ERK1/2 Mediate Wounding- and G-protein-Coupled Receptor Ligands–Induced EGFR Activation via Regulating ADAM17 and HB-EGF Shedding

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PURPOSE. Previous studies have shown that wounding of human corneal epithelial cells (HCECs) results in the release of G-protein-coupled receptor ligands such as ATP and lysophosphatidic acid (LPA), which in turn transactivate epidermal growth factor (EGF) receptor (EGFR) through ectodomain shedding of heparin-binding EGF-like growth factor (HB-EGF). In the present study, the role of extracellular signal-regulated kinases 1/2 (ERK1/2) in regulating EGFR transactivation was investigated.

METHODS. SV40-immortalized HCECs were wounded or stimulated with ATP and LPA. EGFR and ADAM17 activation was analyzed by immunoprecipitation followed by Western blot analysis with phospho-tyrosine or phospho-serine antibodies, respectively. Phosphorylation of ERK and AKT was analyzed by Western blot analysis. HB-EGF shedding was assessed by measuring the release of alkaline phosphatase (AP) in a stably transfected human corneal epithelial (THCE) cell line expressing HB-EGF-AP. ADAM17 and ERK interaction was determined by coimmunoprecipitation.

RESULTS. Early, but not late, ERK1/2 phosphorylation in response to wounding, LPA, and ATP was EGFR independent, but sensitive to the inhibitors of calcium influx, protein kinase C and Src kinase. Wounding, LPA, and ATP-induced HB-EGF shedding and EGFR activation were attenuated by the MAPK/ERK kinase (MEK) inhibitors PD98059 and U0126, as well as by ADAM10 and -17 inhibitors. ADAM17 was found to be physically associated with active ERK and phosphorylated at serine residues in an ERK-dependent manner in wounded cells.

CONCLUSIONS. Taken together, our data suggest that in addition to functioning as an EGFR downstream effector, ERK1/2 also mediates ADAM-dependent HB-EGF shedding and subsequent EGFR transactivation in response to a variety of stimuli, including wounding and GPCR ligands. (Invest Ophthalmol Vis Sci. 2009;50:132–139) DOI:10.1167/iovs.08-2246

Corneal epithelium, like other epithelial barriers in the human body, is continuously subjected to physical, chemical, and biological insults, often resulting in tissue or cell injury and a loss of barrier function. Proper healing of corneal wounds is vital for maintaining a clear, healthy cornea and preserving vision. The wound repair process involves cell adhesion, migration, proliferation, matrix deposition, and tissue remodeling.1 Many of these biological processes are mediated by growth factors, cytokines, and other mediators released in the injured tissues or cells.2 We and others have shown that epithelial wounding induces epidermal growth factor (EGF) receptor (EGFR) transactivation via ectodomain shedding of heparin-binding EGF-like growth factor (HB-EGF) in human corneal epithelial cells (HCECs), and this wound-induced activation of EGFR and its coreceptor erbB2 are required for epithelial migration and wound closure.3–6 HB-EGF is synthesized as a type-I transmembrane protein that can be cleaved to release a soluble 14- to 20-kDa growth factor via ectodomain shedding.7–9 which has emerged as an important posttranslational mechanism to regulate the functions of various membrane proteins.10,11 Several members of a family of membrane-anchored metalloproteinases (MMPs), known as ADAM (a disintegrin and metalloproteinase), have been shown to mediate ectodomain shedding of EGF ligands and transactivation of EGFR.12–16 ADAM9, -10, -12, and -17 have been implicated in the cleavage of HB-EGF.17–20 The released HB-EGF acts via the stimulation of specific cell-surface receptors.21 Four related receptor tyrosine kinases include all the EGF receptors, and their ligands are specific to the cell type in which they are expressed.21,22 Shed HB-EGF acts in an autocrine/paracrine fashion to stimulate its activation. Phosphorylation of EGFR creates docking sites for adaptor proteins such as Grb2, Shc, and Gab1 and leads to the activation of the cytoplasmic domain of the receptor.23–27

We recently showed that lysophosphatidic acid (LPA) and adenosine triphosphate (ATP), released by wounded corneal epithelial cells, promote wound healing by inducing metalloproteinase-dependent HB-EGF shedding, subsequent EGFR transactivation, and its downstream signaling.16–19 LPA is a growth factor-like lipid mediator and an important serum component that affects cell adhesion, migration, proliferation, and survival by binding to its receptors LPA1–3.30–31 ATP was first thought solely to be an intracellular energy source, but later proved to be an important extracellular signaling molecule32 that enhances wound healing via its P2Y receptors.29 LPA and P2Y receptors belong to the seven-transmembrane, G-protein-coupled receptor (GPCR) superfamily.33–35 Transactivation of EGFR by LPA and ATP represents a convergent signaling pathway accessible to stimuli, such as growth factors and ligands of GPCR in response to pathophysiological challenges. However, the intracellular signals linking GPCRs to HB-EGF shedding and EGFR signaling remain elusive.

Mitogen-activated protein kinases (MAPK) are serine/threonine kinase proteins that are activated by diverse stimuli ranging from cytokines, growth factors, neurotransmitters, hormones, cellular stress, to cell adhesion.36 Several recent studies...
have shown that MAPK cascades contribute to corneal wound healing by promoting cell proliferation and migration.\(^{37-40}\) The ERK1/2 pathway is a major downstream signaling pathway of receptor tyrosine kinase or growth factor receptors and is involved in the regulation of mitosis, mitosis, and postmitotic functions in differentiated cells.\(^{41}\) Recently, the ERK1/2 pathway has been implicated in regulating ectodomain shedding of transmembrane proteins.\(^{9,42,43}\) In these studies, exogenous trypsinized, and the cells were then collected by centrifugation. Primary

overnight Dispase treatment at 4°C. The dissected epithelial sheet was Bank. The epithelial sheet was separated from underlying stroma after

factor starved in KBM for 16 hours before experiments. Primary HCECs

grown in keratinocyte SFM in a humidified 5% CO\(_2\) incubator at 37°C and growth-

stained with transwell inserts (Transwell; Corning, Inc., Cambridge, MA) in the presence of defined keratinocyte SFM. Cultures were stimulated with extensive wounding, ATP, and LPA, and ERK1/2 were immunoprecipitated with rabbit antibody against PY99. The membrane was then stripped and reprobed with an antibody against EGFR to evaluate the total amount of EGFR precipitated. Phosphorylation of ERK1/2 and AKT was determined using monoclonal antibodies against phospho-

ERK1/2 and phospho-AKT; antibodies against ERK2 and AKT were used to detect equal protein loading of the respective phosphopro-

teinase inhibitor cocktails, and 0.1 mM phenylmethylsulfonyl fluoride.

Measurement of HB-EGF-AP Shedding

Growth factor–starved THCE cells expressing HB-EGF-AP were cul-
tured in six-well plates, pretreated with different inhibitors for 1 hour, and then stimulated with extensive wounding, ATP-γ-S, or LPA for 15 minutes. Culture medium was collected and alkaline phosphatase (AP) activities in the collected media were measured by chemiluminescence detection (Great EscApe SEAP Kit; BD Biosciences, San Diego, CA) according to the manufacturer’s instructions. Briefly, 15 μL of the collected culture medium was heated with dilution buffer at 65°C for 30 minutes in a 96-well plate, followed by addition of assay buffer and substrate. Chemiluminescence was quantified on a fluorometer (GE-Nios; Tecan Ltd., Durham, NC). The readings, after background luminescence in KBM was subtracted, were normalized against those of cellular protein concentration and expressed as the increased level versus the control level. The results are presented as the mean ± SEM (n = 3). Statistical parameters were ascertained by commercial soft-

ware (SigmaStat, Tulsa, OK) with Student’s t-test; *P < 0.05 was con-
sidered significantly different.

Determination of ADAM17 Serine Phosphorylation and Association with Active ERK

Growth factor–starved THCE cell monolayers on 100-mm dishes were pretreated with different inhibitors for 1 hour and then extensively wounded, as described earlier. The cells were lysed with RIPA buffer and precleared with protein A/G-agarose beads. For each sample, 1 mg protein was immunoprecipitated with 2 μg rabbit antibody against ADAM17, followed by addition of 20 μL protein A/G-agarose beads. Precipitants were immunoblotted with an antibody against PY99. The membrane was then stripped and reprobed with an antibody against EGFR to evaluate the total amount of EGFR precipitated. Phosphorylation of ERK1/2 and AKT was determined using monoclonal antibodies against phospho-

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serine and phospho-ERK1/2. Membrane was then stripped and reprobed with ADAM17 antibody, to evaluate the total amount of ADAM17 precipitated.

RESULTS

Effect of EGFR Inhibition on Wound-, ATPγS-, and LPA-Induced ERK Activation

We have shown in previous work that wounding, ATPγS, and LPA triggers rapid activation of EGFR, ERK1/2, and AKT in transfected human corneal epithelial (THCE) cells.\(^{3,28,29}\) To determine the role of EGFR in the activation of ERK1/2 and AKT, we pretreated cells with EGFR neutralizing antibody or control mouse IgG antibody (Fig. 1A). Phosphorylation of
ERK1/2 and AKT was rapidly increased as early as 5 minutes after wounding (pw) and was still elevated 30 minutes pw. Enhanced AKT phosphorylation was significantly attenuated by EGFR neutralizing antibody at all time points. ERK phosphorylation at an early stage (5 and 10 minutes pw), however, was unaffected by EGFR neutralizing antibody, whereas its later activation (20 and 30 minutes pw) was sensitive to EGFR inhibition. To confirm the insensitivity of early ERK activation to EGFR inhibition, the cells were pretreated with EGFR inhibitor AG1478 and then stimulated with wounding, ATP\textsubscript{S}/H9253S, and\textsubscript{S} LPA (Figs. 1B, 1C, 1D, respectively). As we reported previously, ATP\textsubscript{S} and LPA induced rapid ERK1/2 and AKT phosphorylation, seen at 5 and 30 minutes post stimulation. All three stimulus-induced AKT activation was attenuated by AG1478, whereas ERK activation at 5, but not 30, minutes after stimulation was insensitive to AG1478, confirming that ERK, but not PI3K, can be activated independently of EGFR in response to wounding signals.

**Regulation of Wound-Induced, EGFR-Independent ERK Phosphorylation by Ca\textsuperscript{2+}, Src Kinase, and PKC**

We next examined the regulatory mechanisms of wound-induced, EGFR-independent ERK activation. Because we had demonstrated that cell activation in response to wounding and ATP\textsubscript{S} requires Src\textsuperscript{46} and Ca\textsuperscript{2+} signaling,\textsuperscript{29} respectively, and that selective PKC isoforms may be involved in corneal in-
**Effect of MEK Inhibitors on Wound-, ATPγS-, and LPA-Induced HB-EGF Shedding**

Using an epithelial wound model and a THCE cell line transfected with HB-EGF-AP, we have shown that wounded elicits HB-EGF ectodomain shedding and that the released HB-EGF acts as an endogenous agonist for EGFR activation in an autocrine/paracrine manner. In addition, we recently demonstrated that ATPγS and LPA induce HB-EGF shedding and subsequent EGFR transactivation. To determine whether ERK1/2 mediates the ectodomain shedding of HB-EGF in response to wounding, ATPγS, and LPA, we pretreated cells with the MEK inhibitors PD98059 and U0126 and measured AP activity in the culture media, as an indication of HB-EGF cleavage. Basal HB-EGF shedding was slightly attenuated by PD98059. Wounding resulted in a 15-fold increase in HB-EGF shedding, which was significantly downregulated by PD98059 and U0126 (Fig. 3A). Similarly, ATPγS, and LPA induced a 2.5- and a 7.4-fold increase in HB-EGF shedding, which were attenuated by both MEK inhibitors (Fig. 3B), confirming that MEK-ERK1/2 may regulate the cleavage of HB-EGF.

**Effect of MEK Inhibitors on Wound-, ATPγS-, and LPA-Induced EGFR Activation**

We next sought to determine the effects of MEK inhibitors on EGFR activation, indicated by its tyrosine phosphorylation. Because the PI3K-AKT pathway is another known EGFR downstream effector, the PI3K inhibitor LY294002 was included to compare with MEK inhibition. As shown in Figure 4A, wounding-induced rapid EGFR phosphorylation (5 minutes pw), as well as ERK activation, was attenuated by PD98059 and U0126, but not by LY294002 in THCE cells. AKT phosphorylation, however, was almost completely inhibited by LY294002 and was also downregulated by inhibiting MEK. When the cells were treated with HB-EGF, EGFR phosphorylation and degradation were insensitive to inhibitor treatment, suggesting that HB-EGF bypasses MEK inhibition and functions downstream of ERK activation after wounding. Similar results were observed in primary HCECs (Fig. 4B). Inhibitors of p38 (SB203580, 10 μM) and JNK (SP600125, 10 μM) exhibited no effects on wound-induced EGFR phosphorylation (data not shown), suggesting that these two MAPK pathways are not involved in wound-induced EGFR activation. To determine whether the effect of MEK inhibition on wounding-induced EGFR activation is transient or sustained, a time-course study was performed with PD98059 (Fig. 4D). Consistent with a previous report, wounding induced a prominent increase in EGFR phosphorylation at 5 and 20 minutes pw, which was significantly attenuated by PD98059. After 60 minutes pw, the EGFR activation at this time seemed unaffected by PD98059. The inhibition of MEK, but not PI3K, also attenuated ATPγS- and PIP3-enhanced EGFR activation (Figs. 4E, 4F).
The mean increase in AP activities, respectively. The GPCR ligand-in-

FIGURE 5. Wound-, ATPγS, and LPA-induced HB-EGF shedding was sensitive to ADAM inhibitors. Growth factor-starved HB-EGF-AP cells were pretreated with vehicle, 4 μM GI254023X (ADAM10 inhibitor), or GW280264X (ADAM10 and -17 inhibitor) for 1 hour before stimulation with extensive wounding (A), 100 μM ATPγS (B), or 10 M LPA (C) for 20 minutes. HB-EGF shedding was measured and expressed as the mean increase ± SEM (n = 3). *P < 0.05 and **P < 0.01 in (B) and (C) indicate a significant decrease in HB-EGF shedding compared with ATPγS and LPA, respectively.

-17 activities with equal potency.44 As shown in Figure 5A, extensive wounding resulted in a substantial increase in AP activity, and both basal and enhanced HB-EGF shedding were significantly downregulated by GW, but not by GI, suggesting that ADAM17 may be involved in HB-EGF cleavage in response to injury. HB-EGF-AP cells were also challenged with ATPγS (Fig. 5B) and LPA (Fig. 5C), which resulted in 3- and 8.8-fold increases in AP activities, respectively. The GPCR ligand-induced HB-EGF shedding was attenuated by GI and GW by approximately 30% and 60%, respectively, indicating that both ADAM10 and -17 are involved in THCE cell response to ATPγS and LPA.

Association of Serine-Phosphorylated ADAM17 with Active ERK after Wounding

ADAM17 can be phosphorylated at its serine, threonine, or tyrosine residues.50–52 To determine whether ADAM17 is activated in response to wounding, its serine phosphorylation was assayed by immunoprecipitation. As shown in Figure 6A, wounding and PMA (serving as a positive control) induced strong serine phosphorylation of ADAM17, whereas the total ADAM17 protein precipitated remained unchanged. ERK1/2 phosphorylates substrate proteins on serine or threonine residues50 and has been shown to mediate ADAM17 phosphorylation in transfected cell lines stimulated with PMA or growth factors.50–52 We next sought to determine whether ERK1/2 regulates ADAM17 serine phosphorylation after wounding by using the MEK inhibitors PD98059 and U0126. As shown in Figure 6B, inhibiting ERK1/2 activity significantly attenuated wounding-induced ADAM17 serine phosphorylation, raising the possibility that ERK1/2 may directly use ADAM17 as a substrate. Furthermore, we showed that substantial more phospho-ERK was immunoprecipitated with ADAM17 in wounded or PMA-treated HCECs (Fig. 6C), confirming a role of ERK1/2 in wounding-induced ADAM17 activation.

DISCUSSION

We have demonstrated that wounding of HCECs induces HB-EGF shedding and subsequent EGFR activation.5 We have also shown that ligands for GPCR including ATP and LPA contribute to the regulation of wound healing through EGFR transactivation.28,29 To identify the intracellular pathway(s) participating to the regulation of wound healing through EGFR transactivation,28,29 we previously demonstrated that EGFR and its coreceptor ErbB2 serve as a paradigm for inter-receptor cross talk.12,16,53–56 We next sought to determine whether ERK1/2 is involved in ATP- and LPA-induced HB-EGF cleavage. ADAM17 was found to be phosphorylated and physically associated with active ERK in wounded cells. Taken together, these findings provide new insights into the regulatory role of ERK1/2 in transmembrane protein shedding and EGFR activation in response to wounding and GPCR ligands.

Inter-receptor cross talk is a well established concept in understanding complex signaling networks and in the translation of environmental conditions into appropriate cell responses.12,53 Transactivation of EGFR by GPCR combines the broad diversity of GPCRs with potent EGFR signaling and serves as a paradigm for inter-receptor cross talk.12,16,53–56 We previously demonstrated that EGFR and its coreceptor ErbB2 are critical for corneal epithelial wound healing.5,4 More recently, we showed that ATP and LPA, released on corneal wounding, enhance wound closure via transactivation of EGFR.28,29 ATP P2Y and LPA receptors belong to the GPCR superfamily.35,57 However, the initial mediators that link wounding, ATP, and LPA-induced early ERK1/2 activation was insensitive to EGFR inhibition by its neutralizing antibody or antagonist AG1478. This transient ERK activation, however, was sensitive to the inhibition of calcium influx, Src kinase, and PKC, but not to the inhibition of MMP. We then demonstrated that HB-EGF shedding and subsequent ERK1/2 activation in response to wounding and GPCR ligands were inhibited by MEK inhibitors PD98059 and U0126. Although ADAM17 mediated wounding-induced HB-EGF shedding, both ADAM10 and -17 were involved in ATP- and LPA-induced HB-EGF cleavage.

We have demonstrated that ADAM17 may be involved in HB-EGF cleavage in response to injury. HB-EGF-AP cells were also challenged with ATPγS (Fig. 5B) and LPA (Fig. 5C), which resulted in 3- and 8.8-fold increases in AP activities, respectively. The GPCR ligand-induced HB-EGF shedding was attenuated by GI and GW by approximately 30% and 60%, respectively, indicating that both ADAM10 and -17 are involved in THCE cell response to ATPγS and LPA.
ERK1/2 Mediate Wound-Induced EGFR Activation

**FIGURE 6.** ADAM17 was serine-phosphorylated and associated with active ERK after wounding. Growth factor-starved THCE cells were pre-treated with 50 µM PD98059 or 10 µM U0126 for 1 hour (B) before stimulation with extensive wounding or 1 µM PMA (A, C) for 10 minutes. Cell lysates were immunoprecipitated with ADAM17 antibody, immunoblotted with phospho-serine antibody (A, B) or phospho-ERK antibody (C), and re-probed with ADAM17 antibody, to assess the amount of ADAM17 precipitated.

main shedding of membrane proteins may be cell-type- and substrate-specific and related to the activation of different sheddases. Raf has been suggested to play a role in the EGFR autocrine loop by inducing HB-EGF expression and secretion. However, such regulation occurs at the transcription level at a much later time (in a matter of hours) in transformed cells. In the pathophysiological wounding and GPCR ligand stimulation situations of the present study, ERK-mediated HB-EGF shedding and EGFR activation did not require new protein synthesis and happened much faster (in a matter of minutes). In addition, contrary to the EGFR overexpression and autocrine loop signaling commonly seen in cancer cells,

wounding-induced EGFR phosphorylation was rapid and transient in wounded HCECs, as at 60 minutes pw the level of EGFR phosphorylation was similar to that of the control. The fact that PD98059 had no effect on EGFR phosphorylation at 0 and 60 minutes pw suggests that ERK1/2 is not involved in the maintenance of basal EGFR activity.

Ectodomain shedding has emerged as an important post-translational mechanism to regulate the functions of various membrane proteins. Ectodomain shedding of EGFR ligands has been linked to the ADAM family of proteins and among the family members, ADAM10 and ADAM17/TACE (tumor necrosis factor-α-converting enzyme) are particularly important in the context of ectodomain generation. Using GI254023X, which preferentially blocks ADAM10 but not -17, and GW280264X, which antagonizes both ADAM10 and -17 with equal potency, we showed that both basal and wounding-induced release of HB-EGF was sensitive to GW280264X but not GI254023X, suggesting that constitutive and wound-induced ectodomain shedding of HB-EGF requires ADAM17 but not -10 in HCECs. ATP-PyS and LPA-enhanced HB-EGF cleavage, on the other hand, is diminished by both GI254023X and GW280264X, with the latter to a greater extent, indicating the involvement of both ADAM10 and -17 in mediating GPCR ligand-induced sheddase activity. The underlying mechanism for differential regulation of ADAMS by wounding and by GPCR ligands remains elusive, and siRNA targeting ADAMs yields no conclusive evidence for the involvement of these enzymes in EGFR transactivation (data not shown).

ADAM17 has been shown to be phosphorylated at its serine or threonine residues by ERK1/2 in transfected cells treated with PMA or growth hormones. More recently, *Staphylococcus aureus* protein A was found to activate ADAM17 in an ERK-dependent manner. Our study is the first to document that wounding of HCECs is more potent than PMA in inducing ADAM17 serine phosphorylation in a MEK inhibitor-sensitive manner. In addition, we demonstrated that ADAM17 is associated with phosphorylated ERK in response to wounding and PMA, confirming that ERK may interact with and phosphorylate ADAM17 and therefore regulate HB-EGF shedding.

MAPKs are serine-threonine protein kinases that are activated by diverse stimuli ranging from cytokines, growth factors, neurotransmitters, hormones, cellular stress, to integrin-mediated cell adhesion. The mammalian MAPK can be subdivided into five families: ERK1/2, p38, JNK, ERK3/4, and ERK5, among which ERK1/2 is the best characterized. The ERK1/2 pathway includes three kinases establishing a sequential activation pathway of Raf-MEK-ERK, which is believed to be the primary activator and a downstream effector of EGFR signaling.

ERK1/2 mediated the shedding of transmembrane proteins including HB-EGF, and wound-induced HB-EGF shedding and EGFR activation did not require new protein synthesis and happened much faster (in a matter of minutes). In addition, contrary to the EGFR overexpression and autocrine loop signaling commonly seen in cancer cells, wounding-induced EGFR phosphorylation was rapid and transient in wounded HCECs, as at 60 minutes pw the level of EGFR phosphorylation was similar to that of the control. The fact that PD98059 had no effect on EGFR phosphorylation at 0 and 60 minutes pw suggests that ERK1/2 is not involved in the maintenance of basal EGFR activity.

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In conclusion, our results suggest that ERK1/2 mediates HB-EGF shedding and subsequent EGFR transactivation in response to a variety of stimuli including wounding and GPCR ligands via regulating phosphorylation and activation of ADAM17. The present study provides a novel understanding of ERK function and insights into the regulatory mechanisms of transmembrane protein shedding and EGFR activation. Future research is needed to address the specific roles of mediators upstream to ERK and to determine the interaction between ERK and other ADAMs.

References

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