STAT3-mediated Coincidence Detection Regulates Noncanonical Immediate Early Gene Induction

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Background: Simultaneous EGF and thrombin signaling elicits synergistic gene induction in endothelial cells, but the mechanism of signal integration is unknown.

Results: GSK-3α/β-mediated STAT3 activation increases pro-angiogenic immediate early gene expression.

Conclusion: STAT3 is required to detect simultaneous EGF and thrombin signaling.

Significance: Understanding how disparate signals are integrated within signaling networks is critical to understanding complex signaling events in pathological microenvironments.

Signaling pathways interact with one another to form dynamic networks in which the cellular response to one stimulus may depend on the presence, intensity, timing, or localization of other signals. In rare cases, two stimuli may be simultaneously required for cells to elicit a significant biological output. This phenomenon, generally termed “coincidence detection,” requires a downstream signaling node that functions as a Boolean AND gate to restrict biological output from a network unless multiple stimuli are received within a specific window of time. Simultaneous activation of the EGF receptor (EGFR) and a thrombin receptor (pro tease-activated receptor-1, PAR-1) increases the expression of multiple immediate early genes (IEGs) associated with growth and angiogenesis. Using a bioinformatic comparison of IEG promoter regions, we identified STAT3 as a critical transcription factor for the detection of coincident EGFR/PAR-1 activation. EGFR activation induces classical STAT3 Ser727 phosphorylation but also initiates an inhibitory signal through the PI3K-AKT signaling axis that prevents STAT3 Ser727 phosphorylation. Coincident PAR-1 signaling resolves these conflicting EGF-activated pathways by blocking AKT activation and permitting GSK-3α/β-dependent STAT3 Ser727 phosphorylation and STAT3-dependent gene expression. Functionally, combinatorial EGFR/PAR-1 signaling suppresses EGF-induced proliferation and thrombin-induced leukocyte adhesion and triggers a STAT3-dependent increase in endothelial cell migration. This study reveals a novel signaling role for STAT3 in which the simultaneous presence of extracellular EGF and thrombin is detected at the level of STAT3 post-translational modifications. Collectively, our results describe a novel regulatory mechanism in which combinatorial EGFR/PAR-1 signaling regulates STAT3-dependent IEG induction and endothelial cell migration.

Cell type and spatiotemporal signaling contexts are critical determining factors for the propagation of signaling cascades from cell surface receptors. Rather than having defined pathways of signaling, receptors may activate unique complements of pathways depending on the presence, intensity, timing, or localization of other signals. This nuanced view of cellular signaling requires integration of multivariate extracellular information via coordinated cross-talk between signaling pathways. In this manner, signaling interactions enable cells to process disparate extracellular information at downstream signaling nodes and formulate appropriate biological responses based on specific combinations of signaling inputs. Cross-talk is an important factor in a number of biological processes, including cellular migration, proliferation, gene expression, and calcium influx. However, the specific molecular mechanisms by which cells integrate signals from multiple cell surface receptors are poorly understood.

In many cases, the output of signaling interactions is merely the sum of individual signaling inputs. Less often, multiple signaling inputs induce moderate synergisms of phosphorylation, calcium influx, or gene expression. In rare cases, however, simultaneous signals may induce marked synergisms, where the response to combined stimuli is far greater than the sum of responses to individual inputs. This type of superadditive response can be expressed quantitatively as a synergism ratio (SR) and calculated by dividing the response to combined signals by the product of individual responses. The abbreviations used are: SR, synergism ratio; IEG, immediate early gene; PAR-1, protease-activated receptor-1; GPCR, G-protein-coupled receptor; EC, endothelial cell; MKP-1, mitogen-activated protein kinase phosphatase-1; STAT3, signal transducer and activator of transcription 3; GSK-3α/β, glycogen synthase kinase-3; CREB, cAMP-response element-binding protein; CHX, cycloheximide; GAS, γ-interferon-activated sequence; EGFR, EGF receptor; HUVEC, human umbilical vein endothelial cell; MVEC, microvascular EC; qRT, quantitative RT.

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stimulation by the sum of individual responses. As SR increases, signaling interactions begin to function as cellular coincidence detectors, where simultaneous stimuli are required for cells to elicit a significant biological response. Coincidence detection may occur by several mechanisms, including multiple phosphorylation events, cooperative binding of regulatory molecules, multivalent binding of modular protein domains, two-state allosteric regulation, protein scaffolding, and simultaneous alterations in relative activity between antagonistic enzymes. Many research has focused on signaling interactions between G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). The classical model of RTK/GPCR cross-talk involves GPCR-mediated transactivation of RTKs via metalloprotease-mediated cleavage of membrane-bound growth factor precursors and subsequent autocrine activation of RTKs. Although this model of RTK-GPCR interactions constitutes a cross-talk between cell surface receptors, it only describes a form of linear information transfer where an RTK is essentially a downstream effector of GPCR signaling. As such, this model does not address mechanisms by which intracellular pathways integrate extracellular information following coincident activation of RTKs and GPCRs.

Previous work in our laboratory focused on an RTK-GPCR interaction in endothelial cells (EC) involving the epidermal growth factor (EGF) receptor and the thrombin receptor, protease-activated receptor-1 (PAR-1) (7). EC are situated in direct contact with the bloodstream and are constantly exposed to a wide variety of stimuli, including growth factors, hormones, biomechanical forces, microbial pathogens, and inflammatory agents. Responses to these stimuli are mediated, at least in part, by the induction of immediate early genes (IEGs). As a family of transcription factors, cytokines, phosphatasies, and other enzymes, IEGs are important determinants of delayed gene expression and phenotypic outcomes. We previously reported that simultaneous exposure of EC to EGF and thrombin caused a synergistic induction of the IEG mitogen-activated protein kinase phosphatase-1 (MKP-1) and enhanced migration of EC in vitro (7). EGF is a well known stimulator of cell growth and induces EC proliferation, migration, and angiogenesis. Thrombin is a serine protease that regulates blood coagulation, but it also acts as a signaling molecule by cleaving the NH2 terminus of protease-activated receptors (PARs). We previously showed that synergistic induction of MKP-1 by EGF and thrombin was mediated specifically by PAR-1 activation (7). An increased understanding of signaling interactions between EGF and PAR-1 may yield valuable insight into the mechanisms by which EC signaling networks integrate multivariate extracellular information in pathological microenvironments.

In this report, we describe a detailed, novel molecular mechanism of EGF/PAR-1 cross-talk. We show that STAT3 is a critical point of convergence of signals from EGF and PAR-1, and it functions as a cellular coincidence detector to enhance IEG expression following simultaneous EGF/PAR-1 activation. Importantly, combinatorial receptor signaling coincides with a suppression of EGF-induced proliferation and thrombin-induced leukocyte adhesion and causes a STAT3-dependent enhancement of EC migration. Combinatorial activation of STAT3 is therefore a critical event for increasing the magnitude of IEG expression, and it plays an important role in determining the ultimate phenotypic response of EC during simultaneous exposure to disparate signaling inputs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfections, and Treatments**—Human EC were isolated by trypsinization of umbilical veins as described previously. EC were plated on fibronectin-coated cell culture dishes and maintained in MCDB/F-12 media containing 15% FBS, 0.009% heparin, and 0.015% endothelial cell growth supplement. All experiments were carried out using cells between the third and fifth passage. EC were transfected using Targetfect reagents (Targeting Systems) according to the manufacturer’s protocol. Unless otherwise indicated, EC were serum-starved for 2 h prior to treatment with EGF (16 ng/ml), TRAP (100 μM), or thrombin (5 units/ml).

**Generation of EGR1 Promoter Luciferase Reporter and Luciferase Assay**—A 2.1-kb fragment of the EGR1 promoter was PCR-amplified (see supplemental Table S7 for primers) from human genomic DNA. The fragment spanned from −1958 bp upstream of the EGR1 transcription initiation site to +160 bp into the first exon (supplemental Fig. S6) and was cloned into pGL3-Basic firefly luciferase reporter between the MluI and BglII restriction sites. Site-directed mutagenesis of the EGR1 promoter distal GAS element (TTCCCAGGA → gcgcCcCGGAA) and proximal GAS element (TTCCCGGA → gcgcC CGGA) was performed using the GeneArt site-directed mutagenesis kit (Invitrogen) according to the manufacturer’s instructions. Luciferase assays were performed by transfecting EC with EGR1 promoter reporter 24 h prior to treatment. Cells were lysed with passive lysis buffer (Promega), and luciferase activity was assayed using the luciferase assay system (Promega) according to the manufacturer’s instructions.

**SDS-PAGE and Western Blotting**—Total cell lysate from ~105 EC was resolved using BisTris-buffered SDS-polyacrylamide gels ranging from 8 to 12% depending on the protein of interest. Gels were soaked in protein transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.0375% SDS) and transferred to a PVDF membrane using a Bio-Rad semi-dry transfer cell. After transfer, PVDF membranes were washed briefly in TBST (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% Tween 20) and then blocked for 2 h in 5% bovine serum albumin in TBST. After blocking, primary antibodies were diluted 1:1000 in 5% BSA in TBST and incubated overnight at 4 °C. Membranes were washed and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h, and HRP signals were detected by chemiluminescence.

**Quantitative Real Time-PCR**—Total RNA was isolated using Qiagen RNeasy spin columns. First strand cDNA was synthesized using TaqMan reverse transcription reagents (Roche Applied Science). cDNA reactions were diluted 6-fold in deionized water and used as a qRT-PCR template. Reactions were performed using SYBR Green Master Mix (Applied Biosystems). Gene induction was calculated relative to untreated controls using the 2-ΔΔCt method.
EGFR/PAR-1 Cross-talk Regulates STAT3 Activation

Immunoprecipitation—EC were washed once with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 5 mM Na3VO4) supplemented with protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitors (Roche Applied Science). Lysis was allowed to proceed for 30 min at 4 °C under gentle agitation. Lysates were cleared by high speed centrifugation, and primary antibodies were added for over-night incubation. Antibody complexes were precipitated with protein A/G-agarose beads, washed three times with ice cold RIPA, and denatured with 3× Laemmli buffer for SDS-PAGE.

Quantitative Mass Spectrometry—After immunoprecipitation with an anti-STAT3 antibody, an 86-kDa band was cut from Coomassie-stained gel, digested with trypsin, and analyzed by capillary column LC-tandem MS to identify phosphopeptides. The LC-MS system was a Finnigan LTQ linear ion trap mass spectrometer system. The HPLC column was a self-packed 9-cm × 75-µm inner diameter Phenomenex Jupiter C18 reversed-phase capillary chromatography column. The digest was analyzed using the data-dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in successive instrument scans. The data were analyzed by using all collision-induced dissociation spectra collected in the experiment to search the NCBI nonredundant database with the search program Mascot using a human taxon.

Phosphokinase Protein Array—The human phosphokinase antibody array was purchased from R&D Systems. 500 µg of HUVEC protein was incubated with the array membranes overnight at 4 °C under gentle agitation. Membranes were washed and then incubated with a phospho-antibody detection mixture for 2 h at room temperature. Membranes were washed and then incubated with a streptavidin-HRP-containing solution for 30 min at room temperature. After extensive washing, phosphorylation was detected by chemiluminescence, and relative changes were quantified using ImageJ software.

STAT3 DNA Binding Assay—STAT3 DNA binding activity was measured using the TransAM transcription factor ELISA kit from Active Motif according to the manufacturer’s instructions. Briefly, stimulus-treated EC were harvested in hypotonic lysis buffer (25 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl2) and lysed at 4 °C for 30 min to release cytosolic proteins. Cytosolic fractions were collected; EC pellet was washed once with hypotonic lysis buffer, and nuclear proteins were extracted with high salt lysis buffer (25 mM HEPES, pH 7.5, 420 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA, 10% glycerol) to collect the nuclear fraction. Nuclear extract was diluted in DNA binding buffer according to the manufacturer’s instructions. 10 µg of nuclear extract was added to each well of a 96-well plate that was coated with oligonucleotides containing consensus GAS elements (5’-TTCCCG-GAA-3’) and incubated at room temperature for 1 h. Unbound nuclear proteins were washed away, and DNA-bound STAT3 was detected with an anti-STAT3 antibody, followed by colorimetry using 3,3’,5,5’-tetramethylbenzidine as a chromogenic substrate.

Immune Complex Kinase Assay—An AKT kinase assay kit (catalog no. 9840) was purchased from Cell Signaling Technology. Briefly, EC were treated with agonists for 10 min and lysed with a proprietary lysis buffer containing protease and phosphatase inhibitors. Insoluble cellular material was removed by centrifugation (16,000 × g), and the supernatant was used for immunoprecipitation. Total AKT was precipitated with pan-AKT antibody (Cell Signaling, catalog no. 4691). A portion of the immunoprecipitate was diluted 400-fold for the in vitro kinase assay. Recombinant GST-tagged GSK3α was used as an AKT substrate and incubated in the presence of ATP for 30 min. Reaction was stopped with 4× SDS loading buffer, and GSK3α was resolved by SDS-PAGE and blotted with an anti-phospho-GSK3α antibody (catalog no. 9327).

BrdU Proliferation Assay—BrdU incorporation was measured using a BrdU proliferation kit (Cell Signaling Technology, catalog no. 6813) according to the manufacturer’s instructions. EC were serum-starved overnight and then treated with agonists for 8 h. BrdU labeling was performed between 8 and 12 h without removing treatment media.

Endothelial Wound Healing—HUVEC were seeded in 6-well plates in full growth medium. Cells were washed twice with serum-free MCDB and serum-starved for 2 h. Linear wounds were created using a 200-µl pipette tip; cells were washed once with MCDB, and phase contrast images were taken to capture wound size prior to treatment. Cells were then stimulated with agonists and allowed to migrate for 16 h. Wounds were imaged again, and the percent of scratch closure was calculated using ImageJ software and comparing the wound size of specific fields before and after migration.

Monocyte Adhesion Assay—HUVEC were serum-starved for 2 h and treated with agonists for 6 h. U937 monocytes were maintained in RPMI in 10% FBS. U937 cells were washed once and labeled with 2 µM Calcein-AM (Invitrogen) in MCDB for 15 min. U937 cells were washed twice before adding 2 × 106 monocytes to each well of activated EC after removing EC treatment media. Binding was allowed to proceed for 30 min, after which the adhered monocytes were counted by fluorescent microscopy of EC-bound monocytes.

Statistical Analysis—Unless otherwise indicated, data are expressed as means ± S.E. Differences between groups were analyzed by analysis of variance. Bonferroni post hoc tests were performed to evaluate pairwise differences between groups. Synergism ratio (SR) = responsep/responsep + responseEGF, where the response is the stimulatory-inducible value. To calculate the presence of a synergism, a one-sample t test was performed by comparing the mean SR to a hypothetical value of 1.0. Significance is expressed as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

List of Reagents—A comprehensive list of reagents, kits, antibodies, and primers used in this study can be found in supplemental Tables 3–6.

RESULTS
Synergistic Induction of Multiple IEGs Following Simultaneous EGF and PAR-1 Activation—To investigate the effect of simultaneous EGFR/PAR-1 activation on IEG induction, we treated EC with EGF, TRAP, or both for 1 h (30 min for c-Fos).

11990 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 288 • NUMBER 17 • APRIL 26, 2013
and measured IEG mRNA expression. We calculated an SR by dividing the response to combined stimulation with EGF plus TRAP by the sum of individual responses to EGF or TRAP alone. The transcription factor early growth response 1 and 3 (EGR1 and EGR3) and c-Fos were induced with SRs of 2.18, 10.1, and 4.11, respectively (Fig. 1, A–C). The prostaglandin synthase cyclooxygenase-2 (COX-2) was induced with an SR of 1.52 (Fig. 1D), and interleukin-8 (IL-8) displayed an SR of 2.20 (Fig. 1E). Protein levels of EGR1 and MKP-1 were also synergistically increased following dual EGF plus TRAP treatment with SRs of 1.96 ± 0.09 and 2.95 ± 0.84, respectively (Fig. 1F).

We selected EGR1 as an end point to investigate the molecular mechanism of EGFR/PAR-1 cross-talk. In the presence of EGF, thrombin or TRAP displayed similar synergistic induction of EGR1 protein (EGF + thrombin SR = 2.57 ± 0.82; EGF + TRAP SR = 2.57 ± 0.52) (supplemental Fig. S1). The kinetics of EGR1 expression were similar under all conditions, peaking at 1 h and returning toward basal levels by 3 h (Fig. 1G), suggesting that EGFR/PAR-1 cross-talk controls the magnitude, but not the kinetics, of IEG expression. EGR1 is occasionally expressed in both a high molecular mass (75 kDa) and low molecular mass (57 kDa) form (21). We observe the 75-kDa species in all experiments, but the 57-kDa form is rarely induced. Pretreatment of EC with cycloheximide, an inhibitor of de novo protein synthesis, had no effect on EGR1 mRNA induction or the EGFR/PAR-1 synergism (Fig. 1H), demonstrating that EGR1 was induced as an IEG.

**ERK-independent Coincidence Detection Underlies Synergistic EGR1 Induction**—Numerous publications have reported that EGR1 induction is mediated by the MAPK ERK1/2...
response to various agonists, including EGF (22, 23). To determine specific pathways mediating EGR1 induction by EGF plus TRAP, we pretreated EC with the MEK1 inhibitor PD98059 to prevent ERK activation. PD98059 treatment almost completely inhibited EGR1 induction in response to individual treatment with EGF or TRAP (Fig. 2A). However, when cells were treated with EGF and TRAP simultaneously, an ERK-independent portion of EGR1 induction was triggered with a high degree of synergism (SR = 19.88) (Fig. 2C). In a signal transduction network, an SR of this magnitude suggests the presence of a signaling node that effectively functions as a Boolean AND gate; thereby, a cellular output (EGR1 induction) is elicited only when two stimuli (EGF and TRAP) are simultaneously present. This result revealed two important facts about the mechanism

FIGURE 2. ERK-independent pathway functions as a cellular coincidence detector to enhance IEG induction. A, EC were pretreated for 30 min with the MEK1 inhibitor PD98059 (10 μM). EC were treated with EGF (16 ng/ml), TRAP (100 μM), or both for 1 h, and EGR1 was measured by immunoblot. Graph represents the mean ± S.E. from four independent experiments. B, EC were pretreated for 30 min with the EGFR kinase inhibitor AG1478 (1 μM). Treatments and immunoblots were performed as in A. Graph represents the mean ± S.E. from three independent experiments. C, SRs from individual experiments were averaged to calculate a mean SR and S.E. Data represent the synergism ratio of EGR1 induction when the indicated agonist (x axis) was used to stimulate EC in the presence of EGF.
EGFR/PAR-1 Cross-talk Regulates STAT3 Activation

of EGFR/PAR-1 synergism. First, synergistic EGR1 induction occurs via two distinct signaling pathways (ERK-dependent and ERK-independent). Second, at some level, the ERK-independent pathway likely utilizes a mechanism of coincidence detection to induce ERK-independent EGR1 expression only when EGFR and PAR-1 are simultaneously activated.

The ERK-independent pathway was sensitive to the EGFR kinase inhibitor AG1478 (Fig. 2B), which completely inhibited EGF-induced EGR1 and prevented a synergism during simultaneous EGF plus TRAP treatment (SR = 1.15 ± 0.23) (Fig. 2C). This result revealed that in addition to the canonical pathway of ERK activation, EGFR kinase activity is also required to initiate the alternative, synergistic pathway of EGR1 induction. Therefore, combinatorial EGFR/PAR-1 signaling must be integrated at some downstream signaling node that functions as an AND gate to induce EGR1 following coincident receptor activation (supplemental Fig. S2).

**STAT3 Is Required for Detection of Coincident EGFR/PAR-1 Activation**—To identify candidate transcription factors involved in ERK-independent EGR1 induction, we examined the promoter regions of the six gene targets of the EGFR/PAR-1 synergism for common transcription factor-binding sites. Using a combination of literature-based searches and web-based bioinformatics tools (P-match and TFSEARCH), we compiled a list of potential transcription factor-binding sites for each of the six IEGs (supplemental Fig. S3). Several common transcription factors were identified, including ELK-1, the downstream target of the ERK1/2 signaling pathway that mediates EGR1 induction (24, 25), and CREB, most commonly activated by protein kinase A (PKA). PKA inhibition did not affect synergistic induction of EGR1 following EGF plus TRAP treatment (supplemental Fig. S4), so we did not pursue further experiments on CREB signaling. Notably, every synergistically induced gene had at least one potential binding site for a STAT transcription factor. Further examination revealed that these candidate STAT-binding sites were consensus STAT3-binding elements (γ-IFN-activated sequences, GAS elements) (supplemental Table S1). Each of the six IEGs contained a putative STAT3-binding site between ~1.4–1.8 kb upstream to the transcription initiation site (supplemental Fig. S5A), raising the possibility that STAT3 was a component of the ERK-independent coincidence detector.

siRNA-mediated knockdown of STAT3 completely inhibited the synergistic induction of EGR1 protein (Fig. 3, A and B) and mRNA (Fig. 3C) without affecting EGR1 levels induced by EGF or TRAP alone. STAT3 knockdown significantly reduced the SR of inducible EGR1 protein (2.86 to 0.89) and mRNA (2.09 to 1.14) (Fig. 3D), suggesting a complete loss of function of EGFR/PAR-1 cross-talk. Similarly, the induction of another synergistically induced IEG, MKP-1, was inhibited when STAT3 protein was depleted by RNAi (supplemental Fig. S5B). These results provide convincing evidence that STAT3 is a critical transcription factor mediating the EGFR/PAR-1 synergism, and they implicate STAT3 in the mechanism of coincidence detection observed in experiments with PD98059 (Fig. 2, A and C).

STAT3 activity is primarily regulated by two post-translational modifications (26). Phosphorylation of STAT3 at Tyr705 leads to dimerization and permits DNA binding (27). STAT3 Ser727 phosphorylation, a modification within the COOH-terminal transactivation domain, facilitates physical interactions between STAT3 and histone acetyltransferases (28, 29) and is required for full transcriptional activity of STAT3 (30). We hypothesized that if STAT3 is the node of integration for detecting simultaneous EGFR/PAR-1 activation, then there should be some unique regulation of STAT3 during co-treatment with EGF plus TRAP. EGF treatment per se weakly induced Tyr705 phosphorylation (Fig. 3, E and F), and this modification was not significantly affected by co-treatment with TRAP. Strikingly, STAT3 Ser727 phosphorylation did not occur in response to either EGF or TRAP alone, but it was significantly increased when cells were simultaneously stimulated with EGF plus TRAP (Fig. 3, E and G), suggesting that Ser727 phosphorylation is a mediator of coincidence detection following EGFR/PAR-1 activation. To verify the synergistic phosphorylation of Ser727, STAT3 was immunoprecipitated from EC following agonist exposure, and Ser727 phosphorylation was measured by quantitative mass spectrometry (Fig. 3H). Again, neither EGF nor TRAP induced a significant increase in Ser727 phosphorylation. However, Ser727 phosphorylation was markedly increased following co-treatment with EGF plus TRAP, suggesting that STAT3 Ser727 is a point of convergence for pathways of EGFR/PAR-1 cross-talk.

Ser727-phosphorylated STAT3 Positively Regulates Synergistic EGR1 Induction via a Distal GAS Element—We cloned an ~2-kb fragment of the human EGR1 promoter region upstream of a luciferase reporter to investigate the role of STAT3 Ser727 phosphorylation in synergistic EGR1 induction (Fig. 4A, and supplemental Fig. S6). Previous studies of the EGR1 promoter demonstrated that the ELK-1- and SRF-binding sites within the proximal promoter region are critical for promoter activation in response to various stimuli, including thrombin and EGF (25, 31–34). EGR1 promoter activity was synergistically increased in cells transfected with WT-STAT3 in response to EGF plus TRAP. (Fig. 4, B and C). A STAT3 S727A mutation significantly decreased EGF plus TRAP-induced EGR1 promoter activity and prevented a synergism during co-treatment (Fig. 4, B and C), suggesting that STAT3 Ser727 is critical for promoter activation during EGFR/PAR-1 cross-talk. Mutation of the distal GAS element, but not the proximal GAS element, significantly inhibited EGF plus TRAP-induced promoter activity (Fig. 4D). Because MAPK signaling pathways are active in this context, residual promoter activity of the distal GAS mutant is likely due to EGF-induced activation of serum-response elements within the proximal promoter (31, 35). Next, we measured the DNA binding activity of STAT3 in response to EGF plus TRAP treatment using a DNA-binding ELISA. Surprisingly, we found that untreated STAT3 exhibited DNA binding activity (Fig. 4E). Stimulation of EC with EGF or TRAP for 15 min did not affect STAT3 DNA binding, but co-treatment induced an ~2-fold increase in STAT3-specific DNA binding activity. Together, these results demonstrate that simultaneous activation of EGFR and PAR-1 causes synergistic activation of the EGR1 promoter via a distal GAS element in a manner that requires STAT3 Ser727 phosphorylation.
Coincident EGFR/PAR-1 Signaling Prevents EGF-induced AKT Activation—To identify signaling pathways involved in combinatorial regulation of STAT3 Ser727 phosphorylation, we used a phosphokinase signaling array (R&D Systems) to examine the relative activation of various cell signaling intermediates following individual or combined stimulation with EGF and thrombin. The protein array was composed of nitrocellulose membranes spotted with antibodies for cell signaling mediators, and phosphorylation was detected by chemiluminescence with anti-phosphotyrosine, -phosphothreonine, and -phosphoserine antibodies (Fig. 5A).

The results of the phosphokinase array experiment are summarized in the heatmap (Fig. 5B) and detailed in supplemental Table S2. As expected, EGF treatment induced the phosphorylation of numerous signaling molecules, including ERK1/2, MSK1/2, endothelial NOS, AKT, and GSK-3β. TRAP treatment also induced phosphorylations of known thrombin-regulated proteins (c-Src and CREB). The most dramatic feature of the co-treatment condition was a striking inhibition of AKT phosphorylation at Ser473 relative to cells treated with EGF alone (Fig. 5, A, box, lower dots). Further analysis revealed that phosphorylation of known AKT substrates, including endothe-

FIGURE 3. STAT3 is required for synergistic induction of IEGs. A, EC were transfected with nontargeting or STAT3-targeting siRNA. 48 h post-transfection, EC were treated with EGF, TRAP, or both for 1 h, and EGR1 was measured by immunoblot. B, quantification of four immunoblots performed as in A. C, siRNA-mediated STAT3 knockdown and cell treatment was performed as in A. EGFR1 mRNA was measured by qRT-PCR. Data are averaged from three experiments and expressed as fold induction relative to untreated controls and normalized to levels of GAPDH mRNA. D, SRs for experiments using STAT3 siRNA. Dashed line at y = 1 represents an additive response. E, EC were stimulated for 5 min with EGF (50 ng/ml), TRAP (100 μM), or both. STAT3 phosphorylations were detected by immunoblotting with antibodies specific for Tyr705 and Ser727 phosphorylation. F, quantification of STAT3 Tyr705 phosphorylation from four independent immunoblots. N.S., not significant. G, quantification of STAT3 Ser727 phosphorylation as in F. H, quantitative mass spectrometry analysis of STAT3 Ser727 phosphorylation. HUVEC were treated for 5 min with EGF (16, ng/ml), TRAP (100 μM), or both. Phosphorylation was quantified by comparing the relative abundance of STAT3 Ser727 phosphopeptides to unmodified STAT3 peptides. Data are averaged from two independent experiments.
lial NOS and GSK-3β (Fig. 5A, box, upper dots), was also reduced during simultaneous EGF plus TRAP treatment (Fig. 5B and supplemental Table S2). This observation raised the possibility that EGF plus TRAP treatment modulates AKT-mediated signaling pathways by inhibiting AKT activity. Results of the protein array were confirmed by immunoblotting to detect phosphorylation of AKT. EGF treatment strongly induced phosphorylation of AKT Ser473, and this increase was significantly inhibited by simultaneous EGF plus TRAP treatment (Fig. 5C and D). The inhibition appeared to be specific to AKT signaling, as ERK1/2 phosphorylation was not reduced during EGF plus TRAP treatment (Fig. 5B and C).

EGFR does not have direct binding sites for the Src homology 2 domain of PI3K, but it activates the PI3K-AKT signaling axis via adaptor proteins such as GAB1 (36–38). GAB1 binds to phosphorylated EGFR at Tyr(P)1068 and Tyr(P)1086 to activate the PI3K-AKT signaling axis (38). We investigated whether PAR-1 activation could modulate EGF-induced phosphorylation at these sites to prevent AKT phosphorylation in response to EGF. Co-treatment with EGF and TRAP did not significantly affect total tyrosine phosphorylation of EGFR, Tyr(P)1068, or Tyr(P)1086 (Fig. 5E), suggesting that PAR-1 activation inhibits EGF-induced AKT phosphorylation through a downstream signaling mechanism. Next, we systematically inhibited PAR-1 coupled Gα proteins to identify the Gα member responsible for inhibition of EGF-induced AKT activation. Individual knockdown of Gα12 and Gα13, or inhibition of Gαi by pertussis toxin did not significantly affect the ability of PAR-1 to inhibit AKT activation in response to EGF (supplemental Fig. S7, A–D). However, dual knockdown of Gα12 and Gα13 caused an increase in AKT phosphorylation in response to EGF plus TRAP treatment (Fig. 5F). Together, these data suggest that PAR-1 activation inhibits EGF-induced AKT Ser473 phosphorylation in a Gα12/13-dependent manner without any detectable changes in EGFR phosphorylation.

Inhibition of AKT Is Required for STAT3 Ser727 Phosphorylation and Enhanced EGR1 Expression—To test whether the inhibition of AKT signaling was involved in enhancing EGR1
FIGURE 5. PAR-1 activation prevents EGF-induced AKT phosphorylation. A, phosphokinase array (R&D Systems, catalog no. ARY003) was performed according to the manufacturer’s instructions. Briefly, EC were treated with EGF, thrombin, or both for 10 min. EC were lysed with a proprietary lysis buffer, and lysates were incubated with antibody-spotted nitrocellulose membranes. Relative phosphorylation was detected with anti-phospho-Tyr, -phospho-Thr, and -phospho-Ser secondary antibodies. B, heatmap of data from protein array was created after quantification using ImageJ software. Raw data are available in supplemental Table S2. Heatmap was generated using R-project statistical graphing software and expressed as fold induction relative to untreated controls for each antibody. C, EC were treated as in A with TRAP substituted for thrombin, and levels of phosphorylated ERK and AKT were detected by immunoblot. D, quantification of five immunoblots performed as in C. E, EC were treated with EGF (50 ng/ml), TRAP (100 μM), or both for 5 min. Cells were lysed in ice-cold RIPA buffer, and EGFR was immunoprecipitated with anti-EGFR antibody overnight. Immunoprecipitate was resolved by SDS-PAGE, and total or site-specific tyrosine phosphorylation was analyzed by immunoblot. F, EC were transfected with nontargeting or Gα12/13-targeting siRNA. 48 h post-transfection, EC were treated with EGF (16 ng/ml), TRAP (100 μM), or both for 5 min, and AKT Ser473 phosphorylation was analyzed by immunoblot.
expression, we did a side-by-side comparison of EGF plus TRAP-treated cells and cells treated with EGF plus the PI3K inhibitor LY294002. EGF treatment strongly induced AKT Ser\(^{727}\) phosphorylation at 5 min, and both EGF plus TRAP and EGF plus LY294002-treated cells showed little to no AKT phosphorylation relative to EGF-treated cells (Fig. 6A, *top panel*). In both cases, the inhibition of AKT phosphorylation at 5 min preceded an increase in EGR1 induction at 60 min (Fig. 6A, *bottom panel*) relative to cells treated with EGF alone. EGF plus TRAP and EGF plus LY294002 induced EGR1 to similar levels relative to EGF-treated cells (Fig. 6B), suggesting that modulation of EGF-induced AKT signaling by PAR-1 is a critical step in STAT3-dependent EGR1 induction. Furthermore, EGF-induced *EGR1* promoter activity was significantly increased in EC expressing a DN-AKT construct relative to WT-AKT-expressing cells (Fig. 6C).

To investigate whether the inhibition of AKT kinase activity was required for STAT3 Ser\(^{727}\) phosphorylation, EC were incubated for 15 min with either DMSO or the PI3K inhibitor LY294002, after which they were treated with agonists for 5 min, and STAT3 was immunoprecipitated to assay Ser\(^{727}\) phosphorylation by immunoblot. Similar to previous results (Fig. 3, E, and H), individual EGF treatment did not induce Ser\(^{727}\) phosphorylation. However, in the presence of a PI3K inhibitor, Ser\(^{727}\) phosphorylation was EGF-inducible (Fig. 6D), demonstrating that inhibition of AKT activity is a prerequisite for EGF-induced Ser\(^{727}\) phosphorylation. Furthermore, this result demonstrates that the TRAP-mediated inhibition of EGF-activated AKT is likely the mechanism by which STAT3 Ser\(^{727}\) phosphorylation occurs during coincident EGFR/PAR-1 activation.

We next examined the effect of combinatorial EGFR/PAR-1 signaling on AKT kinase activity. Two phosphorylation sites are involved in AKT activation, Thr\(^{308}\) and Ser\(^{727}\) (39), and phosphorylation at both sites was reduced by EGF plus TRAP co-treatment (Fig. 6E, *top panel*). Although the mechanism by which this inhibition occurs is unclear, this result suggests that it is upstream to AKT, rather than a regulation of AKT itself. Next, AKT activity was measured via an immunocomplex kinase assay (Fig. 6E, *bottom panel*). AKT that was immunoprecipitated from EGF-treated cells strongly phosphorylated a GST-GSK-3\(\alpha\) fusion protein, although TRAP-treated AKT showed little to no activity (Fig. 6, E and F). EGF plus TRAP treatment significantly reduced the ability of AKT to phosphorylate the GST-GSK-3\(\alpha\) fusion protein compared with EGF-treated AKT, indicating a significant reduction in AKT kinase activity during co-stimulation with EGF plus TRAP (Fig. 6E and F). Additionally, EGF-induced GSK-3\(\alpha\)/\(\beta\) phosphorylation was completely inhibited by pretreatment with a PI3K inhibitor (Fig. 6G), indicating that EGF-induced AKT activity is likely the primary mediator of EGF-induced GSK-3\(\alpha\)/\(\beta\) phosphorylation in EC.

AKT-mediated phosphorylation of GSK-3\(\alpha\) at Ser\(^{21}\) and GSK-3\(\beta\) at Ser\(^{9}\) leads to autoinhibition and reduced activity toward downstream substrates (40–42). We hypothesized that a reduction in the inhibitory phosphorylation of GSK-3\(\alpha\)/\(\beta\) during EGF plus TRAP treatment could allow GSK-3\(\alpha\)/\(\beta\) to participate in STAT3 Ser\(^{727}\) phosphorylation. The compound CHIR99021, a potent and highly specific inhibitor of GSK-3\(\alpha\)/\(\beta\) (43), reduced Ser\(^{727}\) phosphorylation in response to EGF plus TRAP (Fig. 6, H and I), but roscovitine, a cyclin-dependent kinase inhibitor, and rapamycin, an inhibitor of mammalian target of rapamycin signaling, had no effect (Fig. 6I and supplemental Fig. S8). These results demonstrate that GSK-3\(\alpha\)/\(\beta\) kinase activity is required for EGF plus TRAP-induced Ser\(^{727}\) phosphorylation, thus placing GSK-3\(\alpha\)/\(\beta\) in the STAT3-dependent, noncanonical pathway of EGR1 induction. Simultaneous knockdown of GSK-3\(\alpha\)/\(\beta\) by RNAi significantly reduced EGF plus TRAP-induced EGR1 expression, although individual knockdown of GSK-3\(\alpha\) or GSK-3\(\beta\) did not significantly affect EGR1 induction (Fig. 6J).

Next, we investigated whether GSK-3\(\alpha\)/\(\beta\) activity was required for ERK-independent EGR1 expression. The MEK1 inhibitor PD98059 inhibited ~50% of EGF plus TRAP-induced EGR1 and CHIR99021 inhibited EGR1 induction to an approximately equal extent (Fig. 6K). Simultaneous inhibition of MEK1 and GSK-3\(\alpha\)/\(\beta\) completely inhibited EGR1 induction by EGF plus TRAP. Similar results were seen for the IEG MKP-1, as inhibition of both GSK-3\(\alpha\)/\(\beta\) and ERK1/2 was required to completely inhibit MKP-1 protein induction in response to EGF plus TRAP (Fig. 6K). This result shows that, like STAT3, GSK-3\(\alpha\)/\(\beta\) function in an ERK-independent pathway that is independently capable of inducing EGR1 expression in response to simultaneous EGF plus TRAP treatment. Furthermore, given that GSK-3\(\alpha\)/\(\beta\) is required for increased STAT3 Ser\(^{727}\) phosphorylation during EGF plus TRAP treatment (Fig. 6, H and I, and supplemental Fig. S8), it is reasonable to conclude that altered GSK-3\(\alpha\)/\(\beta\) activity, due to a reduction in AKT-mediated inhibitory phosphorylation (Fig. 5, C, E, and F), is a critical mediator of EGFR/PAR-1 cross-talk and STAT3-dependent gene expression.

**Coincident EGFR/PAR-1 Signaling Enhances EC Migration in a STAT3-dependent Manner**—EGFR and STAT3 are well known regulators of cell growth and proliferation. To investigate how the EGFR/PAR-1 cross-talk affects EC proliferation, serum-starved EC were stimulated with EGF, TRAP, or both for 8 h, and BrdU incorporation was measured between 8 and 12 h after agonist exposure (Fig. 7A, *top panel*). EGF treatment increased BrdU incorporation to ~2.5-fold compared with nonstimulated cells. TRAP treatment had little to no effect on BrdU incorporation. Interestingly, simultaneous stimulation of EC with EGF and TRAP caused an ~50% reduction in BrdU incorporation relative to EGF treatment alone. siRNA-mediated knockdown of STAT3 further reduced EGF plus TRAP-induced BrdU incorporation, suggesting that STAT3 is critical for EGF-induced DNA synthesis in EC during simultaneous EGFR/PAR-1 activation (Fig. 7A, *bottom panel*).

We previously reported that thrombin induces the expression of leukocyte adhesion molecules on EC and increases monocyte-EC interactions in vitro (20, 44, 45). To investigate the effect that combinatorial signaling has on thrombin-induced leukocyte-EC interactions, we performed an in vitro monocyte adhesion assay on EGF, thrombin, or EGF plus thrombin-stimulated EC. We observed very few monocyte-EC interactions under both untreated and EGF-treated conditions (Fig. 7B, *top left and bottom left panels*). Thrombin induced a significant increase in monocyte adhesion to the EC monolayer
EGFR/PAR-1 Cross-talk Regulates STAT3 Activation

There have been considerable discrepancies regarding EGFR expression in EC (19). Several studies indicated that EGFR was expressed on human microvascular EC (MVEC) and that MVEC were responsive to EGF (46, 47), whereas other reports failed to detect EGFR expression on both MVEC (48) and HUVEC (46, 48, 49). Reports from our laboratory and others (7, 50–52) demonstrate that MVEC and HUVEC do indeed express significant levels of EGFR and are responsive to EGF.

Hirata et al. (53) used a mouse corneal micropocket assay to show that EGFR is as potent as VEGF in inducing angiogenesis in vivo. It has also recently been suggested that VEGF-induced EC migration may be mediated via EGFR transactivation (5). Moreover, although the expression of EGFR on EC has been debated, it is clear that tumor-associated EC express EGFR and are responsive to EGF (47, 54). It is important to address the deficiencies in our knowledge of endothelial EGFR signaling so that we may better understand its role in endothelial physiology, thereby providing a basis for determining the relative contribution of endothelial EGFR signaling to disease states like atherosclerosis and cancer.

It is well established that PAR-1 and other GPCRs can transactivate RTKs via inside-out growth factor signaling in a variety of cell types (15, 16, 55, 56). Through this process, GPCRs can induce proliferative, migratory, or invasive phenotypes in an RTK-dependent manner (6, 56, 57). Our results demonstrate that GPCR-mediated transactivation of RTKs may not be identical to RTK activation per se. Inside-out autocrine signaling may coincide with intracellular modulation of RTK-dependent signaling pathways by GPCR-mediated pathways. Although GPCR activation leads to EGFR transactivation in many contexts, our results raise the possibility that the specific combinations of signaling pathways propagated from EGFR are determined by coincident GPCR activation. Therefore, the temporal relationship between GPCR and RTK activation may have an important effect on EGF-induced phenotypic changes such as migration and proliferation.

We have shown that STAT3 is required for a noncanonical, ERK-independent pathway of IEG induction that is triggered by coincident EGFR/PAR-1 activation. EGF induces the classical STAT3 phosphorylation at Tyr705 (Fig. 3E), but it also initiates an inhibitory signal through the PI3K-AKT signaling axis that phosphorylates GSK-3α/β, prevents STAT3 Ser727 phosphorylation, and inhibits STAT3-dependent EGR1 induction. PAR-1 signaling resolves the conflicting EGF-activated pathways by preventing AKT activation, thus permitting GSK-3α/β-dependent STAT3 Ser727 phosphorylation and ERK-independent EGR1 induction (Fig. 8). Similar to our results, others have shown that the coincidence of detection in a network-based setting can occur by the introduction of an activating signal while simultaneously suppressing an inhibitory signal (8). Collectively, our results describe a novel mechanism by which EGFR and PAR-1 cooperate to form a network-based coincidence detector that regulates STAT3-dependent gene expression.

The EGFR/PAR-1 signaling interaction regulates the magnitude of IEG induction without any detectable change in the kinetics of expression. We show that maximal EGR1 induction can be separated into discrete portions, with ERK1/2 and STAT3 mediating independent pathways. It has been shown that alterations in IEG expression, both in magnitude and duration (Fig. 7B, top right panel), and this effect was almost completely inhibited when EC were simultaneously exposed to both EGF and thrombin (Fig. 7B, bottom right panel), suggesting that coincident EGFR signaling can at least partially inhibit thrombin-induced inflammatory signaling. These results provide evidence that the known phenotypic responses of EGFR-induced proliferation and thrombin-induced inflammation are both suppressed during combinatorial EGFR/PAR-1 signaling.

Previous work from our laboratory demonstrated that simultaneous stimulation of EC with EGF and TRAP caused a significant increase in EC transwell migration in vitro (7). We observed a similar synergistic increase in migration when an EC monolayer was mechanically wounded and then treated with EGF, TRAP, or both for 16 h (Fig. 7C). To investigate a potential role for STAT3 in mediating enhanced EC migration, we depleted STAT3 by RNAi and performed in vitro wound healing assays and measured the degree of scratch closure. Scratch closure was enhanced in cells stimulated with both EGF and TRAP compared with either EGF or TRAP alone (Fig. 7D). siRNA-mediated knockdown of STAT3 slightly reduced EGF-induced wound healing and completely inhibited enhanced wound healing by EGF plus TRAP treatment (Fig. 7, D and E). These results suggest that the combinatorial activation of STAT3 causes increased EC migration, and this increase coincides with a suppression of proliferation and inflammation elicited by either EGF or thrombin treatments per se.

**DISCUSSION**

FIGURE 6. Reduced AKT activity causes increased EGR1 expression in a GSK-3α/β-dependent manner. A, EC were serum-starved for 90 min and then incubated with DMSO or LY294002 (right panels) for an additional 15 min before treatment with indicated agonists. AKT phosphorylation and EGR1 induction were determined by immunoblot. B, quantification of two independent experiments performed as in A. C, HUVEC were co-transfected with EGR1-promoter luciferase reporter and WT-AKT or DN-AKT. 24 h post-transfection, EC were serum-starved for 4 h prior to stimulation with EGF, TRAP, or both for 4 h. Luciferase activity was measured using a luminometer. D, HUVEC were transfected with recombinant V5-tagged STAT3. Cells were pretreated with DMSO or LY294002 for 15 min before treatment with EGF for 5 min. Recombinant STAT3 was immunoprecipitated (IP) using anti-V5 antibody, and STAT3 Ser727 phosphorylation was analyzed by immunoblot. E, serum-starved EC were treated with agonists for 5 min. Cells were lysed, and total AKT was immunoprecipitated using a pan-AKT antibody (Cell Signaling, catalog no. 4691). A portion of the lysate (~10%) was diluted 400-fold in kinase assay buffer and incubated with recombinant GST-GSK3α fusion protein. The majority of lysate was used for immunoblot analysis to determine levels of AKT phosphorylation. F, graph representing the mean ± S.E. of densitometry analysis from three immunocomplex kinase assays. G, EC were pretreated with DMSO or LY294002 for 15 min before treatment with EGF (16 ng/ml) for 5 min. GSK-3α/β phosphorylation was analyzed by immunoblot. H, EC were pretreated for 30 min with the GSK-3α/β inhibitor CHIR99021 (1 μM) and then stimulated for 5 min with EGF, TRAP, or both. STAT3 phosphorylations were detected by immunoblot. I, EC were pretreated for 30 min with the GSK-3α/β inhibitor CHIR99021 (10 μM), roscovitine (20 μM), or rapamycin (10 nM), and then stimulated for 5 min with EGF and TRAP. STAT3 phosphorylations were detected by immunoblot. J, EC were pretreated with control siRNA or siRNA targeting GSK-3α, GSK-3β, or both. 48 h post-transfection, cells were treated with EGF, TRAP, or EGR1 plus TRAP for 60 min, and EGR1 induction was measured by immunoblot. Data represent the mean ± S.E. of three independent experiments. K, EC were pretreated for 30 min with the GSK-3α/β inhibitor CHIR99021 (10 μM), PD98059 (10 μM), or both and then stimulated for 60 min with EGF and TRAP. EGR1 and MKP-1 were detected by immunoblot.
tion, can profoundly impact cellular physiology (17, 18, 58). In particular, one recent study showed that the magnitude of EGR1 induction is critical for digitizing inconsistent or weak growth factor signals into all-or-nothing phenotypic outputs (17). As Zwang et al. (17) showed, the degree of EGR1 induction may be a critical determinant of cell cycle progression, where

![](image)

**FIGURE 7.** Combinatorial EGFR/PAR-1 signaling increases EC migration in a STAT3-dependent manner. A, EC were serum-starved for 16 h and then treated with agonists in serum-free medium for 8 h. BrdU was added to wells for 4 h and cells were fixed in a DNA-denaturing proprietary lysis buffer. BrdU incorporation was detected by an anti-BrdU antibody and colorimetry using HRP-conjugated secondary antibody and 3,3',5,5'-tetramethylbenzidene as a chromogenic substrate. B, EC monolayer was treated with indicated agonists for 6 h, after which agonists were washed away with serum-free media. Calcein-AM-stained U937 monocytes were added to the stimulated EC and allowed to adhere for 30 min. Nonadhered monocytes were washed away, and adhered monocytes were imaged and quantified per field with ImageJ software. C, EC monolayer was mechanically wounded with a P200 pipette tip, and dislodged cells were washed away with serum-free media. EC were treated with indicated agonists, and cells were imaged immediately after scratching and 16 h post-treatment. Dashed white lines indicate the boundaries of the wounds before agonist treatment. D, EC wound healing assay was performed as in C. EC were transfected with control or STAT3 siRNA 40 h prior to the experiment. E, SRs from EC wound healing experiments. Dashed line at $y = 1.0$ represents an additive response. One-sample t test was used to assess the presence of a significant synergism compared with $y = 1$ ($p < 0.05$), and a Student’s t test was used to compare differences between two groups ($p = 0.053$).
EGFR/PAR-1 Cross-talk Regulates STAT3 Activation

EGFR expression must exceed a certain threshold in order for cells to pass a restriction point and initiate DNA synthesis.

GSK-3α/β are multifunctional serine/threonine kinases that are constitutively active under basal conditions and undergo rapid inhibition via serine phosphorylation following growth factor stimulation (42, 59). Our data demonstrate that GSK-3α/β activity is critical for a higher order function of cross-talk by regulating the central node of coincidence detection between EGFR and PAR-1. Several studies have demonstrated that GSK-3α/β is critical for cross-talk during cytokine signaling (60–62). Beurel and Jope (60, 63) showed that inhibitors of GSK-3α/β signaling reduced STAT3 Tyr705 phosphorylation in response to interferon-γ and LPS in cell culture and in mouse brain tissue. Interestingly, their signaling studies in Raw 264.7 macrophages showed that STAT3 Ser727 phosphorylation is unaffected by GSK-3α/β inhibition, in contrast to our results, and indicative of differential regulation of STAT3 by GSK-3α/β depending on the signaling context. To our knowledge, our results are the first example of GSK-3α/β-mediated growth factor activation of STAT3 and may represent a novel opportunity for therapeutic inhibition of STAT3 downstream of oncogenic RTKs.

It is particularly interesting to note that although the observed regulation of STAT3 Ser727 phosphorylation resembles a strong synergism (Figs. 3, E and H, and 6H), STAT3-mediated EGR1 expression appears to be almost perfectly gated by combinatorial EGFR/PAR-1 signaling (Figs. 2A, 3A, and 6K). In other words, there appears to be a low level of noise at the signaling node (STAT3) that is not reflected in the cellular output (STAT3-dependent EGR1 induction). The exact mechanism by which this noise is filtered remains unclear. However, it is intriguing to consider the possibility that other transcription factors may also be required for the STAT3-dependent EGR1 expression. In our comparison of promotor elements of synergistically induced genes (supplemental Fig. S3), we identified several transcription factors that may be common to these genes, including NFκB family members and CREB. Le Goff et al. (64) reported that CREB1-dependent expression of the ABCA1 gene required a nearby STAT element, suggesting that cooperation between STATs and CREB may facilitate enhanced gene expression. Our results clearly show that STAT3 is required for EGR1 induction, but they do not rule out the possibility that other factors are required to act in concert with STAT3 signaling to induce EGR1. We speculate that EC may utilize multiple synergistically activated transcription factors, working in parallel but required in combination, to filter noise at signaling nodes and increase the stringency with which STAT3-dependent EGR1 induction is gated by combinatorial signaling.

We have shown that STAT3 essentially functions as a cellular coincidence detector, triggering EGR1 induction only when EGFR and PAR-1 are simultaneously activated. Coincidence detection, in this context, reflects an extreme synergism where the response to multiple agonists is far greater than responses to individual agonists. A large scale analysis of cross-talk in Raw 264.7 macrophages found that although nonadditive events (positive or negative) are relatively common, the prevalence of marked superadditive responses to pairwise combinations of agonists is rare (4). In fact, less than 1.5% of all ligand pairs in the study demonstrated a significant superadditive response, and the authors noted that although many ligands fail to induce cytokine production by themselves, many ligands display at least one nonadditive interaction when analyzed in combination with another input (4). Thrombin is capable of activating a variety of intracellular signaling pathways via activation of PAR-1. However, in many cases, thrombin’s regulation of signaling pathways (e.g. ERK) and cellular outcomes (e.g. gene expression, migration, and angiogenesis) appears to be much weaker than more potent agonists like EGF and VEGF (7, 65). Our results show for the first time that perhaps one of the strongest responses elicited by PAR-1 signaling is a modulation of EGF-induced PI3K-AKT signaling and a triggering of STAT3-dependent gene expression. We therefore propose that in a combinatorial signaling environment, where growth-factors likely predominate as the most potent determinants of phenotypic outcomes, GPCRs like PAR-1 may act as modulators of growth factor-induced signaling by determining the specificity of RTK-dependent responses.

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FIGURE 8. STAT3-mediated coincidence detection regulates a noncanonical pathway of IEG induction. Either EGF or TRAP is sufficient to induce EGR1 expression via the canonical ERK1/2 pathway. EGF induces the activating STAT3 phosphorylation at Tyr705 but prevents STAT3 Ser727 phosphorylation by strongly activating AKT. Activated AKT phosphorylates and inhibits GSK-3α/β. Coincident PAR-1 signaling inhibits EGF-induced AKT activation, thereby reducing AKT-mediated phosphorylation and inhibition of GSK-3α/β, and permitting GSK-3α/β-dependent STAT3 Ser727 phosphorylation. Simultaneous EGF and PAR-1 activation causes increased phosphorylation of both STAT3 Tyr705 and Ser727 and triggers STAT3-dependent IEG induction.
EGFR/PAR-1 Cross-talk Regulates STAT3 Activation

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