Epidermal Growth Factor Receptor Exposed to Oxidative Stress Undergoes Src- and Caveolin-1-dependent Perinuclear Trafficking

The epidermal growth factor (EGF) receptor (EGFR) has been found to be overexpressed in several types of cancer cells, and the regulation of its oncogenic potential has been widely studied. The paradigm for EGFR down-regulation involves the trafficking of activated receptor molecules from the plasma membrane, through clathrin-coated pits, and into the cell for lysosomal degradation. We have previously shown that oxidative stress generated by H$_2$O$_2$ results in aberrant phosphorylation of the EGFR. This leads to the loss of c-Cbl-mediated ubiquitination of the EGFR and, consequently, prevents its degradation. However, we have found that c-Cbl-mediated ubiquitination is required solely for degradation but not for internalization of the EGFR under oxidative stress. To further examine the fate of the EGFR under oxidative stress, we used confocal analysis to show that the receptor not only remains colocalized with caveolin-1 at the plasma membrane, but at longer time points, is also sorted to a perinuclear compartment via a clathrin-independent, caveolae-mediated pathway. Our findings indicate that although the EGFR associates with caveolin-1 constitutively, caveolin-1 is hyperphosphorylated only under oxidative stress, which is essential in transporting the EGFR to a perinuclear location, where it is not degraded and remains active. Thus, oxidative stress may have a role in tumorigenesis by not only activating the EGFR but also by promoting prolonged activation of the receptor both at the plasma membrane and within the cell.

Activation of the epidermal growth factor (EGF) receptor (EGFR) by EGF results in the initiation of signal transduction cascades involved in cellular survival and proliferation. Therefore, to control cellular growth and tumorigenesis, the activation of the EGFR has to be tightly regulated in a process that includes degradation of the receptor. Binding of EGF to EGFR is rapidly followed by internalization of the membrane-bound receptor mainly through clathrin-coated pits and into early endosomes, which develop into late endosomes. There, the EGFR is either either targeted to lysosomes for degradation or recycled to the plasma membrane (1–3). The inability of the EGFR to be down-regulated via clathrin-mediated endocytosis and degradation has been linked to its oncogenicity (4, 5).

H$_2$O$_2$-induced oxidative stress has been shown to activate and aberrantly phosphorylate the EGFR, which impedes the clathrin-mediated endocytosis and subsequent lysosomal degradation of the receptor (6–8). This results in prolonged downstream activation of proliferative molecules such as Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) (9), and the lack of receptor turnover has been shown to mediate tumor promotion in non-neoplastic rat liver epithelial cells (10). To gain more insight into H$_2$O$_2$-induced EGFR signaling and hyperplastic responses, we examined the trafficking of the receptor under oxidative stress.

Huang and Sorkin (11) have recently reported that knock-down of Grb2 by RNA interference inhibits clathrin-mediated endocytosis of the EGFR after exposure to EGF, and this was linked to the recruitment of the RING domain of c-Cbl to EGFR. In agreement with these data, we have shown that both Grb2 and c-Cbl association with EGFR are abolished following exposure to H$_2$O$_2$ (6, 9). Earlier confocal analysis of EGFR exposed to H$_2$O$_2$ also showed that the receptor is not internalized into vesicular structures in the cell the way it is under EGF exposure, suggesting that the receptor cannot enter clathrin-coated pits under oxidative stress (6). In light of these data, we wanted to determine how EGFR was being sorted under oxidative stress.

We began by looking at caveolae, which are specialized flask-shaped subdomains of biochemically defined lipid rafts (12, 13). Caveolae are formed when caveolin-1 and caveolin-2 hetero-oligomerize and are integrated into the lipid raft (14–16). It has been suggested that caveolin-1 can function in caveolae in a manner analogous to the way clathrin adaptors draw membrane receptors to coated pits and/or drive membrane invagination and budding (12). Caveolin-1 is known to interact directly with many signaling molecules through its caveolin-scaffolding domain at residues 82–101 (17, 18). Indeed, EGFR has been reported to interact with the caveolin-scaffolding domain through a caveolin-binding sequence motif located in the intracellular kinase domain (residues 898–905) of the receptor (17, 18).

Our previous data showed that Tyr-845 of the EGFR was hyperphosphorylated under oxidative stress, and this was attributed to Src activation (9). Since Tyr-14 of caveolin-1 has been identified as a target of Src kinase (19), we wanted to examine the role of caveolin-1 in clathrin-independent EGFR trafficking. Our findings suggest that, under oxidative stress, EGFR is able to undergo clathrin-independent endocytosis.
and is sorted to a perinuclear compartment, where it is not degraded and remains active. The mechanism of this trafficking involves activation of Src by H$_2$O$_2$, which subsequently phosphorylates caveolin-1 Tyr-14 and triggers the caveolar endocytosis of EGFR.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—A549 human lung carcinoma cells from ATCC (Manassas, VA) were maintained in F-12K (Kaighn’s modification) nutrient mixture (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. Prior to treatments, cells grown to ~80% confluence were serum-starved overnight in F-12K medium containing 0.5% dialyzed fetal bovine serum. Chinese hamster ovary cells (ATCC) were maintained in a 1:1 mixture of F-12 Ham (Invitrogen) and Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. Prior to treatments, cells grown to ~80% confluence were serum-starved overnight in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 0.5% dialyzed fetal bovine serum. Transient transfections using WT (Cav-1) and Y14A mutant caveolin-1 in A549 cells or WT and K721A or Y845F mutant EGFR (a generous gift from Dr. Sarah J. Parsons, University of Virginia, Charlottesville, VA) in Chinese hamster ovary cells were performed using Lipofectamine transfection reagent (Invitrogen) with the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s protocol. Transfected cells were serum-starved ~34 h after transfection and treated ~48 h after transfection. For clathrin heavy chain (CHC) silencing, A549 cells were transiently transfected with the following CHC-specific siRNA: sense, 5'-GCAUGAGCUGUUUGAAGAUU-3', and antisense, 5'-PUCUCAACACGCUAUUGCUU-3' (Dharmacon RNA Technologies, Lafayette, CO) or mock-transfected using the Dharmafect 1 transfection reagent (Dharmacon) according to the manufacturer’s instructions, with one modification. The cells were transfected at ~70% confluence in 10-cm dishes, passaged 24 h later onto coverslips for confocal studies, or into 10-cm dishes for immunoblot and real-time PCR analyses. After 24 h, the cells were transfected again according to the manufacturer’s protocol, serum-starved 24 h later, and treated ~16 h after serum starvation.

**Real-time PCR**—RNA was purified using the RNeasy mini kit (Qiagen, Valencia, CA) and treated with DNase using the RNase-free DNase...
set (Qiagen). 4 μg of total RNA were used for the reverse-transcriptase reaction performed with the SuperScript first-strand synthesis system for reverse transcription-PCR (Invitrogen). The cDNA was diluted 1:10, and real-time PCR was carried out using SYBR® Green PCR master mix (Applied Biosystems, Foster City, CA). The primers for the PCR were (based on accession number NM_203506): forward primer, 5'-CTG CAG ACG ACG TG-3', and reverse primer, 5'-GAA CTT CAC CAC CCA GAG GA-3'. The product size is 221 bp. Primers for β-actin served as a control for normalization of cDNA. The real-time PCR was run in a 7900HT sequence detection system (Applied Biosystems).

Treatments—To generate H2O2, glucose oxidase (GO; type II from Aspergillus niger, 15,500 units/g; Sigma) was added to serum-free Dulbecco’s modified Eagle’s medium containing 25 mM glucose and 0.5% bovine serum albumin (Sigma). This medium was then preconditioned for 15 min at 37 °C and added to cells for 15 min at 37 °C. For incubation periods greater than 15 min, GO-containing medium was replaced every 15 min with fresh preconditioned medium. For EGF treatments, cells were incubated in the same medium supplemented with 100 ng/ml EGF (Upstate Biotechnology, Inc., Waltham, MA). For inhibition of Src, cells were preincubated with 5 μM PP1 (Biomol, Inc., Plymouth Meeting, PA) for 45 min followed by treatment as indicated in the presence of PP1.

Immunofluorescence—Immunofluorescence was performed as described previously (20). Briefly, A549 cells grown on coverslips (either transiently transfected or not transfected) were treated as indicated and fixed in 4% paraformaldehyde in phosphate-buffered saline. Cells were permeabilized for 15 min at room temperature with phosphate-buffered saline containing 1% bovine serum albumin, 0.2% Triton X-100, and 0.02% sodium azide, and then coverslips were blocked in phosphate-buffered saline containing 1% bovine serum albumin, 0.2% Nonidet P-40, 5% goat serum, and 0.02% sodium azide at room temperature for 1 h. Coverslips were then incubated for 1 h in primary antibody followed by 1 h in secondary antibody. Primary antibodies used were: anti-EGFR clone 528 (generously provided by John Mendelsohn, Memorial Sloan Kettering Cancer Center, New York, NY); anti-caveolin-1 N-20 and anti-EGFR phospho-Tyr-1173 (Santa Cruz Biotechnologies, Santa Cruz, CA); and anti-Cav-1 phospho-Tyr-14 (BD Transduction Laboratories). The secondary antibodies used were Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR), and nuclei were stained with 1 μg/ml DAPI or 1 μg/ml propidium iodide (Molecular Probes) for 3 min after removal of secondary antibodies. Coverslips were mounted onto glass slides using the ProLong antifade kit (Molecular Probes). Confocal microscopy was performed using an Olympus FX1000 Fluoview confocal laser scanning microscope. All images are merged unless otherwise indicated and are representative of at least 100 cells viewed in each of three separate experiments.

Lysate Preparation, Immunoprecipitation, and Immunoblotting—Lysate preparation and protein immunoprecipitation were performed as described by Bao et al. (21). After treatments, cells were extracted in solubilization buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM EGTA, protease inhibitor mixture (Sigma), and phosphatase inhibitor mixture (Sigma). Lysates were cleared by centrifugation, and 400 μg of protein in the supernatant were immunoprecipitated by overnight incubation with 4 μg of anti-EGFR clone 225 (a generous gift from ImClone Systems Inc., New York, NY) at 4 °C followed by protein A (Repligen Corp., Needham, MA) precipitation for 1-2 h at 4 °C. Immunoprecipitates were washed three times with HNTG buffer containing 20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked for 1 h in Tris-buffered saline, pH 7.5, containing 0.5% Tween 20 and 5% nonfat milk and incubated overnight at 4 °C with primary antibodies followed by a 1-h incubation at room temperature with a 1:10,000 dilution of horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoreactive protein bands were detected with the SuperSignal West Pico substrate (Pierce). Blotting antibodies used were: anti-clathrin heavy chain X22 (Affinity Bioreagents, Golden, CO); anti-EGFR RK2 (generously provided by Dr. J. Schlessinger); anti-phosphotyrosine PY-20, anti-Src, anti-caveolin-1

![FIGURE 2. H2O2-activated EGFR remains active in a perinuclear compartment.](image)

![FIGURE 3. Clathrin silencing prevents EGFR internalization under EGF treatment but does not prevent EGFR perinuclear accumulation under oxidative stress.](image)
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Results

EGFR under Oxidative Stress Co-localizes with Caveolin-1 at the Plasma Membrane but Is Internalized over Time into a Perinuclear Region—When the EGFR is activated by EGF, the receptor is rapidly internalized within clathrin-coated pits and undergoes degradation via the lysosomal pathway. This is easily viewed by immunofluorescence staining, in which there is an even distribution of receptor throughout the plasma membrane when the cells are not treated. After treatment with 100 ng/ml EGF for 15 min, EGFR is rapidly redistributed into punctate vesicular compartments within the cell (Fig. 1A). Although H2O2 activates EGFR, as we have previously shown (6), the receptor remains at the plasma membrane, where it co-localizes with caveolin-1 (Fig. 1A). Over time, there is some accumulation of the EGFR in a perinuclear location (Fig. 1B). Furthermore, we were able to track the movement of the EGFR from the plasma membrane to the perinuclear region of the cell by predelaying the receptor with anti-EGFR mAb 528 prior to exposure to 1 unit/ml GO for 45 min or 100 ng/ml EGF for 15 min (Fig. 1C). This indicates that the perinuclear EGFR originates in the plasma membrane and is not the result of receptor recycling or new synthesis. Since the perinuclear EGFR is not degraded, we were also interested in determining whether it was still active. Confocal analysis using a phospho-specific antibody to Tyr-1173 shows that the perinuclear EGFR remains phosphorylated at this trans-autophosphorylation site and is therefore active (Fig. 2).

Perinuclear Accumulation of EGFR under Oxidative Stress Is Clathrin-independent—A recent study reported by Huang and Sorkin (11) demonstrated that Grb2 facilitated the recruitment of the RING domain of c-Cbl to the EGFR following EGF treatment. This, in turn, allowed clathrin-mediated endocytosis of the receptor. Using RNA interference to knock down Grb2, they were able to show that Grb2 was essential for clathrin-dependent endocytosis of the EGFR. We have previously shown that mutation of the EGFR at Tyr-1045, the c-Cbl docking site, did not completely abolish EGFR internalization into early endosomes under EGF exposure. This was possibly due to the ability of c-Cbl to bind to the receptor indirectly via Grb2 and was abolished under oxidative stress, when Grb2 was no longer able to bind to the EGFR (9). This suggested that the perinuclear EGFR under oxidative stress did not traffic there via a clathrin-dependent pathway.

To determine whether perinuclear trafficking of the EGFR is clathrin-dependent, A549 cells were either mock-transfected or transfected with CHC siRNA and exposed to 100 ng/ml EGF for 15 min or 1 unit/ml GO or pretreated with 5 μM PP1 for 45 min followed by treatment with 100 ng/ml EGF for 15 min or 1 unit/ml GO for 30 min (B). Cells were lysed and 50 μg of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted with antibodies against phospho-Src Tyr-416 (1:1,000), Src (1:1,000), phosphotyrosine (PY20, 1:3,000), EGFR (RK2, 1:1,000), and phosphorylated (Tyr-14) and total caveolin-1 (1:1,000). p-Src, phosphorylated Src; p-EGFR, phosphorylated EGFR; p-Cav-1, phosphorylated caveolin-1.

Figure 1. H2O2 induces association of EGFR and phosphorylated caveolin-1. A, serum-starved A549 cells were exposed to 100 ng/ml EGF for 15 min or 1 unit/ml GO for 30 min (upper); cells were also pretreated with 5 μM PP1 for 45 min followed by treatment with 100 ng/ml EGF for 15 min or 1 unit/ml GO for 30 min (lower). EGFR was immunoprecipitated (IP) from 400 μg of cell lysates with 4 μg of anti-EGFR C225 antibody followed by SDS-PAGE separation of proteins and immunoblotting (IB) with antibodies against EGFR (RK2, 1:1,000), phosphorylated (Tyr-14) and total caveolin-1 (1:1,000), and phosphotyrosine (PY20, 1:3,000). NT, not treated; p-Cav-1, phosphorylated caveolin-1; p-EGFR, phosphorylated EGFR. B, A549 cells were transiently transfected with plasmids encoding wild-type (Cav-1) or mutant (Y14A) caveolin-1, serum-starved, and exposed to 100 ng/ml EGF for 15 min or 1 unit/ml GO for 30 min. Cell lysates were immunoprecipitated with anti-EGFR C225 antibody followed by SDS-PAGE separation of proteins, transfer to a nitrocellulose membrane and immunoblotting with antibodies against phosphotyrosine (PY20, 1:3,000), EGFR (RK2, 1:1,000) and phosphorylated (Tyr-14) and total caveolin-1 (1:1,000). Note: in B, the blank area on the blot for IP:EGFR and IB:Cav-1 under GO treatment is due to incomplete stripping of this blot between probing with anti-Cav-1 and later probing with anti-Cav-1.
for 45 min. Real-time PCR and immunoblot analysis show a near total loss of CHC mRNA and protein, respectively, in the siRNA-transfected cells (Fig. 3, A and B). Confocal analysis shows that when cells are exposed to EGF, CHC silencing prevents EGFR from leaving the plasma membrane, where it co-localizes with caveolin-1 (Fig. 3C). Perinuclear accumulation of EGFR under oxidative stress, on the other hand, is unaffected by CHC silencing (Fig. 3C).

H$_2$O$_2$ Induces Src Family Kinase-dependent Phosphorylation of Caveolin-1—In our previous studies, we found that one of the hallmarks of aberrant EGFR phosphorylation induced by H$_2$O$_2$ was hyperphosphorylation of Tyr-845 (6). Since Tyr-845 is a known target for c-Src kinase (24), we examined the activation of Src under H$_2$O$_2$ exposure and found that Src is indeed activated by H$_2$O$_2$ (Fig. 4A). Furthermore, Tyr-14 of caveolin-1 has also been identified as a target of c-Src (19). To test whether H$_2$O$_2$ induces hyperphosphorylation of caveolin-1 Tyr-14 and whether this is dependent on Src kinases, A549 cells were exposed to H$_2$O$_2$ in the absence and presence of 5 μM PP1, a Src family kinase inhibitor. Immunoblot analysis using a phospho-specific antibody for caveolin-1 Tyr-14 shows that H$_2$O$_2$ induced phosphorylation of this site. This phosphorylation was inhibited by PP1, indicating that caveolin-1 Tyr-14 phosphorylation is dependent on Src kinase(s) (Fig. 4B).

Caveolin-1 That Is Associated with EGFR under Oxidative Stress Is Phosphorylated—Having shown that H$_2$O$_2$ induces caveolin-1 Tyr-14 hyperphosphorylation in a Src-dependent manner, the next step was to determine the significance of this event. By immunoprecipitating the EGFR in non-treated, EGF-treated, and GO-treated A549 cells, we examined the ability of EGFR to bind to caveolin-1. We found that EGFR appears to constitutively bind caveolin-1, but only under H$_2$O$_2$ exposure is the bound caveolin-1 phosphorylated (Fig. 5A, upper). Pretreatment of these cells with 5 μM PP1, followed by exposure to H$_2$O$_2$, resulted in the loss of caveolin-1 phosphorylation (Fig. 5A, lower) and again demonstrated the Src dependence of caveolin-1 phosphorylation. To further test the role of caveolin-1 Tyr-14 phosphorylation in the association between EGFR and caveolin-1, we utilized constructs for both wild-type caveolin-1 (WT Cav-1) and a caveolin-1 Tyr-14 mutant (Y14A). Transient transfection of these constructs into A549 cells followed by no treatment or treatment with 100 ng/ml EGF for 15 min or 1 unit/ml GO for 30 min and immunoprecipitation of EGFR shows that although both WT and Y14A Cav-1 are associated with EGFR, only WT Cav-1 is phosphorylated under H$_2$O$_2$ exposure (Fig. 5B). Additionally, although EGFR Tyr-845 is hyperphosphorylated under oxidative stress, phosphorylation of this site does not appear to have an effect on the receptor’s association with caveolin-1 or on perinuclear sorting. A mutant EGFR that cannot be phosphorylated at Tyr-845 (Y845F) is still
able to associate with caveolin-1 and undergo perinuclear trafficking under oxidative stress (supplemental Fig. S1).

Interestingly, although the H2O2-induced perinuclear EGFR is phosphorylated, EGFR kinase activity is not required for the trafficking of the receptor to this region. When Chinese hamster ovary cells were transiently transfected with WT EGFR or the K721A kinase-dead EGFR, there was perinuclear accumulation of the receptor after a 45-min exposure to 1 unit/ml GO (Fig. 6A). A 15-min exposure to 100 ng/ml EGF, however, resulted in the internalization of WT EGFR only. The K721A EGFR, which is not phosphorylated (Fig. 6B) and therefore not able to acquire the necessary protein-protein interactions with c-Cbl and Grb2 required for clathrin-mediated internalization, remained membrane-bound (Fig. 6A). Furthermore, immunoprecipitation of the WT and K721A EGFR shows that caveolin-1 is constitutively associated with both of the receptors and is phosphorylated only under oxidative stress (Fig. 6B).

The Role of Caveolin-1 in EGFR Trafficking under Oxidative Stress—Since caveolin-1 is phosphorylated under oxidative stress, we next wanted to determine whether association with phosphorylated caveolin-1 plays a role in the clathrin-independent sorting of EGFR under oxidative stress. First, A549 cells were pretreated with 5 μM PP1 for 45 min followed by treatment with 1 unit/ml GO for 45 min or 100 ng/ml EGF for 15 min. Confocal analysis shows that EGFR accumulates next to the nucleus only when cells are exposed to GO alone, and this is prevented by the inhibition of Src kinase(s) by PP1 (Fig. 7A). Cells under EGF treatment show internalization of EGFR into punctate vesicular compartments in the absence and presence of PP1 (Fig. 7A). To verify that perinuclear accumulation of EGFR is specifically dependent on phosphorylation of caveolin-1 at Tyr-14, A549 cells were transiently transfected with either the WT or Y14A Cav-1 constructs, serum-starved 32 h after transfection, and exposed for 45 min to 1 unit/ml GO or for 15 min to 100 ng/ml EGF 48 h after transfection. Confocal analysis of the WT Cav-1-transfected cells shows that EGFR under oxidative stress co-localizes with Cav-1 in a perinuclear compartment, whereas in Y14A-transfected cells, the EGFR does not exhibit perinuclear co-localization with caveolin-1 under oxidative stress (Fig. 7B). Futhermore, confocal analysis using an antibody against phospho-Tyr-14 of caveolin-1 indicates that the perinuclear EGFR co-localizes with the phosphorylated form of caveolin-1 under oxidative stress (supplemental Fig. S2). This demonstrates that phosphorylation of caveolin-1 at Tyr-14 is necessary for the movement of the EGFR/caveolin-1 complex to a perinuclear compartment.

DISCUSSION

Oxidative stress in the form of H2O2 has been shown to affect EGFR phosphorylation and trafficking (6, 9). Our previous studies have demonstrated that aberrant phosphorylation of EGFR under oxidative stress results in the loss of c-Cbl-mediated ubiquitination of the receptor, which is necessary for movement out of the early endosomes and progression into lysosomes for degradation (6, 9). The present study
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focuses on our additional observations regarding the fate of the aberrantly phosphorylated EGFR, which fails to be degraded. We found that under oxidative stress, EGFR does not reach the early endosomes (9) but rather traffics to a perinuclear compartment in a non-clathrin-mediated manner. Therefore, herein we concentrate on the mechanism of clathrin-independent trafficking of H$_2$O$_2$-activated EGFR into a perinuclear compartment, allowing for prolonged signaling not only at the plasma membrane but also within the cell.

Huang and Sorkin (11) recently showed that Grb2 is essential for recruiting the RING domain of c-Cbl to the EGFR to allow clathrin-dependent receptor endocytosis. Consistent with these findings, we have shown that under oxidative stress, both c-Cbl and Grb2 cannot bind EGFR, and therefore, the receptor cannot enter into clathrin-coated pits (9). Interestingly, however, EGFR can still be seen by immunofluorescence to accumulate inside the cell at a perinuclear location after H$_2$O$_2$ exposure (Fig. 1B), and silencing of clathrin demonstrates that this mode of trafficking is clathrin-independent (Fig. 3). Several groups have reported that the EGFR is found in caveolae, which have been proposed to sequester inactive signaling molecules and to mediate cross-talk between different signaling cascades (18, 23, 25). Recent publications have indicated that clathrin-independent endocytosis is mediated by caveolae in a Src-dependent manner (13, 26–28). Furthermore, H$_2$O$_2$ has been shown to inhibit phosphatase activity (29), and phosphatase inhibition by okadaic acid causes significant mobilization of static caveolae, presumably into an endocytic pathway (26, 30). This, again, was shown to be inhibited by genistein, a Src-family kinase inhibitor (26). Caveolin-1, a structural protein necessary for caveolae formation, has been shown to interact directly with EGFR (18). To elucidate the mechanism by which EGFR is being trafficked to the perinuclear compartment under oxidative stress, we investigated the involvement of cavin-1 in this process.

Tyr-14 of caveolin-1 is the target for c-Src kinase phosphorylation (19), and we have shown that H$_2$O$_2$ causes a significant activation of Src (Fig. 4A), which also appears to phosphorylate caveolin-1 at Tyr-14 in our model system. By expressing a Tyr-14 mutant (Y14A) caveolin-1 protein in our cell culture model, we were able to show that EGFR constitutively associates with caveolin-1, which is phosphorylated only in the presence of H$_2$O$_2$ (Fig. 5) and no longer accumulates in the perinuclear compartment either when Src is inhibited or when the Y14A caveolin-1 mutant is expressed (Fig. 6). Although there have been reports that other Src family kinases are involved in oxidative stress-induced phosphorylation of caveolin-1 (31, 32), our observation that EGFR Tyr-845, a c-Src target (24), is hyperphosphorylated under oxidative stress suggests that c-Src is the main kinase involved here. Addi-

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