Sustained Activation of the Mitogen-activated Protein Kinase Pathway

A MECHANISM UNDERLYING RECEPTOR TYROSINE KINASE SPECIFICITY FOR MATRIX METALLOPROTEINASE-9 INDUCTION AND CELL MIGRATION*

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Activation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway is required for ligand-dependent regulation of numerous cellular functions by receptor tyrosine kinases. We have shown previously that although many receptor tyrosine kinase ligands are mitogens for keratinocytes, cell migration and induction of the 92-kilodalton gelatinase/matrix metalloproteinase (MMP)-9 are selectively regulated by the epidermal growth factor and scatter factor/hepatocyte growth factor receptors. In this report we present evidence of an underlying mechanism to account for these observed differences in receptor tyrosine kinase-mediated response. Ligands that are mitogenic, but do not induce MMP-9 or colony dispersion, transiently activate the p42/p44 ERK/MAP kinases. In contrast, ligands that stimulate MMP-9 induction and colony dispersion induced sustained activation of these kinases. The functional significance of sustained MAPK activation was demonstrated by inhibition of the MAP kinase kinase MEK1. Disruption of the prolonged signal by addition of the MEK1 inhibitor PD 98059 up to 4 h after growth factor stimulation substantially impaired ligand-dependent colony dispersion and MMP-9 induction. These findings support the conclusion that duration of MAPK activation is an important determinant for certain growth factor-mediated functions in keratinocytes.

Stimulation of receptor tyrosine kinases by ligand results in activation of multiple signal transduction pathways such as the mitogen-activated protein kinase (MAPK) cascade (1–3). The MAPK family of protein kinases includes the extracellular signal-regulated kinases (ERKs) (4), the c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs) (5, 6), and p38'HOG (7). Within the MAPK family, the ERK and JNK pathways have been reported to be stimulated by receptor tyrosine kinases in various cell types; however, p38'HOG is not commonly activated by growth factors (3). The MAPK/ERK cascade is best characterized for its involvement in mitogenic signaling (3, 4), but additionally, this family of kinases has been implicated in diverse cellular responses such as chemical or osmotic stress (5–7), cell differentiation (8), and migration (9, 10).

There is abundant evidence that certain receptor tyrosine kinases are involved in tumor development or progression (2, 11). In addition to promoting mitogenic responses in target cells, these receptors are also capable of regulating cellular functions that are involved in the acquisition of an invasive phenotype such as modulation of cellular attachments, proteolysis of extracellular matrix, and directional migration (2, 11–13). Many growth factors have been reported to stimulate keratinocyte migration, and it has been shown that receptor tyrosine kinase and integrin-induced cell migration involve activation of the Ras/MAPK signal transduction pathway (9, 10). Similarly, receptor tyrosine kinase ligands induce a number of extracellular matrix-degrading proteases including MMP-9 (13–16), and the ERK, JNK, and p38 MAPK pathways have been reported to contribute to MMP-9 gene expression (15, 17–19).

In the course of previous studies, we have shown that although many receptor tyrosine kinase ligands are mitogenic for keratinocytes, only a subset of receptors, the epidermal growth factor (EGF) receptor and c-Met (scatter factor/hepatocyte growth factor receptor), were also motogenic (16). Receptor specificity for stimulating migration coincided with ligand-mediated invasion through a reconstituted basement membrane and induction of MMP-9. An association between MMP-9 expression and keratinocyte migration is suggested by detection of MMP-9 during wound healing and the correlation between MMP-9 expression and squamous cell carcinoma (SCC) invasiveness (13, 20–22). In our studies, we found that MMP-9 plays a functional role in EGF- and SF/HGF-induced migration, as inhibition of MMP-9 activity impaired receptor tyrosine kinase-dependent SCC locomotion.
The present study focuses on the signaling requirements for receptor tyrosine kinase-dependent MMP-9 induction, and we present evidence of an underlying basis for receptor specificity in the regulation of keratinocyte migration and invasion. In particular, we investigated the apparent paradox that although the MAPK pathway is essential for receptor tyrosine kinase-induced mitogenesis, there is demonstrable receptor specificity with regard to regulation of MMP-9 expression and cell migration.

We find that although ligand-dependent activation of the ERK and JNK pathways was readily detected, sustained activation of p42/p44 ERK/MAPK following growth factor stimulation was associated with induction of MMP-9 expression and keratinocyte motility. Furthermore, attenuation of the sustained signal by inhibition of MEK1 abrogated ligand-induced MMP-9 expression and migration. These results suggest that ligand-dependent regulation of cellular responses related to an invasive phenotype is dictated by the capacity of certain receptor tyrosine kinases to stimulate sustained activation of the MAPK signaling cascade.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture—SCC-12F cells were originally derived from a tumor of the facial epidermis (23) and were generously provided by Dr. William A. Toscano, Jr. (Tulane University, New Orleans, LA). SCC-12F cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium: Ham’s F-12 nutrient mixture (DMEM:F-12) containing 5% iron-supplemented defined calf serum (HyClone Laboratories, Logan, UT). For all experiments involving growth factor addition, SCC cells were plated into DMEM:F-12 containing 0.1% bovine serum albumin (BSA) for 24–48 h prior to growth factor addition. Murine EGF was obtained from Biomedical Technologies Inc. (Stoughton, MA); keratinocyte growth factor (KGF) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and scatter factor/hepatocyte growth factor (SF/HGF) was a generous gift from Genentech. All other growth factors were purchased from Life Technologies, Inc. PD 98059 and SB202190 were obtained from Calbiochem and dissolved in dimethyl sulfoxide (Me2SO). The final concentration of Me2SO did not exceed 0.1% (v/v) in any experiment.

**Measurements of Cell Motility—**Evaluation of colony dispersion (cell scattering) was performed as described previously (24). Briefly, cells were subcultured and maintained in growth medium until colonies of greater than 15 cells were established. Cultures were deprived of growth factors and serum for 24 h prior to treatment with or without ligand at the concentrations and times indicated in the figure legends. Colony dispersion was documented by photography. Photographs of cell cultures were taken at a magnification of ×10 or ×25 using a Nikon N2000 camera mounted upon a Nikon Diaphot-TMD inverted phase contrast microscope. Results shown are representative of at least three independent experiments.

**Western Blot Analysis of Activated MAPK—**Activated MAPK species were detected using phosphospecific antibodies directed against the dually phosphorylated, active forms of the proteins according to the vendor’s instructions. Parallel control blots were obtained using phosphorylation state-independent pan antibodies for detection of the MAPK species. SCC-12F cells were serum-deprived for 24 h prior to stimulation with ligand at the concentrations and for the times indicated in the figure legends. Control and treated cells were rinsed with ice-cold phosphate-buffered saline and then lysed in sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% w/v bromphenol blue. Typically, 10 μg of total cell lysate for each sample was separated on a 10% SDS-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Membranes were blocked with 2% milk in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20 (TBST) for 30 min at room temperature. Membranes were then incubated with primary antibody at 1:1000 dilution in TBST containing 0.25% gelatin (TBSTG) overnight at 4 °C for the phospho-specific phospho-p44/42 MAPK kinase (Thr183/Tyr185) monoclonal antibody (New England Biolabs, Beverly, MA). Membranes were washed in TBSTG and then incubated at room temperature, followed by incubation with a 1:2500 dilution of goat anti-rabbit-conjugated horseradish peroxidase secondary antibody (Promega, Madison, WI) for 1 h at room temperature. The membranes were washed and developed using the SuperSignal chemiluminescent detection system (Pierce). Detection of total ERK was accomplished using a pan-ERK antibody (Transduction Labs, Lexington, KY). Membranes were incubated with primary antibody at a 1:5000 dilution in TBSTG for 30 min at 37 °C, washed with TBSTG as above, and then incubated with a 1:10,000 dilution of goat anti-mouse-conjugated horseradish peroxidase secondary antibody (Promega, Madison, WI) for 1 h at room temperature. The membrane was washed with TBST for 1 h at room temperature and developed using the SuperSignal chemiluminescent detection system (Pierce).

**RESULTS**

**Inhibition of MEK1 Activity Impairs Receptor Tyrosine Kinase-Dependent Colony Dispersion and MMP-9 Induction—**Receptor tyrosine kinase activation promotes MMP-9 expression in multiple cell types, including keratinocytes (13, 15, 16). We have shown previously that not all mitogenic ligands for keratinocytes are capable of inducing MMP-9 expression and colony dispersion; rather, only activation of a small subset of receptors (the EGF receptor and c-Met) regulate these cellular processes (17). Further studies have shown that MAPK activity is essential for MMP-9 expression in oncogenic transformed rat embryo cells and in tumorigenic SCC cells that display constitutive activation of both ERK and JNK/SAPK (17, 18). As stimulation of the Ras/MAPK pathway is shared by receptor tyrosine kinases, we investigated whether MAPK activity was essential for growth factor-mediated MMP-9 induction in a human keratinocyte cell line.

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Our previous studies demonstrated that MMP-9 plays a functional role in ligand-dependent colony dispersion (16); therefore, we investigated whether MAPK activation was essential for EGF- or SF/HGF-dependent induction of MMP-9. Gelatin zymography was performed on conditioned media collected 24 h after ligand stimulation, in the presence or absence of increasing concentrations of PD 98059 as indicated. The upper band represents the 92-kDa gelatinase/MMP-9, and the lower band represents the 72-kDa gelatinase/MMP-2.

As shown in Fig. 3, serum-deprived SCC-12F cells displayed low basal MAPK activation in the absence of growth factor and stimulation of cells with EGF rapidly induced ERK1 and ERK2 activation (Fig. 3A). When the activities of different growth factors were compared, EGF, SF/HGF, insulin-like growth factor (IGF) 1, and keratinocyte growth factor (KGF) all induced ERK1 and ERK2 activation (Fig. 3, A—D). Therefore, selective activation of a particular MAPK isoform (p42 or p44 ERK) did not account for the functional differences between motogenic and non-motogenic receptor tyrosine kinases. These findings were confirmed in an in-gel kinase assay using myelin basic protein as a substrate (data not shown).

In addition to activation of specific MAPK isoforms, variations in duration of MAPK activation has been associated with differences in functional outcome in response to receptor tyrosine kinase ligands (8, 31). We have shown that ERK activation is required for EGF- and SF/HGF-dependent MMP-9 induction and colony dispersion (Figs. 1 and 2, data not shown); however, ERK1 and ERK2 were activated by ligands (KGF and IGF-1) that do not induce either of these responses (Ref. 16, Fig. 3). Therefore, we evaluated the duration of MAPK activation following stimulation with motogenic and non-motogenic growth factors. Stimulation of serum-deprived SCC-12F cells by EGF or SF/HGF resulted in a rapid activation of p42 and p44 ERK that was sustained for up to 6 h (Figs. 3 and 4, data not shown). This is in contrast to the response observed after stimulation with IGF-1 or KGF, where the typical duration of MAPK activation did not exceed 1 h before returning to baseline levels (Fig. 3). Thus, ligands that promote keratinocyte migration and MMP-9 induction also stimulate a prolonged duration of ERK1 and ERK2 activation.

Growth Factor Activation of JNK/SAPK and p38HOG1—As shown in Figs. 3 and 4, there is a correspondence between growth factors that promote sustained ERK1/2 activation and those that induce colony dispersion and MMP-9 activity. There
is evidence that the JNK/SAPK and p38 pathways are also
involved in expression of MMP-9 (13, 17–19), and constitutive
activation of JNK/SAPK is detected in UM-SCC-1 cells display-
ing high basal MMP-9 expression (18). Therefore, we wanted to
determine if specific receptor tyrosine kinase ligands differed
in their ability to activate stress-activated members of the
MAPK family.

We monitored activation of JNK and p38 by EGF, IGF-1,
SF/HGF, and KGF by measurement of catalytic activity in
immunocomplex assays (26, 27). The motogenic ligands EGF
and SF/HGF stimulated robust activation of JNK (~10-fold);
KGF was a more modest JNK activator (~5-fold), and IGF-1
did not stimulate JNK activation (Fig. 5). The time courses of
EGF and SF/HGF-stimulated JNK activation were similar
with JNK activity detected within 5 min, and peak activity was
observed between 15 and 30 min, and activity returned to
background levels within 2 h (Fig. 5). KGF also stimulated JNK
activation, with maximal activity apparent at the 15-min time
point.

It has been reported that the p38HOG pathway is not typi-
cally activated by receptor tyrosine kinase-dependent signals
(3, 7); however, EGF-dependent activation of p38 has been
reported (27). Modest activation (3–5-fold) of p38 was detected
in response to EGF, SF/HGF, and KGF, but not IGF-1, in
SCC-12F cells (Fig. 6, data not shown). As with JNK, the
activation was transient with a return to baseline activity
within 2 h for EGF and KGF. In contrast, SF/HGF appeared to
stimulate a low (2–3-fold) but sustained activation of p38. Al-
though phorbol ester-induced MMP-9 expression is eliminated
by the p38 inhibitor SB 203580 (19), EGF-mediated migration
and MMP-9 induction were essentially unimpaired by the p38
inhibitor SB202190 (data not shown). Taken together, these
findings suggest that the p38HOG pathway does not play a
central role in growth factor-induced keratinocyte migration or
MMP-9 expression or account for receptor specificity in the
regulation of these cellular responses.

Sustained ERK Activation Is Required for Receptor Tyrosine
Kinase-dependent Migration and MMP-9 Induction—As shown
in Figs. 3 and 4, prolonged activation of the p42/p44 ERKs
corresponded with the capacity of specific receptor tyrosine
kinase ligands to differ in their ability to stimulate stress-activated members of the MAPK family. We monitored activation of JNK and p38 by EGF, IGF-1, SF/HGF, and KGF by measurement of catalytic activity in immunocomplex assays (26, 27). The motogenic ligands EGF and SF/HGF stimulated robust activation of JNK (~10-fold); KGF was a more modest JNK activator (~5-fold), and IGF-1 did not stimulate JNK activation (Fig. 5). The time courses of EGF and SF/HGF-stimulated JNK activation were similar with JNK activity detected within 5 min, and peak activity was observed between 15 and 30 min, and activity returned to background levels within 2 h (Fig. 5). KGF also stimulated JNK
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Sustained ERK Activation Is Required for Receptor Tyrosine
Kinase-dependent Migration and MMP-9 Induction—As shown
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corresponded with the capacity of specific receptor tyrosine
kinases to promote keratinocyte migration and MMP-9 induc-
tion. In order to determine the potential significance of sus-
tained ERK activation for these two cellular responses, MAPK
activation following EGF or SF/HGF stimulation was disrupted
by inhibiting MEK1 with PD 98059 at various times after
growth factor addition. MMP-9 activity was analyzed in condi-
A

![Image of G protein in a cell](https://i.imgur.com/3Q5Zx.png)

**Fig. 3.** Time course of MAPK activation following exposure to various RTK ligands. SCC-12F cells were grown to near-confluency in DMEM:F-12 containing 5% calf serum and were serum-starved 24 h prior to growth factor stimulation. SCC-12F cells were stimulated in serum-free DMEM:F-12 containing 0.1% BSA with 10 nM EGF (A), 20 ng/ml SF/HGF (B), 10 nM IGF-1 (C), or 10 nM KGF (D) for 5, 15, or 30 min, or 1 or 2 h as indicated. Whole cell lysates were collected, fractionated on 10% SDS-PAGE, and active phosphorylated proteins detected by immunoblot analysis with the phospho-MAPK antibody (New England Biolabs) as described under “Experimental Procedures.”

**Fig. 4.** Extended time course of SF-dependent MAPK activation. SCC-12F cells were grown as described in the legend to Fig. 3 and stimulated with 20 ng/ml SF/HGF in serum-free DMEM:F-12 containing 0.1% BSA for 5 or 15 min or 1, 2, 4, or 6 h as indicated. Whole cell lysates were collected, fractionated on 10% SDS-PAGE, and active phosphorylated proteins detected by immunoblot analysis with the phospho-ERK antibodies (New England Biolabs). Similar findings were obtained following treatment with EGF.

B

![Image of G protein in a cell](https://i.imgur.com/3Q5Zx.png)

C

![Image of G protein in a cell](https://i.imgur.com/3Q5Zx.png)

D

![Image of G protein in a cell](https://i.imgur.com/3Q5Zx.png)
tioned medium collected from SCC-12F cells treated with EGF in the absence or presence of PD 98059 added at various time points after growth factor addition (Fig. 7A). Ligand-dependent induction of MMP-9 was inhibited when PD 98059 was added 1 h before EGF addition, when both were administered concurrently and when PD 98059 was added at time points up to 4 h after EGF stimulation (Fig. 7A). However, addition of the MEK1 inhibitor at time points beyond 4 h after EGF stimulation no longer effectively blocked EGF-dependent MMP-9 induction (Fig. 7A). This suggests that the MEK1-dependent signal required for MMP-9 induction was fully transmitted by this point. In addition, EGF- and SF/HGF-mediated SCC-12F colony dispersion was inhibited by addition of PD 98059 at time points up to 6 h after growth factor addition (data not shown).

To confirm that MAPK activation was inhibited by the MEK1 inhibitor at the extended time points, cell lysates were collected 30 min after treatment with PD 98059, and activated ERKs were detected by immunoblot analysis. As shown in Fig. 7B, PD 98059 treatment after EGF stimulation effectively blocked the sustained MAPK activation. Thus, inhibition of MEK1 transformed the pattern of EGF-dependent MAPK activation to resemble that observed for IGF-1 and KGF (Fig. 3), and importantly, interruption of sustained MAPK activation resulted in loss of the EGF-dependent cellular responses (Fig. 7, data not shown). Together, these findings indicate that sustained activation of the MAPK signaling cascade is required for receptor tyrosine kinase-dependent MMP-9 induction and cell migration.

**DISCUSSION**

We have demonstrated previously that growth factor-regulated keratinocyte migration and MMP-9 induction is selectively mediated by the EGF receptor and c-Met in SCC lines and normal human keratinocytes (16). The mechanisms responsible for specificity of response to different receptor tyrosine kinases remain unclear; however, it has been proposed that the duration of ERK activation may determine cellular responses to ligand. In one well characterized example, nerve growth factor causes sustained ERK activation and promotes PC12 cell differentiation, whereas differentiation is not induced by EGF which only transiently activates ERKs within this cell type (31, 33, 34).

In this report we present evidence that sustained versus transient activation of the ERK/MAPK signaling cascade represents an underlying mechanism to account for receptor tyro-
sine kinase specificity in ligand-induced keratinocyte migration and MMP-9 induction. Both IGF-1 and KGF transiently activate the ERK/MAPK pathway and stimulate keratinocyte proliferation but do not induce colony dispersion or MMP-9 expression (Ref. 16, Figs. 3 and 4). In contrast, EGF- and SF/HGF-mediated motility and MMP-9 induction are associated with sustained duration of MAPK activation (Figs. 3, 4, and 7). In examining the signaling consequences of receptor tyrosine kinase activation, we found that ligand stimulation enhances activity of similar MAPK constituents (p42/p44 ERK) regardless of the functional outcome (Figs. 3 and 4). The distinguishing characteristic of receptors that regulate MMP-9 induction and colony dispersion is their ability to promote prolonged ERK activation (Figs. 3, 4, and 7B). Importantly, interruption of the sustained signal using the MEK1 inhibitor PD 98059 rendered EGF inactive for migration and MMP-9 induction (Fig. 7, data not shown), indicating that prolonged duration of MAPK activation is required for both of these cellular responses.

Several lines of evidence indicate that MMP-9 gene expression is regulated by activation of various MAPK kinase signaling pathways. Oncogenic transformation with v-Src or v-Ras up-regulate MMP-9 expression, as does diverse stimuli such as EGF, tumor necrosis factor-α, and phorbol ester tumor promoters (35, 36). Phorbol ester stimulation of MMP-9 expression was reported through the p38HOG pathway as use of the p38 inhibitor SB203580 blocked phorbol ester-dependent MMP-9 induction and cell invasion but not mitogenesis (19). In addition, disruption of the ERK- or JNK-dependent signaling decreases endogenous MMP-9 expression in UM-SCC-1 cells (18). Although the JNK and p38 MAPK cascades may be necessary for induction of MMP-9 in response to various stimuli, our data suggest that neither pathway is sufficient for growth factor-stimulated MMP-9 expression. JNK and p38 activities were induced by KGF (Figs. 5 and 6) even though KGF does not induce MMP-9 in these cells (16). Duration of ERK activation corresponds to this receptor-mediated response, and it is intriguing that MAPK-dependent up-regulation of MMP-9 expression is apparent in several systems where MAPK is constitutively activated either through oncogenic transformation or endogenously as part of the transformed phenotype (17, 18, 35, 36).

These studies, together with our findings, suggest that prolonged activation of MAPK activity may be a general requirement for induction of MMP-9 expression. Constitutive up-regulation of MMP-9 through oncogenic transformation by v-Ras has been reported to be through a MEK1-independent activation of MAPK (36). In contrast, our results demonstrate that PD 98059 interferes with growth factor-induced ERK activation and MMP-9 expression, suggesting that receptor tyrosine kinase-mediated MMP-9 induction requires a MEK1-dependent pathway. The requirement for MEK1 activity in the sustained MAPK activation as detected in this system suggests that input from upstream signaling effectors, such as MEK1, is involved in the duration of MAPK activation.

Interestingly, there is also evidence that prolonged MAPK activation may play a role in cell migration. Sustained ERK2 activation has been correlated with Ret- and fibroblast growth factor-mediated scattering in SK-N-MC cells (37). This finding, in conjunction with our results, suggests that sustained ERK2 activation is a common requirement for receptor tyrosine kinase-regulated colony dispersion. One possible consequence of sustained ERK activation might be the induction of gene products specifically required for migration. We have shown that MMP-9 activity is involved in growth factor-induced colony dispersion (16), and therefore, MMP-9 may represent one such gene product.

Analysis of the MMP-9 promoter has identified potential cis-acting elements such as the 12-O-tetradecanoylphorbol-13-acetate-response element, Ets, and NF-kB that are likely to confer induction of MMP-9 in response to growth factors or oncogenic transformation (17, 35, 36). Interestingly, in one model system where cells displayed constitutive activation of MAPK as part of the transformed phenotype, the accompanying up-regulation of MMP-9 expression was dependent upon AP-1/12-O-tetradecanoylphorbol-13-acetate response element sites within the MMP-9 promoter region and one of the essential 12-O-tetradecanoylphorbol-13-acetate response elements bound a Jun D/c-Fos heterodimer (18). A possible mechanism by which sustained MAPK activation could result in MMP-9 induction is through regulation of essential transcription factors such as c-Fos. Expression of this immediate early gene is dependent on MAPK activation, and furthermore, phosphorylation of c-Fos by MAPK enhances its activity (38). Thus, in one possible signaling scenario, initial activation of MAPK may be required to induce c-Fos, and sustained activation may serve to enhance c-Fos transcriptional activity and AP1-dependent expression of the MMP-9 gene (18, 38). It is possible that a sequence of MAPK-dependent induction followed by MAPK-dependent phosphorylation of specific transcription factors may represent a mechanism whereby duration of MAPK activation distinguishes between a mitogenic or motogenic response in target cells. Analysis of the contributions of sustained MAPK activation to differential gene expression will require further study.

The ERK/MAPK signal transduction cascade plays an important role in mediating cellular responses to growth factor receptor activation. In a limited number of cellular systems including PC12 cells and K562 megakaryocytes, sustained activation of MAPK kinases is a requirement for cell differentiation (8, 31, 34, 39). The findings presented in this paper support the conclusion that sustained activation of MAPK kinase signaling cascades may play an important role in dictating other cellular responses such as migration, metalloproteinase expression, and invasive capacity that require de novo protein and RNA expression. Additionally, the ability of a particular receptor tyrosine kinase to promote sustained MAPK activation may serve as an underlying biochemical mechanism to account for receptor specificity in cellular responses to growth factors.

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