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A specific combination of substrates is involved in signal transduction by the *kit*-encoded receptor

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The *kit* protooncogene encodes a transmembrane tyrosine kinase related to the receptors for the platelet derived growth factor (PDGF-R) and the macrophage growth factor (CSF1-R), and was very recently shown to bind a stem cell factor. To compare signal transduction by the *kit* kinase with signaling by homologous receptors we constructed a chimeric protein composed of the extracellular domain of the epidermal growth factor receptor (EGF-R) and the transmembrane and cytoplasmic domains of *kit*. We have previously shown that the chimeric receptor transmits potent mitogenic and transforming signals in response to the heterologous ligand. Here we demonstrate that upon ligand binding, the ligand-receptor complex undergoes endocytosis and degradation and induces short- and long-term cellular effects. Examination of the signal transduction pathway revealed that the activated *kit* kinase strongly associates with phosphatidylinositol 3'-kinase activity and a phosphoprotein of 85 kd. In addition, the ligand-stimulated *kit* kinase is coupled to modifications of phospholipase C_γ and the Raf1 protein kinase. However, it does not lead to a significant change in the production of inositol phosphate. Comparison of our results with the known signaling pathways of PDGF-R and CSF1-R suggests that each receptor is coupled to a specific combination of signal transducers.

Key words: growth factor/oncogene/stem cell factor/tyrosine kinase

Introduction

Receptors for polypeptide growth factors constitute a family of related membrane glycoproteins with intrinsic protein tyrosine kinase activity (Yarden and Ullrich, 1988). Once activated, each receptor interacts by means of tyrosine phosphorylation with several cytoplasmic proteins that are believed to undergo concomitant changes in their state of activation or in their cellular distribution (reviewed in Heldin and Westermark, 1990; Ullrich and Schlessinger, 1990). As a result of these alterations the affected proteins probably act as signal transducers and elicit a pleiotropic cellular response that culminates in mitogenesis. One of the subtypes of receptor tyrosine kinases (RTKs) includes the receptors for the platelet derived growth factor (PDGF-R), the receptor for the colony stimulating factor 1 (CSF1-R), and the product of the *kit* protooncogene. The latter was very recently shown to bind a stem cell factor (Huang *et al.*, 1990; Nocka *et al.*, 1990a; Williams *et al.*, 1990; Zsebo *et al.*, 1990). These receptors share a common structure including an extracellular domain that is composed of five immunoglobulin-like loops and a cytoplasmic portion that includes split tyrosine kinase sequences. Despite extensive structural homology, the three receptors display heterogeneity in their cytoplasmic non-catalytic domains such as the kinase insertion stretches and the carboxy-termini. These regions participate in the interactions between the activated receptor and cytoplasmic proteins (Escobedo and Williams, 1988; Coughlin *et al.*, 1989; Kazlauskas and Cooper, 1989; Morrison *et al.*, 1989; Taylor *et al.*, 1989; Kaplan *et al.*, 1990; Kazlauskas and Cooper, 1990; Kazlauskas *et al.*, 1990; Morrison *et al.*, 1990; Severinsson *et al.*, 1990; Shurtleff *et al.*, 1990; Reedijk *et al.*, 1990), and thus may confer distinct substrate specificities to the homologous tyrosine kinases.

A currently open question related to the mechanism of signal transduction is the extent of functional overlap of mitogenic signals arising at the cell surface from homologous receptors. Although it appears that all RTKs are linked to accelerated cell proliferation, it seems that the identity of each mitogenic signal is preserved intracellularly. For example, gene expression patterns elicited by various growth factors differ at least kinetically or quantitatively (Bravo *et al.*, 1987; Orlofsky and Stanley, 1987; Koskinen *et al.*, 1990). This may be attributed to receptor-specific tyrosine kinase substrate(s) or to unique combinations of substrate proteins which may be shared, but only in part, with other receptors. To address this question, we studied the signal transduction pathway elicited by the *kit* receptor.

The c-*kit* protooncogene was originally isolated by virtue of its structural relatedness to an oncogene of a feline leukemia virus (Besmer *et al.*, 1986; Yarden *et al.*, 1987; Qiu *et al.*, 1988). The finding that c-*kit* and the murine *White* spotting locus (W) are allelic and the localization of specific W mutations to portions of the *kit* coding regions (Tan *et al.*, 1990; Reith *et al.*, 1990; Nocka *et al.*, 1990b) strongly supported the possibility raised by the structure of the gene product that it functions as a receptor for a growth factor. To test this prediction we constructed a chimeric *kit* receptor in which the extracellular domain was replaced by the ligand binding portion of the epidermal growth factor (EGF) receptor. As expected, the chimeric receptor (termed EKR for EGF-*kit* receptor) transmitted a strong mitogenic signal and also mediated a ligand-dependent appearance of a transformed phenotype (Lev *et al.*, 1990). Direct proof to the receptor hypothesis was recently provided through the identification of a 30 kd glycoprotein produced by fibroblasts, stromal and liver cells as the ligand of the *kit*-encoded receptor.

Employing an EGF-receptor-*kit* chimera here we describe the interactions of the ligand-stimulated *kit* kinase with potential cytoplasmic signal transducers. The latter include phosphatidylinositol 3-kinase, phospholipase C_γ and Raf1. Comparison of the *kit* substrates with those of the related receptors for PDGF and CSF1 suggests that each kinase

utilizes a specific combination of cytoplasmic transducers, which are shared in part among the activated receptors.

Results

Endocytosis and degradation of the ligand-receptor complexes

In our previous paper we described the construction of a ligand-stimulatable *kit* kinase (EKR; Lev *et al.*, 1990) in which we replaced the extracellular portion of $p145^{kit}$ with the ligand binding domain of EGF-receptor. Figure 1A shows that ligand binding to EKR-expressing cells was followed by rapid clearance of the ligand-receptor complexes from the cell surface, as determined by the inaccessibility of the complexes to acid wash of the cells. Concomitantly, the radiolabeled ligand accumulated within the cell and underwent degradation that was reflected by a time-dependent increase in the acid-soluble radioactivity (data not shown). This behavior of the chimeric receptor was comparable to that exhibited by the wild-type receptor for EGF (Figure 1A). The apparently normal physiology of the chimeric receptor was reflected also in the rapid ligand dependent down-regulation of the receptor, that was revealed by metabolic labeling of the cells followed by variable length chase periods in the presence or absence of the ligand (Figure 1B). Interestingly, the chimeric receptor, like wild-type $p145^{kit}$ (Yarden *et al.*, 1987), appeared as a doublet band of which the heavier component underwent down-regulation more rapidly (half life = 75 min) than the smaller species (half life = 3 h). We do not know the reason for this differential kinetics but it could be due to tighter binding of the ligand to the higher molecular weight species. This possibility is supported by covalent crosslinking of radiolabeled EGF to the chimeric receptor: The higher band was labeled almost exclusively (Figure 1C). Importantly, we did not observe dimers of the chimeric receptors after chemical crosslinking. Such dimers are clearly seen when the wild-type receptor for EGF (on A431 cells) is analyzed (Figure 1C and Cochet *et al.*, 1988). Possibly lack of detectable dimerization of the chimera is due in part to the relatively rapid dissociation of the heterologous ligand (Lev *et al.*, 1990) and participation of the cytoplasmic portion of EGF-R in dimer stabilization.

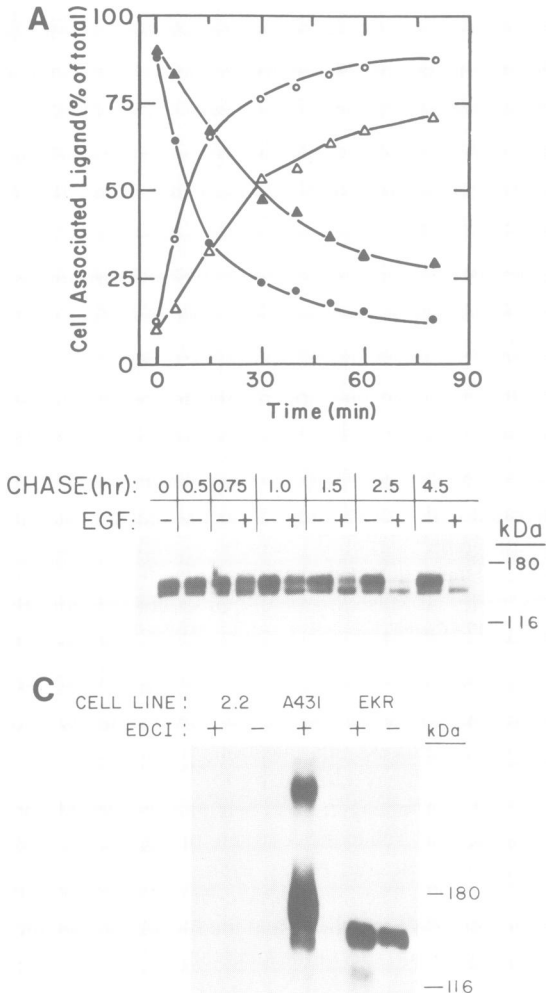

Fig. 1. A Ligand internalization. Cells that overexpress the chimeric *kit*/EGF-R protein (triangles) or the wild-type EGF-R (circles) were incubated with [^{125}I]-EGF for 90 min at 4°C, and then incubated for different periods of time at 37°C. The cell-surface bound and the internalized ligand was determined using acetic acid washing. Cell associated radioactivity (open symbols) and acid dissociable radioactivity (closed symbols) were determined as described under Materials and methods. **B** Ligand-induced degradation of the EKR protein. EKR cells were labeled for 12 h with [^{35}S]methionine and then chased in the absence (−) or presence (+) of EGF (1 μg/ml) for the indicated periods of time at 37°C. The chimeric EKR protein was immunoprecipitated with an antibody to EGF-receptor and analyzed on 7.5% SDS−polyacrylamide gel. **C** Crosslinking of ^{125}I-labeled ligand to EKR cells. [^{125}I]EGF was incubated with EKR, A431 or control untransfected (2.2) cells for 1 h at 4°C. Cells were then transferred to room temperature and incubated in the presence (+) or absence (−) of the chemical crosslinker 1-ethyl-3(3-dimethyl-amino propyl) carbodiimide (EDCI, Cochet *et al.*, 1988). After 1 h cells were lysed, and the chimeric EKR protein immunoprecipitated. The immunocomplexes were resolved by SDS−gel electrophoresis (4.5% acrylamide gel) and autoradiography.

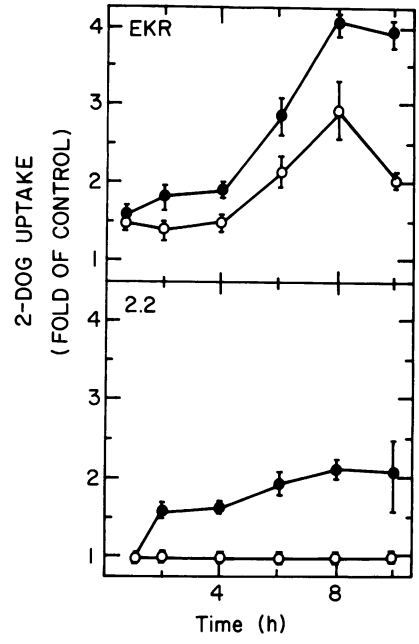

Fig. 2. The effects of EGF and serum on 2-deoxyglucose (2-DOG) uptake. Serum-starved EKR and 2.2 cells were treated at 37°C with either EGF (200 ng/ml; open circles) or with 10% serum (closed circles) for the indicated periods of time. The uptake of 2-DOG is expressed relative to control untreated cells. Each value represents the mean value of three independent experiments.

Long term cellular effects of the chimeric kit receptor

Cultures of the cell expressing the *kit* chimera were maintained in medium containing no serum but supplemented with EGF (100 ng/ml). After 6 days these cultures contained 3-fold more cells relative to control cultures untreated with EGF. In addition, EGF treatment (80 ng/ml, 7 days) of similar cultures but in medium containing 2% bovine serum resulted in marked acidification of the growth medium (pH 7.0) as compared with cultures that were not treated with EGF (pH 7.47). These results reflect an accelerated rate of cellular proliferation and metabolism that are evoked by ligand-stimulation of the *kit* kinase, and they are expected to be accompanied by an increased uptake of nutrients from the growth medium. Indeed, the chimera-expressing cells exhibited an increased uptake of labeled hexose in response to EGF that was added to the growth medium (Figure 2). The control parental untransfected cell line was unresponsive to EGF but responded to serum stimulation.

Ligand-dependent association of the chimeric receptor with an 85 kd protein and PI 3-kinase activity

We attempted next to detect substrate proteins that undergo phosphorylation on tyrosine residues in response to stimulation of the *kit*-kinase by heterologous ligand (Lev *et al.*, 1990). Western blot analysis with antibodies to phosphotyrosine failed to detect any major protein substrate except for the chimeric receptor itself which underwent self-phosphorylation (data not shown). To increase the sensitivity of the analysis we examined phosphoproteins that potentially coprecipitate with the activated receptor. To this end we applied a method that was developed by Kaplan *et al.* (1987). Accordingly, EKR cells were first challenged with the ligand and then the receptor was immunoprecipitated and subjected to self-phosphorylation with [γ-^{32}P]ATP. The phosphorylated proteins were mildly eluted from the immunocomplexes, reprecipitated with antibodies to phosphotyrosine and resolved by gel electrophoresis. The results of these experiments are shown in Figure 3A. Evidently, a major phosphoprotein of 85 kd was specifically associated with the receptor upon stimulation by the ligand. The other band of 140 kd corresponds to the self-phosphorylated receptor itself.

Several studies have correlated between phosphatidylinositol 3′ kinase (PI3K) activity and an 81−85 kd polypeptide that associates with activated tyrosine kinases (Courtneidge and Heber, 1987; Kaplan *et al.*, 1987). We tested therefore immunoprecipitates of the EKR chimera for associated PI3K activity. Cells were stimulated for 10 min at 22°C with saturating concentrations of EGF and then the receptor was subjected to immunoprecipitation with three different antibodies. As demonstrated in Figure 3B, an increased level of PI kinase activity was associated with the receptor after stimulation with the ligand. All three antibodies precipitated PI3K activity at variable efficiency that parallels the amount of receptor in the corresponding immunocomplexes.

To examine the specificity of the PI kinase activity we first tested its susceptibility to inhibition. As expected for type I PI3K activity (Whitman *et al.*, 1987), NP-40 completely blocked the phosphorylation of PI whereas adenosine (0.3 mM) had no inhibitory effect when added to the PI-kinase reaction mixture (Figure 3C). Thus, it was expected that the kinase activity phosphorylates the D3 position of the inositol ring of PI (Whitman *et al.*, 1988).

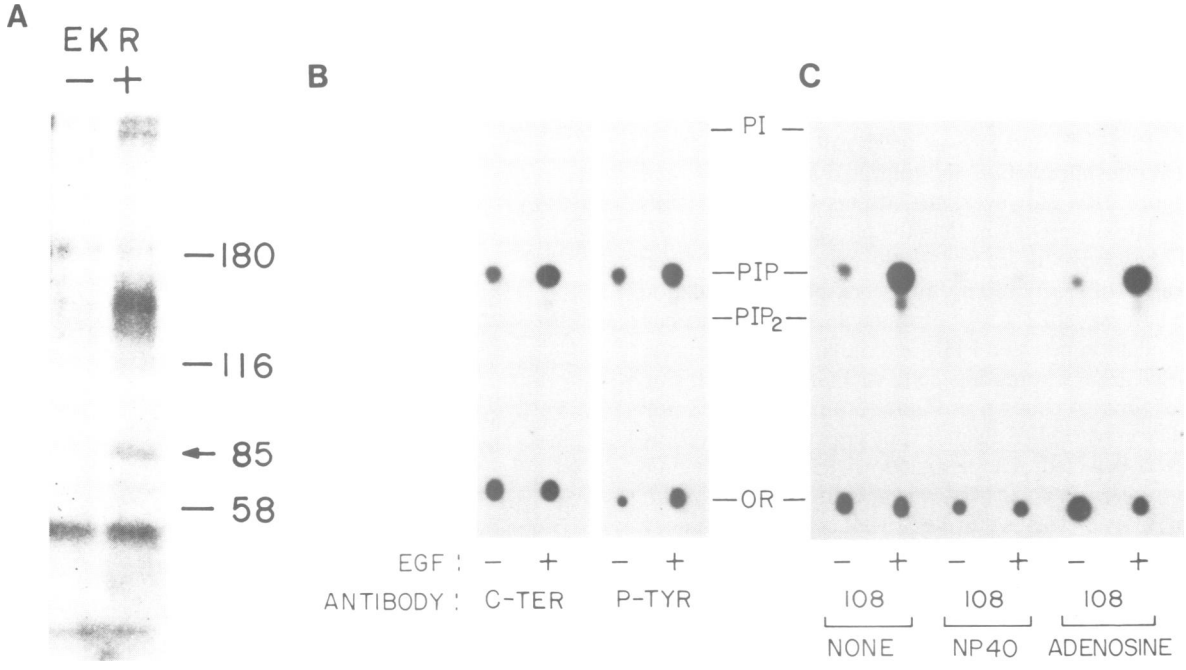

Fig. 3. A. Association of p85 with EKR protein. Quiescent EKR cells were treated for 15 min with (+) or without (−) EGF (200 ng/ml) at 22°C. Cell lysates were then prepared and the chimeric receptor subjected to immunoprecipitation. The immunoprecipitates were washed and subjected to *in vitro* kinase assay. The phosphoproteins were eluted as described (Kaplan *et al.*, 1987), and the eluates were re-immunoprecipitated with an antiserum of phosphotyrosine and separated by SDS−PAGE. The 85 kd phosphoprotein is indicated by an arrow. The locations of molecular weight marker proteins are shown in kd. **B.** PI kinase activity in EKR cells stimulated with EGF. Autoradiogram of TLC separated PIP generated by anti-phosphotyrosine (P-TYR) and anti-*kit* C-terminus antibody (C-TER) immunoprecipitates from EKR cells that were stimulated by EGF for 15 min at 22°C. **C.** Adenosine and NP-40 sensitivity of PI-kinase activity associated with the EKR protein. Lysates of ligand-stimulated EKR cells were subjected to immunoprecipitation with mAb108 and assayed for PI-kinase activity in the presence of 0.5% NP-40 or 300 μM adenosine.

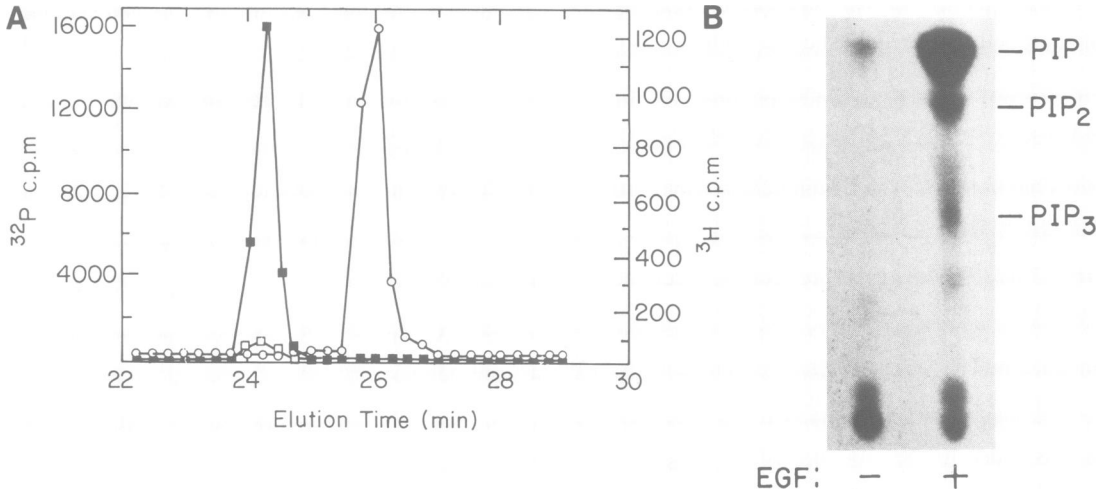

Fig. 4. A. HPLC analysis of phosphatidylinositol-phosphate generated in ligand stimulated EKR cells. Ligand stimulated EKR cells were solubilized, the receptor immunoprecipitated and assayed for PI-kinase activity. The radioactive PIP products from ligand-stimulated (closed squares) and unstimulated (open squares) cells were deacylated and separated from [^{3}H]glycerolphosphoinositol 4-phosphate standard (open circles) by HPLC chromatography as detailed under Materials and methods. **B.** TLC analysis of the products generated by lipid kinase assay on EKR immunoprecipitates using a mixture of phosphoinositides: PI, PI(4)P and PI(4,5)P_2, as substrates. The locations of unlabeled standards are indicated.

This was supported by HPLC analysis of the phosphatidylinositol phosphate (PIP) product. The lipid was extracted from the thin layer plate, deacylated and chromatographed on a strong anion exchange column (Vartiocovsky *et al.*, 1989). Figure 4A depicts the results of this chromatography. The deacylated PIP eluted earlier than a glycerophosphoinositol 4-phosphate standard at a rate corresponding to the 3′ isomer, thus confirming the specificity of the PI kinase to position D3.

In addition to phosphorylation of PI, type I PI3K phosphorylates phosphatidylinositol 4-phosphate (PI-4P) and phosphatidylinositol 4,5-bisphosphate (PI-4-5-P_2, Auger *et al.*, 1989). We tested therefore the capacity of the PI3K activity that associates with the activated *kit* kinase to phosphorylate other phosphatidylinositol lipids. A mixture of PI-4P, PI-4,5-P_2 and PI was used. All three substrates underwent phosphorylation and migrated as PIP, PIP_2 and PIP_3 (Figure 4B). However, the extents of PIP and PIP_2 phosphorylation were lower than the phosphorylation of PI.

In all the above described PI3K experiments a fixed concentration of EGF (100 ng/ml) was used for stimulation that lasted 15 min at 22°C. To examine the time dependency of the association between PI3K and the activated chimera, we exposed EKR-expressing cells to EGF for increasing periods of time. As shown in Figure 5 already after 2 min of stimulation with EGF a significant association with PI3K activity was detected. The association increased with time to reach a peak after 20 min, and then decreased slowly. A similar analysis was performed to determine dependency on the concentration of the stimulating ligand (EGF), and revealed an apparent saturation of the effect at 10 nM concentration of EGF (Figure 5). This value corresponds to saturation of the chimeric receptor and parallels the extent of receptor self-phosphorylation on tyrosine residues (Lev *et al.*, 1990).

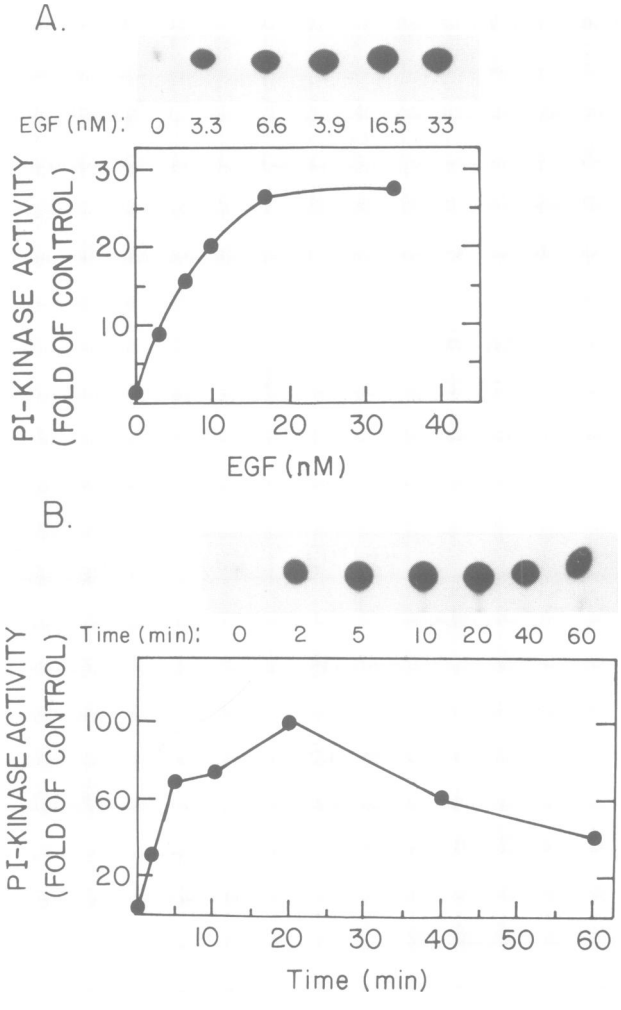

Fig. 5. Time course and dose–response of ligand-stimulated PI kinase activity in EKR cells. Quiescent EKR cells were stimulated for 15 min at 22°C with the indicated concentrations of EGF (**A**) or with 150 ng/ml EGF for the indicated periods of time also at 22°C (**B**). Cell lysates were immunoprecipitated with mAb108 and assayed for PI-kinase activity. The phospholipids were separated by TLC and the radioactivity of the PIP spots (shown above each panel) was determined by β-emission counting.

Tyrosine phosphorylation of phospholipase C_γ in response to ligand stimulation of the chimeric receptor

To further investigate the interaction of the *kit* kinase with inositol lipid metabolism we tested the effect of ligand

stimulation of the chimeric EKR protein on phospholipase C. It has been previously shown that EGF and PDGF stimulate tyrosine phosphorylation of PLC_γ (Wahl *et al.*, 1989; Kumjian *et al.*, 1989; Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989a; Wahl *et al.*, 1989b; Margolis *et al.*, 1990a; Morrison *et al.*, 1990). In addition, EGF was reported to induce membrane association of the enzyme (Todderud *et al.*, 1990), but these changes appear not to affect the catalytic activity of PLC_γ (Kim *et al.*, 1990). Stimulation of EKR-expressing cells with EGF (150 ng/ml, 15 min at 22°C) was followed by cell lysis and immunoprecipitation of PLC_γ with specific anti-peptide antibodies. The immunoprecipitates were resolved by gel electrophoresis and Western blotting. As shown in Figure 6A, blotting with antibodies to phosphotyrosine revealed that the anti-PLC_γ antibodies precipitated a phosphotyrosine-containing protein band of 145 kd from EKR cells but only after stimulation with EGF. The untransfected fibroblasts did not show this protein band in response to EGF. In parallel experiments by Western blotting with an antibody directed to the C-terminus of $p145^{kit}$, we excluded the possibility that this band represented the chimeric receptor rather than PLC_γ. Comparison of the extent of tyrosine phosphorylation of PLC_γ in EKR cells to the extent observed in cells that overexpress the EGF receptor (HER cells) revealed that the effect of EGF on the EKR cells was 5- to 10-fold smaller (Figure 6). This may suggest that the interaction of the activated *kit* receptor with PLC_γ is relatively limited. To test this possibility we examined the association of the EKR protein with PLC_γ. It has been previously shown that EGF receptor and PDGF receptor associate non-covalently with PLC_γ after stimulation with the corresponding ligand (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Kumjian *et al.*, 1989). Although by using Western blotting we could readily detect association between the EGF-receptor and PLC_γ in stimulated A431 cells, we have not been able to similarly detect association between the chimeric *kit* receptor and PLC_γ in EGF-treated EKR cells. An alternative attempt to detect association is shown in Figure 6B, in which we employed an *in vitro* assay (Margolis *et al.*, 1990a). The chimeric receptor or wild-type EGF receptor were immunoprecipitated from unstimulated cells, phosphorylated *in vitro* and allowed to react with whole cell lysates. Receptor-associated proteins were then analyzed by Western blotting with an antibody to PLC_γ. Whereas the wild-type EGF receptor from A431 cells associated with PLC_γ, under these conditions the chimeric *kit* receptor from EKR cells did not display such an association. In conclusion, activation of the *kit* kinase leads to phosphorylation of PLC_γ on tyrosine residues, but this interaction appears to be limited relative to the effect of another tyrosine kinase receptor.

Stimulation of the kit chimera is not coupled to phosphoinositide metabolism

The observed interaction between the chimeric *kit* receptor and two substrate enzymes involved in the metabolism of inositol lipids led us to examine the effect on the overall cellular metabolism of phosphoinositides. EKR-expressing cells were labeled with [^{3}H]inositol, stimulated for various time periods with either serum or EGF, and the relative cellular content of inositol phosphate (IP) determined as described under Materials and methods. Serum stimulation,

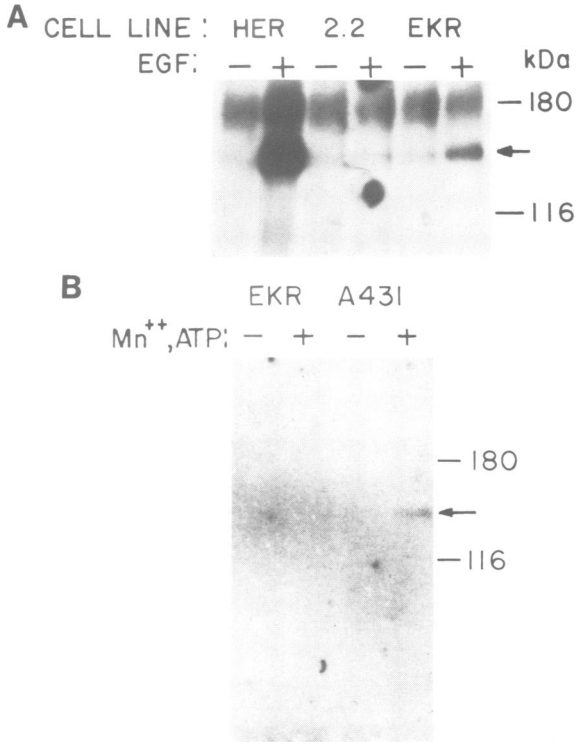

Fig. 6. A. Tyrosine phosphorylation of PLC_γ in response to ligand stimulation. Quiescent EKR, HER or untransfected control cells (2.2), were stimulated with EGF, solubilized and immunoprecipitation with antiserum to PLC_γ. After transfer to nitrocellulose the proteins were blotted with antiserum to phosphotyrosine and probed with [^{125}I]goat anti-rabbit IgG. The band corresponding to the PLC_γ protein (145 kd) is marked by an arrow. **B.** Association between PLC_γ and phosphorylated receptor *in vitro*. Quiescent A431 and EKR cells were solubilized and the receptor immunoprecipitated. The immunoprecipitates were subjected to *in vitro* kinase assay in presence (+) or absence (−) of 10 mM $MnCl_2$ and 50 μM ATP for 20 min at 22°C. The immunoprecipitates were then washed and incubated for 1 h at 4°C with lysates of quiescent 2.2 cells, followed by additional washing and separation by SDS−PAGE. The separated proteins were transferred to nitrocellulose and blotted with an antiserum to PLC_γ. The 145 kd PLC_γ protein is indicated by an arrow.

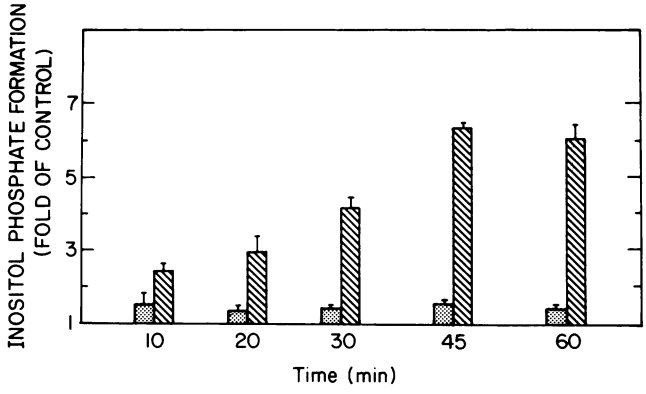

Fig. 7. Effect of EGF and serum on inositol phosphate formation. Serum starved EKR cells were labeled with [^{3}H]inositol, treated with LiCl as described under Materials and methods and then incubated at 37°C with EGF (150 ng/ml, stippled boxes) or fetal calf serum (10%, hatched boxes) for the indicated periods of time. Inositol phosphates were then extracted from the cells as described (Seuwen *et al.*, 1988) and quantified by liquid scintillation. Error bars indicated SEM of duplicate determinations. This experiment was repeated three times with essentially the same results.

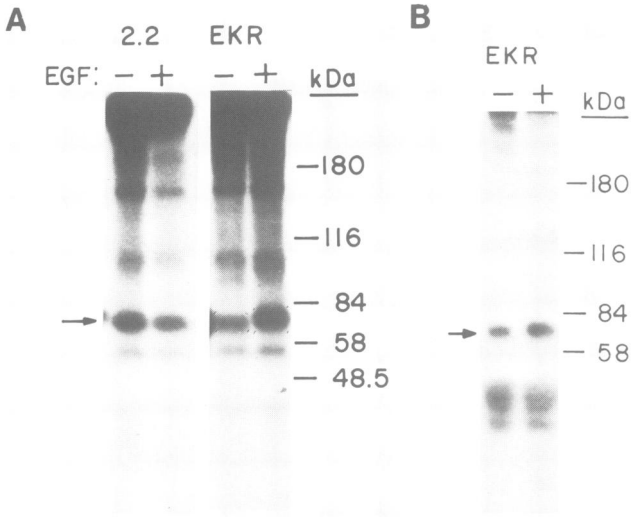

Fig. 8. Modification of the Raf1 protein in response to ligand stimulation. Panel **A**. Quiescent EKR or 2.2 cells were labeled for 4 h with [^{32}P]orthophosphate (0.5 mCi/ml), stimulated for 15 min with EGF, solubilized and subjected to immunoprecipitation with an anti-peptide antiserum directed to the Raf1 protein. The arrow indicates the location of p75$^{c\text{-}raf}$ band. Panel **B**. As in Panel A except that unlabeled cells were analyzed and the Raf1 protein was detected by immunoprecipitation followed by Western blotting with the same antiserum to Raf1.

under the conditions we used, resulted in a gradual accumulation of radioactive IP, up to 6-fold of the basal level after 45 min (Figure 7). In contrast, stimulation of the EKR cells with EGF resulted in no significant change in the level of inositol phosphate. The effects of both serum and EGF on the levels of IP_2 and IP_3 were small as compared to the effects on IP (not shown).

Activation of the EKR protein leads to modification of Raf1

The serine/threonine kinase Raf1 has been implicated in signal transduction mediated by receptor tyrosine kinases (Morrison *et al.*, 1988; Morrison *et al.*, 1989; Baccarini *et al.*, 1990). To determine if activation of the *kit*-encoded tyrosine kinase is also coupled to modification of Raf1, we labeled EKR cells with [^{32}P]orthophosphate and stimulated the cells with EGF. Subsequent immunoprecipitation of the Raf1 protein showed that EGF treatment of EKR cells, but not untransfected cells, resulted in an increase in the apparent molecular weight of the immunoprecipitated p75^{raf} and to an increased phosphorylation (Figure 8A). A similar shift in the Raf1 protein band was detectable also by Western blot analysis of the stimulated cells (Figure 8B). Phospho-amino acid analysis of the phosphorylated Raf1 showed that most of the increased phosphorylation took place on serine residues (not shown). However, due to the faint signals obtained we cannot exclude threonine or tyrosine phosphorylation.

Discussion

Chimeric receptors composed of the extracellular, ligand binding domain, of one receptor, linked to the cytoplasmic domain of another receptor have proven to be useful experimental tools, especially in the case of receptors with no identified ligand molecules (Lee *et al.*, 1989; Lehvaslaiho *et al.*, 1989). The general lesson emerged from the study of chimeric receptor tyrosine kinases is that the identity of the biological signal delivered to the cell is determined by the cytoplasmic, rather than the extracellular portion of each receptor (reviewed in Yarden and Ullrich, 1988). Based on this observation we employed a chimeric *kit* protein to study the intracellular function of the *kit*-encoded tyrosine kinase. As at the time our experiments were performed the ligand of the *kit* receptor was unknown; the only way to address this question was to use a heterologous ligand.

The results obtained with the chimeric *kit* protein indicate the following cellular physiology which is characteristic for a growth factor receptor: Upon ligand binding the chimera undergoes endocytosis that results in intracellular degradation of both the receptor and the complexed ligand. Self-phosphorylation of the receptor is followed by a series of cytoplasmic events that culminate in accelerated cell growth. The most striking effect we found is the association of the ligand-stimulated kinase of *kit* with a PI3K activity. This activity appears to be analogous to the catalytic function that associates with other activated receptors. This is based on the specificity of the kinase to the D3 position of the inositol ring of PI (Whitman *et al.*, 1988), the ability to use PI, PI(4)P and PI(4,5)P_2 as substrates (Auger *et al.*, 1989), its susceptibility to inhibition (Whitman *et al.*, 1987), and correlation between the activity and an 85 kd polypeptide (Courtneidge and Heber, 1987; Kaplan *et al.*, 1987). The association of PI3K with the receptor was ligand-dependent and gradually increased with time. The *kit* receptor thus joins CSF1-R and PDGF-R which are known to directly interact with PI3K (Kaplan *et al.*, 1987; Varticovsky *et al.*, 1989; Shurtleff *et al.*, 1990; Kazlauskas and Cooper, 1990; Reedijk *et al.*, 1990).

Although our results indicate that stimulation of the *kit* receptor leads to modification of the Raf1 protein kinase, at present we cannot determine if this interaction is direct, and whether it leads to activation of the catalytic function of Raf1. However, in analogy to both PDGF-R and CSF1-R which were shown to activate Raf1 (Morrison *et al.*, 1989; Baccarini *et al.*, 1990), it is expected that the interaction with *kit*/SCF-receptor will also result in Raf1 activation. Unlike PDGF-R (Morrison *et al.*, 1989) that directly interacts with Raf1, the CSF1-R appears to indirectly affect the serine/threonine kinase (Baccarini *et al.*, 1990). Preliminary results and our inability to detect phosphotyrosine in the modified Raf1 protein also suggest indirect *kit*-Raf1 interactions.

In contrast to the interaction of the chimeric *kit* protein with PI3K and Raf1, its interaction with PLC$_\gamma$ appears to be rather limited. This was reflected by a 5−10-fold lower apparent stoichiometry of tyrosine phosphorylation of PLC$_\gamma$, as compared with the effect of EGF-R, and also by the lack of stable association between PLC$_\gamma$ and the EKR protein. Tyrosine phosphorylation of PLC$_\gamma$ appears to indirectly affect phosphoinositide metabolism (Kim *et al.*, 1990; Margolis *et al.*, 1990b) through a mechanism that involves association of the enzyme with membranes (Todderud *et al.*, 1990). Our inability to detect significant changes in the production of inositol phosphate in EGF-treated EKR cells (Figure 7) may therefore be attributed to the relatively limited modification of PLC$_\gamma$ in these cells. Indeed, we observed only a 2-fold stimulation of PI-turnover upon EGF-treatment of EGF-R overexpressing cells (data not shown). As the latter cells exhibit much higher ligand-induced tyrosine phosphorylation of PLC$_\gamma$ than EKR cells, parallel quantitative relations between PI-turnover and

PLC_γ phosphorylation would result in an undetectable inositol phosphate signal in EKR cells.

The data presented in this study and in our previous paper (Lev *et al.*, 1990) suggest that the c-*kit*-encoded receptor is not only structurally homologous but also functionally related to the receptors for CSF1 and PDGF. Although each of the three receptors induces mitogenesis, they differ in their tissue distribution and details of their primary structures. These differences may constitute the basis for the selective interaction of each receptor with cytoplasmic effectors. This selectivity was addressed by the present study and previous analyses of the CSF1-R (Downing *et al.*, 1989; Baccarini *et al.*, 1990; Shurtleff *et al.*, 1990;). Figure 9 schematically illustrates the interaction of *kit*/SCF-receptor with cytoplasmic effectors in comparison with the effects mediated by CSF1-R and PDGF-R. Thus, the receptor for PDGF (β-type) interacts with PI3K (Kaplan *et al.*, 1987), PLC_γ (Wahl *et al.*, 1989b; Meisenhelder *et al.*, 1989; Kumjian *et al.*, 1989; Morrison *et al.*, 1990) and Raf1 (Morrison *et al.*, 1989) and leads to accelerated PI turnover (Habenicht *et al.*, 1981) that resultts in Ca^{2+} fluxes and activation of protein kinase C. In comparison, the effects of the homologous CSF1-R and SCF-receptor are more limited: The c-*kit*/SCF-receptor interacts with PI3K, Raf1 and to some extent with PLC_γ, whereas CSF1-R similarily interacts with PI3K (Varticovsky *et al.*, 1989; Shurtleff *et al.*, 1990) and Raf1 (Baccarini *et al.*, 1990) but not with PLC_γ (Downing *et al.*, 1989). In addition the two latter receptors do not lead to an accelerated PI turnover (Figure 7 and Whetton *et al.*, 1986). Thus the c-*kit*/SCF-R is functionally closer to CSF1-receptor than to PDGF-R.

The significance of the apparently distinct signal transmission pathway of each of the type III RTKs is not known. Certainly more extensive analysis, that will include other candidate substrates and analysis of actual activation of substrates rather than interaction, will be required in order to examine to what extent each signaling pathway is distinct. However specific combinations of signal transducers may result in ligand-specific patterns of cytoplasmic second messengers. Dosage effects, as exemplified here by *kit* interaction with PLC_γ, may further extend the number of possible combinations. Potentially this may define a 'cytoplasmic code' that would enable preservation of the identity of hormonal signals in the cytoplasm and results in receptor-specific programs of gene expression. This model may explain such phenomena as differential potencies of various mitogens and also the synergistic effects seen when growth factors are applied together (Rozengurt, 1986). Clearly, more information will be required to test the validity of this model and its applicability to other pathways of cellular activation.

Materials and methods

Cell culture

NIH3T3 fibroblasts (clone 2.2) devoid of endogenous EGF-R were transfected with an EKR-DNA construct as described (Lev *et al.*, 1990). Cells were grown in Dulbecco's modified Eagle (DME) medium supplemented with 10% bovine calf serum.

Antibodies and immunoprecipitation

Cells were lysed in buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulferic acid (HEPES pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid (EGTA), 10 μg leupeptin/ml, 10 μg aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride (PMSF), 200 μM sodium orthovanadate and 100 mM sodium fluoride. Immunoprecipitations were performed using protein A−agarose (Sigma) coupled to specific antibodies. The antibodies used were a monoclonal antibody to EGF-receptor (mAb108, Lax *et al.*, 1989), rabbit anti-*kit* antibody (Ab212, Yarden *et al.*, 1987), rabbit anti-phosphotyrosine antibody prepared as described (Kamps and Sefton, 1988), antibodies to PLC_γ which were raised in rabbits immunized with a synthetic peptide corresponding to residues 1257−1275 of the molecule (Stahl *et al.*, 1988) and an antibody to the Raf1 protein which was raised by using the synthetic peptide Sp63 as described (Schultz *et al.*, 1985). Immunoprecipitates were washed either with HNTG' solution (20 mM HEPES buffer at pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 100 mM sodium fluoride, 200 μM sodium orthovanadate) or successively with H' solution (50 mM Tris−HCl pH 8, 500 mM NaCl, 0.1% SDS, 0.2% Triton X-100, 5 mM EGTA, 100 mM NaF, 200 μM sodium orthovanadate), M' solution (50 mM Tris−HCl pH 8, 150 mM NaCl, 7.5 mM EDTA, 0.1% SDS, 0.2% Triton X-100, 100 mM NaF, 200 μM sodium orthovanadate) and L' solution (10 mM Tris-HCl pH 8, 0.1% Triton X-100, 100 mM NaF, 200 μM sodium orthovanadate). The washed immunoprecipitates incubated for 5 min with gel sample buffer at 100°C and analyzed by SDS−PAGE.

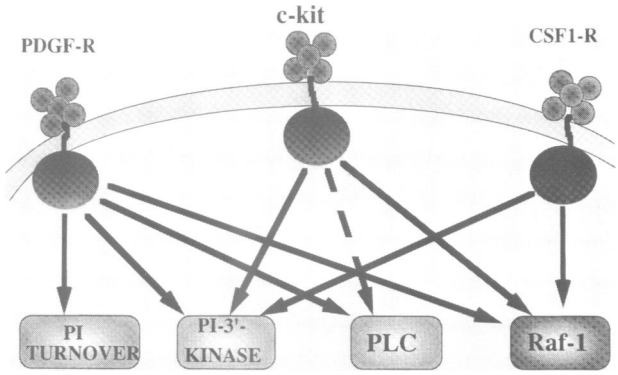

Fig. 9. Schematic illustration of the interaction of the *kit* protein with cytoplasmic effectors in comparison with the homologous receptors for PDGF and CSF1. Arrows do not necessarily indicate catalytic activation. The references for PDGF-R and CSF1-R are given in the text.

Ligand-internalization

The cell-surface bound and internalized ligand were measured as described (Haigler *et al.*, 1979) on confluent monolayers of cells in 24 well dishes.

Receptor degradation

Cells were labeled for 12 h with methionine free DME-medium containing 50 μCi/ml [^{35}S]methionine (Amersham) and 10% dialyzed fetal calf serum. After labeling the cells were incubated for 30 min in fresh DME-medium at 37°C and then maintained at 37°C with or without EGF (1 μg/ml) for various periods of time. The EKR protein was then immunoprecipitated and visualized by autoradiography.

2-DOG uptake

The uptake of 2-deoxyglucose (2-DOG) was measured as described (Flier *et al.*, 1987). The 2-DOG uptake was normalized to the total protein content of the cell lysates. Induced transport is reported as fold induction over the control ± S.E.

Assay of EKR associated phosphoproteins

The protocol of Kaplan *et al.* (1987), was used on serum starved EKR cells.

PI-kinase assay

PI kinase activity was measured according to Whitman *et al.* (1985). For experiments including NP-40, immunoprecipitates were washed with PBS containing 1% NP-40 and assayed in the presence of 0.5% NP-40. Adenosine, where applied, was included in the reaction buffer at final concentration of 300 μM. For separation of phosphatidylinositol-bisphosphate (PIP_2) and PI and phosphatidylinositol-phosphate (PIP) we used a TLC plate which was precoated with 1% (W/V) potassium oxalate and baked for 1 h at 80°C. The developing solvent contained n-propanol/2 M acetic acid (13:7, V/V).

Deacylation and HPLC analysis

The ^{32}P-labeled PIP products were scraped from the TLC plates and treated with methylamine (along with a ^{3}H-labeled PI-4-P standard) to remove the fatty acyl side chain as described by Whitman *et al.* (1988). The deacylated products were separated by HPLC using a partisphere Sax anion-exchange column (Whatman) as described by Auger *et al.* (1989). Samples were loaded in water and eluted with 1 M $(NH_4)_2HPO_4$, pH 3.8, at 1 ml min^{-1}. A gradient from 0 to 1 M $(NH_4)_2HPO_4$, pH 3.8 was developed in 130 min by using dual pumps [pump A H_2O:pump B 1 M $(NH_4)_2HPO_4$ pH 3.8]. B was run at 0% for 10 min, then to 25% B with a duration of 60 min and then to 100% B for 50 min. Eluted fractions were quantitated by liquid scintillation counting.

PI turnover

The method described by Seuwen *et al.* (1988) was used. Quiescent EKR cells in 35 mm dishes were labeled for 16 h with myo-2-[^{3}H] inositol (Amersham, 2 μCi/ml). The medium was then replaced with a fresh DME medium containing 20 mM HEPES (pH 7.5) and 20 mM LiCl. 10 min later EGF (150 ng/ml) or fetal calf serum (10%) were added. At the end of the indicated periods of time the water-soluble inositol phosphates were separated by ion exchange chromatography using AG1-X8 columns (BioRad). Inositol mono-, bis- and tris-phosphates were separately determined by β-counting. Shown are only the values for inositol phosphate. The others showed relatively small changes and are not shown. Each value represents the mean of duplicate determinations in four independent experiments.

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