+} entering the cytosol is likely to be directly related to the size of the lesion.

A particularly appealing model was proposed recently for the membrane repair role of AnxA5, which along with Anx4, Anx6, AnxB12 and the (AnxA2-p11)₂ tetramer complex, self-organizes into two dimensional arrays on biological membranes [46]. It was proposed that upon local influx of Ca^{2+} , AnxA5 molecules bind tightly to torn membrane margins where intracellular PS is exposed, forming a platform that increases membrane rigidity and prevents the wound from expanding. Remarkably, extracellular addition of AnxA5 rescued the severe membrane repair defect of AnxA5 null cells, an effect that was not observed with AnxA5 carrying mutations that prevent formation of two-dimensional arrays on membranes [44]. These results suggest that in the presence of Ca^{2+} annexins can rapidly associate with the exposed inner layer of torn membranes, forming a structure that facilitates repair. It is tempting to speculate that a membrane-associated annexin platform, which has been often observed on wounds [43,44,47], may go beyond the proposed role of preventing lesion expansion. Annexins are highly reactive proteins with binding sites for various molecules, making them ideal scaffolds for the recruitment of additional cytosolic proteins. In particular, annexins are strongly cross-linked by transglutaminase, following assembly into PS-containing membranes in response to Ca^{2+} [48,49]. Transglutaminases belong to a family of enzymes lacking a secretory signal peptide that catalyze Ca^{2+} -dependent acyl transfer reactions, which result in cross-linking between free amine groups and peptidyl glutamine residues of proteins [50]. Interestingly, transglutaminase 2 was implicated in plasma membrane resealing after mechanical damage in lung cancer cells [51]. Thus, the rapid assembly of an annexin platform at sites of membrane damage, coupled to extensive cross-

linking by transglutaminases or other Ca^{2+} -reactive cytosolic proteins, may create a transient protein-based barrier to prevent cytosolic loss (Fig. 2). Such a “clot” mechanism, referred to as a surface precipitation reaction, was actually described in one of the very early studies of plasma membrane repair [1]. This proposed mechanism is very consistent with previous imaging studies showing dense annexin clusters associated with wound sites, and with the suggestion that these protein aggregates prevent cytosol loss while allowing the more definitive membrane-mediated repair to proceed [24,43]. Future studies should clarify additional intriguing aspects of the mechanism by which annexins facilitate plasma membrane repair, including their interaction with S100A11, a protein up-regulated in metastatic tumors that was suggested to facilitate resealing by stimulating cortical actin polymerization [52].

In earlier work we identified Syt VII as a member of the synaptotagmin family of Ca^{2+} sensors present on the membrane of lysosomes, and showed that its cytoplasmic Ca^{2+} -binding C2 domains regulate lysosomal exocytosis and plasma membrane repair [53,14]. Syt VII null mice develop inflammatory myopathy, consistent with the importance of membrane repair in maintaining the integrity of skeletal muscle fibers, a tissue under high risk of mechanical injury [54]. Further reinforcing this concept, another study described plasma membrane repair defects in muscle fibers carrying mutations in dysferlin, a synaptotagmin-like protein that is highly expressed in skeletal muscle and associated with human muscular dystrophy [55]. That study was followed by a flurry of investigations on the mechanism by which dysferlin would promote wound repair, and the initial proposal was that it serves a vesicular Ca^{2+} sensor to facilitate the fusion of membrane “patches” to sites of injury on the sarcolemma [18,56]. However, the steady state localization of dysferlin on the sarcolemma and transverse tubules of intact fibers and its detection on endocytic vesicles after wounding [57] suggests alternative explanations, including a role as a sarcolemma-resident Ca^{2+} sensor that promotes lysosomal exocytosis in wounded myocytes [58]. Nonetheless, the role of dysferlin may be more complex, given its many reported interactions with other proteins implicated in muscle wound repair, such as mitsugumin-53 (MG53) and caveolin-3 [59,60]. Another interesting twist is that the Ca^{2+} -dependent cytosolic protease calpain, which has been implicated in plasma membrane repair [61,62], can cleave dysferlin generating a C-terminal fragment that is specifically recruited to wound sites [63]. Regardless of what emerges regarding the precise mechanism of action of dysferlin, the long array of potential Ca^{2+} -binding sites in the form of seven C2 domains suggests that this protein is part of an expanded machinery to sense Ca^{2+} and increase the efficiency of sarcolemma repair in skeletal muscle.

6. Look outside: a role for extracellularly released proteins in remodeling the plasma membrane and facilitating repair

The rapid exocytosis of lysosomes observed in wounded cells was, until recently, assumed to facilitate repair by adding endomembranes to the cell surface. However, lysosomes contain more than fifty different hydrolases, and many of the assays to quantify lysosomal exocytosis are actually based on the extracellular detection of such enzymes [64]. A possible extracellular function of lysosomal enzymes in plasma membrane repair started to emerge

after it became apparent that exocytosis of lysosomes in wounded cells is followed by massive formation of uncoated endocytic vesicles [11,26,27]. Searching for the origin of these injury-dependent endosomes, an exciting connection was established with an earlier study describing peripheral endosomes of very similar morphology in cells treated with bacterial sphingomyelinase [65], an enzyme that converts the abundant plasma membrane lipid sphingomyelin into ceramide [66,67]. Lysosomes contain acid sphingomyelinase (ASM), which was actually shown in several studies to function extracellularly generating ceramide-enriched domains on the cell surface [68–70]. Such unexpected detection of extracellular, active ASM led to the suggestion that this enzyme might exist in two forms, one lysosomal and another secreted [71]. However, the origin of the secreted form of ASM remained puzzling, given that only a single transcript is found in mammalian cells, and the majority of the enzyme is inside lysosomes [68]. Cell wounding and repair experiments clarified this issue, revealing that ASM is promptly secreted through Ca^{2+} -triggered lysosomal exocytosis, and that deficiency in ASM inhibits plasma membrane resealing [25]. Strikingly, extracellular addition of human recombinant ASM rescued the plasma membrane repair defect of ASM-deficient cells, providing for the first time a link between ASM release from lysosomes, formation of ceramide-enriched endosomes, and membrane resealing [25]. Ceramide-enriched microdomains were shown experimentally to cause invagination of lipid bilayers [66], suggesting a mechanism by which ASM released from lysosomes in wounded cells may act extracellularly to induce rapid formation of endosomes. Such a mechanism also raises the intriguing possibility that additional lysosomal hydrolases such as proteases may also be involved in remodeling the surface of wounded cells and facilitating resealing (Fig. 3).

Recent studies in fibroblasts, epithelial cells and skeletal muscle fibers showed that the endocytic vesicles that accumulate at wound sites consist of caveolae [32]. This was an exciting finding, because a hallmark of caveolae is their enrichment in cholesterol and sphingolipids [30]. Cholesterol extraction potently inhibits both injury-induced endocytosis and plasma membrane repair [11], and sphingomyelin is converted into ceramide by ASM, which promotes membrane repair [25,32]. Ceramide has been detected in association with caveolae [72], making these structures strong candidates for the site of action of ASM in triggering endocytosis and wound removal. Caveolins, proteins required for caveolar formation, are required for efficient membrane resealing in NRK cells and myofibers, in agreement with the extensive reports of muscle pathology associated with mutations in caveolins [33] and also cavin, another structural protein of caveolae [34].

This novel concept, that extracellular events may drive the process by which injured membranes are ultimately resealed, can also shed light on a number of earlier observations that remain poorly understood. For example, although annexins lack a signal sequence for membrane translocation and are therefore largely cytosolic, many of the best-characterized functional roles of annexin occur in the extracellular milieu, where they have been shown to promote anti-inflammatory, pro-fibrinolytic and anti-thrombotic responses [46]. One interesting possibility is that cytosolic annexins may gain access to the cell surface through wounds on the plasma membrane, where they might mask membrane areas with exposed PS and thus prevent uptake of injured cells by phagocytes. Intriguing evidence suggests that MG53, a cytosolic TRIM family E3 ubiquitin ligase involved in muscle fiber wound repair,

might also function extracellularly. Recent studies reported a marked improvement in acute injury animal models by intravenous injections of recombinant MG53 [73–75], suggesting that its mechanism of action may not require that the protein enter cells and join the cytosolic vesicular “patch” repair machinery, as originally proposed [60].

7. Concluding remarks: how far are we from a unified plasma membrane repair theory?

The field of plasma membrane repair has grown considerably in recent years, with many laboratories reporting novel findings that are refining our understanding of this fundamental process. It is now important to determine how the experimental observations relate to each other, and whether there are independent mechanisms or a single resealing pathway that springs into action regardless of the type of lesion. We suggest that the vesicular “patch” hypothesis may have arisen from misinterpretation of endocytic vesicles as exocytic, and emphasize the importance of recent evidence for wound removal, and for an extracellular role of factors released from wounded cells. Another aspect that should be incorporated into future working models is the role of Ca^{2+} -triggered cross-linking and aggregation of cytosolic proteins, which might form a temporary “clot” important for preventing the loss of cytosol from injured cells—as actually suggested in very early studies [1]. Whether shedding of membrane blebs is a major mechanism of lesion removal also deserves further investigation, using biochemical methods to determine the size and content of the released vesicles. Mirroring the spectacular progress seen in the broader field of membrane traffic, it is time to move from static images of fixed cells, which can be misleading, to high resolution live imaging and biochemistry approaches to arrive at more robust models of plasma membrane repair. Caution should also be taken regarding experimental wounding methods; one concern is that the widely used laser wounding approach generates very high local temperatures, which can cause protein denaturation and thermal diffusion artifacts. Despite the challenges of working with a complex process that involves sequential steps very hard to reproduce in cell-free systems, the new perspectives that are emerging will certainly result in more advanced views of the plasma membrane repair mechanism. In a quote attributed to Max Planck, “science advances one funeral at a time”. Now that the field of plasma membrane repair has grown and matured, we may have to go through some funerals to reach a better understanding of this fundamental and fascinating process.

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Fig. 1.

Diagram showing that addition of extracellular tracers identifies the vesicles accumulating next to wound sites as endocytic, and not an exocytic “patch”. (A) In earlier experiments performed without endocytic tracers in the medium, the numerous vesicles observed next to wound sites were thought to undergo Ca^{2+} -dependent homotypic fusion and form a large exocytic patch to reseal the membrane. (B) When wounding experiments were performed in the presence of BSA-gold the vesicles next to wound sites incorporated the extracellular tracer, showing that they originated by endocytosis. These endocytic vesicles subsequently merge generating larger endosomes that join the endocytic pathway, trafficking deeper into the cells and fusing with lysosomes [28].

Fig. 2.

Diagram of a mechanism by which Ca^{2+} -dependent annexin platforms and cross-linked cytosolic proteins could prevent cytosol loss from wounded cells. (A) Intact plasma membrane, with soluble annexin molecules in the low Ca^{2+} environment of the cytoplasm. (B) When the plasma membrane is wounded, Ca^{2+} flows in and cytosol starts to leak. (C) Cytosolic Ca^{2+} elevation recruits annexin to the inner leaflet of the plasma membrane, where it binds to exposed (PS) on the edge of the wound. (D) The membrane-associated

annexin platform serves as a scaffold for the aggregation of cross-linked cytoplasmic proteins, forming a clot that stops cytosol leakage.

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Fig. 3.

Diagram illustrating the role of secreted lysosomal hydrolases in remodeling the plasma membrane and promoting lesion endocytosis. Upon wounding and Ca^{2+} influx, peripheral lysosomes rapidly fuse with the plasma membrane, releasing their contents extracellularly. The lysosomal enzyme acid sphingomyelinase (ASM) converts membrane sphingomyelin into ceramide, promoting membrane invagination and formation of endosomes that remove lesions and promote membrane resealing. Additional lysosomal hydrolases may regulate this process.