Dependence of EGF-Induced Increases in Corneal Epithelial Proliferation and Migration on GSK-3 Inactivation

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PURPOSE. This study was designed to determine in human corneal epithelial cells (HCEC) whether the balance between epidermal growth factor (EGF)-induced increases in proliferation and migration is dependent on the duration and magnitude of extracellular signal-regulated kinase (Erk)1/2 activation.

METHODS. Western blot analysis evaluated the phosphorylation status of Erk1/2 and phosphoinositide 3-kinase (PI3-K) along with cell cycle kinases, paxillin, and mitogen kinase protein phosphatase (MKP)-1. Proliferation and migration rates were determined by [3H]-thymidine incorporation and scratch wound healing assay, respectively.

RESULTS. EGF induced increases in paxillin Ser-126 phosphorylation and cyclin D1 expression through transient Erk1/2 phosphorylation. However, preinhibition of glycogen synthase kinase (GSK)-3 activation with 20 μM SB415286 prolonged and augmented this Erk1/2 response to EGF but decreased cyclin D1 expression, whereas p27Kip1 levels rose. In turn, the mitogenic response fell, whereas paxillin phosphorylation occurred 45 minutes sooner than without SB415286. In contrast, blocking PI3-K activation with LY294002 (50 μM) eliminated EGF-induced GSK-3 inhibition and Erk1/2 phosphorylation as well as increases in proliferation and migration. SB415286 or U0126 (10 μM) suppression of Erk1/2 phosphorylation blocked EGF-induced MKP-1 phosphorylation. Inhibition of EGF-induced increases in proliferation and migration by LY294002 was associated with sustained MKP-1 phosphorylation induced by GSK-3. Prolonging PI3-K phosphorylation by LY294002 increased p27Kip1, whereas cyclin D1 levels fell.

CONCLUSIONS. GSK-3-induced MKP-1 phosphorylation mediates negative feedback control between EGF receptor–linked PI3-K and ERK signaling pathways. Inhibition of such control prolongs Erk1/2 activation and alters the balance between EGF-induced increases in proliferation and migration. Therefore, these responses to EGF can be modulated through altering the feedback between these two pathways. (Invest Ophthalmol Vis Sci. 2009;50:4828–4835) DOI:10.1167/iovs.08-2983

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example, in corneal epithelial cells, the aforementioned increases in migration can occur from PP-mediated inhibition of either EGF- or HGF-induced ERK pathway activation. One of the PPs mediating such crosstalk (i.e., negative feedback) control is a specific dual threonine tyrosine mitogen kinase phosphatase (MKP)-1. This PP is one of four different PPs in this class identified in HCEC with gene microarray analysis whose substrates are components of the MAPK cascade.

In other tissues, MKP-1 expression is stabilized through phosphorylation by either p38 or Erk1/2. Its role in mediating communication between the ERK and p38MAPK pathways was validated in RCEC by showing that EGF-induced increases in migration were enhanced by prolonged p38 activation after MKP-1 siRNA knockdown. Another indication that PPs play a role in eliciting feedback control between p38 and Erk1/2 is that HGF stimulation of HCEC migration was enhanced after nonselective inhibition of PPs activity. Therefore, in corneal epithelial cells changes in PPs activity can modulate migratory responses to injury-induced increases in cytokine expression.

We report here that EGF-induced increases in HCEC migration and proliferation are affected by changes in the extent of negative feedback control between the P3-K and the ERK pathways. GSK-3 is a negative regulator of Erk1/2 activation since GSK-3 inhibition decreases MKP-1 phosphorylation and prolongs Erk1/2 phosphorylation. Prolonging Erk1/2 activation leads to more rapid Ser-126 paxillin phosphorylation and cell migration as well as declines in cell proliferation. Therefore, changes in duration of Erk1/2 phosphorylation determine the balance between EGF-induced mitogenic and migratory responses.

Materials and Methods

Materials

The following antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA): phospho-Ser 21/9 GSK-3α/β, phospho-MKP-1, cyclin D1, p27, anti-GSK-3, and rabbit polyclonal IgG. Anti-paxillin (p-Ser126) antibody was purchased from Upstate Laboratories (Saranac Lake, NY). Anti-Erk1, phospho-Erk1/2, goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP antibody, and anti-(H196) actin, anti-Erk1/2, anti-p38, and β-actin antibodies were used to test for protein loading equivalence.

Cell Culture

SV40-adenovirus immortalized human corneal epithelial cells (HCEC) were obtained as a generous gift from Kaoru Araki-Sasaki. The cells were cultured at 37°C in an incubator with 5% CO2 and 95% ambient air in DMEM/F12 medium, supplemented with 6% FBS, 5 ng/mL EGF, and 5 μg/mL insulin. Cell cycle arrest was achieved by culturing cells in serum-free and EGF-free DMEM/F12 medium and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen (Carlsbad, CA).

Western Blot Analysis

Western blot experiments were performed as described. In brief, the HCEC were gently washed twice in cold phosphate-buffered saline (PBS) and harvested in 0.5 mL cell lysis buffer. Cell lysates were centrifuged at 13,000g for 15 minutes and supernates were collected. Protein content was measured with a bicinchoninic acid assay (BCA) protein assay kit (Pierce Biotechnology, Chicago, IL), and 200 μg proteins were diluted with an equal volume of 2× Laemmli buffer.

Results

EGF-Induced Changes in GSK-3 and Erk1/2 Phosphorylation

EGF activates the PI3-K/Akt signaling pathway in RCEC. In both HCEC and RCEC, HGF-induced PI3-K activation leads to GSK-3 phosphorylation (i.e., inhibition) and suppresses apoptotic-induced signaling. We determined whether GSK-3 inactivation by phosphorylation induces a negative feedback effect on the MAPK pathway through enhancing Erk1/2 phosphorylation. On the other hand, activated (i.e., dephosphorylated) GSK-3 suppresses Erk1/2 phosphorylation in colon cancer epithelial cells. Figure 1A shows the transient changes in Ser-21/9 GSK-3 phosphorylation (i.e., inhibition) during exposure to 10 ng/mL EGF. Within 5 minutes, GSK-3 phosphorylation became maximal followed by an approximately 50% decline after 90 minutes. We determined whether there is an association between SB415286-induced GSK-3 (i.e., inhibition) and Erk1/2 phosphorylation status. Figure 1B shows that 20 μM SB415286 by itself increased Erk1/2 phosphorylation within 5 minutes. To assess whether this effect results in prolongation of Erk1/2 phosphorylation, we determined the
Phosphorylation of Erk1/2.

SB415286 suggest that prolonged GSK-3 inhibition blocks dephosphorylation status.

Figure 2 shows that after 15 minutes, EGF-induced increases in the phosphorylation status of GSK-3 and Erk1/2 were similar to one another. On the other hand, EGFR-induced increases in the phosphorylation status of GSK-3 and Erk1/2 directly increased to a maximal level at 5 minutes followed by a decline to their baseline level after 120 minutes. On the other hand, GSK-3 inhibition based on changes in Ser-21/9 phosphorylation. A 30-minute preincubation with SB415286 prolonged and augmented the transient increase in EGF-induced Erk1/2 phosphorylation status.

Figure 1 demonstrates that without a GSK-3 inhibitor, SB415286 (20 μM) induction of GSK-3 inhibition based on changes in Ser-21/9 phosphorylation. Western blot analysis determined Erk1/2 and GSK-3 phosphorylation status induced by EGF.11 We determined if EGF induces increases in MKP-1 phosphorylation with a time course that can account for declines in Erk1/2 activity. Under baseline conditions (0 min), MKP-1 and p-MKP-1 were identified in HCEC cells (Figs. 3A, 3B). Both total MKP-1 and p-MKP-1 proteins increased with time. At 5 minutes, MKP-1 phosphorylation had not increased (Fig. 3B) and Erk1/2 activation maximized (Fig. 1C), whereas GSK-3 was maximally inhibited (Fig. 1A). After 60 minutes, MKP-1 phosphorylation increased by nearly fourfold (Fig. 3B). These changes were accompanied by a 25% drop in Erk1/2 phosphorylation and nearly a 50% decline in GSK-3 phosphorylation from their maximal levels at 45 minutes (Figs. 1A, 1C). The phosphorylation status of GSK-3 and Erk1/2 reached their maximal levels at the same time, an agreement consistent with the hypothesis that GSK-3 elicits a negative feedback effect on Erk1/2 phosphorylation. The reciprocal relationship between the time-dependent changes in the phosphorylation status of GSK-3 and MKP-1 suggests that changes in the Erk1/2 phosphorylation status are dependent on GSK-3 mediated stabilization MKP-1 by phosphorylation.18 In other words, Erk1/2 dephosphorylation by MKP-1 could be regulated through changes in the phosphorylation status of GSK-3. This would mean that inhibition of GSK-3 phosphorylation suppresses Erk1/2 phosphorylation, since in its activated dephosphorylated state GSK-3 stabilizes MKP-1. MKP-1 stabilization would then prevent Erk1/2 phosphorylation.

MKP-1 Mediates Negative Feedback Control

MKP-1 expression is detectable in RCEC and is one of the PPs controlling the increases in Erk1/2 and p38 phosphorylation status induced by EGF.11 We determined if EGF induces increases in MKP-1 phosphorylation with a time course that can account for declines in Erk1/2 activity. Under baseline conditions (0 min), MKP-1 and p-MKP-1 were identified in HCEC cells (Figs. 3A, 3B). Both total MKP-1 and p-MKP-1 proteins increased with time. At 5 minutes, MKP-1 phosphorylation had not increased (Fig. 3B) and Erk1/2 activation maximized (Fig. 1C). The phosphorylation status of GSK-3 and Erk1/2 reached their maximal levels at the same time, an agreement consistent with the hypothesis that GSK-3 elicits a negative feedback effect on Erk1/2 phosphorylation. The reciprocal relationship between the time-dependent changes in the phosphorylation status of GSK-3 and MKP-1 suggests that changes in the Erk1/2 phosphorylation status are dependent on GSK-3 mediated stabilization MKP-1 by phosphorylation.18 In other words, Erk1/2 dephosphorylation by MKP-1 could be regulated through changes in the phosphorylation status of GSK-3. This would mean that inhibition of GSK-3 phosphorylation suppresses Erk1/2 phosphorylation, since in its activated dephosphorylated state GSK-3 stabilizes MKP-1. MKP-1 stabilization would then prevent Erk1/2 phosphorylation.

To clarify the dependence of MKP-1 phosphorylation on GSK-3 activation, we determined whether changes in the GSK-3 phosphorylation status could be associated with MKP-1 activation at 30 minutes. EGF increased p-MKP-1 expression fourfold (Fig. 3C), whereas SB415286 suppressed its formation in the presence of EGF (n = 3, P < 0.001). On the other hand, irrespective of the presence or absence of EGF, 50 μM LY294002 enhanced p-MKP-1 formation sixfold (n = 3, P < 0.001) whereas this PI3-K inhibitor dephosphorylated GSK-3 and Erk1/2 (Fig. 2). This suggests that dephosphorylated GSK-3...
elicted this p-MKP-1 increase. In contrast, 10 μM U0126 nearly completely suppressed EGF-induced p-MKP-1 formation. Therefore, both Erk1/2 and GSK-3 activation contribute to p-MKP-1 formation.

GSK-3 Modulates Paxillin Phosphorylation Status

Erk1/2 also functions to control cytoskeletal remodeling in different tissues. Its activation contributes to remodeling of focal adhesions and actin filaments during cell spreading as well as tubulin polymerization. Paxillin is a component of the scaffold supporting focal adhesion kinase (FAK), and increases in migration are associated with their phosphorylation. Since Erk1/2 activation mediates paxillin phosphorylation in renal epithelial cells and raf-transformed fibroblasts, we determined whether Erk1/2 activation elicits this response in HCEC. Figure 4A shows that EGF increased Ser-126 phosphorylation on paxillin by 1.6-fold above the control value after 60 minutes. To assess if prolongation of Erk1/2 activation by EGF altered the time course for EGF-induced paxillin, the cells were preincubated for 50 minutes with 20 μM SB415286. Figure 4B indicates that Erk1/2 phosphorylation reached a maximal level after 5 minutes and was sustained for up to 120 minutes. On the other hand, GSK-3 inhibition caused paxillin phosphorylation to reach a maximal level at 15 minutes and return to its basal level at 60 minutes (n = 3, P < 0.001). Therefore, SB415286-induced maximal paxillin phosphorylation occurred 45 minutes sooner than with EGF. This time course shift in paxillin phosphorylation was associated with prolongation of Erk1/2 phosphorylation. These changes suggest that blocking GSK-3 activation suppressed MKP-1 stabilization and hastened paxillin phosphorylation. However, other PPs in addition to MKP-1 also contribute to eliciting paxillin dephosphorylation, since paxillin still underwent dephosphorylation despite suppression of MKP-1 stabilization after GSK-3 phosphorylation.

Paxillin Phosphorylation Modulation by PI3-K and ERK Pathway Inhibition

To affirm the role of Erk1/2 in mediating paxillin phosphorylation, the association between changes in the phosphorylation status of Erk1/2 and paxillin was determined. EGF-induced Erk1/2 activation was varied by selectively inhibiting PI3-K, GSK-3, or Mek1/2. The results shown in Figure 4C indicate that irrespective of GSK-3 inhibition with either EGF and/or SB415286, paxillin phosphorylation reached maximal levels that were similar to one another. On the other hand, inhibition of EGF-induced PI3-K phosphorylation with LY294002 caused paxillin phosphorylation to decline by 53% (n = 3, P < 0.01). Interestingly, blockage of Erk1/2 activation with U0126, irrespective of the presence or absence of EGF, caused paxillin phosphorylation to decline by 75% below its baseline value (n = 3, P < 0.001). These results indicate that changes in Erk1/2 activation have a corresponding effect on paxillin phosphorylation.

PI3-K and ERK Pathway Control of Cyclin D1 and p27Kip1 Expression

EGF-induced increases in cell cycle progression occur subsequent to increases in substrate levels that promote this response, whereas the expression levels of other inhibitory factors are suppressed. One substrate promoting this response is cyclin D1, whereas p27Kip1 has the opposite effect. To determine whether GSK-3 modulation of Erk1/2 phosphorylation status affects EGF-induced increases in cell cycle progression, we evaluated whether changes in GSK-3 activation alter EGF-induced changes in cyclin D1 and p27Kip1 expression. After exposure to EGF (10 ng/mL) for 1 hour, cyclin D1 levels increased ninefold. At 3 hours, this trend reached 11-fold above baseline and then declined to 5.5-fold above the baseline at 6 hours (data not shown). These changes were concomitantly associated with declines in p27Kip1 formation. Figure 5 shows the individual effects of SB415286 (20 μM), LY294002 (50 μM), and U0126 (10 μM) on cyclin D1 and p27Kip1 expression levels either in the presence or absence of 10 ng/mL EGF at 3 hours. EGF and/or SB415286 induced up to a 3.3-fold increase in cyclin D1 expression, whereas p27Kip1 levels remained unchanged. On the other hand, the individual effects of LY294002 and U0126 on cyclin D1 and p27Kip1 were the reverse of EGF and SB415286. LY294002 by itself increased p27Kip1 to a higher level than that obtained with U0126, whereas U0126 had a larger inhibitory effect on cyclin D1 formation than LY294002 (n = 3, P < 0.001). These changes suggest that GSK-3 activation prevents p27Kip1 degradation, whereas it suppresses cyclin D1 formation.
Mitogenic Responses to EGF Suppressed by PI3-K and GSK-3 Inhibition

The association was determined between reciprocal changes in cyclin D1 and p27Kip1 levels and cell proliferation. Figure 6 shows that EGF induced a twofold increase in cell proliferation. However, blocking PI3-K activation with 20 μM LY294002 suppressed proliferation by 48% below the control level (n = 3, P < 0.001). When LY294002 was applied with EGF, LY294002 abolished the mitogenic response to EGF. On the other hand, 10 μM SB415286 by itself increased proliferation by 25% above the control level. Nevertheless, this response was smaller than that obtained with EGF alone. Such modulation indicates that the GSK-3 phosphorylation status contributes to the regulation of proliferation through modulation of the inverse relationship between cyclin D1 and p27Kip1 levels; namely, blocking EGF-induced GSK-3 inactivation increased p27Kip1 levels and obviated proliferation, whereas inhibition of GSK-3 activation led to prolonged activation of Erk1/2 and a somewhat blunted mitogenic response to EGF.

Control of EGF-Induced Increases in Migration by Modulation of PI3-K and ERK Phosphorylation Status

The association was assessed among EGF-induced changes in GSK-3, PI3-K, and Erk1/2 phosphorylation with their individual effects on cell migration. Normalized rates of wound closure were compared through measurements of cell migration over a 24 hour period (Fig. 7). After 24 hours, the remaining wound area was 30% in the untreated control cell cultures. On the other hand, wound closure was completed with either EGF alone or in combination with SB415286. With SB415286 alone, wound closure also occurred more rapidly than under control conditions since the remaining area was smaller (i.e., 18%) either SB415286 (20 μM), LY294002 (50 μM), or U0126 (10 μM) for 30 minutes. Subsequently, cells were incubated with or without 10 ng/mL EGF for 60 minutes. Western blot analysis detected Ser-126 phosphorylation on paxillin.
than its control ($P < 0.01$). On the other hand, LY294002 decreased EGF-induced wound closure since after 24 hours, the remaining wound area was still 60% ($n = 3$, $P < 0.001$).

With U0126, EGF-induced wound closure also declined, since after 24 hours, the remaining area was 42%. The greater inhibitory effect of LY294002 than that of U0126 ($P < 0.001$) suggests that GSK-3 may mediate paxillin phosphorylation through control of Erk1/2 as well as another kinase. These results suggest that EGF-induced increases in cell migration are modulated by changes in the duration of Erk1/2 activation, since direct inhibition of GSK-3 by SB415286 increased migration nearly as much that obtained by EGF alone.

**DISCUSSION**

Figure 8 provides a summary showing how differences in the duration of GSK-3 inhibition affect the balance between EGF-induced increases in proliferation and migration. Transient stimulation by EGF induces simultaneous stimulation of the ERK and PI3-K pathways.8,11 The duration and magnitude of ERK pathway activation by EGF is modulated through GSK-3 mediated MKP-1 phosphorylation. This response stabilizes MKP-1 phosphorylation and its phosphatase activity causes Mek1/2 and Erk1/2 to remain only transiently phosphorylated. Transient Erk1/2 phosphorylation occurs, since we assume that either GSK-3 is initially not maximally inhibited or its inhibition is slightly delayed relative to Erk1/2 phosphorylation. Transient Erk1/2 phosphorylation leads to increases in cyclin D1 levels, cell cycle progression, and proliferation. If Erk1/2 is only transiently activated and then dephosphorylated by stabilized MKP-1, the mitogenic response to EGF is larger than that resulting from prolonged Erk1/2 activation. On the other hand, prolonged constitutive GSK-3 activation induced by inhibition of PI3-K with LY294002 prevents Erk1/2 phosphorylation and obviates the mitogenic response to EGF. Activated GSK-3 stabilizes p27Kip1 accumulation by causing its phosphorylation, leading to suppression of cell migration. Therefore, our results indicate that the rapidity and duration of
GSK-3 inhibition affects the balance between EGF-induced increases in proliferation and migration. Prolonged GSK-3 inactivation by SB415286 leads to sustained Erk1/2 phosphorylation and decreases in cyclin D1 formation, whereas p27Kip1 expression increases. Such reciprocal changes in cyclin D1 and p27 Kip1 are known to inhibit cell cycle progression and proliferation in human pancreatic ductal carcinoma cells.21 Our results are in agreement with those results. In addition, during rapid and prolonged GSK-3 inhibition with SB415296, p-MKP-1 levels fall, causing Erk1/2 to remain activated. Its persistent activation mediates more rapid phosphorylation of Ser126 on paxillin. Phosphorylation of specific paxillin residues by Erk1/2 during exposure to EGF or hepatocyte growth factor in renal epithelial cells and mIMCD-3 epithelial cells in turn enhances paxillin focal adhesion kinase association and cell migration.19,22 Regarding the relationship between paxillin phosphorylation status and increases in migration by either EGF or injury, induced, such an association has been recently described in HCEC.23,24

MKP-1 is expressed under control conditions and is rapidly stabilized by EGF through post-translational phosphorylation. This is evident since increases in its phosphorylation were detected as soon as 5 minutes after EGF exposure and reached a maximal value in 60 minutes followed by a return to its baseline after another 120 minutes (Fig. 3B). A similar rapid time course for increases in MKP-1 expression was described in macrophages.25 Our results indicate that either GSK-3 or Erk1/2 mediates MKP-1 phosphorylation, since EGF-induced MKP-1 stabilization or GSK-3 activation was inhibited by SB415286 and U0126, respectively. Consistent with a role for GSK-3 in mediating MKP-1 stabilization is that inhibition of PI3-K with LY294002 prevented GSK-3 phosphorylation, leading to decreases in p-MKP-1 expression irrespective of the presence or absence of EGF (Fig. 3C). Therefore, the kinetics of EGF-induced GSK-3 inhibition can modulate the duration and magnitude of Erk1/2 phosphorylation. Such control in turn affects the balance between EGF-induced increases in cell migration and proliferation.

GSK-3 plays a pivotal role in the regulation of a multitude of responses. For example, in rat adenocarcinoma cells, EGF stimulates lamellipodia formation as a consequence of PI3-K–mediated GSK-3 inhibition and changes in cytoskeletal architecture.26 In fact, more than 40 proteins, including 18 transcription factors, are substrates of GSK-3.27 One of its kinase substrates is FAK, which is a component of the scaffold protein, paxillin.28,29 Paxillin modulates cell adhesion and migration through control of FAK phosphorylation status. In HCEC, wounding increases migration through JNK-SAPK–mediated paxillin phosphorylation of Ser-178.24 As wounding upregulates EGF expression resulting in Erk1/2 activation, Erk1/2 may induce the phosphorylation of other residues on paxillin than those elicited by JNK-SAPK activation. As in HCEC, in rat kidney epithelial cells and RAW264.7 cells, EGF-induced ERK pathway stimulation increases paxillin phosphorylation on Ser-126.19,20

The mediators controlling cell cycle progression are also under the control of GSK-3. Cyclin D1 and p27Kip1 expression levels contribute to such control in corneal epithelial cells.30 GSK-3–mediated cyclin D1 phosphorylation earmarks it for proteasomal degradation, whereas p27Kip1 phosphorylation increases its stability.31,32 After GSK-3 inhibition with SB415286 or EGF, p27Kip1 levels fell, whereas cyclin D1 levels at 3 hours were threefold above their control level (Fig. 5). On the other hand, suppression of GSK-3 inactivation with LY294002 or Erk1/2 activation with U0126 reversed the effects of EGF on cyclin D1 and p27Kip1 expression. In this case, cyclin D1 expression declined to a sub-basal level, whereas p27Kip1 expression increased up to twofold regardless of the presence or absence of EGF. Taken together, the modulation by EGF of cyclin D1 and p27Kip1 levels is dependent on GSK-3–mediated regulation of Erk1/2 phosphorylation status. Such regulation in turn affects the balance between EGF-induced increases in proliferation and migration.

Our findings are unique regarding how the balance between EGF-induced proliferation and migration is modulated. In some other tissues, there is evidence that growth factor modulation of the duration of Erk1/2 phosphorylation affects the type of biological response induced by its activation.33 For example, in PC12 cells, fibroblasts, macrophages, and T lymphocytes such a dependence has been described. In these tissues, sustained Erk1/2 activation is required for inducing differentiation. In PC12 cells, EGF only induces a weak proliferative response. On the other hand, mutations occurring in cancer leading to sustained ERK activation correlate with carcinogenesis. In renal epithelial cells and fibroblasts, one of the substrates phosphorylated by activated Erk1/2 is paxillin, but the relationship between duration of Erk1/2 phosphorylation and migration was not delineated.12,19 Therefore, our results are unique in that sustained Erk1/2 phosphorylation causes increases in the cell migration response to EGF, whereas proliferation declines. On the other hand, transient Erk1/2 phosphorylation suppresses migration, but instead increases the mitogenic response to EGF.

In summary, we show in HCEC that the negative feedback between GSK-3 and Erk1/2 determines the balance between EGF-induced increases in cell proliferation and migration. This balance is determined by GSK-3–mediated MKP-1 stabilization, which in turn determines the duration of Erk1/2 phosphorylation. GSK-3 inhibition shifts the balance toward ERK pathway promotion of migration. In contrast, constitutive active GSK-3 instead shortens the duration of EGF-induced Erk1/2 activation and in turn delays paxillin phosphorylation.

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