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Redox Regulation of β-Actin during Integrin-mediated Cell Adhesion*

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Redox sensitivity of actin toward an exogenous oxidative stress has recently been reported. We report here the first evidence of *in vivo* actin redox regulation by a physiological source of reactive oxygen species, specifically those species generated by integrin receptors during cell adhesion. Actin oxidation takes place via the formation of a mixed disulfide between cysteine 374 and glutathione; this modification is essential for spreading and for cytoskeleton organization. Impairment of actin glutathionylation, either through GSH depletion or expression of the C374A redox-insensitive mutant, greatly affects cell spreading and the formation of stress fibers, leading to inhibition of the disassembly of the actinomyosin complex. These data suggest that actin glutathionylation is essential for cell spreading and cytoskeleton organization and that it plays a key role in disassembly of actinomyosin complex during cell adhesion.

Actin is the main component of the cytoskeleton and exists as monomeric G-actin, able upon extracellular stimuli to polymerize into filamentous F-actin. Different structures of F-actin are produced by elongation of preformed filaments by accessory proteins, such as profilin, actin-related protein 2/3, actin-depolymerizing factor/cofilin complexes, and many others. These actin-interacting proteins may act through different mechanisms, as forming transversal branching filaments or blocking the extensive elongation at the cell margins (1, 2).

Actin cytoskeleton is modified by integrin signal in response to extracellular matrix (ECM)[3] transforming actin architecture from a cortical ring to a plain structure of stress fibers in complete adherent cells. Integrin-mediated cell adhesion proceeds in a step-like program: cells contact the substratum, integrins ligate to the ECM proteins and clusterize, focal contacts form, the cell spreads and finally organizes the actin filaments in stress fibers for mechanical resistance. The control of cytoskeleton organization is mainly due by the action of the members of the family of the small GTPases Rho like Cdc42, Rho, and Rac. In response to growth or chemotactic factors and cell-cell or cell-matrix interaction, Rho is activated thus inducing the formation of actin stress fibers. In contrast, activated Rac provokes actin-dependent membrane ruffling and Cdc42 causes protrusion of actin-rich microspikes from the cell surface (1).

Much evidence supports the idea that reactive oxygen species (ROS) act as second messengers thus modulating the activity of signaling proteins upon growth factors and cytokines stimulation. The downstream effect of ROS production is the reversible oxidation of redox-sensible proteins through a direct modification of the thiol group of reactive cysteines (3). Redox-regulated proteins include tyrosine phosphatases, several transcription factors, p53, and the $p21^{Ras}$ family of proto-oncogenes (4). Recently, Chiarugi *et al.* (5) proposed a redox regulation of integrin signaling during cell adhesion. In this new model, integrin activation is joined to generation of ROS specifically acting on cytoplasmic target proteins as low molecular weight-phosphotyrosine phosphatase (5) and the tyrosine kinase Src (6). The reversibile oxidation of both these proteins has profound effect on cell spreading onto ECM, through indirect activation of both Focal Adhesion Kinase and the small GTPase Rho (5, 7), thus suggesting that ROS act as second messengers in the organization of cytoskeleton in response to integrin engagement.

Herein, we report evidence that ROS produced by integrins upon ECM contact are essential mediators for actin fibers formation during cell spreading. In particular, ROS acts on mixed disulfide with glutathione which is a key step for actin stress fibers.

EXPERIMENTAL PROCEDURES

Assay of Intracellular H_2O_2—Three minutes before assaying 2′,7′-dichlorofluorescein diacetate (Molecular Probes) was added to a final concentration of 5 μM. Cells were lysed in 1 ml of RIPA buffer containing 1% Triton X-100 and analyzed immediately by fluorescence spectrofotometric analysis using a Perkin Elmer Fluorescence Sprectrofotometer 650-10S equipped with a xenon power supply (excitation wavelength: 488 nm, emission wavelength: 510 nm).

Cell Culture and Transfection—Murine fibroblasts NIH-3T3 (from American Type Culture Collection) were routinely cultured in Dulbecco's modified Eagle's medium supplemented

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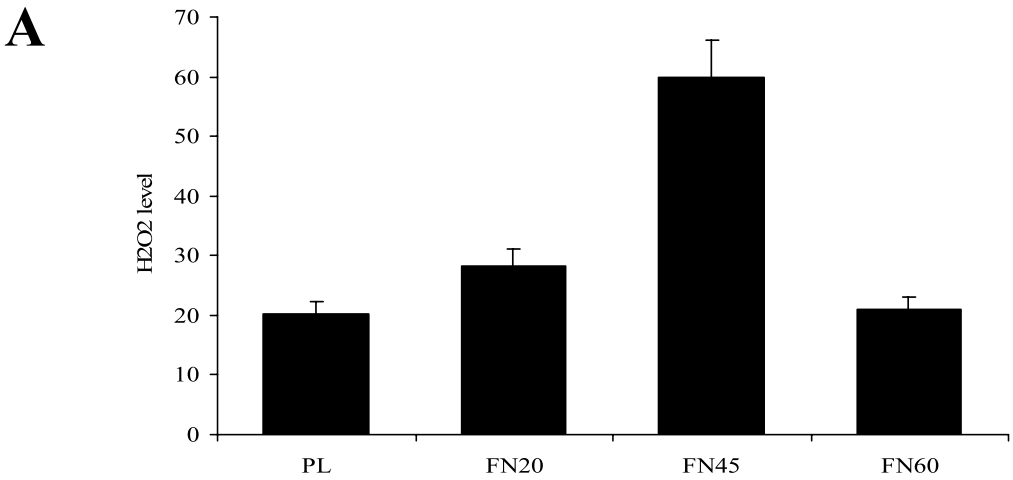
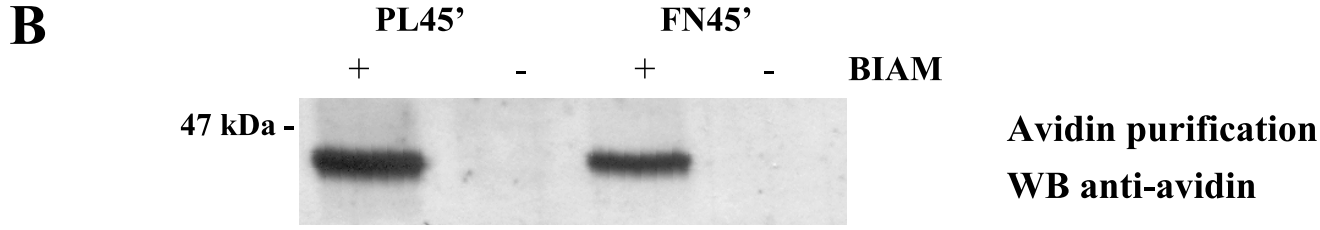
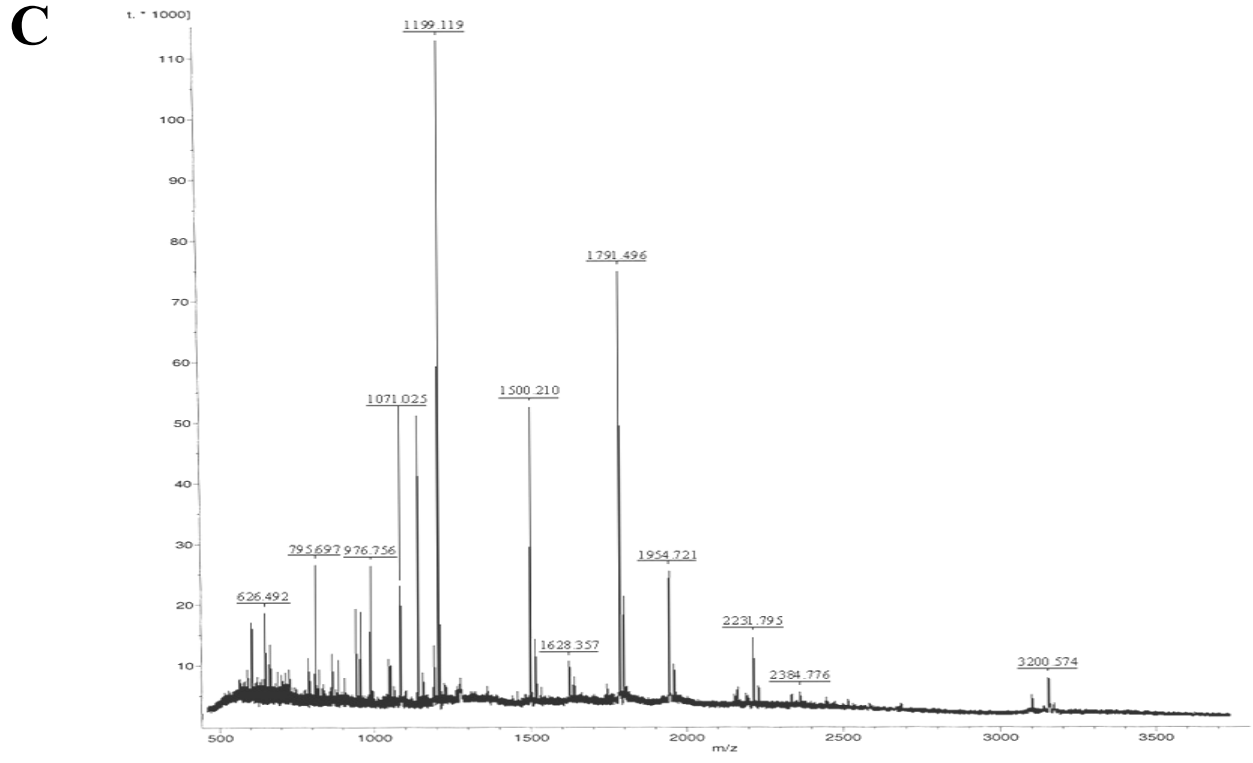
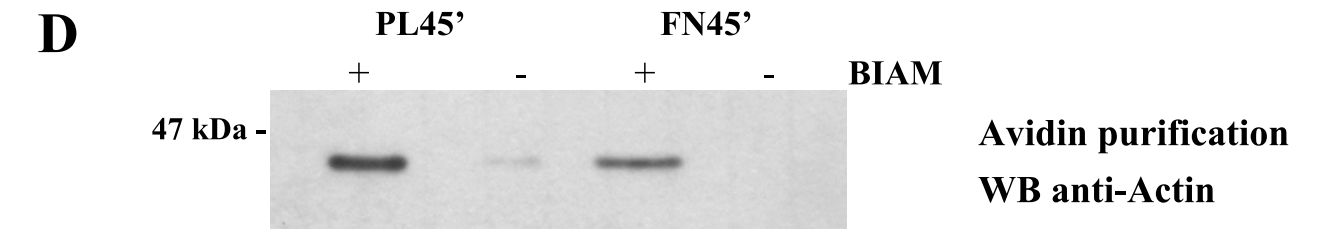
[3] The abbreviations used are: ECM, extracellular matrix; BIAM, *N*-(biotinoyl)-*N*′-(iodoaceyl)ethylenediamine; BSO, buthionine sulfoximine; LOX, lypoxygenase; FN, fibronectin; PL, polilysine; nmMHC, non-muscle myosin heavy chain; NDGA, nordihydroguaretic acid; PTP, protein-tyrosine phosphatases; ROS, reactive oxygen species; RIPA, radioimmune precipitation assay; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; wt, wild-type; TRITC, tetramethylrhodamine isothiocyanate.









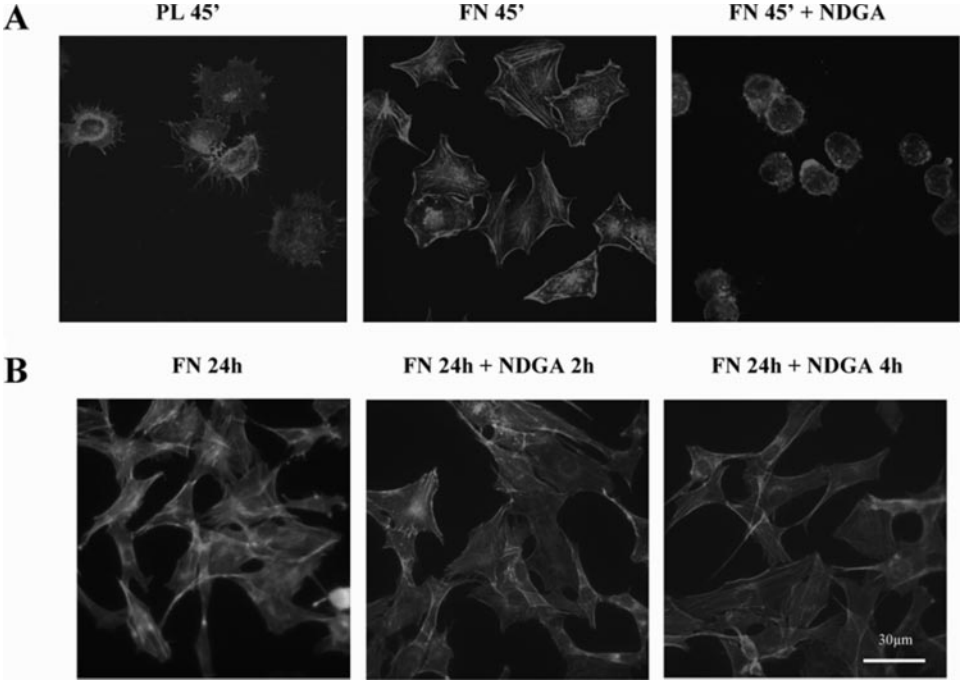


FIGURE 2. **Effect of integrin-generated ROS on actin cytoskeleton organization during cell spreading.** *A*, cytoskeleton organization during cell adhesion was revealed by the use of TRITC-phalloidin, which binds to F-actin. Serum-starved NIH-3T3 murine fibroblasts were maintained in suspension for 30 min and then plated on PL- or FN-coated coverslips for 45 min. The cells were treated, as indicated, with NDGA (10 μM) for the period indicated. *B*, for the samples in adhesion 24 h, the cells were plated on FN-treated coverslips, and NDGA (where indicated) was added the next day for the period indicated.

with 10% calf serum at 37 °C in a 5% CO_2 humidified atmosphere. For transfection experiments, confluent cells were transfected with 4 μg of plasmid using Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instruction. The pSG5-FLAG-β-actin and pSG5-FLAG-β-actin C374A was a generous gift of Prof. C. Stournaras.

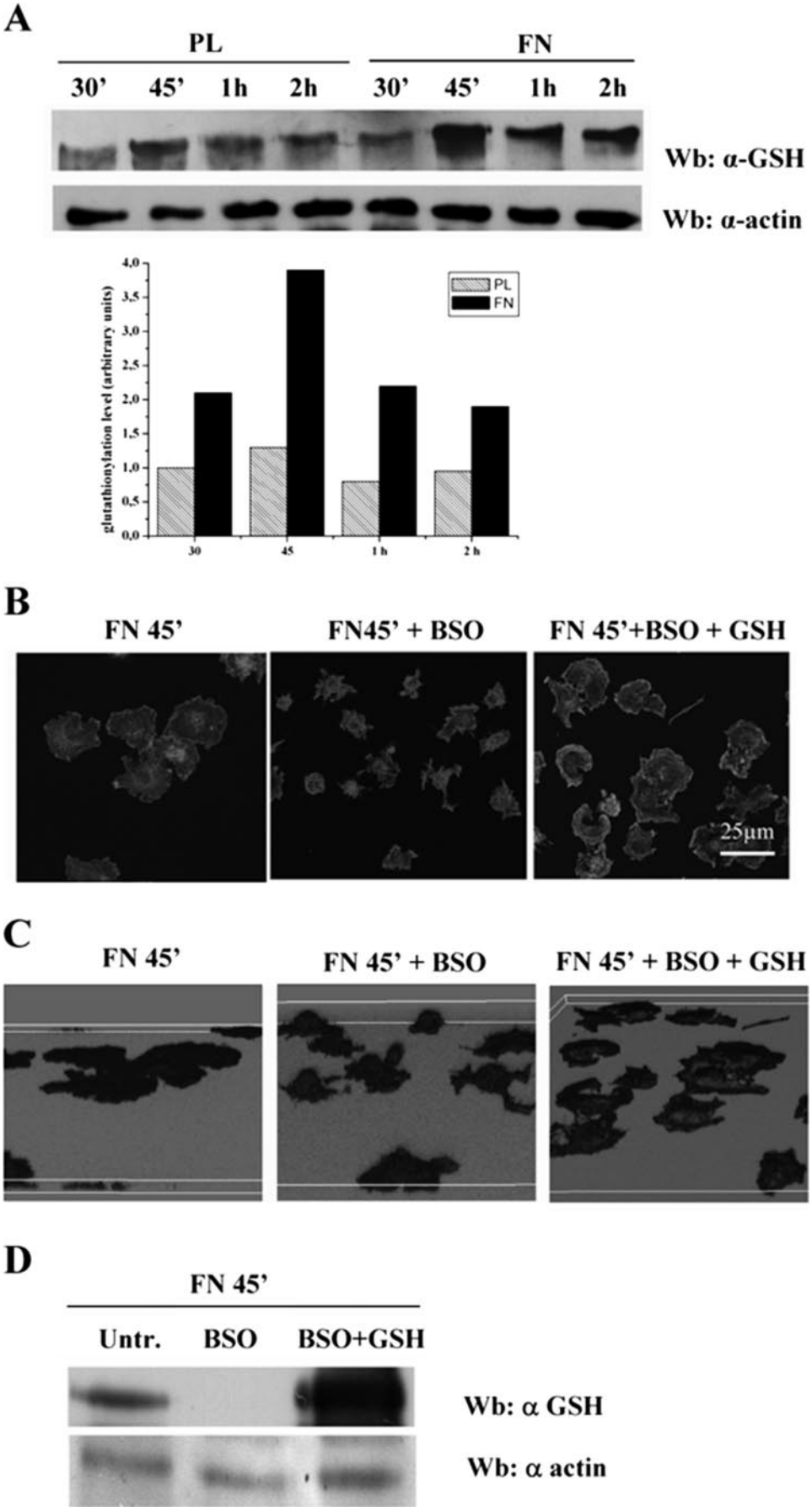
Cell Adhesion Assay—Cells were serum-starved for 24 h and then detached with 0.25% trypsin for 1 min. Trypsin digestion was then blocked by the use of 0.5 mg/ml soybean trypsin inhibitor (Sigma). Cells were centrifuged, diluted in fresh culture medium, incubated for 30 min in gentle agitation at 37 °C, and finally seeded for 45 min on polylysine (PL)-treated or fibronectin (FN)-treated dishes for the period indicated. For *N*-(biotinoyl)-*N*′-(iodoaceyl)ethylenediamine (BIAM) (Molecular Probes) labeling of proteins, cells were lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton, 2 mM EGTA) supplemented with BIAM (100 μM final concentration) and protease inhibitors mixture (Sigma). Lysates were then maintained on ice for 15 min and then centrifuged at 13,000 rpm for 15 min. For the binding of BIAM-labeled proteins with immobilized avidin (Pierce), 30 μl of resin were added to the clarified samples and maintained overnight at 4 °C in gentle agitation. The resin was first washed four times with RIPA buffer and then resuspended in Laemmli sample buffer. The pattern of BIAM-labeled proteins were visualized by a Western blot using horseradish peroxidase-avidin conjugate (Bio-Rad), washed, and developed with the enhanced chemiluminescence kit.

Immunoprecipitation—PL- or FN-plated cells were lysed for 20 min on ice in 500 μl with RIPA lysis buffer. Lysates were clarified by centrifugation and immunoprecipitation was performed for 4 h at 4 °C with 1–2 μg of the specific antibodies. Immunocomplexes were collected on protein A-Sepharose, separated by SDS-PAGE, and transferred onto polyvinylidene difluoride membrane. Immunoblots were incubated in 2% milk and 0.1% Tween 20 in PBS buffer for 1 h at room temperature, probed first with specific antibodies and then with secondary antibodies. Quantity-One software (Bio-Rad) was used to perform quantitative analysis.

MALDI-TOF Analysis—BIAM-labeled lysates were run on SDS-PAGE. The gel was then stained by Coomassie Blue solution, subjected to destain solution for 24 h and finally washed in water until completely equilibrated. The bands of interest were excised, transferred to an Eppendorf tube, and then washed twice with 50 mM NH_4HCO_3/acetonitrile (1:1) and shrunk with acetonitrile. After drying, samples were subjected to a reduction reaction in a buffer containing 10 mM dithiothreitol, 25 mM NH_4HCO_3 for 45 min at 56 °C followed by an alkylation step in a buffer containing 55 mM Iodoacetic acid, 25 mM NH_4HCO_3 for 30 min at room temperature in the dark. After a final washing step, samples were dried up and trypsin digested for 24 h at 37 °C. The peptides were then extracted from gel bands by sonification and by supplementing 50% acetonitrile and 1% trifluoroacetic acid (1:1 proportion with sample), and the supernatants were recovered and then dried. Spectrometric analysis was conducted on an Ultraflex MALDI-TOF (Bruker Daltonics) using a Scout ion source and operating in a positive reflectron mode. Samples were mixed with α-cyano-4-hydroxycinnamic acid (1:1). An 0.8 picomol/μl sample was deposed with the dry droplet technique on an AnchorChip target. Peptides were identified within an error of 120 parts/million.

Confocal Microscopy—Presuspended NIH-3T3 were seeded onto coverslips, washed with phosphate-buffered saline and

FIGURE 1. **Identification of actin as redox-regulated protein during integrin-mediated cell adhesion.** *A*, serum-starved NIH-3T3 cells were detached, maintained in suspension for 30 min at 37 °C, and then seeded on PL- or FN-treated dishes for the periods indicated. Hydrogen peroxide evaluation was performed by adding to the cells, 5 μg/ml ROS fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes) 3 min before the end of the adhesion period. *B*, affinity chromatography showing the different redox state of the protein of about 47 kDa of PL- or FN-seeded cells. *C*, MALDI-TOF analysis of the digested peptides of the protein showed in *B* identified it as β-actin. The spectrum shows the matched peaks. *D*, the same results of MALDI-TOF analysis was obtained by a Western blot experiment performed on the eluate of chromatography using specific antibodies against β-actin.



then fixed in 3% paraformaldehyde for 20 min at 4 °C. Fixed cells were permeabilized with three washes with TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) and then blocked with 5.5% horse serum in TBST for 1 h at room temperature. Cells were then incubated with specific primary antibodies or fluorescent phalloidin, diluted 1:100 in TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) overnight at 4 °C. When necessary, cells were incubated with secondary antibodies (diluted 1:100) for 1 h at room temperature in TBST with 3% bovine serum albumin. After extensive washes in TBST cells were mounted with glycerol plastine and observed under a laser scanning confocal microscope (Bio-Rad MRC 1024 Es) equipped with a krypton/argon laser source for fluorescence imaging and with differential interference contrast optics. The argon (488 nm) and krypton (568 nm) laser lines were used to simultaneously excite the cells, and the emitted fluorescence signals were collected with a Nikon Plan Apo 60×/1.4 oil immersion objective. A series of optical sections (512 × 512 pixels) were taken through the depth of the cells with a thickness of 1 μm at intervals of 0.2 μm.

Wound Healing Migration Assay—Confluent NIH-3T3 cells were serum-starved for 24 h, and dishes were scored with a sterile 200-μl micropipette tip and photographed. Thereafter cells were treated with 30 ng/ml platelet-derived growth factor. After 8 h transfected cells were visualized by a colorimetric reaction using the Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. Then the wounds were photographed again to visualize incoming cells.

RESULTS

Actin Is Oxidized by ROS Produced by Integrin Receptors during Cell Adhesion—Besides our identification of low molecular weight-phosphotyrosine phosphatase and the tyrosine kinase c-Src as two target proteins of the integrin-induced ROS (5, 6), we now have continued with a further screening for redox-sensitive proteins during integrin receptor signaling, using BIAM, which is a sulfhydryl-modifying reagent that selectively probes the thiolate form of reactive cysteine residues (8). ECM adhesion of NIH-3T3 murine fibroblasts was assayed 45 min after seeding onto coated dishes, in accordance with the peak of ROS during ECM-induced cell spreading (5) (Fig. 1*A*). Western blot analysis of affinity-purified BIAM-labeled lysates of PL- and FN-adhering cells shows an integrin-dependent redox sensitivity of a protein of about 47 kDa, which was identified by MALDI-TOF analysis as β-actin (Fig. 1, *B* and *C*). This identification was confirmed by anti-β-actin immunoblot analysis (Fig. 1*D*).

Parallel to previous findings indicating actin as a redox-sensitive protein during induced oxidative stress (9), we aimed to investigate the function of integrin-elicited ROS on the organization of the overall actin cytoskeleton. Previous studied indicated that, following engagement of integrin receptors, the small GTPase Rac-1 plays a key role in ROS production though the activation of 5-lypoxigenase (LOX) (5). We now find that the depletion of oxidants using specific inhibitor for LOX, nordihydroguaretic acid (NDGA), greatly affects actin organization during cell spreading (Fig. 2*A*). FN-spreading cells treated with this inhibitor display a morphology very similar to PL-adhering cells, with a disorganized peripheric actin ring and a rounded shape. Administration of this antioxidant when the spreading is completed does not affect the overall actin cytoskeletal architecture (Fig. 2*B*), suggesting that ROS play a role in actin organization only during the dynamic process of cell spreading in response to binding to ECM proteins.

Cells Spreading and Cytoskeleton Rearrangements Require Actin Glutathionylation—Mild oxidation can result in the formation of mixed disulfides between protein sulfydryl groups and glutathione (GSH), giving rise to *S*-glutathionylated proteins. Furthermore, actin has already been indicated as a glutathionylated protein during epidermal growth factor stimulation (10). With the goal to analyze whether during integrin-mediated cell adhesion actin undergoes glutathionylation upon oxidation, we investigated its binding to GSH during a time course experiment. Anti-GSH immunoblot analysis of actin revealed that there is a transient increase in actin glutathionylation upon engagement of integrin receptors (Fig. 3*A*). Moreover, we find that GSH is greatly implicated in actin regulation during cytoskeleton rearrangement due to cell spreading. Actually, depletion of GSH causes a dramatic impairment of actin cytoskeleton organization leading to a rounded cell shape, similar to the effect observed in adhering cells treated with NDGA. In addition, the effect of buthionine sulfoximide (BSO) on actin cytoskeletal organization and cell shape can be relieved by re-administration of GSH (Fig. 3, *B* and *C*), thus indicating the reversibility of the inhibition of actin organization upon GSH depletion. Although we cannot exclude that BSO affects the glutathionylation of other cytoskeletal proteins, we speculate an effect on β-actin glutathionylation. We thereafter checked the level of actin glutathionylation in the above conditions (Fig. 3*D*). Actually, gluthathionyated actin correlates with a normal cell shape (untreated and BSO+GSH-treated cells), while complete loss of actin glutathionylation is linked to an unusual cell shape (BSO-treated cells).

Involvement of Cysteine 374 of Actin in Cytoskeleton Organization during Cell Spreading—ROS modify protein function by the specific alteration of the redox state of reactive cysteine residues (3). Although actin contains six highly conserved cysteine residues, the three-dimensional structure reveals that only the thiol of cysteine in position 374 is exposed on the sur-

FIGURE 3. **Role of actin glutathionylation in cytoskeleton rearrangements and cell spreading.** NIH-3T3 cells are serum-deprived for 24 h, maintained in suspension for 30 min, and then plated on PL or FN matrix for the period indicated. *A*, specific antibodies anti-GSH (Virogen) were used for the quantification of glutahionylated actin in PL- or FN-plated cells at different times of adhesion; the *bar plot* below indicates the normalized glutathionylation level of actin. *Wb*, Western blot. *B*, cytoskeleton organization as revealed by TRITC-phalloidin. Where indicated, an inhibitor of GSH synthesis, BSO 40 μM, was added when cells were serum-depleted and maintained during the experiment. Where indicated, GSH 5 mM was added to BSO-treated cells during the suspension phase and maintained during the adhesion period. *C*, three-dimensional view of the cells in *A* showing a different ability to adhere on FN matrix. *D*, lysates of untreated and BSO- and BSO+GSH-treated cells were used in an immunoblot analysis using antibodies against GSH to detect the glutathionylation level of actin 45 min after adhesion on FN-coated dishes.

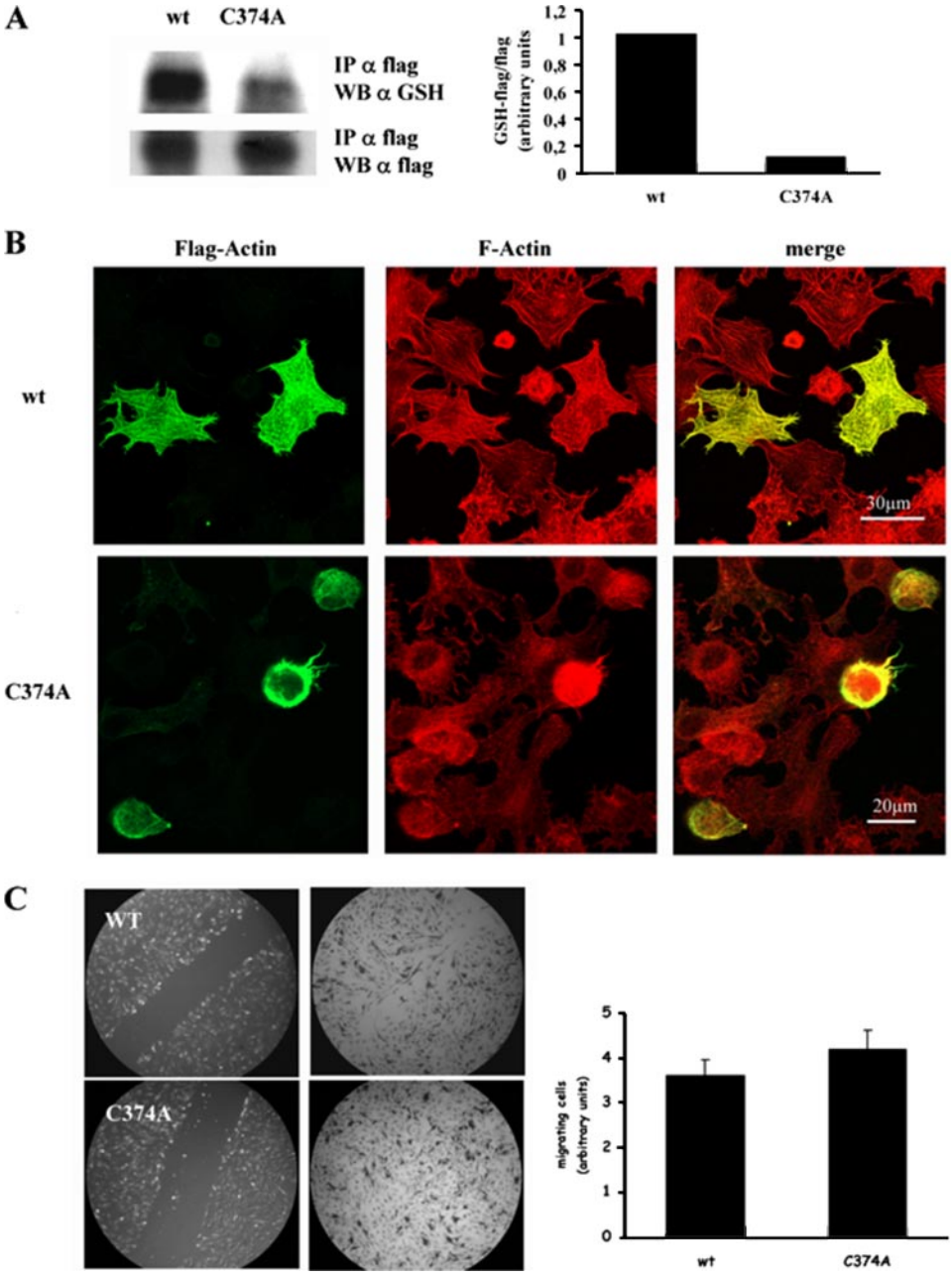


FIGURE 4. **Actin cysteine 374 is involved in actin reorganization and cell spreading.** NIH-3T3 cells were transiently transfected with the plasmid expressing the wild-type form of FLAG-β-actin (FLAG-wt) or with the mutant C374A (FLAG-C374A). At 24 h after transfection cells are serum-deprived for 24 h, detached, maintained in suspension for 30 min, and then seeded on FN-treated cover dishes for 45 min. *A*, the amount of glutathionylated FLAG-actin was revealed by a Western blot (*Wb*) using anti-GSH antibodies on anti-FLAG immunoprecipitates; the *bar plot* beside indicates the normalized glutathionylation level of actin. *B*, the experiment was performed as described in *A*; then, after plating the cells on FN-treated coverslips, double-labeling was performed with Alexa 488 secondary fluorescent antibodies and fluorescein isothiocyanate-phalloidin. Co-localization (merging) of F-actin and exogenous FLAG-actin is shown. *C*, wound healing assay of NIH-3T3 cells transiently transfected with the plasmids expressing the wild-type (FLAG-wt) or the mutant C374A (FLAG-C374A) of actin. When the cell cultures have reached a saturation density, the cells were scratched. Wound regions were then allowed to heal by cell migration in the presence of platelet-derived growth factor for 8 h. Transfected cells were visualized by a colorimetric reaction using Vectastain ABC kit. The histogram shows the number of migrated cells expressing wild-type actin or C374A mutant obtained by the mean of three independent experiments. This parameter was calculated by the ratio between the transfection efficiency and the number of transfected cells that migrated in the wound.

face of the molecule. The *in vitro* study of Wang *et al.* (10) indicated that cysteine 374 is a glutathionylated residue that undergoes deglutathionylation upon epidermal growth factor stimulation. We therefore looked at the role of cysteine 374 in GSH disulfide formation during cell adhesion, using FLAG-tagged wild type (wt) and the cysteine 374 to alanine (C374A) mutant of β-actin. We observed that the C374A mutant glutathionylation is greatly impeded upon integrin engagement (Fig. 4*A*), thus confirming C374A as the main GSH-reactive sulfydryl in actin. To analyze the role of actin redox regulation during cell spreading, a double labeling analysis of FN-adhering cells was carried out to reveal whole actin organization (*red*) and recombinant FLAG-tagged actin (*green*) (Fig. 4*B*). The results demonstrate that wild-type β-actin overexpression does not affect cell spreading or F-actin organization, whereas C374A β-actin expression leads to a severe inability to organize the cytoskeleton and to spread in response to ECM ligands (Fig. 4*B*). Remarkably, cells expressing the redox-insensitive mutant have a rounded shape and a morphology very similar to antioxidant-treated ones (Fig. 2). These findings suggest that *in vivo* C374 probably plays a key role in GSH disulfide formation upon integrin engagement and in cell spreading.

On the other side, we were interested in studying whether cysteine 374 oxidation is involved in other phenomena requiring actin cytoskeleton reorganization. Platelet-derived growth factor-induced cell migration was analyzed by a wound healing assay in NIH-3T3 cells expressing wild-type or C374A β-actin. We found that wild-type and C374A expressing cells have a similar motility after platelet-derived growth factor administration suggesting that cysteine 374 does not affect cell migration (Fig. 4*C*).

Redox Regulation of Actin Is Essential for Actinomyosin Complex Disassembly—Cytoskeleton contractility of both muscle and non-muscle cells is guaranteed by the actinomyosin complex which, in response to extracellular signals, can be rapidly assembled or disassembled to allow cell shape changes (11). We therefore analyzed the redox sensitivity of the association between actin and non-muscle myosin heavy chain (nmMHC) in cell spreading due to ECM contact. Fig. 5*A* shows

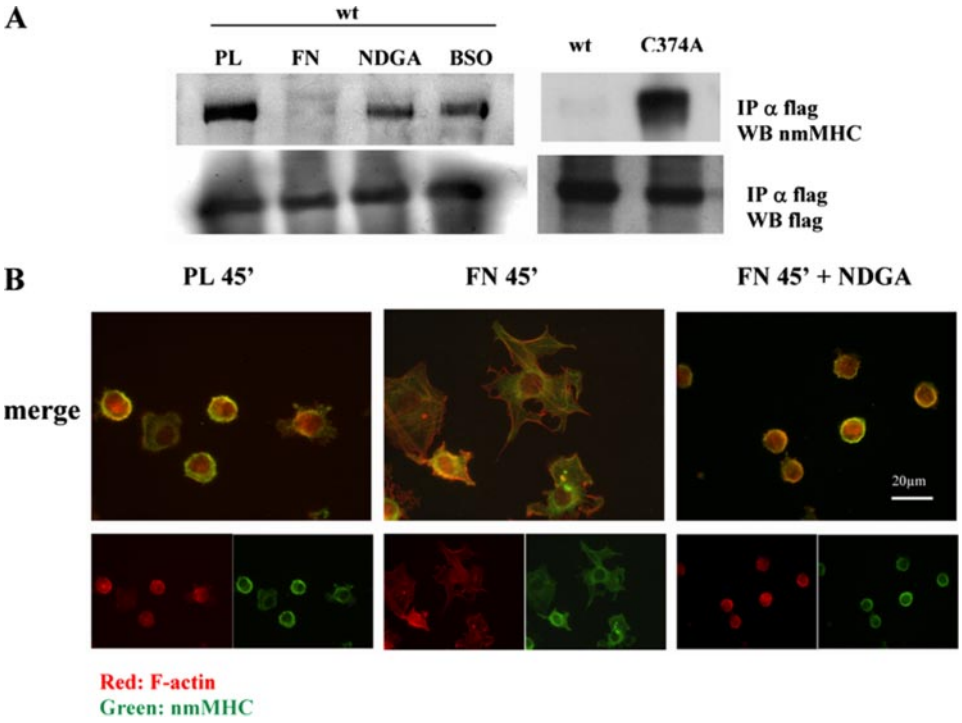


FIGURE 5. **ROS-dependent association between actin and nmMHC.** *A*, cells were transfected with FLAG wt β-actin or, where indicated, with the C374A mutant. At 24 h after transfection cells were serum-deprived for 24 h, detached, maintained in suspension for 30 min, and then seeded on FN- or PL-coated cover dishes for 45 min. Association between nmMHC and overexpressed actin was detected by immunoblots using anti-nmMHC antibodies (Biomedical Technologies Inc.) or anti-FLAG antibodies (Sigma) performed on anti-FLAG immunoprecipitates. *B*, co-localization of endogenous actin and nmMHC. Fixed cells were double-labeled with fluorescein isothiocyanate-phalloidin and with Alexa 488 secondary fluorescent antibodies to probe nmMHC.

that successful and completed cell adhesion is associated with the disassembly of the actinomyosin complex, as indicated by the disappearance of the association between myosin and actin upon FN contact. GSH depletion and ROS removal both impede the dissociation between actin and nmMHC, confirming a role for integrin-induced ROS production and GSH content in the dynamics of actinomyosin complex. Moreover, this association is greatly enhanced in C374A-expressing cells, suggesting that glutathionylation of cysteine 374 is involved in the disassembly of the actinomyosin complex upon completion of cell spreading. The ROS-dependent association between actin and nmMHC is further confirmed by confocal analysis performed on endogenous actin (Fig. 5*B*).

Hence, whatever the methods used for impeding or limiting actin glutathionylation (PL seeding, antioxidant treatment, GSH depletion, or mutation of actin glutathionylation site) the effect on actinomyosin complex is the inhibition of its disassembly.

DISCUSSION

We report here that: (i) during integrin-mediated cell adhesion actin is a redox-sensitive protein and undergoes a direct redox regulation by a physiological source of ROS; (ii) inhibition of 5-LOX-driven oxidants completely impedes actin fiber formation during cell adhesion leading to a ring-like actin cytoskeleton; (iii) actin undergoes glutathionylation on Cys^{374} during cell adhesion and the impairment of this redox modification leads to the inability of cells to spread and to organize the cytoskeleton in response to ECM; (iv) impairment of actin glutathionylation leads to inhibition of the disassembly of the actinomyosin complex, suggesting that this redox modification is a key step in the dynamic contraction of cytoskeleton during cell spreading.

The recent literature opened an intriguing scenario in which signal transduction by oxygen species, through reversible regulation of PTPs, cytosolic protein-tyrosine kinases and receptor protein-tyrosine kinases, and cytoskeletal proteins, represents a widespread and conserved component of the biochemical machinery triggered by several extracellular stimuli to transmit their signals (3, 4). Integrin-mediated adhesion causes the transient inactivation of low molecular weight-PTP and SH2-protein-tyrosine phosphatase 2.[4] This inactivation guarantees for the increase in tyrosine phosphorylation of the PTP substrates, *i.e.* focal adhesion kinase and $p190^{Rho}$ GTPase-activating protein (12). Beside the regulation of PTPs, anchorage-dependent ROS can regulate protein tyrosine phosphorylation through a direct action on protein-tyrosine kinases. We recently reported a direct redox regulation of the tyrosine kinase Src, which undergoes a strong activation mediated by an intramolecular disulfide bond formation in response to integrin receptor engagement (6). The results described herein correspondingly support the novel idea that ROS regulate cytoskeleton dynamics through both indirect and direct systems. Indirect regulation is translated through soluble redox-sensitive enzymes, as low molecular weight-phosphotyrosine phosphatase or Src, whose oxidation gives rise to a signal culminating in cytoskeleton rearrangement (5, 6). Besides this indirect regulation, ROS can act on actin cytoskeleton through a shorter way, *i.e.* the direct oxidation of a cytoskeletal protein as actin.

Previous studies indicated that, following engagement of integrin receptors, the small GTPase Rac-1 plays a key role in ROS production though the activation of 5-LOX (5). We now find that the depletion of 5-LOX driven oxidants greatly affects actin organization and hence cell spreading. ECM-spreading cells treated with this inhibitor display a morphology very similar to PL-adhering cells showing a round profile. Herein we report that ROS produced during integrin-mediated cell adhesion affect the ability to spread of ECM-seeded cells targeting the actin cytoskeleton. Indeed, antioxidant-treated cells display chaotic actin fibers and a disorganized peripheric actin ring.

[4] M. L. Taddei, personal communication.

We therefore reasoned that ROS are not involved in actin oxidation as housekeeping mediators, in agreement with their role as inducible second messengers of integrin receptor signaling. In fact, administration of this antioxidant when cell spreading is completed does not influence the whole cytoskeletal structure, signifying that ROS participate in actin reorganization only during the dynamic process of ECM-induced cell spreading.

We highlight that ROS are not merely by-products of 5-LOX during leukotriene synthesis and may be included among primary products of this enzyme. Actually, based on several preliminary reports describing leukotrienes produced by LOX as key molecules involved in cell adhesion regulation (13, 14), we can speculate that both lipid and oxidant LOX products synergize in the regulation of cell adhesion.

Intracellular oxidation can result in the formation of mixed disulfides between protein sulfydryl groups and GSH resulting in *S*-glutathionylated proteins. Actin glutathionylation has been already reported during epidermal growth factor (10) and in hepatocytes and hepatoma cells exposed to oxidative stress (15). The present findings indicate an increase in actin glutathionylation upon engagement of integrin receptors, suggesting that GSH is heavily involved in actin regulation during cytoskeleton rearrangement due to cell spreading. Indeed, GSH-depleted cells show lesser spreading and great impairment of cytoskeleton organization, similar to cells treated with NDGA. In particular, the effect of BSO on cytoskeletal organization and cell spreading can be relieved by re-administration of GSH, suggesting that the inhibition of cell spreading in response to GSH depletion, and hence to the induced oxidative stress, is reversible. Hence we correlated actin glutathionylation with cell spreading and the impairment of this glutathionylation, due to GSH depletion, to a rounded shape and a ring-like actin cytoskeleton. This statement is in keeping with the previous findings of Wang *et al.* (10), describing that cell rounding upon growth factor-induced mitosis is correlated with actin de-glutathionylation.

In vivo actin glutathionylation is a simple way in which oxidants exert their role in protein post-translational modification. It has been reported *in vivo* and *in vitro* that the oxidation of a sulfhydryl group to sulfenic acid (SOH) rapidly and spontaneously leads to further oxidation products such as sulfinic and sulfonic acid (SO_2 and SO_3) (16). These further *S*-oxidation states are probably terminal products on which cellular reduction systems cannot operate, leading to permanently modified proteins. In intracellular systems with low oxidation grade to redox-sensitive proteins, the formation of disulfides, either between intra- or intermolecular protein cysteines or with a mixed disulfide with GSH, retains a key role (16). This post-translational modification has been recently suggested as a possible means of redox regulation of protein function and activity and, therefore, one of the mechanisms whereby oxidants exert a regulatory effect on cell function (17–19). In this light actin glutathionylation may be a mechanism that protects cells against irreversible organization of microfilaments, regulating actin polymerization and preserving microfilaments dynamics.

Although actin contains six highly conserved cysteine residues, the three-dimensional structure reveals that only the thiol of cysteine in position 374 is exposed on the surface of the molecule, so as to be accessible to solvents and highly mobile (20). In addition, surface exposure of Cys^{374} is not influenced by actin polymerization into F-actin (21). Our findings suggest that *in vivo* actin oxidation involves cysteine 374 and that this residue probably plays a key role in GSH disulfide formation upon integrin engagement and in cell spreading. In fact, the removal of cysteine 374 causes redox insensitivity of actin and completely impedes actin fiber formation and cell spreading but not cell motility. These results support the idea that cysteine 374 plays a role only during cell spreading and whole actin cytoskeleton organization but not during the dynamic of cytoskeleton reorganization of a moving cells, thus suggesting that actin dynamics are dissimilar during adhesion of cells or during cell movement. Furthermore, the key role of Cys^{374} in actin polymerization is further indicated by its ability to act as an *in vivo* dominant negative mutant, since it is able to almost completely suppress the organization of endogenous actin, obstructing filament formation. On the other side we exclude that the C374A mutant affects actin function as a result of a conformational change, as indicated by its ability to completely support cell motility.

Although actin has already been indicated as a redox-sensitive protein during induced oxidative stress (9), we report here the first evidence of an *in vivo* actin oxidation by a physiological source of ROS. Exogenous oxidative stress has been implied in the disruption of cytoskeleton both during diamide treatment and in fibroblasts of Friederich ataxia patients, a disease correlated with iron-mediated oxidative stress (20, 22–24). In both reports the exogenous oxidative stress is correlated with a stable high level of actin glutathionylation and impairment of cytoskeleton function. On the contrary, our data propose actin glutathionylation as a key signaling event downstream of integrin receptor engagements. In this context ROS act as physiological messengers transiently increasing upon ECM contact and peaking in concomitance of cell spreading. The transitory redox regulation of actin during integrin receptor engagement

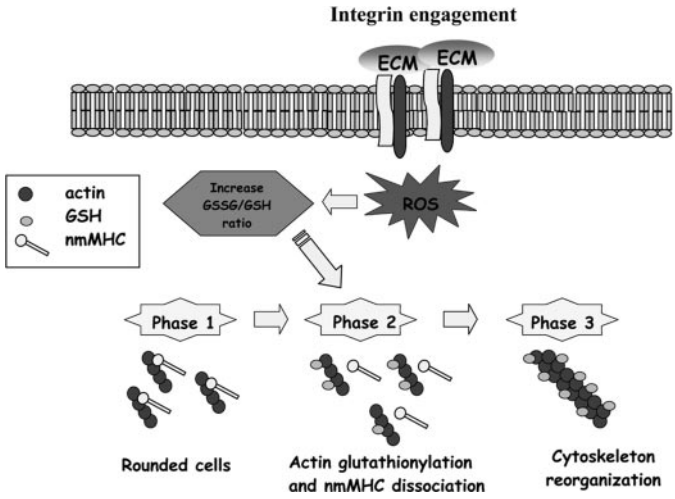


FIGURE 6. **Proposed model for actin organization during cell adhesion.** ROS produced by integrins upon ECM contact directly drives actin polymerization, leading to actin glutathionylation on cysteine 374. This redox modification of actin permits the disassembly of the actomyosin complex leading to the lack of cell contractility. So, cytoskeleton reorganization and cell spreading can occur.

leads to cytoskeleton organization and cell spreading. We suggest that a continuous oxidative stress, as in diamide-treated or ataxia cells, can greatly differ from a transient signaling event, and this can account for the different effect observed on actin cytoskeleton organization.

Myosin II is the major motor protein in both muscle and non-muscle cells, and its main role is to regulate contraction in association with actin filaments. nmMHCII is a part of the actinomyosin cytoskeleton, which is subject to continuous remodeling in response to ECM contact to allow cell shape changes (25–28). We report herein that glutathionylation of actin causes the disassembly of the actinomyosin complex. Although a high resolution structure of the actomyosin interface is not yet available, we suggest that the redox modification to which actin undergoes during cell spreading negatively regulates the binding between actin and myosin, thus exerting a crucial effect on the dynamic cycles of assembly/disassembly of the contractile machinery. We further speculate that the disassembly of the actinomyosin complex caused by actin glutathionylation could strongly contribute to impairment of microfilament function in diamide treated or Friederich ataxia cells (24).

Cytoskeleton dynamics leading to cell spreading through loss of contractility and organization of actin fibers involve the two antagonistic small GTPases Rho and Rac through three synergic pathways, two of which are redox-based. First, activation of Rac-1 regulates the phosphorylation of myosin heavy chain and actinomyosin disassembly, leading to loss of contractility and to cell spreading (29). Second, the Rac-mediated ROS production during cell adhesion leads to a down-regulation of Rho activity finally culminating in loss of contractility and permitting cell spreading (7). Our herein presented data point to a third mechanism regulating cytoskeleton assembly in response to ECM contact, through a redox-based actin reorganization due to glutathionylation (Fig. 6). We emphasize the role of a direct regulation though oxidation of actin function, but the precise mechanism by which oxidants change the structure and the spatial organization of actin filaments remains to be elucidated.

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Redox Regulation of β-Actin during Integrin-mediated Cell Adhesion

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