Epidermal Growth Factor Receptor (EGFR) Signaling Requires a Specific Endoplasmic Reticulum Thioredoxin for the Post-translational Control of Receptor Presentation to the Cell Surface*1

Received for publication, November 1, 2014, and in revised form, February 9, 2015. Published, JBC Papers in Press, February 9, 2015, DOI 10.1074/jbc.M114.623207

Aiwen Dong, Dariusz Wodziak, and Anson W. Lowe1
From the Department of Medicine, Stanford University, Stanford, California 94305

Background: AGR2 expression supports the transformed properties of many cancer cell lines.
Results: AGR2 protein functions as an endoplasmic reticulum thioredoxin that regulates EGFR presentation to the plasma membrane.
Conclusion: AGR2 controls EGFR-mediated signaling.
Significance: AGR2 represents a novel regulatory mechanism for signal transduction and a therapeutic target for EGFR-dependent cancers.

The epidermal growth factor receptor (EGFR) is a well-characterized receptor-tyrosine kinase that functions in development and serves a vital role in many human cancers. Understanding EGFR regulatory mechanisms, and hence approaches for clinical intervention, has focused on ligand-receptor interactions and tyrosine kinase activity. Here, we show using the NCI-H460 lung and A431 epidermoid human cancer cell lines that EGFR binding to anterior gradient homolog 2 (AGR2) in the endoplasmic reticulum is required for receptor delivery to the plasma membrane and thus EGFR signaling. Reduced AGR2 protein levels or mutation of an essential cysteine in the active site result in decreased cell surface EGFR and a concomitant decrease in signaling as reflected by AREG, EGR1, and FOS expression. Similar to previously described EGFR nulls, an AGR2 null also resulted in embryonic lethality. Consistent with its role in regulating EGFR-mediated signaling, AGR2 expression is also enhanced in many human cancers and promotes the transformed phenotype. Furthermore, EGFR-mediated signaling in NCI-H460 cells, which are resistant to the tyrosine kinase inhibitor AG1478, is also disrupted with reduced AGR2 expression. The results provide insights into why cancer prognosis or response to therapy often does not correlate with EGFR protein or RNA levels because they do not reflect delivery to the cell surface where signaling is initiated. AGR2, therefore, represents a novel post-translational regulator of EGFR-mediated signaling and a promising target for treating human cancers.

EGFR-mediated signal transduction is initiated by soluble growth factors that bind the receptor at the cell surface (1–4).

* This work was supported by the Stanford Division of Gastroenterology and Hepatology.
1 To whom correspondence should be addressed: Alway Bldg., Rm. M211, 300 Pasteur Dr., Stanford, CA 94305-5187. Tel.: 650-725-6764; Fax: 650-723-5488; E-mail: lowe@stanford.edu.
2 The abbreviations used are: EGFR, epidermal growth factor receptor; AREG, amphiregulin; AGR2, anterior gradient homolog 2; ITGB1, integrin β1.
**EXPERIMENTAL PROCEDURES**

**Reagents**—The following antibodies were used for protein immunoblotting: anti-AGR2 (16), anti-EGFR (Cell Signaling, 2232), anti-phospho-EGFR (Abcam, ab40815), anti-EGR1 (Cell Signaling, 4153), anti-FOS (Cell Signaling, 2250), anti-ITGB1 (BD Biosciences, 610467), anti-GAPDH (Cell Signaling, 2118), anti-calnexin (Cell Signaling, 2433S), and anti-ACTB (Sigma, A2066). Antibodies used for immunoprecipitation include anti-AGR2 (Abnova, H00010551-M03), anti-EGFR that binds the mature protein (mAb 528, ATCC, HB-8509), and control mouse IgG2b (Santa Cruz Biotechnology, sc-2762). Other antibodies used for immunofluorescent staining include anti-ITGB (BioLegend, 303002) and anti-EGFR (Millipore, 06-847).

**Cell Culture and Treatments**—The lung adenocarcinoma cell line NCI-H460 and the epidermoid carcinoma cell line A431 (ATCC, Manassas, VA) were cultured according to protocols provided by the vendor. MCF-10A (ATCC) was cultured in DMEM/F-12 supplemented with 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin, 500 ng/ml hydrocortisone, and 100 ng/ml cholera toxin. After retroviral or lentiviral infection, cells were maintained in the presence of puromycin (2 μg/ml). N. gonorrhoeae (ATCC, Manassas, VA) were cultured according to protocols provided by the vendor. MCF-10A (ATCC) was cultured in DMEM/F-12 supplemented with 5% horse serum, 20 ng/ml EGF, 10 μg/ml insulin, 500 ng/ml hydrocortisone, and 100 ng/ml cholera toxin. After retroviral or lentiviral infection, cells were maintained in the presence of puromycin (2 μg/ml) (Sigma). For all N-ethylmaleimide experiments, cells were rinsed twice with ice-cold PBS and incubated with 20 mM N-ethylmaleimide in PBS for 10 min on ice. For the AG1748 (EGFR kinase inhibitor) experiments in the presence of serum, cells were plated 1 day before to reach 80% confluence when drug treatment was started. The culture media was replaced with fresh complete media containing 2% fetal bovine serum and 1% antibiotics and 1% penicillin/streptomycin. 5-FU treatment was started. The culture media was replaced with fresh complete media containing 2% fetal bovine serum and 1% antibiotics and 1% penicillin/streptomycin.

**Co-immunoprecipitation of Mixed Disulfides**—N-Ethylmaleimide (20 mM)-treated cells were lysed in cell lysis buffer (20 mM Tris HCl, pH 8.0, 137 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and 10% glycerol with protease inhibitor mixture) for 1 h. 5 μg of whole cell extracts were incubated with 2 μg of anti-AGR2 antibody (Abnova, H00010551-M03) or control mouse IgG2b (Santa Cruz Biotechnology, sc-2762) for 1 h. The protein complexes were precipitated by the addition of protein G-agarose beads (Millipore, 16-266) with incubation for 16 h at 4 °C. The beads were washed with cell lysis buffer 4 times and boiled for 5 min in sample buffer with or without 0.1% SDS. The eluents were analyzed with protein immunoblotting. Mass spectrometry was used to identify AGR2 binding proteins. Briefly, 30 μg of whole cell extracts were incubated with 10 μg of anti-AGR2 antibody. The beads were washed 4 times, and after the last wash bound proteins were eluted with 0.1 M glycine HCl, pH 2.7, into tubes containing 0.1 M Tris HCl, pH 9.0.

**Real-time PCR Analysis**—3 μg of RNA sample was reverse-transcribed with GoScript Reverse Transcriptase System (Promega, A5003) to generate cDNA, which was subjected to SYBR Green-based real-time PCR analysis. Primers used were: AGR2 forward (5’-ATGAGTGCCCCACACGTCAA-3’) and reverse (5’-GGACATCTGGGCACTAGGA-3’); AREG forward (5’-GTGTTCTGTCGCTCCTTGATA-3’) and reverse (5’-ACTCACAGGGGAAATCTCACT-3’); EGFR forward (5’-CCTACTCGTCCTAAACCCC-3’) and reverse (5’-TGGCACTTCTTACACTTGCGG-3’); EGR1 forward (5’-GTACAGTGTTGTCGCTGCGATTT-3’) and reverse (5’-GAGGATCATACCTGTGTTGTCGT-3’); FOS forward (5’-CATTCCCAGGGAGACACTCAGGA-3’) and reverse (5’-AGGTGATCACGAGGATCTCTT-3’); HERPUD1 forward (5’-TGGAGTGGGACCACCTCAAACAA-3’) and reverse (5’-GGTGTGACGAGCAAGG-3’); HSPA5 forward (5’-CACAGTGATGCTCAGATCTCGG-3’) and reverse (5’-TGGTCTCTCTGCTAGGAGG-3’); HSPA5 forward (5’-AGTGGGACCACCTCAAAG-3’) and reverse (5’-TGCCTCTCTGCTAGGAGG-3’).
Appropriate Alexa Fluor® 594- or 488-conjugated secondary antibody (Invitrogen, 1:500) was added to the slide for 1 h at room temperature in the dark. The slides were then washed 5 times with PBS and mounted using VECTASHIELD antifade reagent (Vector Laboratories, H1200) with DAPI nuclear stain. The slide was imaged using a Nikon TS-1 confocal microscope (Nikon C1 system) with a 60× objective lens.

Subcellular Fractionation—MCF-10A cells transfected with either AGR2 or vector were plated in 150-mm dishes. Isolation of plasma membranes and microsomes was performed as previously described under the following conditions (24). After two washes with ice-cold PBS, cells were carefully harvested into 3 ml of homogenization buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 5 mM Tris-HCl, pH 7.4, 1× protease inhibitor mixture) with a rubber policeman and transferred to a prechilled 5 ml Kontes Potter-Elvehjem homogenizer. Homogenization was performed for 5 strokes with steady and consistent pressure. The homogenate was transferred into a cooled polypropylene tube (Falcon 2059) and centrifuged at 600 g for 3 min (HB-4 rotor, Sorvall). The supernatant (S1) was reserved and centrifuged again at 600 g for 3 min. The resultant supernatant (S2) was then centrifuged for 20 min at 20,000 × g and 4 °C (70Ti rotor, Beckman), which produced a pellet (P3) enriched in plasma membranes. The supernatant was centrifuged at 100,000 × g for 1 h (Ti70, Beckman), which produced a pellet (P4) enriched in microsomal membranes. The pellet was resuspended in 100 μl of 300 mM sucrose, 10 mM Hepes, pH 7.4.

Site-directed Mutagenesis—AGR2-C81A was produced with the QuikChange II XL mutagenesis kit (Stratagene) using human AGR2 cDNA and expressed from the pcDNA3.1 vector (Life Technologies) (16). References to the AGR2 amino acid sequence are derived from NCBI accession code NP_006399.

Flow Cytometry—EGFR expression at the plasma membrane was determined by plating cells in 60-mm dishes. Twenty-four hours later the culture media was replaced with serum-free media for 1 h. The cells were washed with PBS, detached with Cell Dissociation Buffer (Invitrogen, 13151-014), and collected into tubes containing complete media on ice. Cells were washed 4 times with Cell Staining Buffer (BioLegend, 420201) and blocked with 5% BSA in PBS for 15 min at room temperature. After four washes with Cell Staining Buffer, cells were incubated with allophycocyanin-labeled anti-EGFR (BioLegend, 352905) or isotype control (BioLegend, 400121) for 45 min at 4 °C in the dark. Flow cytometry data were acquired using the Scanford (FACScan, Cytek) and analyzed with FlowJo v10 software (TreeStar).

Isolation of Cell Surface Biotinylated Proteins—Cell surface proteins were labeled covalently using a membrane-impermeant biotinylation reagent EZ-Link Sulfo-NHS-Biotin (Pierce, 21217). The following steps were carried out at 4 °C to prevent any trafficking; cells were washed 3 times with PBS2+ (PBS with 0.1 mM CaCl2, 1 mM MgCl2) followed by a 30-min incubation with 1 mg/ml Sulfo-NHS-Biotin in PBS2+, washed twice with PBS2+, and quenched twice with 50 mM glycine in PBS2+ for 5 min. After four washes with PBS2+, the cells were lysed while on the plate (1% Triton X-100, 150 mM NaCl, 10% glycerol, 50 mM HEPES pH 7.5, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM Na3VO4, 2 mM EDTA, and a protease inhibitor mixture (Sigma, P8340)) followed by centrifugation for 10 min at 16,000 × g. The protein concentration of the cleared lysate was determined using the bicinchoninic acid assay (Pierce, 23228 and 23224). 200 μg of protein samples were transferred to new tubes, and the volume was brought up to 400 μl with lysis buffer before streptavidin beads (GE Healthcare, 17511301) were added and agitated at 4 °C for 3 h. The beads were collected by centrifugation (7000 × g) and washed 4 times with lysis buffer at 4 °C followed by an incubation at 95 °C in Laemmli sample buffer. Equivalent volumes were analyzed with protein immunoblotting followed by densitometry of the resultant bands with a flatbed scanner and ImageJ (image.nih.gov). For Fig. 2G, NCI-H460-shAGR2 cells were plated in six 60-mm dishes and transfected with vector control, wild-type AGR2, or the AGR2 C81A mutant with Lipofectamine 2000 (Invitrogen). Forty-eight hours post-transfection, cell surface proteins were labeled with biotin or left unlabeled as described above.

RESULTS

AGR2 Forms a Mixed Disulfide with EGFR—Based on the hypothesis that AGR2 acts as a thioredoxin, AGR2 should form a mixed-disulfide reaction intermediate with its substrate(s). NCI-H460 lung adenocarcinoma cells were used to identify potential substrates because AGR2 expression is necessary for the cells’ transformed properties (16, 17). N-Ethylmaleimide, which alkylates free sulfhydryl moieties, was administered to the NCI-H460 cells to promote the persistence of mixed-disulfides and enhance detection of AGR2 substrates (25). AGR2 antibodies were then employed to isolate mixed-disulfide complexes from homogenates of N-ethylmaleimide-treated or untreated NCI-H460 cells by immune precipitation followed by protein immunoblotting with AGR2 antisera produced in a different species. If the samples were left unreduced to retain the mixed-disulfide complexes, major bands ~170 kDa were detected that disappeared or decreased in intensity if the samples were initially reduced with dithiothreitol, therefore, supporting their identity as AGR2 mixed disulfides (Fig. 1A). EGFR was suspected as a potential AGR2 substrate because of its known mass and the previously characterized effects of AGR2 on EGFR signaling via AREG expression (16). Protein immunoblotting with EGFR antisera of the same AGR2 immune-precipitated mixed disulfide samples revealed bands identical to those previously visualized with the AGR2 antisera, therefore, identifying EGFR as a potential AGR2 substrate (Fig. 1B). The AGR2-EGFR mixed disulfide was also detected with protein immunoblotting of N-ethylmaleimide-treated NCI-H460 whole cell lysates by protein immunoblotting (Fig. 1C).
EGFR Delivery to the Cell Surface Requires AGR2

The experiments were repeated with A431 cells, a cell line derived from an epidermoid carcinoma that is often used as a model system because of its high EGFR expression (26). Previous studies have shown that AREG expression is also strongly induced by EGFR signaling (27, 28). Whether A431 cells are also dependent on AGR2 for EGFR signaling and AREG expression was evaluated. Protein immunoblotting established that A431 cells also express AGR2 (Fig. 1D). Transduction of the A431 cells with shAGR2 led to an 15.8-fold decrease in AGR2 RNA along with a concomitant 10.2-fold decrease in AREG expression and established that it is influenced by AGR2 expression in A431 cells. N-Ethylmaleimide treatment of A431 cells also preserved AGR2 mixed disulfides with EGFR that were disrupted with a reducing agent (Fig. 1, E and F). AGR2 immune precipitates from A431 cells were also analyzed by mass spectrometry, which identified EGFR as the top candidate with respect to the number of total unique peptides and the total spectrum count (supplemental Table S1). No other ErbB family members were detected in the mass spectrometry analysis. Together, the results support a physical interaction between AGR2 and EGFR.

AGR2 Expression Regulates EGFR Expression at the Plasma Membrane—The most common role ascribed to endoplasmic reticulum-based thiredoxins is the folding or assembly of proteins through the formation of disulfide bonds (29). Secreter proteins that have attained the appropriate conformation are then allowed to progress to the Golgi apparatus and the remainder of the secretory pathway. Proteins that are unable to achieve the appropriate conformation are exported from the endoplasmic reticulum and destroyed in the proteasome. An evaluation was, therefore, initiated to determine whether AGR2 interactions with EGFR influence receptor delivery to the plasma membrane. Our previous studies showed that transduction with shAGR2 is able to reduce AGR2 RNA and protein in NCI-H460 cells by 11- and 9-fold (16). Two different shAGR2 sequences were used to decrease AGR2 expression, which resulted in similar results on AGR2 expression.

Immunocytochemistry was performed using non-permeabilized NCI-H460 and A431 cells that were labeled with a monoclonal antibody (mAb528) that binds the mature EGFR ectodomain at the cell surface (30, 31). Compared with vector controls, a striking decrease in EGFR cell surface staining in both NCI-H460 and A431 cells was observed after AGR2 expression was reduced with shAGR2 (Fig. 2A). As a control, no changes in cell surface expression of integrin, β1 (ITGB1) were observed in either cell line after AGR2 knockdown (Fig. 2B). Immunocytochemistry was also performed on detergent-per-
EGFR Delivery to the Cell Surface Requires AGR2

A

EGFR (mAb 528)/DAPI

vector

shAGCR2

H460

A431

B

ITGB1/DAPI

vector

shAGCR2

H460

A431

C

EGFR (COOH)/DAPI

vector

shAGCR2

H460

A431

D

Events (Modal)

H460

A431

E

H460

Vector

shAGCR2

Biotin

Strept -IP

ITGB1

cell lysate

EGFR

ACTB

A431

Vector

shAGCR2

Biotin

Strept -IP

ITGB1

cell lysate

EGFR

ACTB

F

EGFR mRNA

H460

Vector

shAGCR2

A431

Vector

shAGCR2

G

NCI-H460-shAGCR2

vector

shAGCR2

DMSO

Tunicamycin

Vector

shAGCR2

H

HSPAG mRNA (ΔCt)

DNDS

Tunicamycin

Vector

shAGCR2

HERPUD1 mRNA (ΔCt)

DNDS

Tunicamycin

Vector

shAGCR2

p = 0.0113

p = 0.0992

p < 0.0001

p = 0.3986
meabilzed NCI-H460 cells with an anti-cytoplasmic domain EGFR antibody, which also revealed a decrease in cell surface EGFR with shAGR2 reduction of AGR2 expression. The anti-cytoplasmic antibody was also able to detect significant amounts of intracellular EGFR (Fig. 2C).

Further validation of decreased cell surface EGFR expression with shAGR2 was obtained with flow cytometry. Flow cytometry with anti-EGFR antibodies determined that the mean cell surface EGFR expression decreased by 5- and 58-fold compared with vector controls in NCI-H460 and A431 cells, respectively, after AGR2 expression was reduced with shRNA (Fig. 2D). A third approach biotinylated all cell surface proteins, which was followed by their isolation with streptavidin beads. The relative amount of biotinylated cell surface EGFR was determined by protein immunoblotting. A 4- and 10-fold decrease in plasma membrane EGFR after transduction with shAGR2 was observed in NCI-H460 and A431 cells, respectively, compared with vector controls (Fig. 2E). ITGB1 was used as a plasma membrane control and displayed a 0- and 1.8-fold decrease in NCI-H460 and A431 cells, respectively. Quantitative RT-PCR for EGFR transcripts revealed no significant difference between wild-type and shAGR2-treated cells (Fig. 2F). Likewise, an evaluation for changes in total EGFR protein secondary to shAGR2 also revealed no significant changes when compared with β-actin (Fig. 2, E and G). After transduction with shAGR2, EGFR is present within the cell and not on the cell surface (Fig. 2, C and G). Additional control experiments assessed for the presence of endoplasmic reticulum stress in the context of transduction with shAGR2. Analysis of HSPA5 and HERPUD1 revealed no signs of endoplasmic reticulum stress (Fig. 2H), which is consistent with our published work and that of others (32–34). The data, therefore, demonstrate that cell surface EGFR was determined by its cellular distribution rather than the total amount of receptor in the cell.

As a control for potential off target effects of shAGR2, rescue experiments were performed with NCI-H460-shAGR2 cells that express low AGR2 levels. Transduction of NCI-H460-shAGR2 cells with wild-type AGR2 cDNA that is insensitive to inhibition at the cell surface. Consistent with previous studies (37), the EGFR-specific tyrosine kinase inhibitor, AG1478, was ineffective at concentrations up to 50 μg/ml in NCI-H460 cells in inhibiting EGFR expression in the A431 cells (Fig. 3, D and E). Reduction of AGR2 expression with shAGR2 dramatically reduced EGFR-stimulated EGFR phosphorylation and EGR1 and FOS protein and RNA expression (Fig. 3, A–C). Similar reductions in EGFR phosphorylation and EGFR and FOS protein and RNA were observed. The reduction in AGR2 expression with shAGR2 did not result in a significant change in total EGFR protein, which is consistent with an effect on subcellular redistribution (Fig. 3A). EGFR-mediated signaling is, therefore, dependent on AGR2 expression.

Before serum, signaling was also induced with the addition of EGF (100 ng/ml) to the media of serum-starved cells, which resulted in marked induction of EGR1 and FOS expression. The EGFr-specific tyrosine kinase inhibitor, AG1478, significantly inhibited the EGF-induced expression in the A431 cells (Fig. 3, D and E). Reduction of AGR2 expression with shAGR2 dramatically reduced EGFR-stimulated EGR1 and FOS expression (Fig. 3, F and G).

Decrease AGR2 Expression Effectively Reduces EGFR Signaling in Cells Resistant to a Tyrosine Kinase Inhibitor—Reducing EGFR transport to the plasma membrane is a potentially more effective approach for disrupting EGFR signaling than receptor inhibition at the cell surface. As a control for potential off target effects of shAGR2, rescue experiments were performed with NCI-H460-shAGR2 cells that express low AGR2 levels. Transduction of NCI-H460-shAGR2 cells with wild-type AGR2 cDNA that is insensitive to shAGR2 was obtained with flow cytometry. Flow cytometry with anti-EGFR antibodies determined that the mean cell surface EGFR expression decreased by 5- and 58-fold compared with vector controls in NCI-H460 and A431 cells, respectively, after AGR2 expression was reduced with shRNA (Fig. 2D). A third approach biotinylated all cell surface proteins, which was followed by their isolation with streptavidin beads. The relative amount of biotinylated cell surface EGFR was determined by protein immunoblotting. A 4- and 10-fold decrease in plasma membrane EGFR after transduction with shAGR2 was observed in NCI-H460 and A431 cells, respectively, compared with vector controls (Fig. 2E). ITGB1 was used as a plasma membrane control and displayed a 0- and 1.8-fold decrease in NCI-H460 and A431 cells, respectively. Quantitative RT-PCR for EGFR transcripts revealed no significant difference between wild-type and shAGR2-treated cells (Fig. 2F). Likewise, an evaluation for changes in total EGFR protein secondary to shAGR2 also revealed no significant changes when compared with β-actin (Fig. 2, E and G). After transduction with shAGR2, EGFR is present within the cell and not on the cell surface (Fig. 2, C and G). Additional control experiments assessed for the presence of endoplasmic reticulum stress in the context of transduction with shAGR2. Analysis of HSPA5 and HERPUD1 revealed no signs of endoplasmic reticulum stress (Fig. 2H), which is consistent with our published work and that of others (32–34). The data, therefore, demonstrate that cell surface EGFR was determined by its cellular distribution rather than the total amount of receptor in the cell.

As a control for potential off target effects of shAGR2, rescue experiments were performed with NCI-H460-shAGR2 cells that express low AGR2 levels. Transduction of NCI-H460-shAGR2 cells with wild-type AGR2 cDNA that is insensitive to inhibition at the cell surface. Consistent with previous studies (37), the EGFR-specific tyrosine kinase inhibitor, AG1478, was ineffective at concentrations up to 50 μg/ml in NCI-H460 cells in inhibiting EGFR expression in the A431 cells (Fig. 3, D and E). Reduction of AGR2 expression with shAGR2 dramatically reduced EGFR-stimulated EGFR phosphorylation and EGR1 and FOS protein and RNA expression (Fig. 3, F and G).

Decrease AGR2 Expression Effectively Reduces EGFR Signaling in Cells Resistant to a Tyrosine Kinase Inhibitor—Reducing EGFR transport to the plasma membrane is a potentially more effective approach for disrupting EGFR signaling than receptor inhibition at the cell surface. Consistent with previous studies (37), the EGFR-specific tyrosine kinase inhibitor, AG1478, was ineffective at concentrations up to 50 μg/ml in NCI-H460 cells in inhibiting EGFR expression in the A431 cells (Fig. 3, D and E). Reduction of AGR2 expression with shAGR2 dramatically reduced EGFR-stimulated EGFR phosphorylation and EGR1 and FOS protein and RNA expression (Fig. 3, F and G).

Decrease AGR2 Expression Effectively Reduces EGFR Signaling in Cells Resistant to a Tyrosine Kinase Inhibitor—Reducing EGFR transport to the plasma membrane is a potentially more effective approach for disrupting EGFR signaling than receptor inhibition at the cell surface. Consistent with previous studies (37), the EGFR-specific tyrosine kinase inhibitor, AG1478, was ineffective at concentrations up to 50 μg/ml in NCI-H460 cells in inhibiting EGFR expression in the A431 cells (Fig. 3, D and E). Reduction of AGR2 expression with shAGR2 dramatically reduced EGFR-stimulated EGFR phosphorylation and EGR1 and FOS protein and RNA expression (Fig. 3, F and G).

Decrease AGR2 Expression Effectively Reduces EGFR Signaling in Cells Resistant to a Tyrosine Kinase Inhibitor—Reducing EGFR transport to the plasma membrane is a potentially more effective approach for disrupting EGFR signaling than receptor inhibition at the cell surface. Consistent with previous studies (37), the EGFR-specific tyrosine kinase inhibitor, AG1478, was ineffective at concentrations up to 50 μg/ml in NCI-H460 cells in inhibiting EGFR expression in the A431 cells (Fig. 3, D and E). Reduction of AGR2 expression with shAGR2 dramatically reduced EGFR-stimulated EGFR phosphorylation and EGR1 and FOS protein and RNA expression (Fig. 3, F and G).

Decrease AGR2 Expression Effectively Reduces EGFR Signaling in Cells Resistant to a Tyrosine Kinase Inhibitor—Reducing EGFR transport to the plasma membrane is a potentially more effective approach for disrupting EGFR signaling than receptor inhibition at the cell surface. Consistent with previous studies (37), the EGFR-specific tyrosine kinase inhibitor, AG1478, was ineffective at concentrations up to 50 μg/ml in NCI-H460 cells in inhibiting EGFR expression in the A431 cells (Fig. 3, D and E). Reduction of AGR2 expression with shAGR2 dramatically reduced EGFR-stimulated EGFR phosphorylation and EGR1 and FOS protein and RNA expression (Fig. 3, F and G).
EGFR Delivery to the Cell Surface Requires AGR2

FIGURE 3. EGFR-mediated signaling is dependent on AGR2 expression. A, protein immunoblots for EGFR, phosphorylated EGFR (EGFR-P), EGR1, FOS, and β-actin (ACTB) of whole cell lysates derived from A431 cells propagated in serum-containing media after transduction with a vector control, shEGFR, or treated with the EGFR tyrosine kinase inhibitor AG1478 (2 μM). B and C, quantitative real-time-PCR analysis of EGR1 (B) and FOS (C) RNA in A431 cells similarly treated with shEGFR, AG1478 (2 μM), and shAGR2. D and E, quantitative real-time-PCR analysis of EGR1 (D) and FOS (E) RNA in A431 cells similarly treated with EGF (100 ng/ml) in serum-free conditions with and without EGF (100 ng/ml). F and G, quantitative real-time-PCR analysis of EGR1 (F) and FOS (G) RNA in A431 cells transduced with vector control or shAGR2. Cells were first serum-starved for 16 h followed by a 20-min treatment with 100 ng/ml EGF. Data are presented relative to β-actin and shown as the mean ± S.D. of triplicates. p value, two-tailed unpaired t tests.

FIGURE 4. Reduced AGR2 expression decreases EGFR signaling in tyrosine kinase inhibitor-resistant cells. A and B, quantitative real-time-PCR analysis of EGR1 (A) and FOS (B) RNA in NCI-H460 cells treated with AG1478 (2 μM) and shAGR2. C and D, effect of AG1478 on EGR1 (C) and FOS (D) expression in serum-free conditions with and without EGF (100 ng/ml). E and F, response of EGR1 (E) and FOS (F) expression to EGF stimulation in NCI-H460 cells transduced with a vector control or shAGR2. Cells were first serum-starved for 16 h followed by a 20-min treatment with 100 ng/ml EGF. Data are presented relative to β-actin and shown as the mean ± S.D. of triplicates. p value, two-tailed unpaired t tests.

Similar results were obtained when EGF was employed in lieu of serum to stimulate EGFR-mediated signaling. EGF addition to the media resulted in a 77- and 5-fold increase of EGR1 and FOS mRNA expression that was not affected by the tyrosine kinase inhibitor, AG1478 (Fig. 4, A and B). In contrast, shAGR2 significantly reduced the effects of EGF stimulation (Fig. 4, C and D). Manipulating AGR2, therefore, exhibited greater efficacy in inhibiting EGFR signaling than the tyrosine kinase inhibitor.

Recombinant AGR2 Expression Alone Is Sufficient to Stimulate EGFR Plasma Membrane Delivery—The experiments presented thus far showed that reducing AGR2 expression in transformed cells decreases cell signaling secondary to EGFR’s absence from the cell surface. The converse experiment was also performed in which AGR2 was expressed in two non-transformed cell lines MCF-10A and CHO-K1 that do not express AGR2 (38, 39). MCF-10A cells are a non-transformed epithelial cell line derived from human fibrocystic breast tissue. Immunocytochemistry revealed that the MCF-10A cells are...
EGFR Delivery to the Cell Surface Requires AGR2

AGR2 Null Mice Are Not Viable—A homozygous EGFR null mutation most often results in embryonic lethality, although strain-dependent differences may result in a small percentage (5–16%) that live as long as 3 weeks (41, 42). If AGR2 is necessary for EGFR-mediated signal transduction, then the AGR2 null mutant should exhibit features similar to that of the EGFR null. Three previously published studies by different laboratories, including our own, generated conditional AGR2 null mice that resulted in viable mice with histologic changes in the stomach and intestines that preferentially affected secretory cell lineages (32, 33, 43). One potential source for the different outcomes of the AGR2 null from that of the EGFR null mice was that all three prior studies utilized floxed AGR2 exons whose excision were dependent on the constitutive expression of Cre recombinase. Therefore, a definitive non-conditional null mouse was generated with germ line deletions of AGR2 exons 2–8. Heterozygotes alone compared with heterozygote with wild-type matings resulted in average litter sizes of 4.4 and 6.2, respectively. Heterozygote matings resulted in only 2 (3.8%) viable offspring that were homozygous AGR2 null mice (Table 1). One homozygote died at 3 weeks. The remaining homozygote that was sacrificed at 6 weeks was 27% smaller by weight than its heterozygous littermate and manifested many of the gastrointestinal changes previously observed with the conditional AGR2 null (32). Overall, the outcomes of the AGR2 null mirrored that of the published EGFR null mice.

DISCUSSION

This study presents a novel mechanism for controlling EGFR-mediated signal transduction. We demonstrate that an endoplasmic reticulum-based thioredoxin is able to dictate signaling activity by determining whether EGFR is delivered to the plasma membrane from the secretory pathway. Consistent with the endoplasmic reticulum’s established role, proteins must attain the appropriate conformation before they can proceed to the Golgi apparatus and the plasma membrane. Protein folding and assembly in the endoplasmic reticulum is largely facilitated by the heat shock and thioredoxin protein families (29). AGR2’s homology to other thioredoxins, its formation of mixed disulfides with EGFR, and loss of AGR2 function after mutating the putative active site cysteine are consistent with a role in establishing the appropriate disulfide bonds in EGFR. Appropriately assembled EGFR is then able to exit the endoplasmic reticulum to the plasma membrane where signaling is transduced (44). AGR2 enables EGFR transport to the cell surface and thus impacts cell signaling without affecting receptor RNA or protein levels.

The discovery of EGFR as a substrate is consistent with our previous observations that characterized AGR2’s effects on signal transduction (16). Induced AREG expression by AGR2 is compatible with the activation of EGFR-mediated signaling. Recent gene expression studies revealed that AREG is one of the most highly induced genes by EGFR signaling and thus may serve as a marker of signaling activity (27, 28). With respect to the previously reported impact of AGR2 expression on YAP1 activity, recent studies of Drosophila and human cells demonstrated that EGF signaling induces YAP1 activation by inhibiting its phosphorylation through the Ajuba proteins (45). It is

heterogeneous and that very few cells express AGR2 (0.5%, n = 598 cells; Figs. 1D and 5D). A

EGFR protein expression was evaluated with an anti-cytosolic domain antibody that detected strong EGFR staining within detergent-permeabilized MCF-10A cells. After transduction with AGR2 cDNA, the same antibody detected EGFR at the plasma membrane (Fig. 5A). Immunocytochemistry of detergent-permeabilized MCF-10A cells was also performed with monoclonal antibody 528, which detects mature EGFR that is endoglycosidase H-resistant, and revealed an increase in EGFR cell surface expression after transduction with AGR2 cDNA (Fig. 5A, bottom). In contrast, monoclonal antibody 528 detected much less intracellular EGFR compared with the anti-cytosolic domain antibody in vector control MCF-10A cells, suggesting that much of the protein possesses immature glycosylation.

The results with monoclonal antibody 528 are consistent with AGR2 expression facilitating EGFR delivery to the Golgi apparatus where complex glycosylation occurs (40). Protein immunoblotting of MCF-10A cell lysate that was resolved with a low percentage SDS-PAGE gel showed that the EGFR mass increased with AGR2 expression, which is consistent with maturation in glycosylation that occurs with progression in the secretory pathway (Fig. 5B). In addition, several bands between 50 and 100 kDa that are believed to represent EGFR degradation were detected mainly in cells transduced with the vector control.

Subcellular fractionation was also employed to show the transfer of EGFR protein from the endoplasmic reticulum to the plasma membrane after AGR2 expression. EGFR protein in the plasma membrane fraction increased 4.9-fold after AGR2 transduction of MCF-10A cells (Fig. 5C).

AGR2 mixed disulfides were also purified from the MCF-10A in a manner similar to that described for the NCI-H460 and A431 cells. A ~170-kDa AGR2 reactive band was visualized in non-reduced conditions only for AGR2-transduced MCF-10A cells, which disappeared after reduction with dithiothreitol (Fig. 5D). Protein immunoblotting confirmed that the band was EGFR (Fig. 5E). The data again demonstrated that EGFR is the major AGR2 mixed disulfide (Fig. 5D).

Cell surface biotinylation was used to biochemically demonstrate that significant plasma membrane delivery of EGFR was achieved only after MCF-10A cells were transduced with AGR2 (Fig. 5F). Transduction with AGR2 harboring the C81A active site mutant did not promote EGFR delivery to the plasma membrane. Cell surface ITGB1 did not change with AGR2 expression. Significant amounts of EGFR protein were detected whether or not AGR2 was present in the MCF-10A cells (Fig. 5F).

AGR2-induced EGFR delivery to the plasma membrane was also reconstituted in CHO-K1 cells, which are negative for both EGFR and AGR2 expression. Transient transfection of CHO-K1 cells with an EGFR-GFP construct alone resulted in an intracellular distribution. Co-transfection with EGFR-GFP and AGR2 cDNA resulted in EGFR cell surface expression (Fig. 5G).

3 A. Dong, D. Wodziak, and A. W. Lowe, personal observation.
**EGFR Delivery to the Cell Surface Requires AGR2**

**FIGURE 5.** Recombinant AGR2 expression in MCF-10A and CHO-K1 cells results in EGFR plasma membrane delivery. A, immunocytochemistry of permeabilized MCF-10A cells with anti-cytoplasmic (red, top panels) or mAb 528 (green, bottom panels) EGFR antibodies. The cells were transduced with either vector control or AGR2 cDNA. The scale bar = 10 μm. B, 20 μg of whole cell extracts from AGR2 or vector control-transfected MCF-10A cells were resolved with a 7% SDS-PAGE gel and immunoblotted with anti-EGFR antibodies. C, protein immunoblots of subcellular fractions (10 μg/lane) from MCF-10A cells transfected with a vector control or AGR2 cDNA. Antibodies for EGFR, ITGB1, calnexin (CANX), and AGR2 were used. Calnexin serves as a marker for the microsomes. homo, homogenate; PM, plasma membrane; Mic, microsomes. D, immune precipitates (IP) from N-ethylmaleimide (NEM)-treated or untreated MCF-10A cells transfected with AGR2 or vector control cDNA were collected with control IgG or mouse anti-AGR2 antibodies and detected with protein immunoblotting using rabbit anti-AGR2 antibodies. Before SDS-PAGE, the samples were unreduced (−, left panel) or reduced (+, right panel) with 0.1M DTT. The band at ~180 kDa in the unreduced IgG lane most likely represents nonspecific binding to intact immunoglobulin. Arrowhead, AGR2-EGFR mixed disulfide; arrow, AGR2. E, AGR2 immune-precipitated mixed disulfides from N-ethylmaleimide-treated MCF-10A cells transfected with AGR2 or vector control and detected by protein immunoblotting with anti-EGFR antibodies. Arrowhead, EGFR. F, MCF-10A cells were transfected with cDNA representing AGR2, AGR2-C81A, or a plasmid vector control followed by cell surface labeling with biotin. 200 μg of cell lysates were subjected to streptavidin affinity purification. Fifteen percent of the purified proteins were assessed for EGFR and ITGB1 with protein immunoblotting. The whole cell lysates (10 μg, WCL) represent 10% of the starting material used for the affinity purification and were probed with antibodies to EGFR, AGR2, and β-actin (ACTB). G, immunocytochemistry of CHO-K1 cells transiently transfected with EGFR-GFP alone (left panel) or EGFR-GFP + AGR2 (right two panels). Green, GFP signal from EGFR-GFP fusion protein; red, anti-AGR2; blue, DAPI. Scale bar = 10 μm.
also likely that induced AREG expression by EGFR is mediated by YAP1 activation as previously described (16).

The absence of another dominant AGR2 mixed disulfide (Figs. 1, A, C, and D, and 5D) suggests that EGFR is a major substrate for AGR2. None of the proteins previously reported to bind AGR2 such as LYPD3 (C4.4a), DAG1, or MUC2 (33, 46) were detected in the mass spectrometry analysis of AGR2 mixed disulfides. Re-evaluation of MUC2 in a recent study also concluded that it is not a AGR2-binding protein and that AGR2 served no role in its folding (47). The present study differed from previously identified AGR2 binding proteins by the ability to measure a biologic outcome that is shared by its substrate. In the present study similar outcomes were achieved whether AGR2 or EGFR expression was reduced, thus supporting an essential functional relationship between the two proteins. Other AGR2 binding proteins are expected such as potential oxidases that will recycle AGR2 or other chaperones that may also participate in EGFR folding. We conclude, however, that AGR2’s major biological effect is mediated through EGFR signaling because of the overlap in cellular effects.

EGFR’s dependence on AGR2 is also supported by the newly generated knock-out mice. The approach used to generate AGR2 null mice in the present study ensured that AGR2 expression was disrupted, which resulted in a high rate of embryonic lethality, and closely resembled the outcome of previously generated EGFR null mice (41, 42). It is likely the previously published conditional null mice expressed some AGR2 at early developmental stages that increased viability (32, 33, 43). In summary, the results of the null mice support an essential role for AGR2 in EGFR-mediated signaling.

AGR2’s expression pattern is also consistent with that of a regulated process. In contrast to other endoplasmic reticulum-based thioredoxins, AGR2 is not constitutively expressed in all cells. In fact, AGR2 is rarely expressed in most normal cells, indicating that its expression is regulated. In contrast, AGR2 expression is present in cell lines and tissues where EGFR-mediated signaling is active. Considering the absence of AGR2 protein and transcript in most normal tissues and the widespread expression of EGFR transcripts, AGR2 expression represents a novel and possibly dominant post-translational mechanism for regulating EGFR-mediated cell signaling. The identification of EGFR as an AGR2 substrate provides insights into why cancer prognosis or response to therapy often does not correlate with EGFR protein or RNA levels because such measures do not reflect delivery to the cell surface (7, 48).

The number of publications reporting AGR2 expression in tumors has increased significantly and includes non-small cell lung cancer, colon cancer, breast adenocarcinoma, ovarian cancer, papillary thyroid tumors, prostate adenocarcinoma, glioblastoma, pancreatic adenocarcinoma, oral cancer, and esophageal cancers (9–15, 49–52). EGFR-mediated signaling has been reported to serve a significant role in all of these tumors.

Based on this study’s findings, we hypothesize that AGR2 is expressed in all tumors with active EGFR-mediated signaling and serves as a major regulatory factor.

The present work also features AGR2 as a novel therapeutic target because it regulates EGFR at a very early stage in its life cycle. Recent studies have suggested that most, if not all, EGFR-mediated signaling occurs at the plasma membrane (5, 6), which this study demonstrates can only be achieved with AGR2 expression. Manipulation of AGR2 expression successfully disrupted EGFR-mediated signaling in cells that were resistant to a tyrosine kinase inhibitor. Other factors that may also weaken the effectiveness of current therapies include tyrosine kinase-independent EGFR functions that affect DNA synthesis, glucose uptake, and cell proliferation (53, 54). AGR2 as a therapeutic target is positioned to impact both kinase-dependent and -independent EGFR functions and represents a novel approach for inhibiting all EGFR-related activities.

Acknowledgment—We thank the Stanford Cancer Center Cores (Grant P30CA124435) for mass spectrometry, flow cytometry, and transgenic mouse services.

| TABLE 1 Viable offspring of AGR2+/− heterozygote matings |
| --- |
| n | Males | Females | +/+ | +/− | −/− |
| 52 | 28 | 24 | 22 (42.3) | 28 (58.8) | 2 (3.8) |

REFERENCES

1. Cohen, S., Fava, R. A., and Sawyer, S. T. (1982) Purification and characterization of epidermal growth factor receptor/protein kinase from normal mouse liver. Proc. Natl. Acad. Sci. U.S.A. 79, 6237–6241

2. Cohen, S., Carpenter, G., and King, L., Jr. (1980) Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. J. Biol. Chem. 255, 4834–4842

3. Ulrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., and Schlessinger, J. (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309, 418–425

4. Yarden, Y., Harari, I., and Schlessinger, J. (1985) Purification of an active EGFR receptor kinase with monoclonal antireceptor antibodies. J. Biol. Chem. 260, 315–319

5. Brankatschk, B., Wichert, S. P., Johnson, S. D., Schaad, O., Rossner, M. J., and Gruenberg, J. (2012) Regulation of the EGFR transcriptional response by endocytic sorting. Sci. Signal. 5, ra21

6. Sousa, L. P., Lax, I., Shen, H., Ferguson, S. M., De Camilli, P., and Schlessinger, J. (2012) Suppression of EGFR endocytosis by dynamin depletion reveals that EGFR signaling occurs primarily at the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. 109, 4419–4424

7. Yarden, Y., and Pines, G. (2012) The ERBB network at last; cancer therapy meets systems biology. Nat. Rev. Cancer 12, 553–563

8. Persson, S., Rosenquist, M., Knoblach, B., Khosravi-Far, R., Sommarin, M., and Michalak, M. (2005) Diversity of the protein disulfide isomerase family: identification of breast tumor induced Hag2 and Hag3 as novel members of the protein family. Mol. Phylogenet. Evol. 36, 734–740

9. Hao, Y., Triadafilopoulos, G., Sabbaie, P., Young, H. S., Omary, M. B., and Lowe, A. W. (2006) Gene expression profiling reveals stromal genes expressed in common between Barrett’s esophagus and adenocarcinoma. Gastroenterology 131, 925–933

10. Lowe, A. W., Olsen, M., Hao, Y., Lee, S. P., Taek Lee, K., Chen, X., van de Rijn, M., and Brown, P. O. (2007) Gene expression patterns in pancreatic tumors, cells, and tissues. PLoS ONE 2, e323

11. Thompson, D. A., and Weigel, R. J. (1998) HAG-2, the human homologue of the Xenopus laevis cement gland gene XAG-2, is coexpressed with estrogen receptor in breast cancer cell lines. Biochem. Biophys. Res. Commun. 251, 111–116

12. Zhang, J. S., Gong, A., Cheville, J. C., Smith, D. L., and Young, C. Y. (2005)
EGFR Delivery to the Cell Surface Requires AGR2

AGR2, an androgen-inducible secretory protein overexpressed in prostate cancer. *Genes Chromosomes Cancer* **43**, 249–259.

13. Fritzschke, F. R., Dahl, E., Dankof, A., Burkhartd, M., Pahl, S., Petersen, I., Dietel, M., and Kristiansen, G. (2007) Expression of AGR2 in non small cell lung cancer. *Histol. Histopathol.* **22**, 703–708.

14. Zhu, H., Lam, D. C., Han, K. C., Tin, V. P., Suen, W. S., Wang, E., Lam, W. K., Cai, W. W., Chuang, L. P., and Wong, M. P. (2007) High resolution analysis of genomic aberrations by metaphase and array comparative genomic hybridization identifies candidate tumour genes in lung cancer cell lines. *Cancer Lett.* **245**, 303–314.

15. Armes, J. E., Davies, C. M., Wallace, S., Taheri, T., Perrin, L. C., and Au-bauer, H., and Sumoy, L. (2011) Multiple platform assessment of the EGF receptor tyrosine kinase activity in living cells and EGF-stimulated cell proliferation. *J. Biol. Chem.* **264**, 14503–14509.

16. Lyall, H. D., Maloney, T. M., Woldman, S. R., Peterson, W. D., Jr., Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F., and Brooks, S. C. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res.* **50**, 6075–6086.

17. Soule, P. T., Cieciura, S. J., and Robinson, A. (1958) Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. *J. Exp. Med.* **106**, 945–956.

18. Slier, L. J., and Lane, M. D. (1985) Post-translational processing of the epidural growth factor receptor. *Glycosylation-dependent acquisition of ligand-binding capacity. J. Biol. Chem.* **260**, 687–690.

19. Threadgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D., Maniata, C., Moulton, T., Herrup, K., and Harris, R. C. (1995) Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* **269**, 230–234.

20. Anelli, T., and Sittia, R. (2008) Protein quality control in the early secretory pathway. *EMBO J.* **27**, 315–327.

21. Reddy, B. V., and Irvine, K. D. (2013) Regulation of Hippo Signaling by EGFR-AMAPK signaling through ajuba family proteins. *Dev. Cell* **24**, 459–471.

22. Bergström, J. H., Berg, K. A., Rodríguez-Piñeiro, A. M., Stecher, B., Johansson, M., and Hultberg, H., and Storling, K. (2009) Anterior gradient 2: a novel sensitive tumor marker for metastatic oral cancer. *PLoS ONE* **5**, e91086.

23. Arteaga, C. L. (2002) Epidermal growth factor receptor dependence in human tumors: more than just expression? *Oncologist* **7**, 31–39.

24. Chen, Y. T., Ho, C. L., Chen, P. K., Chen, Y. L., and Chang, C. F. (2013) Anterior gradient 2: a novel sensitive tumor marker for metastatic oral cancer. *Cancer Lett.* **339**, 270–278.
Tang, X., and Tang, F. (2014) N,N’-dinitrosopiperazine-mediated AGR2 is involved in metastasis of nasopharyngeal carcinoma. *PLoS ONE* **9**, e92081

51. Di Maro, G., Salerno, P., Unger, K., Orlandella, F. M., Monaco, M., Chiappetta, G., Thomas, G., Oczko-Wojciechowska, M., Masullo, M., Jarzab, B., Santoro, M., and Salvatore, G. (2014) Anterior gradient protein 2 promotes survival, migration and invasion of papillary thyroid carcinoma cells. *Mol. Cancer* **13**, 160

52. Hong, X. Y., Wang, J., and Li, Z. (2013) AGR2 expression is regulated by HIF-1 and contributes to growth and angiogenesis of glioblastoma. *Cell Biochem. Biophys.* **67**, 1487–1495

53. Rauch, J., Volinsky, N., Romano, D., and Kolch, W. (2011) The secret life of kinases: functions beyond catalysis. *Cell Commun. Signal* **9**, 23

54. Weihua, Z., Tsan, R., Huang, W. C., Wu, Q., Chiu, C. H., Fidler, I. J., and Hung, M. C. (2008) Survival of cancer cells is maintained by EGFR independent of its kinase activity. *Cancer Cell* **13**, 385–393