Signal Integration and Coincidence Detection in the Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase (ERK) Cascade

**CONCOMITANT ACTIVATION OF RECEPTOR TYROSINE KINASES AND OF LRP-1 LEADS TO SUSTAINED ERK PHOSPHORYLATION VIA DOWN-REGULATION OF DUAL SPECIFICITY PHOSPHATASES (DUSP1 AND -6)**

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Diverse stimuli can feed into the MAPK/ERK cascade; this includes, for example, the extracellular matrix, cell-cell interactions, growth factors, hormones, and some agents that can affect cell density or cell shape. These signals are often transduced into the nucleus of cells through MAPKs, including ERK, which is one of the most widely studied members of the MAPK family. The MAPK/ERK cascade plays a central role in the regulation of cell growth, differentiation, and survival.

This article has been withdrawn by Nishamol Geetha, Judit Mihaly, Alexander Stockenhuber, Francesco Blasi, Pavel Uhrin, Michael Freissmuth, and Johannes M. Breuss. The withdrawing authors regret that Bernd R. Binder passed away. In Fig. 3A, The EGF/LF lanes did not accurately represent the experimental results. The tERK lanes were duplicated in Fig. 3C. The LF lanes were derived from a different immunoblot in Fig. 3D. The pERK and tERK immunoblots from PDGF/LF treatment in Fig. 4B were reused in Fig. 4C for EGF/LF treatment. The DUSP1 and actin immunoblots from untreated cells in Fig. 7A were reused in Fig. 8C. The DUSP6 immunoblot from untreated cells in Fig. 7B was reused in Fig. 8D. In addition, the actin immunoblot from untreated cells in Fig. 7B was reused in Figs. 7C and 8D for the untreated samples. The corrected blots are available upon request.

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mounted in Permafluor (Lab Vision Corp., Fremont, CA). Images were captured at 40- and 100-fold magnification on a Zeiss LSM 510 META confocal microscope in multitrack mode, using pinhole sizes between 0.7 and 1.5 μm and the appropriate standard laser-filter combinations and were digitized using the built-in software.

Statistics—Values are reported as means ± S.E. of three experiments done in duplicate (ERK phosphorylation) or in triplicate to quintuplicate (adhesion assay). Statistically significant differences were verified by paired or unpaired t test and by analysis of variance followed by a Bonferroni post hoc test or Dunnett’s test for multiple comparison, as appropriate.

RESULTS

Combined Stimulation of Cells with EGF and Lactoferrin Induces Sustained ERK2 Activation—The shape and duration of receptor-induced ERK stimulation are highly variable. We surmised that the upstream cascade can integrate additional signals that are translated into distinct time-dependent activity profiles. This conjecture was tested by incubating HT1080 cells (a human fibrosarcoma endowed with several receptor tyrosine kinases) in the presence of the LRP ligand lactoferrin, which per se does not stimulate ERK phosphorylation (Fig. 1A, top, lanes on the right). EGF-induced stimulation of ERK phosphorylation peaked at 5–10 min and then declined to a low residual activity that could be still detected after 2 h (Fig. 1A, top, lanes on the left). However, when EGF was combined with lactoferrin, a sustained activation was observed over >60 min (Fig. 1A, bottom, lanes on the left). In contrast to incubations with EGF alone, the combination of EGF and lactoferrin resulted in ERK phosphorylation that was still detectable after 4 and 16 h. In fact, the time course was comparable with that seen with persistent stimulation of protein kinase C isoforms by the phorbol ester PMA (Fig. 1A, bottom, lanes on the right).

This response was not unique to the fibrosarcoma cell line because it was recapitulated in primary cultures of human skin fibroblasts (Fig. 1B). This suggests that the ERK cascade acted as a coincidence detector and signal integrator that translated the simultaneous occupancy of the EGF receptor and an LDL receptor-related protein into a sustained response. We verified
Occupancy of LRP-1 Promotes Sustained ERK Activation

**FIGURE 2. Combined stimulation of HT1080 cells with EGF and lactoferrin does not induce a sustained activation of ERK**

HT1080 fibrosarcoma cells were stimulated with EGF (25 ng/ml), IGF2 (100 ng/ml), and VEGF 165 (50 ng/ml) (data not shown). Comparable findings were also obtained with PDGF (10 ng/ml), IGF2 (100 ng/ml), and VEGF 165 (50 ng/ml) (data not shown).

EGF activates many additional pathways other than ERK. We determined whether the amplifying effect of lactoferrin was limited to EGF-dependent activation of the MAPK pathway by assessing other pathways (i.e. PI3K-dependent activation of AKT and stimulation of the non-receptor tyrosine kinase SRC). EGF induced phosphorylation of AKT both on Ser473 (Fig. 2A) and Thr308 (Fig. 2B). The combination of EGF and lactoferrin neither caused any additional stimulation nor affected the time course of AKT phosphorylation (Fig. 2A and B). Similarly, the phosphorylation of SRC elicited in response to the combination of EGF and lactoferrin was comparable with that caused by the addition of EGF alone (data not shown). Although we did not examine additional signaling pathways (e.g. activation of phospholipase Cγ), these observations suggest that the signal amplification caused by lactoferrin does not indiscriminately involve all possible pathways but appears to be confined to MAPK activation.

**Sustained ERK Activation Depends on LRP-1**—The association of ligands with LRP can be blocked by receptor-associated protein (RAP) (10). If the action of lactoferrin and of LDL were mediated via interaction with an LRP family member, it ought to be blunted by preincubation of the cells in the presence of RAP. This was the case (Fig. 3A). There are several family members of the LDL receptor-related protein family. Earlier observations indicated complex formation between LRP-1 and the PDGF receptor (2, 11). Hence, we substantiated the conjecture that LRP-1 might be required also for supporting the lactoferrin-promoted augmentation of EGF-induced ERK phosphorylation by using MEFs isolated from mice deficient in LRP-1 and/or LDL receptor. MEFs lacking LRP-1 did not respond to the combination of EGF and lactoferrin to induce ERK activation (Fig. 3C). In fact, in the absence of lactoferrin blunted the response to EGF. A possible underlying mechanism is currently not understood. In the presence of LDL receptor-deficient MEFs, EGF and lactoferrin

that this coincidence detection was not limited to the pair EGF and lactoferrin; the combination of EGF and basic fibroblast growth factor (FGF2) also resulted in sustained ERK phosphorylation (Fig. 1C, cf. left-hand bFGF-labeled lanes with lanes labeled bFGF/LF). Similarly, incubation in the presence of LDL (100 μg/ml) augmented the levels of phospho-ERK regardless of whether cells were treated concomitantly with EGF or FGF; LDL per se did not change the levels of ERK phosphorylation (Fig. 1C). Comparable findings were also obtained with PDGF (10 ng/ml), IGF2 (100 ng/ml), and VEGF 165 (50 ng/ml) (data not shown).

We defined the required region of uPAR by employing two monoclonal antibodies, R3 and R2, directed against uPAR domains 1 and 3, respectively. Cells were pretreated with these antibodies and then challenged with a receptor tyrosine kinase receptor agonist and lactoferrin. Fig. 3B shows the results for stimulation of cells with PDGF; blockage of the uPAR domain 1 with the R3 antibody precluded sustained ERK phosphorylation in response to the combination of PDGF and lactoferrin (Fig. 4B). This is consistent with the fact that the antibody blocks the association of uPA and uPAR (13). A similar observation was made with HT1080 cells that had been challenged by a combination of EGF and lactoferrin in the presence of the R3 antibody (Fig. 4C). In contrast, monoclonal antibody R2, which does not interfere with uPAR-uPA binding, did not interfere with sustained ERK2 activation (Fig. 4D). Similar findings were obtained with FGF, VEGF, and IGF (data not shown).
Cytoplasmic Accumulation of Phospho-ERK Translates into Accelerated Adhesion on Vitronectin—ERK phosphorylation promotes nuclear translocation of the enzyme (14). This was also seen if HT1080 cells were stimulated by EGF (Fig. 5, cf. first and second row). The sole addition of lactoferrin did not affect the distribution of ERK (Fig. 5, row 3). Surprisingly, the combination of EGF and lactoferrin resulted in a delayed translocation of active phosphorylated ERK into the nucleus; for the first 60 min, there was little appreciable translocation of phospho-ERK into the nucleus (Fig. 5, cf. rows 4–6 with control rows 1 and 2), whereas it accumulated in the perinuclear region and most prominently at submembraneous spots. After prolonged stimulation with EGF and lactoferrin, phosphorylated ERK did accumulate in the nucleus (Fig. 5, bottom row).

The observations suggested that the stimulation of cells by the combination of EGF and lactoferrin initially redirected ERK signaling to cytosolic targets. Cytosolic ERK has many targets, and several of these are involved in actin dynamics (15), such that ERK plays a prominent role in integrin-dependent adhesion (16). Accordingly, we evaluated whether the combined addition of EGF and lactoferrin promoted cell adhesion. This was the case; at early time points (10 min), the number of cells that adhered to vitronectin-coated dishes was augmented (Fig. 6A, white bars). In contrast, neither EGF nor lactoferrin by themselves affected cell adhesion at this early time point. Thus, increased cytosolic phosphorylated ERK (levels) translated into a relevant biological response, namely accelerated cell adhesion promoted by the combination of EGF and lactoferrin. Blockage of the MAPK cascade ought to blunt the response to the combination of EGF and lactoferrin if there were a cause-and-effect relation between cytosolic accumulation of MAPK and enhanced adhesion. This prediction was verified by employing the MEK1 inhibitor PD98059. Pretreatment of cells with 25 μM PD98059 for 30 min reduced the number of adherent cells by about 50% if cells were stimulated by EGF and lactoferrin; in contrast, PD98059 had no appreciable effect on cell adhesion.
under any of the other conditions tested (Fig. 6A, black bars). EGF (17) and uPAR (18) stimulate cell migration via stimulation of the MAPK cascade. Accordingly, we allowed HT1080 cells to adhere on vitronectin and subsequently examined over the next 15 h by time lapse microscopy whether the combination of EGF and lactoferrin had a stronger chemokinetic effect than the sole addition of EGF. In fact, trajectories of cells were significantly longer in the presence of EGF and lactoferrin than when each compound was added separately (Fig. 6B). We also examined directed migration induced by wounding a cell monolayer. The combination of EGF and lactoferrin also resulted in accelerated migration (data not shown).

Proteasomal Degradation of DUSPs Accounts for Sustained ERK Activation in Response to EGF and Lactoferrin—The magnitude and duration of MAPK signaling is dependent on the balance between the activities of upstream activators and deactivation by phosphatases. Because phosphorylation of both threonine and tyrosine residues is required for activity, dephosphorylation of either is sufficient for inactivation. This is achieved by dual specificity (threonine/tyrosine) protein phosphatases (DUSPs) (19). DUSPs differ in their affinity for individual MAPKs. ERK1/2 can be dephosphorylated by the inducible DUSP1, DUSP4, and DUSP5, which are found primarily in the nucleus, and by the cytosolic isoforms DUSP6, DUSP7, and DUSP9 (20). Sustained activation of ERK2 via the combined input from a tyrosine kinase receptor and LRP-1 may be due to a decline in DUSP levels. We tested this hypothesis by assessing the effect of combined stimulation with EGF and lactoferrin on the amount of DUSP in lysates of HT1080 cells. Incubation with EGF and lactoferrin led to a pronounced and sustained downregulation of the nuclear DUSP1 (Fig. 7A) and of the cytosolic DUSP6 (Fig. 7B). The capacity of LRP-1 ligands to synergize with tyrosine kinase receptor ligands was not limited to lactoferrin but was also recapitulated in the presence of LDL (cf. Fig. 1C). Thus, when combined with EGF, LDL was predicted to cause a similar decline in DUSP levels if the decline in DUSP levels and the change in ERK phosphorylation were causally related. This was the case (Fig. 7, A and B). In contrast, stu-
lation with EGF alone caused only a transient reduction in the levels of DUSP1 and DUSP6, whereas lactoferrin alone did not affect the expression levels of DUSP1 and DUSP6 (open diamonds in Fig. 7, A and B). We also verified that the combination of EGF and lactoferrin did not result in an indiscriminate loss of all DUSP isoforms. There was, for instance, no appreciable change in the levels of the nuclear isoform DUSP5 (Fig. 7C), DUSP7, and DUSP9 (data not shown).

The down-regulation of DUSP1 and DUSP6 occurred swiftly (i.e. it was detectable within 10 min). An obvious explanation

**FIGURE 5. Localization of phosphorylated ERK by immunocytochemistry in cells stimulated by EGF and lactoferrin.** Serum-starved HT1080 cells were stimulated with EGF (25 ng/ml), lactoferrin (70 μg/ml), and a combination thereof for the indicated time points. Thereafter, the cells were fixed with paraformaldehyde (4%), permeabilized with 0.2% Tween 20, and stained for total ERK (green, first column) and phospho-ERK (red, second column). Confocal images were captured on a Zeiss LSM 510 microscope using the settings outlined under "Materials and Methods." The third column shows merged confocal images of the first and second columns. Arrowheads mark phosphorylated ERK in the cytoplasm and in basal adhesion contacts. Images were captured, micrographs were printed, and cells were scored for the presence of phospho-ERK in the cytosol and in the nucleus. 90 cells from three independent experiments were scored for each condition. Error bars, S.E.
for this rapid decline was proteolytic degradation via the proteasome. In fact, when cells had been pretreated with the proteasome inhibitor MG132 (10 μM), DUSP levels were not decreased upon combined stimulation with EGF (25 ng/ml) and/or lactoferrin (70 μg/ml), or LDL (100 μg/ml) for the indicated time periods. Expression of DUSP1 (A), DUSP6 (B), and DUSP5 (C) was assessed from immunoblots, quantified densitometrically, and normalized to actin. The blots shown are representative of three independent experiments; relative levels (initial control = 100%) are shown in the graphs (error bars, S.E.).
The combination of EGF and lactoferrin increased DUSP degradation and triggered sustained ERK activation. Thus, down-regulation of DUSPs ought to lead to a sustained EGF response. We focused on DUSP6 because this is the cytosolic isoform, the down-regulation of which ought to phenocopy the combined effect of lactoferrin and EGF. DUSP6 levels were reduced by using an appropriate siRNA (Fig. 9A). Silencing of DUSP6 led to a sustained activation of ERK with EGF alone; there was not any appreciable difference in the time course of phospho-ERK accumulation in cells that had been stimulated by the sole addition of EGF or by the combination of EGF and lactoferrin (Fig. 9B). This was not the case in cells transfected with a control siRNA (Fig. 9C) or untransfected cells, which were examined in parallel (Fig. 9D).

If sustained ERK activation induced by combined stimulation with EGF and lactoferrin were indeed the result of proteasomal degradation of DUSP1 and/or DUSP6, blockage of the proteasome ought to prevent sustained activation. This prediction was verified by treating cells with the proteasome inhibitor MG132 prior to stimulation by EGF, lactoferrin, or a combination thereof. Inhibition of the proteasome by MG132 did not affect the response to EGF (Fig. 10, cf. A and B, left). In contrast, in cells challenged by the combined addition of EGF and lactoferrin, pretreatment with MG132 precluded sustained ERK phosphorylation (Fig. 10B, right). Phospho-ERK levels declined more rapidly after the initial peak than in untreated cells (Fig. 10A, right).

**DISCUSSION**

The ERK cascade plays a central role in mitogenic signaling. It has attracted interest because input via extracellular signal is translated into both a spatial and a temporal dimension. This concept was originally developed with the study of PC12 differentiation (14) and probed rigorously by rewiring the signaling network (21); the rat pheochromocytoma cell line PC12 is differentiated by NGF because this agonist causes a very long lasting stimulation of ERK activity. In contrast, the kinetics of EGF-induced ERK phosphorylation supplies a stimulus for cell division. In addition, the ERK cascade displays hysteresis and bistability. It is therefore capable of storing information and converting an analog signal (the graded concentration of mitogen) to a digital output; regardless of whether differentiation or

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**FIGURE 8. Degradation of DUSP1 (A and C) and DUSP6 (B and D) induced by the combination of FGF and lactoferrin (A and B) or of EGF and lactoferrin (C and D) in the absence and presence of MG132 (1 μM) for 30 min or in the absence of MG132 for the indicated intervals. The levels of DUSP1 (A and C) and DUSP6 (B and D) were assessed by immunoblotting and quantified. The blots shown are representative of three independent experiments; relative levels (initial control = 100%) are shown in the graphs (error bars, S.E.).**

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**Supplementary Material**

1. [Supplementary Figure 1](#).
2. [Supplementary Table 1](#).
3. [Supplementary Methods](#).
4. [Supplementary References](#).
cell division is examined, if the activity has reached a critical threshold, an irreversible decision is made. The cell undergoes replication or differentiates. It is therefore interesting to understand how the kinetics of ERK activation is controlled. Because the ERK signaling pathway receives input from several pathways, it allows for signal integration; irrespective of the source of the upstream input, it is converted into an analog signal, namely the enzymatic activity of the dually phosphorylated ERK. Thus, the integral of the input is encoded in the strength of the output signal. Because ERK is subject to deactivation by dephosphorylation and because it drives irreversible decisions, it also allows for coincidence detection; thresholds may only be reached if two signals are present at the same time. Thus, in the original model of PC12 cell differentiation, the ERK cascade also acted as a coincidence detector; the simultaneous presence of cAMP converted the EGF-driven output from proliferation to differentiation (14).

Previous investigations have focused on the cross-talk with G protein-coupled receptors. These studies have provided evidence for trans-activation (22), for release of growth factors via the release of latent membrane-bound growth factors (23), and for second messenger-driven loops (14, 24). More recently, the
Occupancy of LRP-1 Promotes Sustained ERK Activation

Cooperation between two receptor tyrosine kinases has been subjected to a rigorous analysis (25). Here we show that input via LRP-1 and uPAR can substantially alter the temporal shape of the ERK response. These observations underscore the function of the cascade as a signal integrator and coincidence detector; our experiments demonstrated that the absence or presence of two additional signals (i.e. engagement of the LRP-1 receptor and input via the urokinase receptor uPAR) produced a temporally and spatially distinct pattern of enzyme activation (i.e. sustained phosphorylation of ERK that was initially confined to the cytoplasm). Although these findings are consistent with the predictions arising from the analysis of the network properties (26), there are, to the best of our knowledge, no earlier reports that document this type of cooperation between LRP-1 and receptor tyrosine kinases. However, the results from our experiments are unequivocal. The simultaneous activation of the EGF receptor and the engagement of LRP-1 profoundly affected the temporal and spatial pattern of ERK phosphorylation. This response was not confined to the ErbB family of receptor kinases but was found with all receptor tyrosine kinases examined, indicating that it is a universal phenomenon. The very mechanism by which this effect is brought about is also consistent with the following generalization. The occupancy of LRP-1 triggers degradation of DUSP1 and DUSP6 via proteasomal degradation. This effect is predicted to enhance ERK phosphorylation by receptor tyrosine kinases regardless of the nature of the upstream input.

Interestingly, the sustained response required input via both the urokinase/plasminogen activator receptor uPAR and LRP-1; although we did not study the detailed mechanism underlying this cooperation, our experiments document that neither sustained ERK phosphorylation nor LRP-1-triggered degradation of DUSP1 and DUSP6 occurred if signaling either via uPAR or via LRP-1 was abrogated. Both DUSP1 (MAPK phosphatase-1, MKP1) and DUSP6 (MKP-3) are subject to regulation by the ERK cascade because phosphorylation of these enzyme inhibitors renders them susceptible to ubiquitination and hence to proteasomal degradation. This effect is predicted to enhance ERK phosphorylation by receptor tyrosine kinases regardless of the nature of the upstream input.

The very mechanism by which this effect is brought about is also consistent with the following generalization. The occupancy of LRP-1 triggers degradation of DUSP1 and DUSP6 via proteasomal degradation (27, 30). The exact reason for this unmasking of DUSP1 and DUSP6 is not yet clear, but it appeared to be subject to rapid dephosphorylation, resulting in enhanced nuclear accumulation of phospho-ERK. The very mechanism by which this effect is brought about is also consistent with the following generalization. The occupancy of LRP-1 triggers degradation of DUSP1 and DUSP6 via proteasomal degradation. This effect is predicted to enhance ERK phosphorylation by receptor tyrosine kinases regardless of the nature of the upstream input.

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and thus promote the emergence of clinically manifest cancer. uPA/uPAR may also enhance MAPK signaling by growth factor
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