Reversible Glutathionylation Regulates Actin Polymerization in A431 Cells*

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In response to growth factor stimulation, many mammalian cells transiently generate reactive oxygen species (ROS) that lead to the elevation of tyrosine-phosphorylated and glutathionylated proteins. While investigating EGF-induced glutathionylation in A431 cells, paradoxically we found deglutathionylation of a major 42-kDa protein identified as actin. Mass spectrometric analysis revealed that the glutathionylation site is Cys-374. Deglutathionylation of the G-actin leads to a 6-fold increase in the rate of polymerization. In vitro studies revealed a 12% increase in F-actin content 15 min after EGF treatment, and F-actin was found in the cell periphery suggesting that in response to growth factor, actin polymerization in vivo is regulated by a reversible glutathionylation mechanism. Deglutathionylation is most likely catalyzed by glutaredoxin (thioltransferase), because Cd(II), an inhibitor of glutaredoxin, inhibits intracellular actin deglutathionylation at 2 μM, comparable with its IC_{50} in vitro. Moreover, mass spectral analysis showed efficient transfer of GSH from immobilized S-glutathionylated actin to glutaredoxin. Overall, this study revealed a novel physiological relevance of actin polymerization regulated by reversible glutathionylation of the penultimate cysteine residue mediated by growth factor stimulation.

The mechanisms involving extracellular stimuli-induced ROS generation in cells and subsequent signal propagating events are under active investigation (1–5). In response to growth factor, ROS, such as O_2^• or H_2O_2, effectively inhibit protein-tyrosine phosphatases (PTPs) by converting the active site cysteines to sulfenic acids. The sulfenic derivatives are readily S-glutathionylated by GSH, which is abundant in mammalian cells (6), thereby avoiding further oxidation to irreversible sulfenic or sulfonic derivatives (5). PTP inhibition leads to elevation of tyrosine-phosphorylated proteins, thereby promoting signal transduction cascades. Reactivation of glutathionylated PTP is most likely catalyzed by glutaredoxin, characterized as the primary enzyme for protein-SSG deglutathionylation in cells (7, 8). As the intracellular redox equilibrium shifts toward oxidation and protein phosphorylation in response to extracellular stimuli, cytoskeletal changes also occur. Actin, an abundant and ubiquitous cellular protein, plays a major role in mediating the infrastructure and dynamics of the cytoplasmic matrix (9, 10). Actin polymerization is a dynamic process implicated in growth factor-mediated cytoskeletal changes (11). Here we report both in vitro and in vivo data that indicate EGF-induced deglutathionylation of the penultimate cysteine residue of actin facilitates its polymerization and cytoskeletal changes.

EXPERIMENTAL PROCEDURES

Materials—The human epithelial A431 cell line was from ATCC; bovine muscle actin, EGF, DTNB, NADPH, GSSG reductase, carboxymethyl-BSA, CM-Sepharose, Q-Sepharose, phenyl-Sepharose, GSSG, and GSH were from Sigma; rabbit muscle actin was a generous gift from Dr. Xiong Liu; monoclonal anti-GSH antibody was obtained from Virogen; pYFP-actin vector was from CLONTECH; DCF-Ac and Phalloidin-Oregon Green 488 were from Molecular Probes; precast SDS gels and polyvinylidene difluoride membranes were from Invitrogen; Fugene-6 was from Roche Molecular Biochemicals; H_2O_2 was from Fisher; CdCl_2 and 2-vinyl pyridine were from Aldrich. [14C]Iodoacetamide was from American Radiolabeled Chemicals. Recombinant human glutaredoxin was expressed in Escherichia coli (12) and purified as described (13). Typical specific activity of pure enzyme is 100 units/mg in the standard assay with cysteinyl-glutathione disulfide and GSH as substrates (12).

Effect of EGF or H_2O_2 on Protein Glutathionylation—A431 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum to 90% confluency. After washing and incubating with PBS (30–60 min at 37 °C), the cells were incubated with H_2O_2 or EGF (at the indicated concentrations) at room temperature for 30 or 10 min, respectively. Cells were lysed with 50 mM of buffer A (125 mM sodium phosphate, pH 7.5, 3 mM EDTA) containing 1% Triton X-100. Western blot analysis using anti-GSH antibody.

Generation of ROS in A431 Cells upon EGF Treatment—A431 cells were grown as described to 70% confluency. Then they were starved in Hanks’ balanced salt solution (containing 2 mM Mg^{2+} and sodium phosphate, pH 7.5, 3 mM EDTA) containing 1% Triton X-100. Western blot analysis using anti-GSH antibody.

Changes in the [GSSG]/[Total GSH] Ratio in Treated A431 Cells—A431 cells were grown to 90% confluency. After starvation in PBS for 2 h, cells were exposed to EGF (5 μM) or H_2O_2 (10 mM) or PBS (control) for 30 min at 37 °C, followed by lysis in 50 mM of buffer A (125 mM sodium phosphate, pH 7.5, 3 mM EDTA) containing 1% Triton X-100. Measurement of the [GSSG]/[Total GSH] ratio was adapted from Griffith (14).

Analysis of Cysteine pK_a—To determine cysteine pK_a values, pH-dependent radioactive incorporation into rabbit muscle actin from [45S]iodoacetamide was carried out analogous to previous studies (15). Buffers (10 mM) (MES (pH 5–7), HEPES (pH 7–9), CAPS (pH 9–10)) were adjusted to 0.5 μM concentration and contained carboxymethyl-BSA as carrier.
EGF-induced Deglutathionylation and Actin Polymerization

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**RESULTS AND DISCUSSION**

Monoclonal anti-GSH antibody was used to investigate growth factor-mediated protein glutathionylation in *vivo*. Fig. 1A shows that 

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**FIG. 1. Glutathionylation and deglutathionylation of a 42-kDa protein upon 
H$_2$O$_2$ and EGF treatment, respectively.** A431 cells were incubated at room temperature in PBS containing H$_2$O$_2$ (A) or EGF (B) at various concentrations. Incubation time was 30 min for H$_2$O$_2$ or 10 min for EGF. Then the cells were lysed with SDS sample buffer, and the glutathionylation state of the lysate proteins was analyzed by Western blot using monoclonal anti-glutathione antibody. The wide band in A at the top of lane 9 is likely derived from aggregates of highly glutathionylated proteins formed at very high H$_2$O$_2$ concentrations.

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In contrast, treatment of the same cell line with increasing concentrations of EGF surprisingly resulted in a progressive decrease in the extent of glutathionylation of the same protein band (Fig. 1B). At 0.5 μM EGF (lane 5), the glutathionylated 42-kDa protein was diminished to approximately one-third of the basal level. When EGF was raised to 2 μM or higher, glutathionylation was no longer observable (lanes 7 and 8). The unexpected decrease in glutathionylation of the 42-kDa protein prompted us to examine ROS generation in A431 cells in response to EGF. Using the membrane-permeable fluorescent ROS indicator DCF-DA, we found that 0.17 μM EGF induced a rapid ROS production as indicated by enhancement of fluorescence intensity (data not shown). This is in agreement with Bae et al. (2). Because the [GSSG]/[Total GSH] redox ratio affects the state of glutathionylated proteins, we also examined the effect of EGF-induced ROS production on the redox state of GSH (Fig. 2). For control cells, the redox ratio was 3.4 ± 0.1%. When cells were treated with 1 μM EGF or 10 mM H$_2$O$_2$ for 30 min, the [GSSG]/[Total GSH] ratio increased to 6.2 ± 1% or 17 ± 2%, respectively. These results indicate that EGF did shift the cellular redox equilibrium toward more oxidative, so the observed deglutathionylation of the 42-kDa protein most likely is due to an active compensatory process that is required for cellular response. This unexpected dichotomy was also observed with HeLa cells.

To identify the 42-kDa protein, we used HeLa cells to obtain a larger quantity of the protein. The protein was purified to homogeneity using consecutive FPLC chromatographic separations, which included CM-Sepharose, Q-Sepharose, and phenyl-Sepharose, followed by two-dimensional electrophoresis. The purified protein was subjected to trypsin digestion and peptide sequence analysis (Michigan State University Core Facility) and was thereby identified as the cytoplasmic actin. Glutathionylation of actin, among other proteins, has been shown to be under stress, its high purity and sequence homology. When the actin was served with HeLa cells.

MS Analysis of Glutathione Residue Transfer—Rabbit muscle actin (~100 μg) was immobilized onto 200-μl dry volume of DNAse-coupled resin and glutathionylated with 1 μM GSSG in PBS for 1 h at room temperature. After thorough washing with PBS, the resin was suspended in 100 μl of PBS containing 0.2 nmol (2.3 μg) of glutaredoxin and incubated at 37 °C for 1 h before centrifugation. The supernatant was subjected to LC-MS analysis with a Finnigan LCQ (Thermoquest, San Jose, CA) mass spectrometer.

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**FIG. 2. [GSSG]/[Total GSH] ratios in A431 cells treated with H$_2$O$_2$ and EGF.** The ratios of GSSG, the oxidized form of GSH, to total GSH concentration were determined according to the 2-vinylpyridine/DTNB assay (see “Experimental Procedures”). For A431 cells incubated 30 min in PBS alone (PBS), in the presence of 1 μM EGF/PBS (EGF), or in 10 mM H$_2$O$_2$ for 30 min (H$_2$O$_2$), the ratios were determined to be 3.4 ± 0.1, 6.2 ± 1, and 17 ± 2%, respectively.
incubated with 1 mM GSSG for 30 min, the modified actin showed a molecular mass increase of 305 Da relative to untreated actin, based on LC-MS analysis, indicating incorporation of one GSH per actin. The modified actin was then subjected to CNBr cleavage followed by mass spectrometric analysis of the peptides. The 305-Da GSH moiety was found to be associated with a peptide (WITKQEDAEAPSIVHRKCF) encompassing the C-terminal penultimate cysteine. This result identified the penultimate cysteine (Cys-374) as the most likely glutathionylation site in vivo, consistent with findings that this cysteine appears to be the most reactive; it was preferentially labeled at physiological pH by thiol-modifying reagents (10, 18). Cysteine appears to be the most reactive; it was preferentially labeled at physiological pH by thiol-modifying reagents (10, 18). This suggests that the relative reactivity of the cysteines in actin may not be determined by unusually low pKa values, but by their accessibility. Accordingly, we investigated the pH dependence of the reaction of actin with [3H]IAM. The data (not shown) revealed a single inflection point corresponding to a normal pKa of 8.4, and only one carboxamido moiety was incorporated per actin molecule when iodoacetamide was maintained at a 3-fold molar excess relative to actin. However, when actin was treated with a 12-fold molar excess of IAM at pH 11, nearly six carboxamido moieties were incorporated, corresponding to the six cysteine residues per actin monomer.

Several reports have addressed the relationship between the states of thiol groups on actin and its state of polymerization (16, 17, 21, 22), however, no clear understanding of the effect of oxidative modification can be derived from those studies. Therefore, we studied the relative rates of polymerization of the glutathionylated and non-glutathionylated forms of bovine muscle G-actin induced by 2 mM Mg2+ and 100 mM KCl. Monitored by light scattering, the steady-state rate for non-glutathionylated actin was at least 5.6-fold faster than that of the glutathionylated actin (Fig. 3). These data are consistent with the finding that a small cleft exists in the actin molecule in the vicinity of Cys-374, and the size of this cleft is reduced upon polymerization (23). Accordingly, the GSH moiety at the penultimate cysteine is expected to hinder the rate of actin polymerization. Furthermore, in vitro modifications of the highly conserved C-terminus of actin including cysteine S-glutathionylation and C-terminus truncation of the last two residues led to destabilization of filamentous actin (24, 25). The physiological effect of EGF-mediated actin de glutathionylation on F-actin formation was investigated both in batch and at the single cell level. When A431 cells, with or without 1 μM EGF, were incubated for 20 min and then fixed, permeabilized, and stained with the filamentous actin indicator, phalloidin Oregon Green 488, the results revealed that the F-actin increased by more than that observed with glutathionylated actin.

To address the in vivo role of glutaredoxin, we investigated the effects of Cd2+, a known glutaredoxin inhibitor that has been known to inhibit intracellular deglutathionylation (8). Here, A431 cells were preincubated with 2 μM CdCl2, the IC50 range observed in vitro, for 1 h prior to treatment with EGF. Western blot analysis (Fig. 6) revealed that Cd2+ itself had little effect on actin glutathionylation prior to EGF treatment (lanes 1 and 3). However, Cd2+ inhibited EGF-induced actin de glutathionylation (lanes 2 and 4). Together, these results
**Fig. 5.** Transfer of glutathione moiety from actin to glutaredoxin. Actin (100 μg) was immobilized on DNase I-coupled resin and treated with 1 mM GSSG in PBS at room temperature for 1 h to give selective glutathionylation at the penultimate cysteine. After thorough washing with PBS, the resin was suspended in 0.2 nmol of human recombinant glutaredoxin was added and incubated at 37 °C for 1 h. The supernatant was subjected to LC-MS analysis. Comparison of panel B to panel A shows transfer of the glutathione moiety (305 Da) to the glutaredoxin.

**Control**

**EGF**

**Cd²⁺**

**Cd²⁺/EGF**

**Fig. 6.** Deglutathionylation of actin is blocked by Cd²⁺. After incubating for 1 h at 37 °C with PBS (lane 1) or with CdCl₂ (2 μM) in PBS (lane 3), the A431 cells were further incubated at 37 °C for 20 min in the absence (lanes 1 and 3) or presence of 1 μM EGF (lanes 2 and 4). The medium was removed, and the cells were lysed in 2X SDS sample buffer and analyzed for actin glutathionylation by Western blot with the monoclonal anti-glutathione antibody. The results indicate that glutaredoxin is responsible for the deglutathionylation of actin in response to growth factor stimulation in A431 cells.

Because conventional actins serve to provide a framework for cellular structure and mobility, constant movement and morphological changes of cells are achieved by a rapid rearrangement of actin filaments (11, 26). To achieve these multiple cellular functions, actin polymerization, translocation, polarity, and rate of assembly are rigorously regulated by a large number of actin-binding proteins as well as by covalent modifications that may also target the actin-binding proteins (10). Actin phosphorylation (27) and ADP-ribosylation (28) have been reported, and these events are linked to the state of polymerization, although details of the covalent modifications in the signal transduction pathways have not been fully investigated. Previously, it was reported that the EGF receptor is associated specifically with actin filaments in A431 cells (29). Our findings directly link this growth factor-mediated signaling pathway to deglutathionylation of actin at its penultimate cysteine residue, catalyzed by glutaredoxin, which leads to enhancement of the rate of actin polymerization and translocalization of the polymerized actin to the cell periphery. These results also highlight the importance of reversible glutathionylation of protein as a physiologically relevant regulatory mechanism. It is remarkable that the same EGF stimulus results in opposite changes in the intracellular state of glutathionylation of PTP1B (5) and actin (this report). In light of the likely role of cytoskeletal arrangements in transducing extracellular signals (30), regulation of the state of glutathionylation of actin and corresponding changes in its state of polymerization may modulate the localization and dynamic assembly of specific signal transduction scaffolds.

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