of this process is under way, and members of the SNARE family (membrane proteins thought to be required for many fusion events) are implicated in resealing (8). However, as an emergency response it must be rapid and Ca\(^{2+}\)-responsive and yet capable of being activated with a great level of temporal and spatial flexibility. Therefore, it has been hypothesized that resealing-based fusion may utilize, in addition to canonical elements such as the SNAREs, a specialized subset of fusion components (9). Dysferlin, a Ca\(^{2+}\)-activated membrane-binding protein that mediates exocytotic fusion events in nematodes (10), is one candidate emergency fusion component. Thus, it was recently shown that skeletal muscle cells from dysferlin null mice fail to reseal disruptions (3).

One protein shown by immunolocalization and immunoprecipitation to associate with dysferlin in normal, undisturbed skeletal muscle is annexin A1 (11), a member of the annexin family of Ca\(^{2+}\)-regulated membrane-binding proteins (12). This observation, as well as the following additional points, suggested that annexin A1 might be another component of the repair machinery. First, tissues expressing annexin A1 include those mentioned above as known locales of plasma membrane disruption. Second, humans with Duchenne muscular dystrophy, in which the frequency of sacrolemma disruption is greatly increased, exhibit enhanced levels of skeletal muscle annexin (several family members) expression (13), and humans with dysferlin-related muscular dystrophy specifically exhibit an increase in annexin A1 expression (14). Third, Ca\(^{2+}\)-activated annexin A1 can aggregate and, in some cases, fuse phospholipids bilayers (12). Finally annexin A1 was shown recently to participate in a specific fusion event, the inward vesiculation in multivesicular endosomes, which is driven by budding and fusion of the organelle-limiting membrane (15). Clearly, however, the question of whether annexin A1 is in fact a component of the resealing machinery can only be answered by direct functional and structural such as are described here.

### EXPERIMENTAL PROCEDURES

#### Expression Vectors, Transfection, and Cloning—HeLa and BS-C-1 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and penicillin/streptomycin. The annexin A1 green fluorescent protein (GFP) expression vectors have been described and were originally characterized elsewhere (16). For transfection, subconfluent
HeLa and BS-C-1 cells were scraped from 35-mm dishes in 200 μl of PBS containing 20 μg of vector. Enrichment of GFP-expressing cells was then achieved by antibiotic-based selection followed by subcloning.

Population Wounding and Reagent Loading—For the peptide inhibition studies, subconfluent HeLa cells were scraped at 37 °C from 35-mm dishes with a rubber policeman in 200 μl of PBS alone or in PBS containing peptide. Cmanx1-GFP-positive HeLa cells and nontransfected HeLa cells were scraped in the same way, except that Texas Red dextran (TRDx, 70 kDa, Molecular Probes) was added (1 mg/ml) to all PBS solutions. For the antibody inhibition studies, the scrape-loading technique was modified so that we could maximize antibody introduction into cell cytosol. HeLa populations were scraped 1–2 h after being seeded into 96-well plates (instead of 35-mm dishes) with the narrow end of a standard 200-μl laboratory pipette (“yellow”) tip (instead of a rubber policeman) in 5 μl (instead of 200 μl) of fluorescein dextran (FDx, 156 kDa, Molecular Probes) containing the same concentration (250 μg/ml) of mouse monoclonal anti-annexin A1 antibody (BD Biosciences, whole human annexin A1 as antigen) or the same isotype mouse antibody (provided as a gift by L. Ignatowicz, Medical College of Georgia, Augusta).

FACS Analysis—HeLa and BS-C-1 cells were centrifuged after scraping or other wounding procedures for 5 min at 1000 rpm. The cell pellet was resuspended in 200 μl of PBS. Samples were kept on ice until analysis, and propidium iodide (PI; 0.05 mg/ml) was added to cell suspensions immediately prior to FACS analysis of the dye (PI, FDx, TRDx) staining level carried out as described previously (17). Histograms were obtained by analyzing 10,000 cells with CellQuest Pro (BD Biosciences).

Laser Wounding and Image Analysis of Resealing—Cells cultured in 35-mm dishes were subjected to laser wounding at 37 °C in the presence of FM-143 dye, and dye uptake and/or GFP fluorescence were monitored by time lapse image acquisition and image analysis, all as described previously (18).

Immunostaining—Subconfluent cultures of cells grown in glass slide wells were scratched with a syringe needle (31-gauge) in the presence of FDx (1 mg/ml, 10 kDa). Fixative (4% formaldehyde in PBS) was then added immediately, and the cells were processed further by standard methods for intracellular immunostaining with the anti-annexin A1 antibody (see above) as described previously (19).

RESULTS

Inhibition of Plasma Membrane Resealing by Annexin A1-interfering Reagents—We first employed anti-annexin A1 antibody to interfere specifically with intracellular activities of the protein. The most common approach for utilizing an antibody to disrupt protein functioning in vivo is to load it into cell cytosol, for example by microinjecting or scrape-loading it, and then to assess the effect on the cell response in question. For this purpose we developed a miniaturized version of the scrape-loading technique, applicable to cell cultures growing in 96-well plates and consuming less than 10 μl of a valuable reagent such as an antibody. Its efficiency, measured as the percentage of cells successfully “loaded” with a membrane-impermeant dye, FDx (10 kDa), was comparable with that obtained using a much larger culture format (6-well plate) and volume (200 μl) of loading reagent (Fig. 1A). Moreover, in terms of its consumption of reagent, measured as the mean fluorescence level obtainable in a population cultured in the larger or smaller format plates and scraped in identical amounts of this dye, it was clearly superior: a 3-fold higher level of fluorescence was obtained when the miniaturized technique was employed (Fig. 1B). We suggest that scrape loading is now comparable in its economic consumption of a valuable reagent to microinjection, yet scrape loading remains capable of rapidly loading thousands of cells in one simple maneuver that requires a minimal amount of equipment and skill. This miniaturized scrape-loading technique could therefore find widespread use as a superior method for economically loading into living cells membrane-impermeant reagents such as antibodies, DNA and RNA.

When we attempted scrape loading of annexin A1 antibody mixed with a 156-kDa FDx as a loading marker, no brightly fluorescent, FDx-positive BS-C-1 cells were observed microscopically 3–5 h later (data not shown). By contrast, in control populations scraped in control antibody, numerous heavily labeled cells could be seen; these had suffered large disruptions, admitting a significant quantity of a large macromolecule (156-kDa), and resealed, trapping that macromolecule in their cytosol. The failure to obtain FDx-positive cells when the scraping was carried out in the presence of anti-annexin A1 antibody prevented us, as intended, from using a microscopic, laser-based resealing assay (18); the requisite population, heavily loaded with anti-annexin A1 antibody, was not present. However, it suggested that the antibody might have an effect on repair of the disruption through which it was entering and that the most heavily wounded cells in the population were failing to reseal and hence to trap the FDx. To test this possibility quantitatively, cells were scraped in saline containing anti-annexin A1 antibody and FDx or containing FDx only, and the populations analyzed immediately by FACS. Wounded cells that failed to reseal could be stained post-injury by the addition of PI, a membrane-impermeant nuclear stain that enters through the disruption. Contrastingly, those wounded cells that succeeded...
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at resealing retained, behind a restored permeability barrier, FDx that entered into cell cytosol through the disruption. FACS revealed that populations scraped in the presence of antibody contained almost twice as many PI-positive cells as those scraped in a control, irrelevant antibody (Fig. 2A). Moreover, cells scraped in anti-annexin A1 antibody failed to trap FDx (Fig. 2B). Thus two independent measures, cell permeability to PI and FDx trapping, indicated that antibody-mediated perturbation of annexin A1 potently inhibits resealing.

Annexin A1 has two well characterized domains of potential importance for its normal functioning. One is the “N-terminal interaction domain,” an N-terminal sequence of ~40 residues that is unique in annexin A1 and binds several proteins including S100A11, a member of the S100 family (12). Peptides of the N-terminal interaction domain have previously been shown to disrupt interactions of annexins with specific protein ligands, and perhaps most importantly for the present study, the N-terminal domain is required for annexin A1 membrane aggregation activity (20). Therefore, we next employed as a potential function-inhibiting reagent a peptide comprising the N-terminal 1–13 residues of annexin A1. This peptide contains the S100A11 binding site and has been shown to interact specifically with S100A11 (21). Resealing of HeLa cells after scraping was Ca$^{2+}$-dependent (Fig. 3A, left panel). When HeLa cells were scraped in the presence of annexin A1 peptide (Ac1–13), the level of PI staining was significantly increased above control levels, and this differential effect was also Ca$^{2+}$-dependent (Fig. 3A, right panel). The inhibitory effect of the N-terminal peptide on resealing was not observed when cells were scraped in a control peptide consisting of a scrambled sequence of the amino acid residues present in the inhibitory peptide (Fig. 3B). These results further support the possibility that annexin A1 is involved in the resealing response and suggest, moreover, that its interaction with other proteins, mediated through its N-terminal sequence, is crucial to this role.

A Ca$^{2+}$-insensitive Annexin A1 Mutant Protein Dominantly Inhibits Plasma Membrane Resealing—A second annexin A1 domain, of special relevance for a Ca$^{2+}$-activated response such as resealing, is the Ca$^{2+}$-binding core domain. To investigate the potential role of this domain, a HeLa cell clone was generated that expressed high levels (Fig. 4A, lower left panel) of a mutant annexin A1 incapable of Ca$^{2+}$ binding (Cmanx1) linked to GFP. Cmanx1-GFP would be predicted to act in a dominant-negative fashion as an inhibitor of endogenous annexin A1 function by engaging in protein interactions through the N-terminal domain but not linking them to Ca$^{2+}$-sensitive resealing (16). We found that, unlike HeLa cells expressing wild type annexin GFP (Wtanx1; Fig. 4A, left upper panel), Cmanx1-GFP expressing cells (accompanying left lower panel) do not display Ca$^{2+}$-sensitive resealing; approximately equal numbers of Cmanx1-GFP cells were PI-positive whether or not Ca$^{2+}$ was present (Fig. 4A, right panel). We also assessed, as described above for the antibody experiments, the ability of

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**FIGURE 2. Inhibition of resealing by an antibody directed against annexin A1.** A. Upper panels, typical FACS analysis of the percentage of PI-positive HeLa cells that failed to reseal following scraping in the presence of a control identical isotype antibody or anti-annexin A1 antibody. Lower panel, averaged results of four independent analyses such as those on the upper panels of cells scraped in the presence of a control or anti-annexin A1 antibody. Error bars indicate ±S.D. (*, p < 0.0001; t test, n = 4). B. FACS analysis of cell fluorescence following scraping in FDx (156 kDa) in the presence of control antibody (green profile) or with anti-annexin A1 antibody (red profile). The mean fluorescence levels of each population are indicated (color-coded, upper right-hand corner) as well as the percent of cells in each population exceeding the resealing threshold (color-coded numbers, above and below threshold bar).

**FIGURE 3. Inhibition of resealing by an annexin A1 N-terminal peptide.** A. Left panels, typical FACS analysis of the percentage of PI-positive cells present after scraping in PBS containing 1.5 mM Ca$^{2+}$ (+Ca) or containing no added calcium (–Ca). Right panel, averaged results of four independent analyses such as those on the left of cells scraped with 1.5 mM Ca$^{2+}$ (red bars) and without Ca$^{2+}$ (green bars) in saline containing increasing concentrations of annexin A1 peptide (Ac1–13 AMYSEFLQAWFI). Error bars indicate ±S.D. B. Typical FACS analysis of cells scraped in the presence of a peptide comprising a scrambled sequence (Ac-FAEVLSWFKMIAQ-OH) of annexin A1 amino acids 1–13 (Control peptide) and in increasing concentrations of the native peptide (Ac1–13).
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Annexin A1 Redistributes to Sites of Membrane Disruption—

Given the apparent importance of the Ca\(^{2+}\)-binding domain in rescaling, and knowing that Ca\(^{2+}\) activates phospholipid binding of annexin A1, we predicted that a redistribution of annexin A1 from the cytosol onto local membranes would occur at disruption sites. Previous studies (11) demonstrate an association of dysferlin and annexin A1 in uninjured skeletal muscle cells but did not test this more important prediction of wound-induced relocalization of annexin A1. To this end, we scratched monolayers of HeLa and BS-C-1 cells with a syringe needle in the presence of FDx and then rapidly (<10 s later) added fixative to the cultures prior to immunostaining with anti-annexin A1 antibody. This injury to the culture, denudation of linear stripes of cells, also caused injury to individual cells, specifically those lining the denuded site, many of whom were hit by the needle but not fully dislodged (22). This antibody stains a single band of the approximate molecular weight of annexin A1 in a Western analysis of whole cell lysates and does not cross-react with annexin A2 (data not shown). No staining was observed when the fluorescently tagged secondary antibody only was used for immunostaining (not shown). Distinct “hot spots” of annexin A1 antibody staining were observed in a subset of the cells in wounded cultures (Fig. 5A). This hot spot-positive subset of cells consisted entirely of FDx-positive, e.g. wounded, cells. Moreover, the hot spots were located within the wounded cells at presumptive sites of membrane disruption, e.g. on the cell boundaries nearest the scratch site where they were hit with the needle. Cells not wounded, e.g. FDx-negative, displayed a very uniform, presumably cytosolic fluorescence. In addition to marking wounded cells, FDx also conveniently served in this experiment as marker of cell thickness. Therefore, in ratio (Fig. 5A, Merged) images of the FDx (green)- and antibody (red)-generated fluorescence, yellow/red labeling indicates sites of annexin A1 concentration and not sites where a change in cell thickness had occurred, an artifact often overlooked in immunostaining studies of this type (23). To examine the kinetics of this wound-induced annexin A1 redistribution, we imaged living BS-C-1 cells expressing annexin A1-GFP during the creation of disruptions with a laser. A transient increase in annexin A1-GFP fluorescence that, curiously, swept as a wave through cytoplasm toward the disruption site (see supplemental movie M1), as well as punctate staining, was observed during the interval ~2–20 s after creation of a disruption (Fig. 5B). Simultaneous imaging of FM-143 influx in such cells (see supplemental movies M2 and M3) indicated that rescaling was taking place during this interval (Fig. 4C) provides a quantitative indication of rescaling kinetics). Importantly, in contrast with cells expressing Wtanx1-GFP, those expressing Cmanx1-GFP (supplemental movie M4) or GFP alone (not shown) did not exhibit a transient increase in brightness at disruption sites; instead, a uniform loss of fluorescence was observed (supplemental movie M4) in the Cmanx1-GFP cells that failed to rescale (as indicated by heavy internal staining with FM-143 dye, supplemental movies M5 and M6; see also Fig. 4C).

Cmanx1-GFP-expressing cells to trap (by resealing a large disruption) a large macromolecule, in this case TRDx (70 kDa). Fluorescence labeling resulting from scraping in TRDx was significantly diminished in Cmanx1-GFP cells relative to nontransfected HeLa cells (Fig. 4B). Finally, using a laser to create disruptions, we compared rescaling of Cmanx1-GFP BS-C-1 cells with that of nontransfected cells. In this assay, staining of internal membranes due to entry of an otherwise membrane-impermeant dye, FM-143, was monitored microscopically over time after the laser disruption (3). Rescaling blocks further dye entry through a disruption and hence halts cell staining. Measured whole cell fluorescence therefore plateaued if a cell succeeds at rescaling. Entry of FM-143 dye into Cmanx1-GFP-positive BS-C-1 cells was far more extensive than into wild type cells (Fig. 4C). Moreover, dye entry into the mutant cells continued throughout the time course measured, whereas in Y-type cells it plateaued at ~20 s post-laser wounding, indicating rescaling completion at this time point. Together, these three measurements in two different cell types strongly confirm the involvement of annexin A1 in rescaling. They additionally suggest that the Ca\(^{2+}\)-binding domain of this protein is crucial for this role.

FIGURE 4.
Inhibition of rescaling in cells expressing a mutant annexin.
A, left upper and lower panels, FACS analysis of cell fluorescence in a clonal population of cells expressing Wtanx1-GFP (Wtanx1) and Cmanx1-GFP (Cmanx1; solid red profiles) and nontransfected HeLa cells (empty green profile). Right panel, inhibition of rescaling in cells expressing Wtanx1-GFP and Cmanx1-GFP. Averaged percentage of PI-positive cells (failed rescaling) in the wtanx1-GFP (Wtanx1) and the Cmanx1-GFP (Cmanx1) clones after syringe wounding in PBS with 1.5 mm added Ca\(^{2+}\) (+Ca) or without (−Ca). Error bars indicate ± S.D. (*, p = 0.0003, **, p = 0.0659; t test, n = 4). B, average percentage of cells retaining TRDx (70 kDa) after scraping of the Cmanx1-GFP clone (Cmanx1, red) and of nontransfected HeLa cells (control, green) that were co-cultured at a 1:1 ratio. Error bars indicate ± S.D. (*, p < 0.0001; t test, n = 3). C, averaged cell fluorescence resulting from uptake of FM-143 at various time points after laser wounding of BS-C-1 cells expressing Cmanx1-GFP (Cmanx1) or wild type annexin (control). Error bars indicate ± S.E.

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Annexins, as we have already mentioned, are known to promote Ca\(^{2+}\)/H\(_{11001}\)-dependent membrane aggregation and fusion in vitro, and annexins A1 and A2 have been found to associate with dysferlin. This has lead to speculation that annexins might have a role in resealing (11), but no functional and structural evidence was available to support this hypothesis. Such functional data are now provided by three independent approaches. Using an inhibitory antibody, a peptide competitor, and a dominant-negative mutant protein to disrupt annexin A1 function, as well as three independent resealing assays, we demonstrate here a requirement for annexin A1 in resealing responses.

We propose that Ca\(^{2+}\) entering through a plasma membrane disruption causes cytosolic annexin A1 to bind to membranes surrounding the disruption site and thereby initiates the homotypic and exocytotic membrane fusion events of resealing.

Of particular relevance to our work, because they document a role for annexins in homotypic and exocytotic fusion events involving membranes of relevance to resealing, are the following studies. Annexin A1 was identified as the cytosolic factor that, in a Ca\(^{2+}\)-dependent fashion, aggregates isolated neutrophil-specific granules and promotes exocytotic fusion with the plasma membrane (24–28). Annexin A2 has been shown, using inhibitory antibodies and peptide competitors, to be required for Ca\(^{2+}\)-activated, homotypic fusion of endosomes, which have been implicated as an internal membrane source for resealing (29). Moreover, inhibitors of phospholipase A2 block fusion among endosomes both in vitro and in vivo, and this block that can be overcome in vitro by the addition of arachidonic acid (30). Finally, annexin A1 is required for the generation of internal vesicle of multivesicular endosomes, which requires budding and fusion events at the limiting membrane (15).

**FIGURE 5. Localization of annexin A1 after plasma membrane disruption.** A, images of FDx (FITC Dextran), annexin A1 immunostaining (Cy-3 Annexin A1), and a merge (Merged) of these two acquired from BS-C-1 and HeLa cultures that were scratched (along dotted line) in PBS (1.5 mM Ca\(^{2+}\)) containing FDx and then fixed immediately for intracellular immunostaining. Arrows mark probable plasma membrane disruption sites. These exhibit strong staining with the anti-annexin A1 antibody. B, images of a living BS-C-1 cell expressing wild type annexin-GFP that was wounded (arrow marks site) with a laser. The indicated times refer to the interval after laser wounding.

**DISCUSSION**

Annexins, as we have already mentioned, are known to promote Ca\(^{2+}\)-dependent membrane aggregation and fusion in vitro, and annexins A1 and A2 have been found to associate with dysferlin. This has lead to speculation that annexins might have a role in resealing (11), but no functional and structural evidence was available to support this hypothesis. Such functional data are now provided by three independent approaches. Using an inhibitory antibody, a peptide competitor, and a dominant-negative mutant protein to disrupt annexin A1 function, as well as three independent resealing assays, we demonstrate here a requirement for annexin A1 in resealing responses. We propose that Ca\(^{2+}\) entering through a plasma membrane disruption causes cytosolic annexin A1 to bind to membranes surrounding the disruption site and thereby initiates the homotypic and exocytotic membrane fusion events of resealing.

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Ca\(^{2+}\) entering through a disruption, it should be pointed out, is also predicted to activate cytosolic phospholipase A\(_2\), with consequent generation of arachidonic acid, which can promote
annexin-mediated fusion events (31, 32). In fact, it has been known for some time that resealing can be blocked by a cytosolic phospholipase A_2 inhibitor. Therefore, resealing may depend on the activation not only of annexin A1 but also of cytosolic phospholipase A_2.

A unique aspect of the fusion mechanism we are proposing is the cytosolic localization of a key protein component, annexin A1, at the time of receipt of the Ca^{2+} signal. We propose that following the recruitment of annexin A1 from this location onto membranes surrounding the disruption site, it would then promote the homotypic and exocytotic membrane fusion events required for resealing. Importantly, this initial cytosolic location maximizes the availability of annexin A1, a desirable attribute for an emergency fusion response whose location and timing are not predictable. Consistent with this recruitment model, our immunolocalization and GFP analysis clearly show that annexin A1 becomes concentrated during resealing at plasma membrane disruption sites.

This recruitment-based mechanism differs fundamentally from that proposed to mediate rapid Ca^{2+}-regulated fusion events such as synaptic exocytotic events. These and other rapid, regulated exocytotic responses that occur at a predictable location in a polarized cell are hypothesized to depend upon the rapid, regulated exocytotic responses that occur at a predictable location in a polarized cell. These and other events such as synaptic exocytotic events. These and other rapid, regulated exocytotic responses that occur at a predictable location in a polarized cell are hypothesized to depend upon the rapid, regulated exocytotic responses that occur at a predictable location in a polarized cell. These and other rapid, regulated exocytotic responses that occur at a predictable location in a polarized cell are hypothesized to depend upon the rapid, regulated exocytotic responses that occur at a predictable location in a polarized cell. These and other rapid, regulated exocytotic responses that occur at a predictable location in a polarized cell are hypothesized to depend upon the rapid, regulated exocytotic responses that occur at a predictable location in a polarized cell. These and other rapid, regulated exocytotic responses that occur at a predictable location in a polarized cell are hypothesized to depend upon the rapid, regulated exocytotic responses that occur at a predictable location in a polarized cell. These and other rapid, regulated exocytotic responses that occur at a predictable location in a polarized cell are hypothesized to depend upon the rapid, regulated exocytotic responses that occur at a predictable location in a polarized cell.

In conclusion, the simple mechanism proposed here can explain how a rapid, emergency fusion response can be elicited whenever and wherever needed. It does not rely on an elaborate network of interacting proteins between the exocytotic vesicle and plasma membranes (33).

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