A Plasma Membrane Wound Proteome

REVERSIBLE EXTERNALIZATION OF INTRACELLULAR PROTEINS FOLLOWING REPARABLE MECHANICAL DAMAGE

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Cells in mechanically active tissues undergo constant plasma membrane damage that must be repaired to allow survival. To identify wound-associated proteins, a cell-impermeant, thiol-reactive biotinylation reagent was used to label and subsequently isolate intracellular proteins that become exposed on the surface of cultured cells after plasma membrane damage induced by scraping from substratum or crushing with glass beads. Scrape-damaged cells survived injury and were capable of forming viable colonies. Proteins that were exposed to the cell surface were degraded or internalized a few seconds to several minutes after damage, except for vimentin, which was detectable on the cell surface for at least an hour after injury. Seven major biotinylated protein bands were identified on SDS-PAGE gels. Mass spectrometric studies identified cytoskeletal proteins (caldesmon-1 and vimentin), endoplasmic reticulum proteins (ERp57, ERp5, and HSP47), and nuclear proteins (lamin C, heterogeneous nuclear ribonucleoprotein F, and nucleophosmin-1) as major proteins exposed after injury. Although caldesmon was a major wound-associated protein in calpain-damaged subunit knock-out fibroblasts, it was rapidly degraded in wild-type cells, probably by calpains. Lamin C exposure after wounding was most likely the consequence of nuclear envelope damage. These studies document major intracellular proteins associated with the cell surface of reversibly damaged somatic cells. The studies also show that externalization of some proteins reported to have physiologic or pathologic roles on the cell surface can occur in cells undergoing plasma membrane damage and subsequent repair.

Reagents—SurfactAmp® Triton X-100 ampules were obtained from Pierce. Calpeptin was purchased from Calbiochem.

EXPERIMENTAL PROCEDURES

Reagents
**A Plasma Membrane Wound Proteome**

**Cell Lines**—HFL1 normal human fibroblast, C₂C₁₂ mouse myoblast, and IEC-6 rat intestinal epithelial cell lines were obtained from American Type Culture Collection. SV40 large T-antigen transformed Capan1-null mouse embryonic fibroblasts and transformed fibroblasts from wild-type littermates were kindly provided by Dr. Peter Greer (Queen’s University, Kingston, Canada). The transformed wild-type fibroblasts are hereafter called MEFs for ease of discussion. Fibroblast cell lines derived from vimentin knock-out mice and wild-type littermates were generously supplied by Dr. Robert Evans (University of Colorado Health Science Center). The cells were routinely cultured in DMEM containing 10% FBS in a humidified 5% CO₂ atmosphere at 37 °C.

**Protein Biotinylation, Electrophoresis, and Immunolabeling**—The cell-impermeant, thiol-reactive biotinylation reagent, maleimide-PEG₇-biotin (MPB) was obtained from Pierce (catalog number 21902). Maleimide-PEG₇₀₀-biotin (MPB₇₀₀), a polyethylene glycol derivative having an average molecular weight of 7500, was purchased from JenKem Technology (Beijing, China). N-Hydroxysuccinimidylo-S-S-biotin, 8d4 anti-talin mouse monoclonal antibody, goat polyclonal anti-vimentin, goat polyclonal anti-lamin B, FITC-streptavidin, and alkaline phosphatase-labeled ExtrAvidin® were obtained from Sigma. For some experiments, the anti-vimentin antibody was directly labeled with FITC by an established procedure (14). A chicken IgY antibody developed against rat m-calpain large subunit was kindly provided by Dr. Kevin K. Wang (Banyan Biomarkers, Alachua, FL). Rabbit anti-caldesmon (H300) and rabbit anti-lamin A/C (H110) were purchased from Santa Cruz Biotechnology. Rabbit anti-ERP57 (ab10287) and mouse monoclonal anti-HSP47 (ab13510) antibodies were obtained from Abcam. Cy5-goat anti-rabbit IgG, Alexa-488 donkey anti-mouse IgG, and Alexa-555 formaldehyde-fixable dextran with a molecular weight of 10,000 were obtained from Invitrogen.

**Cell Scoring**—Adherent cultured cells were subjected to mechanical damage by scraping from plastic tissue culture dishes under previously described conditions (9). Briefly, the cells were grown as monolayers until 30–50% confluent, transferred to scraping buffer (Dulbecco’s PBS containing 4.5 mM d-glucose, 0.33 mM sodium pyruvate, and 1.5 mM CaCl₂, pH 7.2), and detached from the substratum using a plastic cell scraper. In some experiments, the cells were scraped in 12-well plates and cultured overnight before assessing viability with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reductase assay (15). For clonal survival studies, the scraped cells were plated at a density of 3 cells/cm² in six-well plates. After 8 days in culture, the colonies were detected by crystal violet staining and quantitated. For immunofluorescence localization studies, the cells were labeled with MPB 45 s after scraping as previously described (9). After a 10-min labeling period on ice, the cells were washed four times in cold Dulbecco’s PBS containing 1.5 mM CaCl₂ and plated on number 1 circular glass coverslips precoated with fibronectin at 5 μg/cm². After culturing in DMEM containing 10% FBS at 37 °C for 1 h, the cells were fixed for 10 min in cold 4% paraformaldehyde in PBS. The fixed cells were washed with PBS, with or without 0.5% Triton X-100 as indicated in the specific experiment. The fixed cells were blocked for 10 min with 5% horse serum in PBS and then labeled sequentially with primary antibodies and fluorescent secondary antibodies, with three PBS washes after antibody incubations. The coverslips were mounted on glass slides using Fluoromount G containing DAPI, and confocal microscopy was carried out within a few hours later.

**Biotinylation of Cell Surface-exposed Cytoplasmic Proteins for Proteomic Studies**—A modified biotinylation protocol was employed to provide discrete protein bands on SDS-PAGE that could be used for protein identification by mass spectrometry. The previously described labeling procedure (9) was modified in the following way. Cells cultured in 150-mm dishes to ~70% confluence were scraped at 37 °C from the surface by a single pass of the cell scraper over each region of the dish. MPB was added to 0.4 mM, unless otherwise indicated, at 1 min after scraping. The cells were immediately placed on ice, incubated for 60 min, washed, and resuspended in PBS containing 1% Triton X-100, 0.2 mM PMSF, 20 μM leupeptin, 4 μM pepstatin A, and 5 mM benzamidine. Prior to the addition of 0.2% SDS and heating to 60 °C, the Triton-disrupted cells were incubated at 37 °C for 30 min with 20 Kunitz units DNase I and 100 μg of RNase A/ml. The samples were denatured in SDS, bound to Neutravidin gel, and washed, and biotinylated proteins were eluted as previously described (9).

**Glass Bead Damage**—For immunofluorescence microscopy studies, the cells were cultured on fibronectin-coated coverslips in a 24-well plate, until 70–90% confluent. Glass bead injury was carried out at 37 °C, using an established procedure (16, 17). Briefly, 0.5 ml of scraping buffer, with or without 1 mg of Alexa-555 fixable dextran/ml, was added to each well. Acid-washed glass beads (0.5-mm diameter; Thomas Scientific 566R50) were poured over the cells from a height of ~2–3 cm. The lid of a plastic PCR tube was used for dispensing, and it held ~35 mg of beads. The beads were agitated over the cells for 15 s on a Lab-Line rotator platform (model 1309) at a setting of 6 (160 rpm). Preliminary studies indicated that this resulted in a release of less than 3% of total cellular LDH, therefore producing modest cell damage. Thirty seconds after bead injury, the cells were washed twice with warm PBS and fixed with 4% paraformaldehyde in PBS for 10 min on ice. The cells were then washed and subjected to immunofluorescent labeling.

For proteomic analysis of biotinylated proteins, two 150-mm culture dishes were used per experimental condition, and the cells were harvested in 500 mg of glass beads. Thirty seconds after bead injury, 0.4 mg MPB was added, and the cells were then scraped at 37 °C in the presence of 0.5 ml of scraping buffer/dish. The scraped cells were centrifuged at 200 × g for 30 s and immediately placed on ice. Except where otherwise stated, further steps were carried out at 4–7 °C. The supernatants were discarded, and the cell pellets were resuspended with 40 μl of 20 mM MPB in scraping buffer. After 5 min of labeling, the cells were washed once with 500 μl of scraping buffer and centrifuged at 200 × g.
for 30 s to remove cell fragments. Two of the cell pellets were suspended in 1.0 ml of scraping buffer and transferred to a 37 °C CO2 incubator for 10 or 30 min to allow uptake of biotin-labeled cell surface proteins. The third cell pellet (zero time sample) was not incubated at 37 °C. All of the samples were homogenized in lysis buffer (10 mM Na-Pipes, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 2 mM EGTA, 100 μM sodium vanadate, 0.25 μM microcystin, 20 μM leupeptin, 4 μM pepstatin A, 0.5 mM benzamidine, 0.25 mM PMSF, pH 6.8) The samples were homogenized by passing through a 25-gauge needle eight times. This treatment was just sufficient to allow maximum lysis, assessed by LDH release.

The lysed cells were centrifuged at 200 × g for 5 min to produce a crude nuclear pellet and postnuclear supernatant. The postnuclear supernatant was further centrifuged at 21,000 × g for 30 min to produce a crude mitochondrial/or-ganellar pellet and at 21,000 × g to produce supernatant. In initial studies, the 21,000 × g supernatant was further centrifuged to yield a 100,000 × g (microsomal) pellet and superna-tant (cytosolic fraction). However, biotin-labeled proteins were not detected in the microsomal fraction under the conditions employed for analysis, and in further studies biotinylated proteins found in the 21,000 × g supernatant were designated as cytosolic. The nuclear pellet was suspended in 200 μl of PBS containing 1% Triton X-100, and deoxyribonuclease I was added to 20 Kunitz units/ml. After incubating at 37 °C for 30 min, the deoxyribonuclease-treated samples were sonicated twice for 5 min in a water bath sonicator. The samples were centrifuged at 21,000 × g to produce a crude cytoskeleton Triton-extract fraction (TxE) and a detergent-resistant cytoskeletal pellet. The fractions were immediately heated in SDS-PAGE buffer for electrophoresis and blotting, or they were stored at −20 °C in lysis buffer for use within 3 weeks of preparation.

Proteomic Analysis—An aliquot of purified biotinylated protein fraction derived from one-third of a 150-mm dish of scraped cells was heated to 70 °C in SDS sample buffer and loaded on a NuPAGE 4–12% bis-tris-polyacrylamide gel (Invitrogen). Because less biotinylated protein was recovered after bead damage, the equivalent of one 150-mm dish was loaded per lane for bead-damaged cells. After electrophoresis, the gels were stained with colloidal Coomassie Blue dye, and the bands were excised for proteomic analysis. Applied Biomics performed in-gel trypsin digestion, peptide extraction, and MALDI-TOF/TOF tandem mass spectrometry. Applied Biomics also provided protein identification using GPS Explorer software and the MASCOT search engine.

Protein Blotting—Cell lysates, biotinylated proteins, or subcellular fractions were subjected to SDS-PAGE in Invitrogen NuPAGE® 4–12% bis-tris-polyacrylamide gels and transferred to nitrocellulose using a Bio-Rad miniblot apparatus. The amount of sample derived from one-fifth of a 150-mm dish of 50% confluent cells was loaded, except where noted. The blots were blocked with 4% dried milk in Tris-buffered saline containing 0.05% Triton X-100. The same buffer was used for anti-body dilution, and (without milk) for wash steps. Antigens were detected with alkaline phosphatase-conjugated secondary antibodies, using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium staining protocol (18) or by enhanced chemi-luminescence (Pierce Supersignal® kit), using peroxidase-labeled secondary antibodies. All of the blots shown are representative of results from at least three replicate independent experiments.

Immunofluorescence Microscopy—Confocal images were obtained using the 60× objective of a Leica TCS SP5 multi-photon laser scanning confocal microscope housed in the Advanced Microscopy and Imaging Center of the University of Toledo Health Science Campus. The images presented are summated z-stacks throughout the volume of the cells. Emission and excitation wavelengths were chosen from the provided software for each fluorophore utilized in the studies, and settings used in co-localization studies did not produce overlapping fluorescent signals. Identical instrument sensitivity settings were used for all samples to be compared with each other within a given experiment. Image manipulation using Corel PhotoPaint® was limited to alteration of intensity, contrast, and brightness settings, all of which were identical for data sets to be compared with each other.

RESULTS

Scraped Cells That Recover Sequester Surface-exposed Biotinylated Proteins—Previous studies had shown that cytoplasmic proteins were exposed at the surface of cells injured by scraping from culture dishes and could be biotin-labeled with MPB (9). Although these studies suggested that the damaged cells survive after scrape injury, the fate of the biotinylated proteins was not clear. To address this point, MEFs were scraped, biotinylated, and replated in tissue culture dishes. After 1 h of culture, the cells were harvested and probed for biotinylated proteins as described under “Experimental Procedures.” Cells that had attached to the culture dish displayed a prominent streptavin-HRP-positive band at 57 kDa (Fig. 1A, lane L, asterisk) that co-migrated with vimentin immunoreactivity (data not shown), confirming previous studies that identified this intermediate filament protein as a major cytoplasmic protein detected at membrane injury sites (9). As anticipated, cell fragments generated by scraping were heavily labeled with biotinylated proteins (Fig. 1A, lane F). Washed cells that did not adhere to the culture dish (Fig. 1A, lane D) were weakly labeled, and no labeling was detected in control attached cells that were not scraped prior to the addition of MPB (Fig. 1A, lane NS). Scraped cells that survived appeared to undergo normal attachment to substrate, generating filopodia and lamellipodia (Fig. 1B, arrow). Nearly all of the scraped cells contained biotin-labeled proteins 1 h after scraping (Fig. 1B, lower panels). However, the extent of labeling varied considerably, perhaps reflecting the degree of injury. In less extensively labeled cells, punctate fluorescent labeling was observed (Fig. 1B, arrowheads) that co-localized with structures observed in differential interference contrast images.

In preliminary studies, MEFs that survived scrape damage were capable of forming robust colonies after more than 1 week in culture (9). This suggested that the majority of the cells did not suffer damage that resulted in delayed killing, a phenomenon that has been observed in wounded neurons in vivo (19). In follow-up studies employing several independent experiments, clonal survival of scrape-damaged MEFs was 63 ± 7% S.E. (n =
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previously identified at the site of membrane damage (9, 21): and probed by protein immunoblot analysis for three proteins. Biotinylated proteins were isolated from each fraction

mitochondrial/organellar, Tx, and detergent-resistant cytoskeletal lysates were prepared and fractionated into crude cytosolic, Tx-extractable, and cytoskeletal fractions, but over the next 10 min at 37 °C, it was redistributed from the cytoskeletal fraction to the cytosolic and Tx-extractable fractions. At 30 min after recovery, the total biotinylated talin content in all fractions was reduced, indicating its relatively slow turnover. In contrast, a robust signal for biotinylated vimentin remained in the cytoskeletal fraction for at least 30 min, consistent with the presence of a strong signal for biotinylated vimentin in cells 1 h after labeling (Fig. 1A). Biotinylated m-calpain was detected in the Tx fraction and remained there. It was undetectable 30 min after cell recovery, suggesting a more rapid turnover compared with talin.

Vimentin localization was further explored by immunofluorescence microscopy. In contrast with the majority of biotin labeling, which was redistributed from the injury site (Fig. 1B), vimentin was detected at discrete sites on the surface of replated cells 1 h after damage (Fig. 2A). Thus, at least some vimentin appeared to remain externalized well after the 20–30 s required to restore PM impermeability to dyes. No vimentin was detected on uninjured cells. The nuclear intermediate filament protein lamin B was not found at the surface of scraped cells. The latter observation is significant because both lamin B and vimentin have been shown to be exposed at the surface of apoptotic B-lymphocytes (22), whereas the present studies are consistent with substantial cell survival after scrape injury. The anti-vimentin and anti-lamin B antibodies readily detected their respective targets in detergent-permeabilized cells (Fig. 2A, right-hand column). Clonal survival assays indicated that long term survival after scrape damage in the presence of Ca2+ was compromised in fibroblasts from vimentin-null mice (Fig. 2B), whereas wild-type control fibroblasts displayed typical survival rates of >60%. In three independent experiments, vimentin-null fibroblasts scraped in the presence of Ca2+ displayed less than 40% survival compared with nonscraped cells (data not shown).

In sum, these experiments argue that most cells recovering from scrape damage retained proteins that were exposed to the cell surface and were therefore biotinylated by MPB. The fate of the individual proteins was different, and the degree of biotin labeling varied from cell to cell. Nevertheless, most cells survived and were capable of forming colonies.

Identification of a Set of Intracellular Proteins That Are MPB-biotinylatable after Cell Scarping—Although previous studies had identified vimentin as the major biotinylated protein observed on SDS-PAGE analysis of scraped cells (9), it was clear that other proteins were present but not well resolved on gel electrophoresis. This could be the result of band smearing because of multiple substitution with MPB. Vimentin, having only one cysteine residue (NCBI reference sequence NP_035831.2), would not be subject to this problem. Consistent with this interpretation, vimentin appeared as a single, narrow band on streptavidin-probed blots (Fig. 1A).

The improved biotinylation protocol, described under “Experimental Procedures” and employed in all biotinylation experiments described hereafter, utilized a lower concentration of biotin, allowing detection of multiple bands. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reductase viability assay (15). Thus, cells that initially survive scrape damage are capable of forming viable colonies. These survival rates are consistent with results from early studies investigating scraping as a mechanism for loading cells with macromolecules (20).

To assess the fates of individual biotinylated proteins, MEFs were pulse-biotinylated, washed to remove excess MPB, and allowed to recover at 37 °C. At various times thereafter, the lysates were prepared and fractionated into crude cytosolic, mitochondrial/organellar, Tx, and detergent-resistant cytoskeletal fractions, as described under “Experimental Procedures.” Biotinylated proteins were isolated from each fraction and probed by protein immunoblot analysis for three proteins previously identified at the site of membrane damage (9, 21): talin, vimentin, and m-calpain (Fig. 1C). The three proteins displayed remarkably different patterns of spatiotemporal distribution. Biotinylated talin was initially distributed in the cytosolic, Tx-extractable, and cytoskeletal fractions, but over the next 10 min at 37 °C, it was redistributed from the cytoskeletal fraction to the cytosolic and Tx-extractable fractions. At 30 min after recovery, the total biotinylated talin content in all fractions was reduced, indicating its relatively slow turnover. In contrast, a robust signal for biotinylated vimentin remained in the cytoskeletal fraction for at least 30 min, consistent with the presence of a strong signal for biotinylated vimentin in cells 1 h after labeling (Fig. 1A). Biotinylated m-calpain was detected in the Tx fraction and remained there. It was undetectable 30 min after cell recovery, suggesting a more rapid turnover compared with talin.

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of MPB for labeling. Nuclease treatment was included to minimize possible protein band broadening on SDS-PAGE caused by DNA and RNA viscosity effects. Application of this protocol to MEFs identified seven biotinylated proteins between 38 and 75 kDa in mass that were consistently observed from experiment to experiment (Fig. 3). Other bands were variably labeled, e.g. the major Coomassie-stained protein between bands 5 and 6 was determined by mass spectrometric analysis to be actin. In other experiments it was barely detectable. When blots were probed with streptavidin-alkaline phosphatase to detect biotinylated proteins, several bands were observed within the 38 – 75 kDa range. Strong staining was also observed at higher molecular weights, possibly representing minor protein bands that are highly labeled with biotin.

When scrape damage of calpain small subunit knock-out (Capns1null) and wild-type MEFs was compared, more biotinylated vimentin was isolated from the knock-out cells (Fig. 3, band 4), as previously noted (9). This was interpreted as accumulation of vimentin at the cell surface because of a lack of calpain-mediated proteolysis and compromised membrane repair in the damaged Capns1null MEFs (9). Utilizing the improved MPB labeling protocol, other bands, including the ones marked 1 and 2 in Fig. 3, were also more highly labeled in the Capns1null sample.

PM wounds are rapidly made impermeable to hydrophilic dyes by patching with internal membranes, usually within 20-30 s after injury (12). However, biotinylation of externalized talin, vimentin, and other proteins has been observed upon the addition of MPB several minutes after injury (9). Thus, incubation with the cell-impermeant MPB 1 min or more after injury may label intracellular proteins that remain externalized after the initial, rapid phase of repair. However, it was possible that the reagent could still permeate the wound site and that proteins labeled with MPB were simply intracellular proteins that were most reactive under the conditions of the studies. For example, they might possess cysteine side chains that are more reactive compared with other proteins. To further address this issue, the cells were permeabilized with 1% Triton X-100 and incubated with MPB under conditions that were utilized in the cell scrape injury studies. Exposure to Triton permeabilizes cell membranes and should allow MPB to enter the cells, labeling all accessible thiols. In contrast with the small set of proteins
labeled in scraped cells, permeabilization of cells with Triton X-100 resulted in labeling of many proteins throughout the range of the 4–12% gradient PAGE gel (Fig. 4A). It is not likely therefore, that the selective labeling of proteins after cell scraping is the result of entry of MPB through the site of injury and subsequent distribution throughout the cell.

Possible labeling of intracellular proteins by endocytosis of MPB was addressed by incubating nonscraped cells with MPB for 15 min at 37 °C under the conditions employed for the scrape damage protocol. Only trace amounts of biotinylated proteins were recovered in this experiment (Fig. 4B). As a further test for surface labeling by MPB, a linear polyethylene glycol homolog of MPB with an average molecular weight of 7,500 (MPB7500) was used in biotinylation studies. The large size of MPB7500 should greatly decrease its ability to diffuse through compromised PM. Protein bands labeled with MPB7500 migrated near the seven detected with MPB (Fig. 4C, right panel). The bands were more diffuse, as expected, because MPB7500 consists of a mixture of variable length polyethylene glycol chains. The results of these studies indicate that the proteins labeled with MPB are exposed at PM damage sites.

A limitation of MPB as a probe for cell surface-exposed proteins after PM injury is its requirement for an accessible reduced cysteine side chain in the target protein. Although few intracellular proteins lack a reduced cysteine in their primary sequence, it is less certain that every cysteine residue will be available to react with MPB. It is also possible, although unlikely, that some cysteine residues might be too rapidly oxidized upon exposure to the extracellular environment to be detected by reaction with MPB. As an alternative to MPB, scrape-damaged MEFs were labeled with N-hydroxysuccinimidyld-S-S-biotin, a biotinylation reagent that reacts with lysine residues in proteins. As expected, because cell surface-resident proteins contain more lysine side chains than reduced cysteine side chains, many protein bands were detected in nonscraped, control MEFs (Fig. 4D, left lane). However, more biotinylated protein was found on scraped cells. Importantly, major protein bands were observed co-migrating with the seven MPB-labeled proteins. Additional injury-associated proteins were detected that were not observed in the MPB experiments, including one at 95 kDa (Fig. 4D, band a), one at 47 kDa (Fig. 4D, band b), and a series of proteins migrating between 25 and 38 kDa (Fig. 4D, region c). Thus, although the MPB-labeled bands are not the only proteins expressed at PM wound sites, they appear to constitute a major subset of wound-associated proteins.

Identification of Wound-associated Proteins—The seven major MPB-labeled proteins in MEFs were harvested from colloidal Coomassie-stained PAGE gels and submitted for mass spectrometric analysis. Bands 1–4 and 7 were identified as caldesmon 1, lamin C, ERp57, vimentin, and nucleophosmin 1, respectively (Table 1). Band 5 was a mixture of HSP47, heterogeneous nuclear ribonucleoprotein F (hnRNP F), and ERp5. Band 6 contained annexins A1 and A2. Of the proteins identified, only annexins have been proposed to have a direct role in PM repair (8, 23). The other proteins fell into three categories: cytoskeletal proteins (vimentin, lamin C, caldesmon-1), resident ER proteins (ERp5, ERp57, and HSP47), and nuclear proteins (nucleophosmin-1, heterogeneous nuclear ribonucleoprotein F, and lamin C).

Wound-associated Caldesmon Is Rapidly Cleaved by Calpain during the Initial Stages of PM Wound Repair—Although caldesmon-1 was a major cell surface protein on scrape-injured Capns1+/−MEFs (Fig. 3, band 1), it was barely detectable in scraped wild-type cells. To investigate whether this was also true in other cells that express caldesmon-1, rat IEC-6 epithelial cell line and human HFL1 nontransformed lung fibroblasts were scrape-damaged and analyzed for surface-expressed cytoplasmic proteins (Fig. 5A). Both cell types exposed lamin C, ERp57, vimentin, HSP47, and annexins upon scrape injury (Fig. 5A, bands 2–6). The nontransformed HFL1 fibroblasts had little
TABLE 1
Proteins identified at the surface of scrape-wounded MEFs

| Band | Protein      | Accession number | Molecular weight | Peptide count | Sequence coverage | Protein score | Confidence interval |
|------|--------------|------------------|------------------|---------------|------------------|--------------|-------------------|
| 1    | caldesmon 1  | gi21704156       | 60,417           | 7            | 14               | 64           | 97                |
| 2    | lamin C     | gi1794160        | 65,407           | 28           | 56               | 649          | 100               |
| 3    | ERp57       | gi38382858       | 56,588           | 14           | 39               | 226          | 100               |
| 4    | vimentin    | gi55408          | 53,689           | 27           | 76               | 613          | 100               |
| 5    | HSP47       | gi26348007       | 46,490           | 10           | 34               | 108          | 100               |
| 6    | hnRNPF      | gi23274049       | 45,701           | 5            | 26               | 75           | 100               |
| 7    | nucleophosmin-1 | gi56206422   | 38,654           | 15           | 57               | 405          | 100               |

FIGURE 5. Wound-associated caldesmon was rapidly degraded by calpain. A, IEC-6 cells and HFL1 fibroblasts were scrape-damaged, and biotinylated wound-associated proteins were isolated as described under “Experimental Procedures” (lanes marked S). Nonscraped control samples (NS) were also prepared. The samples, along with IEC-6 cell lysate (Lys), were electrophoresed in SDS-PAGE gels and Coomassie-stained. The labels on the gel are the same as in Fig. 3. B, HFL1 fibroblasts were scraped and put on ice immediately (within 10 s, lanes labeled t0) or after 1 or 12 min, as indicated. One set of samples was pretreated 1 h before scraping with 20 μM calpeptin to inhibit calpains. The cells were MPB-labeled, and the biotinylated proteins were isolated. Caldesmon (Cald) was detected by immunoblot analysis using HRP-conjugated second antibody and ECL staining. C, wild-type and Capns1−/− MEFs were scraped with or without 20 μM calpeptin as indicated and placed on ice after 15 s. The samples were incubated with MPB, and biotinylated proteins were isolated. Caldesmon was detected by immunoblot analysis. As a loading control, the Capns1 wild-type sample was stripped and reprobed for vimentin (Vim).

Biotinylated nucleophosmin-1 (band 7), a protein that is known to be up-regulated in transformed cell lines (24). No signal for caldesmon-1 was apparent in wound-associated proteins isolated from HFL1 fibroblasts. A biotinylated protein migrating at 68 kDa in the IEC-6 cells, indicated as band 1 in Fig. 5A, was shown by tandem mass spectrometric analysis to be lamin A (data not shown). There was no mass spectrum signal for caldesmon-1 in the latter sample. A time course of biotinylatable caldesmon appearance after scrape injury of HFL1 cells indicated that it was present initially but was mostly lost by 1 min after scraping (Fig. 5B). Preincubating HFL1 cells with the calpain inhibitor calpeptin prevented loss of caldesmon. Moreover, caldesmon was lost within 15 s of scrape injury to wild-type MEFs but not Capns1−/− MEFs (Fig. 5C). The loss of biotinylatable caldesmon was very rapid compared with talin (9) and, in the case of MEFs, within the time frame of membrane rescaling to impermanent dyes. This suggests that the loss of biotinylatable caldesmon was probably the result of proteolysis by calpain rather than its shielding from MPB by internalization.

**PM Wound Proteome of Glass Bead-damaged Myoblasts**—All of the studies described so far employed cell scraping to identify cytoplasmic proteins exposed at PM wound sites. To determine whether the same proteins were associated with injury generated by a different model system, the cells were subjected to glass bead crush injury as described under “Experimental Procedures.” For comparison, the C2C12 mouse myoblast cell line used in these studies was also subjected to the same scrape damage protocol employed for the other cell lines investigated. A larger fraction of C2C12 myoblasts survive scrape damage compared with MEFs (25), indicating that they are less susceptible to this type of mechanical damage. Consistent with this notion, weaker signals for biotinylated proteins were observed compared with the other cell types studied (compare right panel in Fig. 6A with Figs. 3 and 5A). Nevertheless, the protein bands migrated with the same relative mobilities as the wound-associated proteins in the other cell types. Wound-associated proteins isolated from glass bead-injured C2C12 myoblasts displayed the same size distribution pattern as the scrape-damaged myoblasts (Fig. 6A, first panel). Sufficient protein was recovered from the 68-, 54-, and 39-kDa bands of the bead-damaged sample to verify their identities as lamin A, vimentin, and annexin-A2, respectively, by tandem mass spectrometric analysis (not shown). Immunoblotting revealed the presence of ERp57 and HSP47 as well (Fig. 6B).

**Lamin C Externalization after Injury Did Not Require Vimentin and May Reflect Damage to the Nuclear Envelope**—Recent studies have shown that the nuclear lamina is connected via lamins A and C to the cytoplasmic vimentin intermediate filament network, providing a continuous cytoskeletal connection between the nucleus and the rest of the cell, including the inner face of the PM (26). This connectivity could explain how lamins A and C accumulate at wounded PM. However, when vimentin-null fibroblasts were subjected to scrape damage, biotinylatable lamin C was still present, and it was in approximately the same amount as in wild-type cells (Fig. 7A). Mass spectrometric analysis confirmed that the bands shown in Fig. 7A were indeed lamin C (not shown). Lamin C was nearly undetectable at the wound site when cells were incubated with MPB 10 min after scrape injury (Fig. 7B). Reprobing the blot for vimentin showed that it was still susceptible to MPB labeling at this time, as.
expected from previous experiments (9). Lamin C labeled 1 min after damage remained in cells and was redistributed to the Tx and mitochondrial/organellar fractions by 30 min after injury (Fig. 7C). This suggests that, like talin (Fig. 1C) (9), lamin C is internalized after wound repair. In bead-damaged C3C12 myoblasts, lamin A/C and vimentin were immunostained when the nucleus was damaged, but only vimentin immunostaining was observed otherwise (Fig. 8, A–C). Thus, MPB labeling of lamin C appeared to be a consequence of nuclear membrane damage and did not depend on association with vimentin. Lamin A/C also partly co-localized with HSP47, at bead injury sites adjacent to nuclei (Fig. 8D). Thus, lamin C exposure may attend ER damage.

**DISCUSSION**

To date, no study has reported identification of the PM wound reparome, i.e. the major proteins that comprise the PM repair machinery, in somatic cells. The present study was initiated as a starting point to identify proteins directly participating in one or more of the as yet poorly defined mechanisms for patching ruptured PM, remodeling the wound site, and eventually replacing it with new PM.

Among the major intracellular proteins associated with PM damage (Table 1), only annexin A1 had been previously shown to participate in repair (8). Annexin A1 is a phospholipid and calcium-binding protein thought to aid in reparative membrane fusion events. It accumulated at sites of membrane damage in BS-C-1 and HeLa cells, and membrane resealing was inhibited by expression of a dominant-negative annexin A1 protein that does not bind calcium (8). The discovery that annexin A1 is abundant at the PM after damage is consistent with it having a major role in the repair process.

Initial studies demonstrated a requirement for the ubiquitous, typical calpains m- and μ-calpain in membrane repair (9, 25). However, the critical calpain substrates, which need to be rapidly proteolyzed (within 20–30 s) to facilitate initial membrane patching, were not explored. The present work identifies caldesmon as a candidate for this function. Because caldesmon is important for cortical cytoskeletal organization (27, 28), its cleavage may facilitate the rapid clearance of local cytoskeleton that is required for wound repair (29).
eral of the ER protein disulfide isomerases, including ERp5 (30, 31) and ERp57 (32, 33) are proposed mediators of cell adhesion, membrane fusion, and cell surface receptor function. Prior to its recognition as a resident ER chaperone protein that specifically controls collagen helix formation, HSP47 (also called col- 

ligin-2) was characterized as a cell surface protein that binds extracellular collagen (34). HSP47 was enriched in sarcolemma of muscle biopsies from Duchenne, Becker, and dysferlin-deficient muscular dystrophy patients compared with healthy controls and may function in repair or regeneration of muscle fibers (35). In Fig. 9A, potential roles of ER proteins in wound repair are illustrated, including the possible blockade of calcium extrusion near the injury site by ERp57-mediated inhibition of smooth endoplasmic reticulum calcium ATPase activity (36). It is known that intracellular calcium concentration around wound sites can remain elevated for several minutes after injury (5), possibly because of calpainolytic activation of PM calcium channels within 5 μm of the wound (37). This could lead to further remodeling of the injury site by calpains and other calcium-requiring enzymes.

Exposure of vimentin at injury sites was prolonged compared with the other proteins (9) (Figs. 1 and 2A). Thus, vimentin may accumulate on the surface of cells that are constantly undergoing membrane repair, perhaps having physiologic or pathologic consequences. Initial studies suggest that vimentin is important in long term survival after PM injury (Fig. 2B). Vimentin and several of the other proteins identified in this study are known autoantigens associated with rheumatoid arthritis (Table 2) (38–40). Cell surface vimentin is a target for picornaviruses (41) and bacterial pathogens, including the Escherichia coli virulence factor IbeA (42), and necrotizing Group A Streptococci (43, 44). Remarkably, in the latter studies, the extracellular vimentin form that associated with Streptococci was a fragment

**FIGURE 8.** Lamin C was detected near the nucleus in bead-damaged C2C12 myoblasts. C2C12 myoblasts were subjected to glass bead damage as described under “Experimental Procedures,” fixed without detergent permeabilization, and immunostained for the various antigens indicated. A and B, results from two representative injury sites. C, enlargement of the boxed area in B. Alexa-555 fixable dextran was included in the bead injury buffer to detect cells that had been damaged (dextran). Lamins A and C (lam A/C) were detected with rabbit anti-lamin A/C serum and Cy5-goat anti-rabbit second antibody (100-fold dilute). Vimentin was detected with FITC-labeled goat anti-vimentin (40-fold dilute), added at the same time as the anti-rabbit second antibody. D, at a third injury site, HSP47 was detected with mouse monoclonal anti-HSP47 and Alexa-488 donkey anti-mouse second antibody (200-fold dilute). The stained cells were mounted in Fluoromount G containing DAPI to stain nuclei. The merged images in A–C also show differential interference contrast light micrographs. Asterisks, damage sites with no apparent lamin A/C exposure. Arrows, damage sites involving nuclei where lamin A/C immunostaining is detected. Bars in A and B, 20 μm; bars in C and D, 10 μm.

**FIGURE 9.** Working model for involvement of ER and nuclei in PM damage. A, mechanical damage of the PM also produces breaks in underlying ER. The resultant influx of extracellular calcium rapidly seals the break at the verges of the ER and PM by previously described mechanisms, including calpain-mediated clearing of damaged cytoskeleton. If the wound is large, vesicle patches might also form between damaged ER tubules. Calpain-mediated cleavage of adjacent calcium channels provides continuous calcium uptake to allow prolonged remodeling of the injury site. Annexin A1 may also contribute by its calcium pore forming capability. ERp57 is predicted to inhibit smooth endoplasmic reticulum calcium ATPase (SERCA) in the presence of millimolar extracellular calcium, thereby preventing rapid clearing of calcium from the wound site. HSP47 may escape from the wound site and assist in remodeling the collagen matrix surrounding the wound. B, nuclei in close proximity to a PM wound site suffer damage to the nuclear envelope. Lamin B remains associated with the wounded membrane, whereas the lamin A/C lamina remains intact to provide a mechanical barrier against nuclear damage. For clarity, F-actin and associated proteins are not shown.

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similar in size to that produced by digestion with calpains. It was recently demonstrated that fibroblast vimentin at the site of scrape injury is processed by calpains during membrane repair (9). The results of the present study provide support for the previously expressed idea that vimentin, exposed by reversible PM injury, could serve as a docking site for Streptococci in the early stages of myonecrosis or necrotizing fasciitis (43).

Exposure of the type A lamins (lamins A and C) upon cell wounding was of special interest. Mutations in the LMNA gene (which encodes both lamin A and lamin C) cause a number of genetic diseases, including Emery-Dreifuss muscular dystrophy (45). Although compromised signal transduction through type A lamins is likely to be involved in at least some of these diseases (46), increased nuclear fragility caused by weakened mechanical properties of nuclear lamina containing mutated lamin A/C (47, 48) may also result in laminopathy-associated cell injury. It should be noted that skeletal muscle fiber nuclei are located in close proximity to the muscle PM (sarcolemma) (49), where they could be exposed to the types of mechanical damage modeled in the present study. Wound-associated lamin B was not found in proteomic analyses of the cell lines employed in the present study. The type A lamins comprise the thick sheet of nuclear lamina facing the nuclear matrix, whereas lamin B appears to be less organized and is directly linked to the inner face of the nuclear envelope (50). Thus, although the absence of lamin B at the site of damage is presently unexplained, it is possible that it remains associated with the damaged nuclear envelope, whereas lamins A/C remain self-associated to form a barrier to the extracellular environment at the wound site (Fig. 9B). The continued presence of a lamin A/C lamina would provide a relatively large surface area for reaction with MPB and subsequent detection by mass spectrometry or immunofluorescence microscopy. Mutations that weaken this barrier might result in greater nuclear damage upon mechanical injury, leading to cell morbidity or mortality.

There are a number of caveats in interpreting the results of the studies presented here. First, the biotinylation method only identifies proteins that are capable of reacting with MPB. As discussed above, these proteins were among the major proteins isolated when an amine-reactive biotinylation reagent was employed for labeling (Fig. 4D). However, a few other injury-related proteins were detected with the latter reagent and not with MPB. Second, some of the proteins that are important for repair may be sequestered at sites that are protected from labeling because of constraints on MPB diffusion. For example, components of the membrane fusion complexes required for patching the torn membrane were not detected in the present work. It may be that their localization between juxtaposed membrane sheets limits MPB diffusion and thereby prevents biotinylation. Last, the present work focused on fibroblasts and explored injury of only a few other cell types (IEC-6 epidermoid cells and C2C12 myoblasts). Thus, cell-specific components associated with PM damage and repair may be missing or over-represented in the analysis. For example, neither dysferlin nor MG53, both clearly important for repair of damaged skeletal muscle, were among the proteins identified in the present work. Moreover, because skeletal muscle does not express caldesmon, calpain effects on sarcolemma repair obviously cannot act through reduction of localized caldesmon levels. Several experiments to investigate the wound proteome in glass bead-damaged C2C12 differentiated myotubes were unsuccessful because of a high labeling background in presumably non-damaged control cultures (not shown). This was not a problem in the other cell models employed (see for example the non-scraped control lanes in Fig. 5A). It will be necessary to develop an improved model system to assess the PM wound proteome in striated muscle.

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