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Cell scattering of SK-N-MC neuroepithelioma cells in response to Ret and FGF receptor tyrosine kinase activation is correlated with sustained ERK2 activation

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The c-*ret* proto-oncogene encodes a receptor tyrosine kinase which plays an important role in kidney and enteric nervous system development. Germline mutations in c-ret are responsible for the dominantly inherited cancer syndromes, multiple endocrine neoplasia types 2A and 2B and familial medullary thyroid carcinoma as well as the developmental disorder Hirschsprung's disease. Using SK-N-MC neuroepithelioma cells stably transfected with an EGFR/Ret chimeric receptor, we have studied cellular consequences and signalling events following activation of exogenous EGFR/Ret and endogenous FGF and PDGF receptor tyrosine kinases in cells of neuroectodermal origin. Here we report that Ret activation led to cell scattering, growth inhibition and loss of anchorage-independent growth. Basic FGF, but not PDGF, evoked similar responses in those cells. Nevertheless, activation of all three receptor tyrosine kinases led to ERK2 activation. Analysis of the kinetics of ERK2 activation and downstream events revealed that Ret and FGF receptor activation led to sustained ERK2 activation and SRE transactivation, while PDGF treatment led to transient ERK2 activation and failed to induce SRE transactivation. Our results suggest that sustained, but not transient ERK2 activation may be involved in cell scattering.

Keywords: Ret; ERK2; signal transduction; transcription; cell scattering; growth inhibition

Introduction

Polypeptide growth factors are involved in the regulation of cell growth, migration, differentiation, survival and morphogenetic processes during embryonic development as well as during maintenance of homeostasis in adult life. Many such factors exert their action through receptors with ligand-inducible intrinsic tyrosine kinase activity (Schlessinger and Ullrich, 1992).

The c-*ret* proto-oncogene encodes a transmembrane receptor tyrosine kinase (RTK) with a cadherin-like motif in the extracellular domain (Takahashi *et al*., 1988; 1989; Takahashi, 1995). It encodes two protein isoforms of 1072 (P9) and 1114 (P51) amino acids differing from each other by having nine or 51 different amino acids in their C-terminus due to alternative splicing (Tahira *et al*., 1990). Recently, a third c-*ret* transcript predicted to encode a protein with an alternative C-terminal 43 amino acids resulting from alternative splicing has been identified (Myers *et al*., 1995).

Ret (acronym for *RE*arranged during *T*ransfection) was originally identified as a transforming gene, by transfection of human T-cell lymphoma DNA into NIH3T3 cells (Takahashi *et al*., 1985). Subsequently, c-*ret* oncogenic rearrangements, designated *RET*/PTCs, were found in patients suffering from papillary thyroid carcinomas (PTC) (reviewed in Jhiang and Mazzaferri, 1994). Recently, germline point mutations in the c-*ret* gene have been found to be the causative genetic event in several human dominantly inherited cancer syndromes including multiple endocrine neoplasia (MEN) types 2A and 2B and familial medullary thyroid carcinoma (FMTC) as well as in the developmental disorder Hirschsprung's disease (HSCR) (reviewed in Takahashi, 1995 and Pasini *et al*., 1996). The germline mutations in c-*ret* found in patients with MEN 2A, FMTC and MEN 2B resulted in 'gain of function' of Ret leading to constitutive activation of its intrinsic tyrosine kinase, and/or to a change in the substrate specificity (Carlson *et al*., 1994; Asai *et al*., 1995; Santoro *et al*., 1995; Songyang *et al*., 1995). On the other hand, germline mutations in c-*ret* found in patients with HSCR (aganglionic megacolon) resulted in 'loss of function' of Ret (Edery *et al*., 1994; Romeo *et al*., 1994; Pasini *et al*., 1995). HSCR is regarded as the consequence of premature arrest of craniocaudal migration of neural crest-derived enteric neuroblasts towards the distal part of the colon, suggesting that c-*ret*, in addition to its role in tumorigenesis, might play a role in the development of the enteric nervous system (ENS).

Analysis of c-*ret* expression during vertebrate embryogenesis and c-*ret* gene ablation studies in the mouse support the idea that Ret is involved in the migration and differentiation of neural crest cells of the enteric nervous system. In the developing nervous system, c-*ret* is expressed in undifferentiated neuroectodermal precursors, neuroepithelial cells of the ventral neural tube and subsets of migrating neural crest cells, as well as in their differentiated postmitotic derivatives (Pachnis *et al*., 1993; Avantaggiato *et al*., 1994; Schuchardt *et al*., 1995; Tsuzuki *et al*., 1995; Lo *et al*., 1995). Mice homozygous for a targeted disruption of the c-*ret* gene die soon after birth showing renal agenesis or severe dysgenesis and a lack of enteric

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neurons throughout the digestive tract, similar to patients with Hirschsprung's disease (Schuchardt *et al*., 1994).

Although the long sought ligand of the Ret RTK has only recently been identified to be Glial cell line-derived neurotrophic factor (GDNF) (Durbec *et al*., 1996; Jing *et al*., 1996; Treanor *et al*., 1996; Trupp *et al*., 1996), studies of Ret signalling have been performed using a chimeric human EGFR/Ret (HERRet) receptor or oncogenic constitutively active isoforms of Ret (i.e. the *RET*/PTCs) (Borello *et al*., 1994; Santoro *et al*., 1994). GDNF uses a multi-subunit receptor complex in which GDNFRα and Ret function as the ligand-binding and signalling components, respectively (Jing *et al*., 1996; Treanor *et al*., 1996). GDNF stimulates the autophosphorylation of Ret, the hallmark of ligand-induced RTK activation (Schlessinger and Ullrich, 1992). Since EGF stimulation also resulted in autophosphorylation of EGFR/Ret (Santoro *et al*., 1994), this chimeric receptor provides a valuable tool to study signal transduction following Ret activation that mimics endogenous Ret activation. Several proteins have been shown to be phosphorylated, activated, and/or associated with Ret. These include rasGAP, PLC-γ, Shc, Grb2, Grb7, Grb10, p21ras, Raf-1, ERK2, paxillin and Enigma (Borrello *et al*., 1994, 1996; Romano *et al*., 1994; Santoro *et al*., 1994; Pandey *et al*., 1995, 1996; van Weering *et al*., 1995; Durick *et al*., 1996). Although a role for PLC-γ and Enigma has been established in mitogenesis (Borrello *et al*., 1996; Durick *et al*., 1996), the role of these and/or other proteins in other biological processes following Ret activation remains to be elucidated.

The specificity of long-term effects of a particular RTK is determined by the substrates that are phosphorylated following activation of this RTK and by the subset of proteins that associate with it. In addition, evidence is emerging that the duration of activation of downstream signalling components is involved. For instance, transient ERK2 activation is observed in pheochromocytoma PC12 cells following EGF stimulation leading to proliferation of the cells, while NGF treatment leads to sustained ERK2 activation and differentiation of the cells (reviewed in Marshall, 1995).

SK-N-MC cells are neuroepithelioma cells of embryonic neuroectodermal origin and are thought to be stem cells or immature intermediates of neuroblasts and epithelial cells (Biedler *et al*., 1973; Ross *et al*., 1983; Dehner, 1986; Ciccarrone *et al*., 1989). Since Ret expression has been found in several cell lineages of neuroectodermal origin, we investigated the long-term cellular consequences and signalling events by Ret in SK-N-MC cells using stably transfected cells expessing chimeric receptors consisting of the ligand-binding and transmembrane domain of the human EGF receptor (HER) and the cytoplasmic domain of the P9 isoform of the human Ret receptor (HERRet) (Santoro *et al*., 1994). In parallel, we studied signalling mediated by endogenous FGF and PDGF receptors in these cells. Previously, we have shown that HERRet chimeric receptors couple to signal transduction pathways in SK-N-MC cells in that EGF stimulation leads to autophosphorylation of the HERRet receptor and activation of the p21ras-ERK signal transduction pathway (van Weering *et al*., 1995). Here, we report that activation of Ret in SK-N-MC cells resulted in sustained ERK2 activation. Activation of endogenous FGF receptors (FGFR) also resulted in sustained ERK2 activation, whereas PDGF treatment evoked transient ERK2 activation in these cells. Ret and FGFR activation led to cell dissociation and migration ('scattering') and growth inhibition, while PDGF treatment did not, indicating that sustained ERK2 activation is correlated with cell scattering and growth inhibition in neuroectodermal-derived SK-N-MC neuroepithelial cells.

Results

Immediate-early gene expression in response to HERRet activation

The lack of a known ligand for the Ret receptor tyrosine kinase (RTK) has hampered the study of ligand-induced signal transduction by this receptor in the past. To circumvent this problem we made use of a chimeric receptor consisting of the ligand-binding and transmembrane domain of the human EGF receptor (HER) and the cytoplasmic domain of the human Ret receptor (HERRet; isoform P9) (Santoro *et al*., 1994) that was stably transfected into human SK-N-MC neuroepithelioma cells. SK-N-MC cells, derivatives of neuroectodermal origin, do not express Ret receptors and do not respond to EGF (Takahashi and Cooper, 1987; van Weering *et al*., 1995). However, EGF stimulation of SK-N-MC cells stably transfected with the HERRet receptor resulted in autophosphorylation of the receptor (Figure 1a) and activation of the p21ras-ERK signal transduction pathway (van Weering *et al*., 1995). Activated ERKs translocate into the nucleus, where they phosphorylate and activate nuclear transcription factors such as Elk-1, resulting in transcription of immediate-early genes (IEG). A well known regulatory target for the ERKs is the serum response element (SRE), which is present in the promoter region of many IEGs, including c-*fos* (reviewed in Hill and Treisman, 1995; Marshall, 1995). Ret-mediated SRE activation was studied in SK-N-MC and HERRet expressing (SKF5 and SKF6) cells using a reporter-construct containing three copies of the c-*fos* SRE fused to the Herpes virus thymidine kinase (tk) promoter driven bacterial chloramphenicol acetyl transferase (CAT) gene (3xSREtkCAT). As shown in Figure 1b, EGF treatment of SKF5 and SKF6 cells resulted in an almost fivefold transactivation of the SRE-dependent CAT reporter gene construct, while EGF treatment of the parental cells did not result in transactivation of this construct. Cells transfected with the control tkCAT vector showed no induced CAT-activity in response to EGF (Figure 1b). In addition, we investigated IEG expression in response to EGF in parental SK-N-MC and HERRet expressing SKF5 cells. EGF stimulation of the stable transfectant SKF5 (similar results were obtained with SKF6; data not shown) resulted in transient expression of the IEGs *egr*-1, c-*fos* and c-*jun* with maximal levels after 30 min, while no induction of expression could

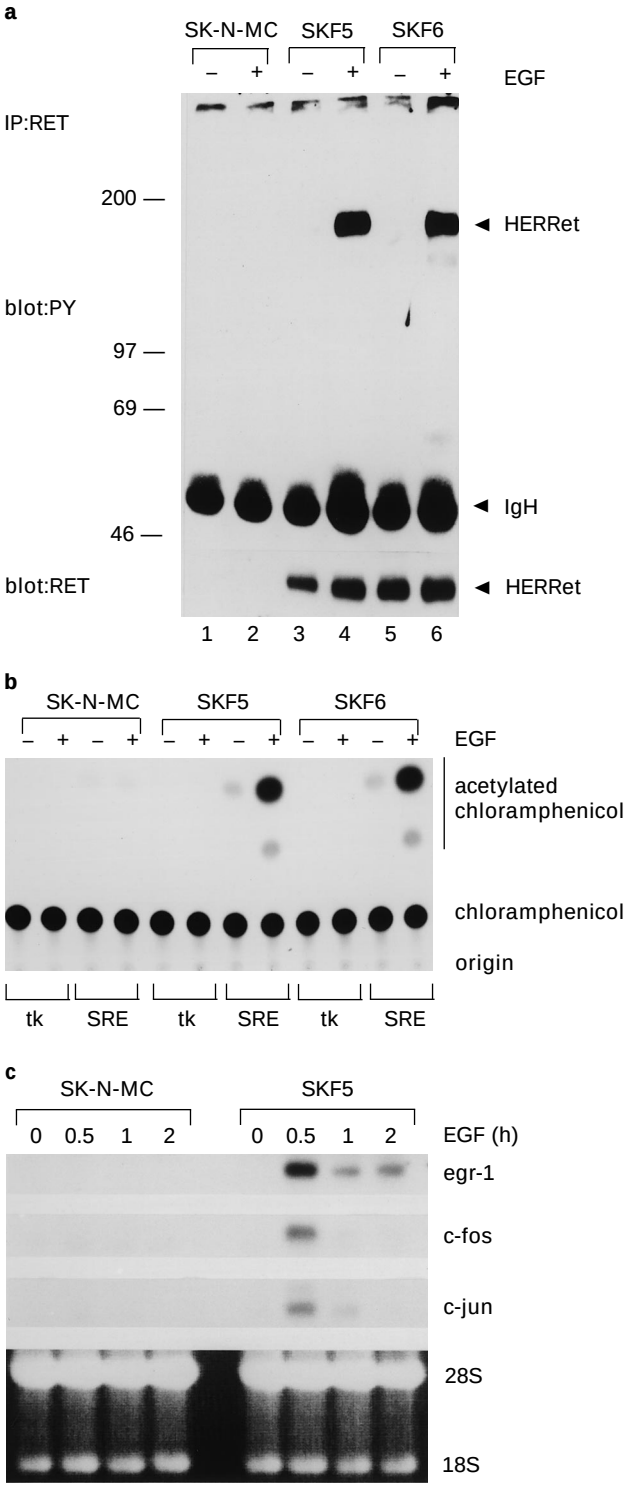

Figure 1 Stably transfected SK-N-MC cells express functional HERRet chimeric receptors. (**a**) Autophosphorylation of the HERRet chimeric receptor upon addition of EGF. Subconfluent serum-starved SK-N-MC and SKF5/6 cells were left untreated (−) or treated (+) for 10 min with EGF (50 ng/ml) as indicated. Cells were lysed in RIPA lysis buffer, after which HERRet was immunoprecipitated and resolved by SDS–PAGE followed by immunoblotting with the anti-phosphotyrosine antibody PY-20 (upper panel). The lower panel shows a reprobe of the same blot with the Ret99 antibody directed against the tyrosine kinase domain of Ret. Detection was with ECL. The molecular weight of marker proteins that were co-electrophoresed with the samples are indicated on the left in kDa. The position of the chimeric HERRet receptor is indicated. The indicated HERRet bands in the lower panel colocalizes exactly with PY-containing HERRet in the upper panel. (**b**) Ret-mediated transactivation of an SRE-dependent CAT-reporter gene construct. SK-N-MC, SKF5 and SKF6 cells were transiently transfected with CAT-reporter constructs as indicated together with a β-galactosidase expression vector (pSV2LacZ) as an internal control for transfection efficiency. Serum-starved cells were left untreated (−) or treated for 6–8 h with EGF (50 ng/ml). Cells were then harvested and CAT assays were performed on β-galactosidase normalized samples. A representative experiment out of three independent experiments is shown. (**c**) Northern blot analysis of Ret-induced immediate-early gene expression. Subconfluent serum-starved cells were left untreated or treated with EGF (50 ng/ml) for the indicated time periods. Twenty micrograms of total RNA was loaded per lane and subsequently probed with ^{32}P-labelled cDNA fragments specific for *egr-1*, c-*fos* and c-*jun* as indicated. To show that equal amounts of RNA were loaded per lane, the RNA was visualized by u.v. illumination after ethidium-bromide staining. The positions of the 28S and 18S ribosomal bands are indicated

be detected in the parental cell line (Figure 1c). These results demonstrate that exogenous HERRet chimeric receptors couple to endogenous signalling pathways, leading to nuclear events, including enhanced gene transcription.

Ret-induced morphological changes in SK-N-MC cells

No marked differences in morphology and growth properties were observed between wild-type and HERRet transfected cells when grown in medium supplemented with 10% fetal calf serum (FCS). In the absence of added growth factors cells grew in clusters with few, short extensions at the periphery of the cluster (Figure 2A and E). However, when SKF5 cells (similar results were obtained with SKF6 cells; data not shown) were treated with EGF or bFGF these growth factors generated a 'scattering' response (Figure 2F and G). In contrast, treatment with PDGF did not lead to cell scattering (Figure 2H). The same responses were observed in the parental cell line (Figure 2B–D), with the exception that EGF treatment did not result in scattering in agreement with the lack of cognate receptors. Cell scattering is characterized by several properties: cells lose their epithelial features, they become fibroblastic, and some cells dissociate and start moving individually. These morphological changes became apparent approximately 6 h after ligand-induced Ret activation in SKF5 cells (Figure 3). Similar results were obtained with bFGF treatment of SK-N-MC, SKF5 and SKF6 cells (data not shown). The failure of PDGF to induce scattering could either be due to the absence of a proper signal for the induction of scattering or to the induction of an inhibitory signal. We therefore stimulated SKF5 cells both with either EGF and PDGF or bFGF and PDGF. In both cases scattering was observed (Figure 4) indicating that the failure of PDGF to induce scattering is not due to an inhibitory signal but to the absence of an inducing signal.

Previously, we have shown that EGF treatment (Ret activation) of SKF5 cells resulted in phosphorylation of ERK2 and that this phosphorylation event correlates with ERK2 activation as measured by an *in vitro* kinase assay using myelin basic protein as a substrate (van Weering *et al*., 1995). Here, we demonstrate that not only EGF, but also bFGF and PDGF treatment resulted in ERK2 phosphoryla-

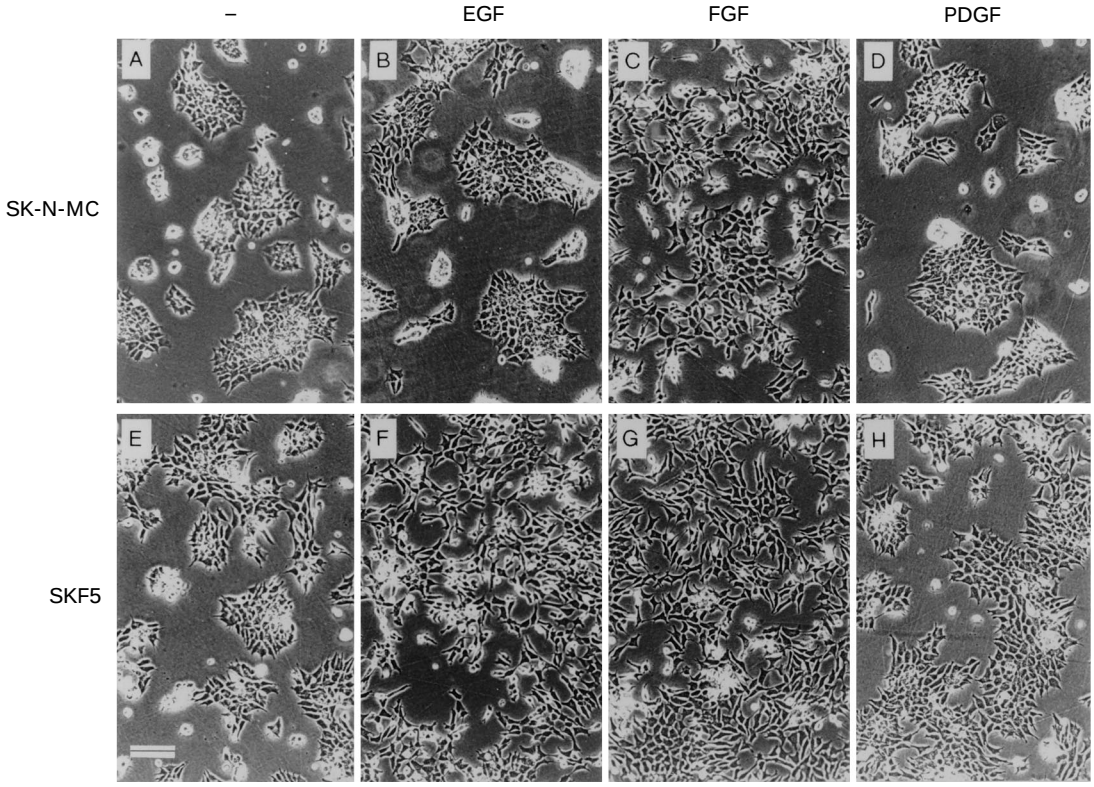

Figure 2 Scattering of SKF5 cells in response to EGF and bFGF, but not PDGF. Phase-contrast micrographs of SK-N-MC (**A**–**D**) and SKF5 (**E**–**H**) cells left untreated (**A**, **E**) or treated for 16 h with EGF (50 ng/ml) (**B**, **F**), bFGF (10 ng/ml) (**C**, **G**) and PDGF (20 ng/ml) (**D**,**H**). Bar represents 150 μm

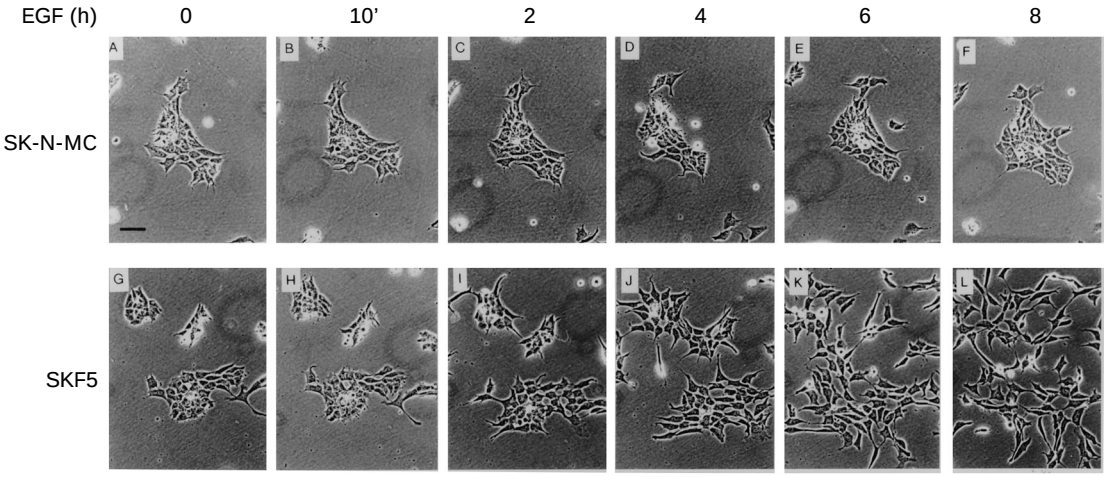

Figure 3 Time-course of EGF-induced scattering of SKF5 cells. Phase-contrast micrographs of SK-N-MC (**A**–**F**) and SKF5 (**G**–**L**) cells left untreated (**A**, **G**) or treated for 10 min (**B**, **H**), 2 h (**C**, **I**), 4 h (**D**, **J**), 6 h (**E**, **K**) or 8 h (**F**, **L**) with EGF (50 ng/ml). Bar represents 150 μm

tion as shown by a mobility shift assay, indicating that SK-N-MC cells contain functional cognate bFGF and PDGF receptors (Figure 5). In conclusion, although HERRet expressing SK-N-MC cells contain functional exogenous Ret and endogenous bFGF and PDGF receptors, with respect to ERK2 activation, only EGF (Ret activation) and bFGF induced scattering in these cells.

Sustained ERK2 activation correlates with cell scattering

Differential responses of the cell, for example proliferation versus differentiation, appear to be defined by differences in the duration of the p21ras-ERK signal, with extending kinetics favouring differentiation (reviewed in Marshall, 1995). In order to determine the effects of EGF, bFGF and PDGF on the

duration of ERK2 activation, serum-starved parental and HERRet transfected cells were stimulated with these growth factors for different periods of time (Figure 6). PDGF treatment of the parental cell line resulted in transient ERK2 activation, whereas bFGF treatment resulted in sustained ERK2 activation which remained high for at least 24 h (Figure 6a). EGF stimulation of the parental cells did not show activation of ERK2 (Figure 5, lane 2), while EGF stimulation of SKF5 and SKF6 cells resulted in sustained ERK2 activation, with the same kinetics as seen for bFGF in the parental and HERRet expressing cells, in that ERK2 activation in response to EGF also remained high for at least 24 h. (Figure 6a–c, and data not shown). PDGF treatment of SKF5 cells resulted in rapid, transient ERK2 activation which peaked at 10 min after stimulation (Figure 6d), similar to the PDGF response in the parental SK-N-MC cells. These results indicate that sustained ERK2 activation correlates with the scattering response described above.

Sustained, but not transient ERK2 activation is associated with transcriptional activation (Marshall, 1995). Therefore, we determined whether differences in the duration of ERK2 activation leads to differences in SRE activation. As shown in Figure 7, EGF or bFGF treatment of SKF5 and SKF6 cells resulted in transactivation of the 3xSREtkCAT reporter gene construct. In contrast, PDGF treatment of the parental and HERRet transfected cells did not result in transactivation of the SRE-dependent construct, as compared with that in untreated cells (Figure 7). Cells transfected with the control tkCAT vector showed no induced CAT-activity in response to all three growth factors (data not shown). Taken together, these data demonstrate that sustained ERK2 activation led to SRE-mediated transactivation, while transient ERK2 activation did not.

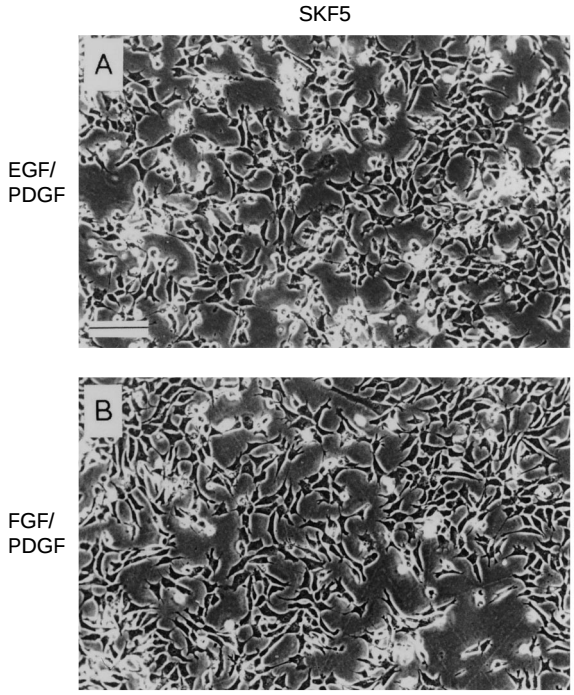

Figure 4 EGF and bFGF-induced scattering of SKF5 cells is not inhibited by PDGF. Phase-contrast micrographs of SKF5 cells treated with either EGF (50 ng/ml) and PDGF (20 ng/ml) (**A**) or bFGF (10 ng/ml) and PDGF (20 ng/ml) (**B**) for 16 h. Bar represents 150 μm

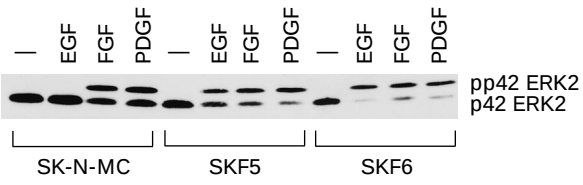

Figure 5 EGF, bFGF and PDGF-induced ERK2 phosphorylation in HERRet expressing SK-N-MC cells. Subconfluent serum-starved SK-N-MC, SKF5 and SKF6 cells were left untreated (−) or treated for 5 min with EGF (50 ng/ml), bFGF (10 ng/ml) and PDGF (20 ng/ml) as indicated. Cells were then lysed in Laemlli sample buffer and resolved by SDS–PAGE followed by immunoblotting with ERK2 antisera. Detection was with ECL. The positions of unphosphorylated (p42ERK2) and phosphorylated ERK2 (pp42ERK2) are indicated

Ret and FGFR activation mediated growth inhibition

Since the scattering response appeared to be accompanied by growth inhibition, we studied several growth

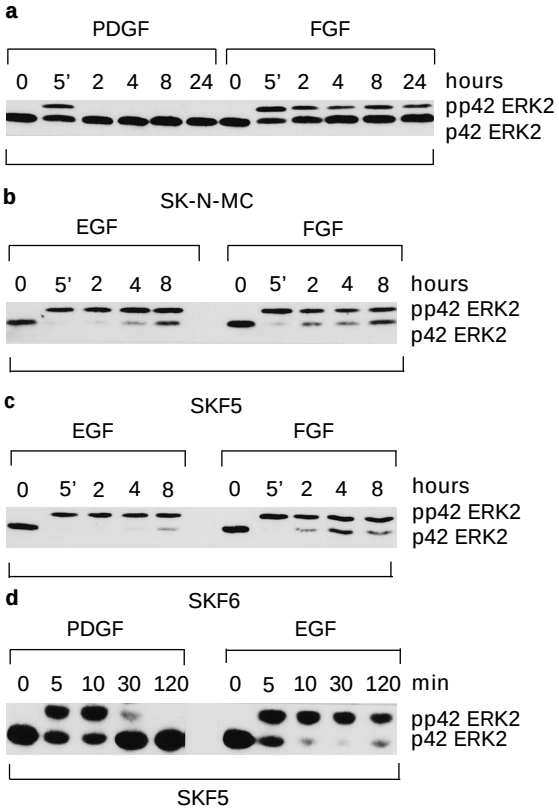

Figure 6 Sustained ERK2 phosphorylation in response to EGF and bFGF, but not PDGF. Subconfluent serum-starved SK-N-MC, SKF5 and SKF6 cells were left untreated (0) or treated for the indicated time periods, varying from 5 min (5′) to 24 h with the appropriate growth factors. The growth factors were used at the following concentrations: EGF (50 ng/ml), bFGF (10 ng/ml) and PDGF (20 ng/ml). Cells were lysed in Laemlli sample buffer and resolved by SDS–PAGE followed by immunoblotting with ERK2 antisera. Detection was with ECL. The positions of unphosphorylated (p42ERK2) and phosphorylated ERK2 (pp42ERK2) are indicated

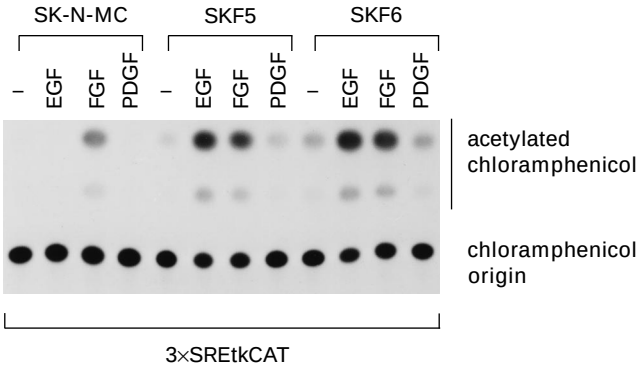

Figure 7 EGF and bFGF, but not PDGF transactivate an SRE-dependent CAT-reporter gene construct. SK-N-MC, SKF5 and SKF6 cells were transiently transfected with CAT-reporter constructs as indicated along with a β-galactosidase expression vector (pSV2LacZ) as an internal control for transfection efficiency. Serum-starved cells were left untreated (−) or treated for 6–8 h with EGF (50 ng/ml), bFGF (10 ng/ml) and PDGF (20 ng/ml) as indicated. Cells were then harvested and CAT assays were performed on β-galactosidase normalized samples. A representative experiment out of at least three independent experiments is shown

properties of the HERRet expressing cell lines in comparison to the parental cell line, including [^{3}H]thymidine incorporation, determination of viability (trypan blue exclusion) and anchorage-independent growth in soft agar. [^{3}H]thymidine incorporation assays were performed to determine the rate of DNA synthesis in the parental, SKF5 and SKF6 cell lines in response to EGF, bFGF and PDGF. In the parental cell line only bFGF treatment resulted in ligand-dependent inhibition of DNA synthesis in a concentration-dependent manner, whereas EGF, as expected, or PDGF treatment did not (Figure 8A). [^{3}H]thymidine incorporation in SKF5 and SKF6 cells was clearly inhibited by EGF in a dose-dependent manner (Figure 8B). bFGF inhibited DNA synthesis of SKF5 and SKF6 cells similar to the parental cells in a dose-dependent manner (Figure 8C).

Upon addition of EGF or bFGF part of the cells detached from the substratum. To rule out the possibility that the inhibition of [^{3}H]thymidine incorporation in response to these growth factors is due to loss of cells, the number of viable adherent and nonadherent cells was determined using the trypan blue stain exclusion method. As shown in Figure 9A, only bFGF treatment of the parental cell line resulted in inhibition of growth rate, while EGF and PDGF treatment had no effect. However, EGF treatment of SKF5 and SKF6 cells resulted in inhibition of growth rate to the same extent as bFGF treatment of these and the parental cells (Figure 9B and C). PDGF treatment did not affect the growth rate of SKF5 and SKF6 cells (Figure 9B and C).

In addition to participating in normal biological processes, cell migration plays a role in the development of many pathological conditions such as invasive and metastatic tumor spread. The tumorigenicity of cells *in vivo* correlates well with the ability of cells to form anchorage-independent colonies *in vitro*. Therefore, anchorage-independent growth was determined in the absence or presence of EGF in the parental, SKF5 and SKF6 cells using a soft agar growth assay. Figure

Table 1 Growth properties of HERRet-expressing SK-N-MC cells

	Growth in soft agar (%)			
Cell line	–	*EGF*	*bFGF*	*PDGF*
SK-N-MC	59.4±4.3[a]	54.2±5.0	0.2±0.2	41.6±2.2
SKF5	52.9±2.4	5.0±0.9	1.1±0.4	30.0±3.4
SKF6	59.5±1.2	2.6±1.3	0.5±0.5	48.2±3.1

To test anchorage-independent growth, 1.0×10^3 cells/cm^2 were plated in 50 mm petri dishes in DF-medium supplemented with 10% FCS in 0.375% agar onto a basal layer of 0.5% agar. Colony formation was tested in the absence or presence of EGF (10 ng/ml), bFGF (10 ng/ml) and PDGF (10 ng/ml). After 10–14 days, colonies were scored counting 20 random fields, corresponding to 2.3% of the total dish area. Colonies larger than ten cells were counted as one. [a]Values represent average ± standard deviation of a representative quadruplicate experiment

10 shows that SK-N-MC and the HERRet expressing clones were able to grow in an anchorage-independent manner. However, colony formation of SKF5 and SKF6 cells in soft agar was clearly inhibited by EGF, already at a concentration of 10 ng/ml, while no effect was observed in the parental cells. In addition, colony formation was tested in the presence of bFGF and PDGF. As summarized in Table 1, colony formation in soft agar in the presence of bFGF was almost completely inhibited in all three cell lines tested, whereas colony formation in the presence of PDGF was only slightly inhibited in these cells. In summary, these results demonstrate that Ret and FGFR, but not PDGF receptor activation led to inhibition of growth rate and anchorage-independent growth of SK-N-MC cells, implicating that Ret and FGFR activation may result in the acquisition of a less transformed or more differentiated phenotype.

Discussion

Receptor tyrosine kinases (RTK) are emerging as key regulators of decisive events during development; i.e. these receptors control cell movement, morphogenesis, proliferation and differentiation (for review see Birchmeier and Birchmeier, 1993). c-Ret, a member of the RTK superfamily (van der Geer *et al*., 1994), has been shown to play a critical role in the development of the enteric nervous system as well as in the excretory system (Pachnis *et al*., 1993; Schuchardt *et al*., 1994). Ret has been implicated as the receptor for a neurotrophic factor, recently identified as GDNF, in the control of proliferation, migration, differentiation and survival of neural-crest cells and their derivatives, as well as in inductive interactions during kidney organogenesis. Using SK-N-MC neuroepithelioma cells, which resemble stem cells or immature intermediates of neuroblasts as a model system, we have studied long-term cellular consequences and signal transduction in response to exogenous Ret activation in cells of neuroectodermal origin. In addition, we have investigated signalling events in these cells provoked by two other endogenous RTKs, FGFR and PDGFR.

Here we report that Ret RTK activation led to cell scattering and inhibition of proliferation of SK-N-MC cells. Activation of FGFRs, but not PDGFRs induced similar responses in these cells. Cell scattering is

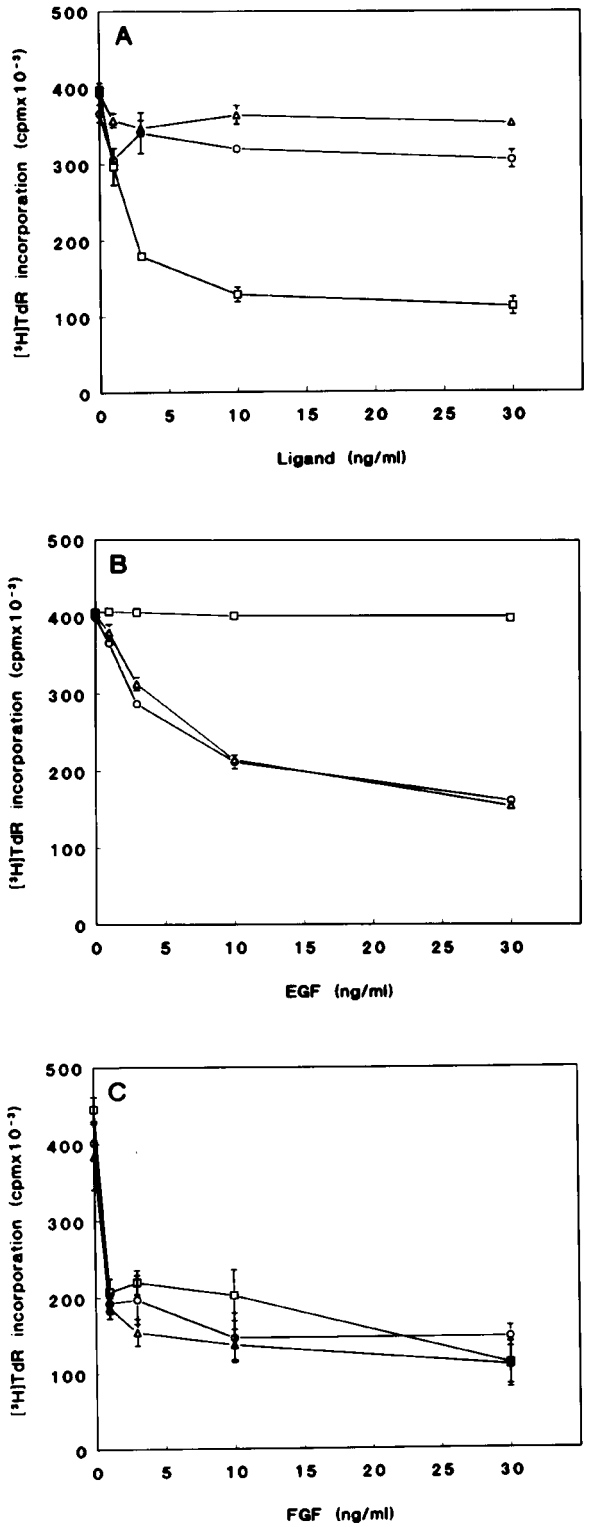

Figure 8 Dose-dependent inhibition of [^{3}H]thymidine incorporation in HERRet expressing cells by EGF and bFGF, but not PDGF. (**A**) [^{3}H]Thymidine ([^{3}H]TdR) incorporation in SK-N-MC cells is inhibited by bFGF, but not by EGF or PDGF. Cells were plated at a density of $2.5 \times 10^4/cm^2$ in 24-well plates in 1 ml bicarbonate-buffered DF-medium supplemented with 10% FCS. After 48 h cells were left untreated or treated for 48 h with increasing concentrations of EGF (△), bFGF (□) and PDGF (○) as indicated. To monitor DNA synthesis, cells were labelled with [^{3}H]TdR for 18 h before termination of the culture. Cells were then fixed, solubilized and the radioactivity incorporated into the DNA was determined by liquid scintillation counting. (**B**) Dose-dependent inhibition of [^{3}H]TdR incorporation in SKF5 and SKF6 cells upon addition of EGF. [^{3}H]TdR incorporation in SK-N-MC (□), SKF5 (△) and SKF6 (○) cells in response to increasing concentrations of EGF was determined as described above. (**C**) Dose-dependent inhibition of [^{3}H]TdR incorporation in SK-N-MC, SKF5 and SKF6 cells by bFGF. [^{3}H]TdR incorporation in SK-N-MC (□), SKF5 (△) and SKF6 (○) cells in response to increasing concentrations of bFGF was determined as described above

characterised by several properties: cells lose their epithelial features, and become fibroblastic, while some cells dissociate and start to migrate individually. The scattering response *in vitro* resembles an epithelium-to-mesenchyme transition (EMT) which occurs during morphogenetic processes in embryonic development *in vivo* (Duband *et al*., 1995). Although the molecular mechanism underlying EMT remains to be elucidated, it has been postulated that RTKs may play an important role in this phenomenon. Our results demonstrate that there is a correlation between RTK-induced cell scattering and sustained ERK2 activation, in that EGF (Ret activation) and bFGF induced cell scattering and sustained ERK2 activation, while PDGF treatment did not result in scattering and led to rapid, transient ERK2 activation. Therefore, it is tempting to speculate that sustained ERK2 activation is involved in the scattering response and possibly in EMT *in vivo* as well.

Recently, Sachs *et al*. (1996) demonstrated that several RTKs induced scattering in Madin-Darby canine kidney (MDCK) epithelial cells. Wild-type trkA receptors and chimeric receptors consisting of the extracellular domain of trkA and the kinase domains of c-*neu* (c-*erb*B2), c-*ros*, c-*met* and KGFR (Keratinocyte growth factor receptor) induced dissociation and scattering of these cells in response to NGF. Mutational analysis of tyrosine residues in the trkA receptor revealed that mutation of the Shc binding site, but not the binding sites for PI-3-kinase or PLC-γ prevented scattering upon addition of NGF (Sachs *et al*., 1996). In addition, SF/HGF-induced cell scattering in MDCK cells requires p21ras activation, since cotransfection of dominant negative p21ras^{ASN17} or microinjection of p21ras neutralizing antibodies abolished scattering (Hartmann *et al*., 1994; Ridley *et al*., 1995). Based on these and several other observations Sachs *et al*. (1996) suggested that cell dissociation and scattering of epithelial cells requires Shc activation and possibly recruitment of Grb2 that activates the p21ras-ERK pathway (Pronk and Bos, 1994; Pawson, 1995). We have previously shown that activation of Ret in SK-N-MC cells leads to autophosphorylation of the receptor and tyrosine phosphorylation of Shc and subsequent association of Shc with Grb2-Sos resulting in activation of the p21ras-ERK signalling cascade (van Weering *et al*., 1995), consistent with the idea that activation of the p21ras-ERK pathway is involved in cell scattering.

Recently, Ridley *et al*. (1995) demonstrated that, although p21ras activity contributes to the SF/HGF induced scattering response in MDCK cells, scattering requires one or more additional signals. The time delay (4–6 h) between addition of SF/HGF and scattering suggests that the signal could involve new gene transcription. In addition, it has been demonstrated that sustained ERK activation leads to translocation of

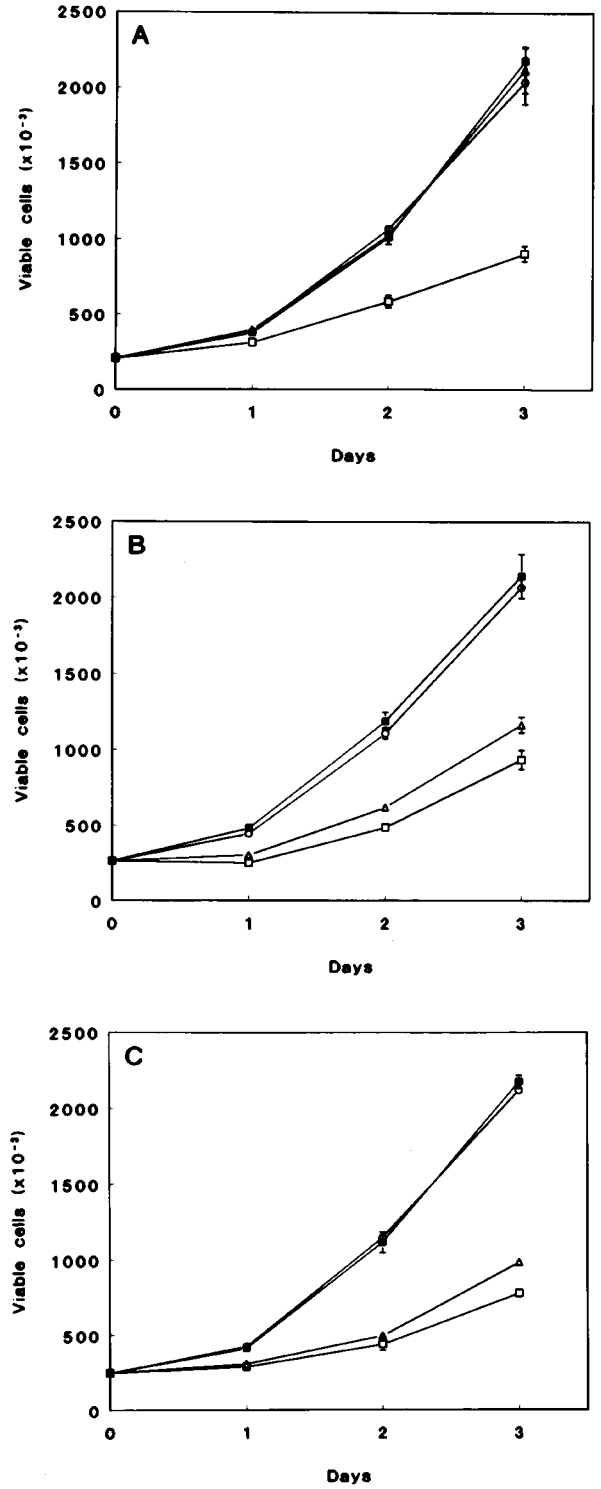

Figure 9 Growth inhibition of HERRet expressing cells by EGF and bFGF, but not PDGF. (**A**) Proliferation of SK-N-MC is inhibited by bFGF, but not by EGF and PDGF. SK-N-MC cells were seeded at a density of 1.5×10^5/well in 6-well plates in DF-medium supplemented with 10% FCS. After 24 h of incubation (day 0) three wells per plate were left untreated (■) or treated with EGF (△; 50 ng/ml), bFGF (□; 10 ng/ml) and PDGF (○; 20 ng/ml) as indicated. After a further 24 h of incubation the number of viable adherent and nonadherent cells was determined by trypan blue exclusion (day 1). This procedure was repeated every 24 h for the next three days. Data represent the mean ± SEM of three cultures for each treatment. Proliferation of SKF5 (**B**) and SKF6 (**C**) cells is inhibited by EGF and bFGF, but not by PDGF. Cells were left untreated (■) or treated with EGF (△; 50 ng/ml), bFGF (□; 10 ng/ml) and PDGF (○; 20 ng/ml) as described above

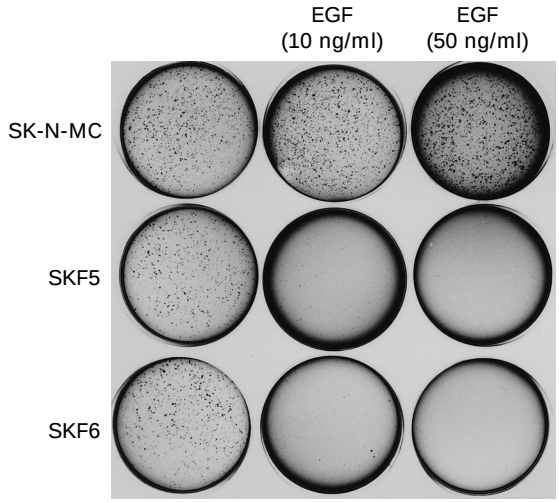

Figure 10 Anchorage-independent growth of HERRet expressing cells is inhibited by EGF. To test anchorage-independent growth, SK-N-MC, SKF5 and SKF6 cells (1.0×10^3 cells/cm^2) were plated on 50 mm petri dishes in DF-medium supplemented with 10% FCS in 0.375% agar onto a basal layer of 0.5% agar. Colony formation was tested in the absence (−) or presence of EGF (10 or 50 ng/ml) as indicated. After 10–14 days, the cells were fixed and stained with trypan blue solution

ERKs to the nucleas where they can subsequently phosphorylate transcription factors resulting in gene transcription (Hill and Treisman, 1995; Marshall, 1995). Our results reveal that EGF and bFGF but not PDGF induced scattering in SKF5 and SKF6 cells that became apparent approximately 6 h after addition of these growth factors. Consistent with the findings described above, we demonstrated that EGF and bFGF, but not PDGF induced transactivation of SRE-containing reporter constructs. In addition, we showed that Ret activation rapidly induced the expression of specific genes, such as *egr*-1, c-*fos* and c-*jun*. In line, oncogenic *RET*/PTC1 was recently shown to activate the *NGFI-A* (*egr-1*) promoter in PC12 cells (Califano *et al*., 1995). Further studies unravelling the identity of newly transcribed genes should provide information on the role of these genes in the scattering response. These genes could encode proteins regulating cell-cell contacts or proteases degrading the extracellular matrix, thereby regulating the scattering response.

The scatter effect in HERRet expressing SK-N-MC cells in response to EGF and bFGF is accompanied by growth inhibition. The reduced growth rate and the altered morphology suggest that Ret activation exerted a differentiative effect on SK-N-MC cells. In agreement with this notion, we showed that the growth in soft agar, an *in vitro* correlate of tumorigenicity, of the HERRet expressing cells was almost completely inhibited in the presence of EGF and bFGF. Although migration is often associated with metastatic tumor spread, our results suggest that Ret activation in SK-N-MC cells may result in the acquisition of a more differentiated phenotype. Consistent with these findings, oncogenic isoforms of Ret have recently been shown to induce the differentiation of human SK-N-BE neuroblastoma cells (D'Alessio *et al*., 1995).

Several lines of evidence suggest that the duration of ERK activation determines long-term cellular re-

sponses (reviewed by Marshall, 1995). For example treatment of PC12 cells with NGF or FGF leads to sustained ERK activation resulting in neurite outgrowth and eventually cessation of proliferation, whereas treatment with EGF leads to transient ERK activation resulting in proliferation. We demonstrate that sustained ERK2 activation correlates well with growth inhibition of SK-N-MC neuroepithelioma cells, which may be linked to the acquisition of a more differentiated phenotype. In addition, it is evident from our studies that treatment of these cells with stimuli that induce sustained ERK2 activation results in cell scattering. Therefore, it is tempting to speculate that sustained ERK2 activation is involved in this response. However, sustained ERK activation is not necessarily sufficient to induce scattering.

In summary, we have demonstrated that Ret and FGFR activation in SK-N-MC neuroepithelial cells resulted in sustained ERK2 activation leading to SRE-mediated transactivation correlating with cell scattering and growth inhibition, whereas PDGF treatment of these cells resulted in transient ERK activation, but not in SRE-mediated transactivation, cell scattering or growth inhibition. Our results suggest that sustained ERK activation may not only be involved in cellular differentiation, but also in cell scattering. Future experiments will lead to better insights into the role of ERKs in motility responses.

Materials and methods

Antibodies and plasmids

Purified EGF, recombinant bovine bFGF and recombinant human PDGF were purchased from Biomedical Technologies Inc., Boehringer Mannheim and Pepro Tech, respectively. Rabbit polyclonal antibodies directed against Ret (Ret99; van Weering *et al*., 1995) and ERK2 (Pronk *et al*., 1993) were described elsewhere. The anti-phosphotyrosine monoclonal antibody PY20 was purchased from Transduction Laboratories. pSV2LacZ (Shen *et al*., 1993), tkCAT (pBLCAT2; Luckow and Schultz, 1987) and 3xSREtkCAT (de Groot *et al*., 1990) have been described.

Cell culture and transient transfections

The human neuroepithelioma SK-N-MC and SKF5/F6 (human EGFR/Ret chimeric receptor stably transfected SK-N-MC cells, van Weering *et al*., 1995) cell lines were maintained as monolayer cultures in a 1:1 mixture of DMEM and Ham's F12 (DF-medium) bicarbonate-buffered (44 mM) medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), in a 7.5% CO_2 humidified atmosphere at 37°C. Cell lines were routinely passaged 3 times a week.

For transient transfection experiments, cells were split 1:10 in 6-well plates (Nunc, Denmark), and 24 h later, the cells were transfected by the calcium phosphate co-precipitation method (Graham and van der Eb, 1973) with a total of 10 μg CsCl-purified DNA, consisting of 6 μg CAT reporter construct together with 4 μg pSV2LacZ as reference plasmid to determine transfection efficiency. Approximately 16–20 h after transfection the medium was refreshed and after an additional 6–8 h the cells were serum-starved overnight in DF-medium supplemented with 0.5% (v/v) FCS. After a 6–8 h stimulation period with different growth factors, as indicated in the Figure legends, the cells were harvested in 0.5 ml PBS, centrifuged at 10 000 r.p.m. for 5 min, and resuspended in 75 μl 250 mM Tris/25 mM EDTA. After three cycles of freeze-thawing followed by centrifugation at 13 000 r.p.m. for 10 min, the supernatants were assayed for CAT activity (Gorman *et al*., 1982) after being normalized for β-galactosidase activity (Pfahl *et al*., 1990).

β-galactosidase activity (LacZ) and CAT assays

LacZ activity was determined by incubating aliquots of CAT-extracts (20 μl) together with 130 μl 100 mM phosphate buffer (pH 7.8) and 90 μl LacZ buffer (30 mM $Na-PO_4$ (pH 7.0), 3 mM KCl, 0.3 mM $MgSO_4$, 15 mM β-mercaptoethanol and 20 μg orthonitrophenyl-β-galactopyranoside (Sigma)) in 96-well microtiter plates. After 5–60 min β-galactosidase activity was determined by measuring the absorbance at 412 nm in a microplate reader (BioRad, Model 3550).

CAT assays were performed by incubating LacZ-corrected supernatants with 125 μl reaction mixture consisting of 50 μl H_2O, 54 μl Tris-HCl (pH 7.5)/EDTA (250 mM/25 mM), 20 μl 4 mM acetyl coenzymeA (Sigma) and 1 μl of [^{14}C]chloramphenicol (0.025 μCi; 1 Ci = 37 GBq; Amersham) for 1–2 h at 37°C. Subsequently, the samples were ethylacetate-extracted, subjected to thin-layer chromatography and exposed to PhosphorImager screens. CAT activity was determined by quantitation of the radioactive spots of [^{14}C]chloramphenicol and its acetylated forms using ImageQuant software (Molecular Dynamics).

Immunoprecipitation and Western blotting

Cells were grown to subconfluency in 60 cm^2 dishes (Nunc) after which the cells were serum-starved for 36 h. During this period the cells were stimulated with EGF (50 ng/ml) for the indicated time periods. Subsequently, the cells were washed twice with ice cold PBS0 (PBS without calcium and magnesium) and lysed in 750 μl RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA 0.5% (w/v) deoxycholic acid, 1% (v/v) NP-40, 0.1% (w/v) SDS, 0.1 μM aprotinin, 1 μM leupeptin, 1 mM orthovanadate, 1 mM PMSF). Lysates were centrifuged for 8 min 13 000 r.p.m. at 4°C to remove cellular debris. For immunoprecipitation of Ret, cleared lysates were incubated with Ret polyclonal antibody for 2 h at 4°C followed by the addition of protein A sepharose (Pharmacia) (2 h at 4°C). Protein-loaded beads were washed three times with RIPA lysis-buffer after which the precipitated proteins were eluted in Laemmli sample buffer, heated for 5 min at 95°C and loaded on 7.5% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P membranes (Amersham) and immunoblotted with the primary antibody for 1 h followed by the appropriate secondary antibody for 1 h. Blots were developed utilizing Enhanced ChemiLuminescence (ECL, Amersham). For reprobing, the blots were stripped with 2% (w/v) SDS, 100 mM β-mercaptoethanol and 62.5 mM Tris, pH 6.7 for at least 30 min at 65°C. Blots were reincubated with the appropriate primary antibodies and developed as described above.

MAP kinase mobility shift assay

Subconfluent cultures of SK-N-MC and SKF5/6 cells grown in 6-well plates (Nunc) were serum-starved overnight (0.5% (v/v) FCS) and stimulated. After stimulation, the cells were washed twice with ice-cold PBS, immediately lysed in Laemmli buffer and heated for 5 min at 95°C. Equal amounts of total cell lysates were resolved on 12.5% SDS–polyacrylamide gels. Proteins were transferred to Immobilon-P-membranes (Amersham) and immunoblotted with polyclonal ERK2 antisera according to standard procedures (as described above). Blots were developed utilizing ECL.

Northern blot analysis

Total RNA was prepared using a guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). RNA was separated on a 0.8% (w/v) agarose/2.2 M formaldehyde gels, transferred to hybond C-extra membranes (Amersham) and baked at 80°C for 2 h. To check whether equal amounts of RNA were loaded per lane, RNA was visualized by u.v. illumination after ethidium-bromide staining. The blots were prehybridized overnight at 42°C in $5 \times$SSC, $2 \times$Denhardts solution, 50 mM NaH_2PO_4 (pH 6.8), 100 mM EDTA, 0.1% (w/v) SDS 50% (v/v) formamide and 0.1 mg/ml salmon sperm DNA, and then hybridized overnight with multiprime random [α-^{32}P]dCTP-labelled (rediprime labelkit, Amersham) cDNA probes at 42°C. The following restriction fragments were used as probes: a 1.0 kb PstI mouse genomic fragment homologous to v-*jun* for c-*jun*; a 0.8 kb *Pst*I fragment of v-*fos* for c-*fos*; a 1.6 kb *Bgl*II fragment of mouse *zif/268* for *egr-1*. Subsequently, the blots were washed twice with $2 \times$SSC, 0.1% (w/v) SDS for 20 min at room temperature (RT) and twice with $0.1 \times$SCC, 0.1% (w/v) SDS and exposed to Kodak X-OMAT-AR X-ray films.

Determination of cell viability

Six-well plates were seeded with 1.5×10^5 cells/well. After 24 h of incubation the number of adherent and non-adherent cells was determined (day 0) by trypan blue stain (Flow Laboratories) exclusion using a Burker cell counting chamber. EGF, bFGF or PDGF was then added to the remaining plates to three wells per plate with three wells as controls. After a further 24 h of incubation the number of adherent and nonadherent cells was determined in triplicate (day 1). This procedure was repeated every 24 h for the next 3 days.

[^{3}H]Thymidine incorporation assay

Cells were plated at a density of 2.5×10^4/cm^2 in 24-well plates (Nunc, Denmark) in 1 ml bicarbonate-buffered DF-medium supplemented with 10% (v/v) FCS. After 48 h the growth factors to be tested were added to the medium. To monitor DNA synthesis, 50 μl serum-free DF-medium containing [^{3}H]thymidine (1.0 μCi, Amersham) was added to the cells for the last 18 h of a 48 h incubation with growth factors. Subsequently, the medium was gently poured off the cells and the cells were fixed by incubating the cells for 30 min in 1 ml 10% TCA at 4°C. The cells were washed twice with 0.5 ml 5% TCA, dried to the air and solubilized by a 30 min incubation in 0.5 ml 0.5 M NaOH at 37°C. Determination of the radioactivity incorporated into the DNA was performed by liquid scintillation counting by adding 3.5 ml of a liquid scintillation cocktail (Lumac-Lsc, The Netherlands).

Soft agar colony growth assay

To test anchorage-independent growth, 1×10^4 cells were plated in a 20 cm^2 tissue culture grade Petri dishes (Nunc) in 2 ml 0.375% (w/v) agarose medium containing DF-medium supplemented with 10% (v/v) FCS onto a basal layer of 0.5% (w/v) agar. Colony formation was tested in the absence or presence of EGF, FGF and PDGF. Visible colonies comprising >10 cells were scored counting 20 random fields, corresponding to 2.3% of the total dish area, after 10–14 days. Subsequently, the cells were fixed with 2% (w/v) paraformaldehyde overnight at 4°C, washed twice with distilled water and stained with 0.04% trypan blue in PBS (Flow Laboratories) overnight at 4°C.

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