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Specificity of Receptor Tyrosine Kinase Signaling: Transient versus Sustained Extracellular Signal-Regulated Kinase Activation

Review

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Summary

A number of different intracellular signaling pathways have been shown to be activated by receptor tyrosine kinases. These activation events include the phosphoinositide 3-kinase, 70 kDa S6 kinase, mitogen-activated protein kinase (MAPK), phospholipase C-γ, and the Jak/STAT pathways. The precise role of each of these pathways in cell signaling remains to be resolved, but studies on the differentiation of mammalian PC12 cells in tissue culture and the genetics of cell fate determination in Drosophila and Caenorhabiditis suggest that the extracellular signal-regulated kinase (ERK-regulated) MAPK pathway may be sufficient for these cellular responses. Experiments with PC12 cells also suggest that the duration of ERK activation is critical for cell signaling decisions.

Introduction

Receptor tyrosine kinases are involved in signaling both cell proliferation and differentiation (reviewed by Schlessinger and Ullrich 1992). Their role in determining cell differentiation rather than proliferation is especially well exemplified from genetic studies in Caenorhabditis elegans and Drosophila melanogaster, in which signaling from the Let-23, sevenless, and torso receptors determines cell fate in the absence of proliferation (reviewed by Perrimon, 1993; Dickson and Hafen, 1994). For mammalian cells in tissue culture, the stimulation of quiescent fibroblasts into DNA synthesis and the differentiation of the PC12 chromaffin cell line into sympathetic neurones (Greene and Tischler, 1976) have been much-used experimental systems to investigate receptor tyrosine kinase signaling. A central issue in attempting to understand receptor tyrosine kinase signaling is whether different receptors activate different signal transduction pathways and whether there are distinct pathways for differentiation and proliferation. Numerous studies show that, depending on where it is expressed, the same receptor can signal proliferation or differentiation; for example, the fibroblast growth factor receptor signals differentiation in PC12 neuronal cells but in fibroblasts stimulates proliferation. Such observations indicate the critical importance of cell context in understanding signaling.

Extensive work has now elucidated the principles of signal transduction pathways from receptor tyrosine kinases. Following ligand binding, receptor dimerization, and autophosphorylation, Src homology 2 (SH2) domain–containing proteins are recruited to phosphorylated tyrosine residues on the receptor. These SH2 domain–containing proteins include the p85 components of the phosphoinositide 3-kinase (PI3-kinase) pathway; phospholipase C-γ in the protein kinase C pathway; Src family kinases; and p120–GAP, Shc, and Grb2 in the Ras pathway (reviewed by Schlessinger, 1994). In addition, receptor kinases are able to activate the p91STAT pathway (Fu and Zhang, 1993; Silvennoinen et al., 1993; Sadowski et al., 1993). Recruitment to phosphorylated tyrosine residues on receptors leads to activation of the signaling molecule by a variety of mechanisms: tyrosine phosphorylation in the case of phospholipase C-γ and STATs (Sadowski et al., 1993); conformational changes induced by binding of the SH2 domain to phosphotyrosine for PI3-kinases (Backer et al., 1992; Carpenter et al., 1993) and SH-PTP2 tyrosine phosphatase (Lechleider et al., 1993); and translocation to the plasma membrane for stimulation of Ras guanine nucleotide exchange by Sos (Quilliam et al., 1994; Aronheim et al., 1994).

Attempts to Determine Critical Signaling Events

The issue regarding which of these signaling components are needed for cell proliferation or differentiation has been a much-studied area. Numerous attempts have been made to delineate the importance of a particular signaling component or receptor phosphotyrosine by constructing mutant receptors that lack individual tyrosine residues and therefore cannot recruit SH2-containing proteins to those sites. With a few exceptions, e.g., Coughlin et al. (1989), experiments with mutant receptors tend to show that deletion of any one single site does not compromise the stimulation of DNA synthesis. Such results suggest that there are either parallel signal transduction pathways, each of which can signal DNA synthesis, or that there is redundancy in signaling. One example of redundancy is clearly exemplified by the experiments of Valius and Kazlauskas (1993), which showed that either of two tyrosine phosphorylation sites in the platelet-derived growth factor receptor is sufficient for activation of $p21^{Ras}$ and stimulation of DNA synthesis. What is more difficult to conclude from such experiments is whether a known SH2-containing protein recruited to a particular site is essential to signaling since it is always possible to argue that there is another unknown protein recruited to the same site that is the critical signaling intermediate.

A different way to approach the question of critical signaling pathways comes from genetic studies. Strikingly for the Let-23, sevenless, and torso signaling pathways, defects in ligand or receptor can be compensated for by gain-of-function alleles of Ras (Fortini et al., 1992), Raf (Dickson et al., 1992; Han et al., 1994), MEK (Tsuda et al., 1993), or extracellular signal–regulated kinase (ERK) (Brunner et al., 1994b), all of which lie in the same signaling pathway (see below). Such results are perhaps not so surprising with Ras or Raf for which it has been known for some time that oncogenic forms can liberate signal trans-

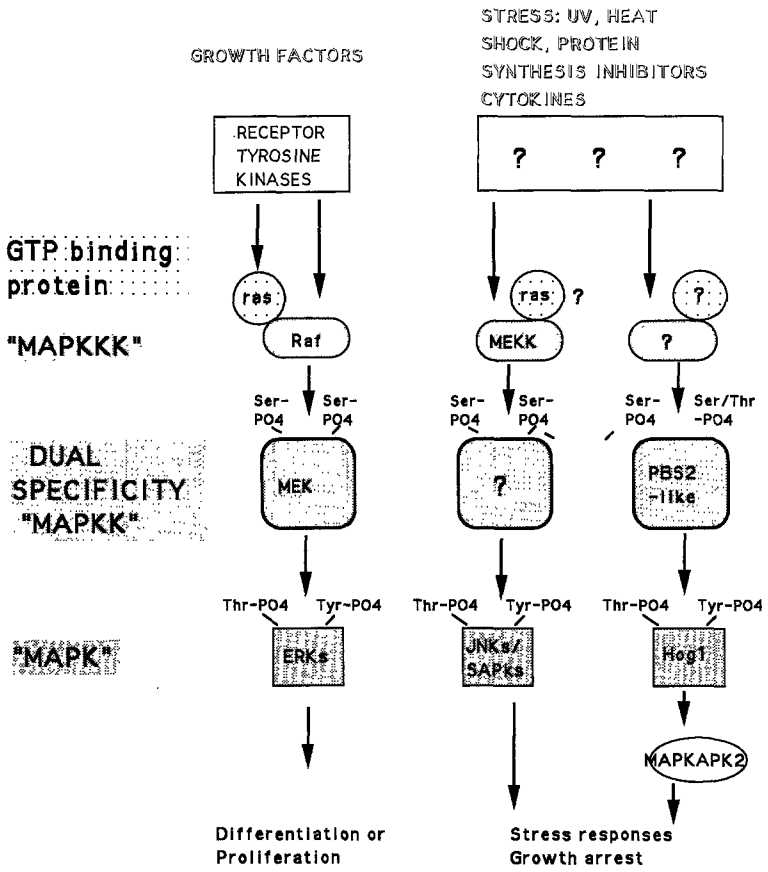

Figure 1. MAPK Pathways in Mammalian Cells

Both the receptor tyrosine kinase pathways and the stress response pathways contain a central core of a serine/threonine kinase, MAPKKK; a dual-specificity kinase, MAPKK; and a serine/threonine kinase, MAPK. Receptor tyrosine kinase signaling ocurrs through $p21^{Ras}$, and there is evidence that some stress responses, e.g., to ultraviolet radiation (UV), may also involve $p21^{Ras}$ (Hibi et al., 1993). Although MEKK-1 activation appears to be Ras-dependent (Lange-Carter and Johnson, 1994), there is no evidence yet for a direct interaction between Ras and MEKK-1.

Note also that not all stimuli leading to Raf activation may be mediated by Ras (Howe et al., 1992; Fabian et al., 1994).

Abbreviations: JNK, Jun kinase; MAPKAPK2, MAPK-activated protein kinase 2; PBS2, a dual-specificity protein kinase in the S. cerevisiae osmotic regulation pathway; PO4, phosphate group; SAPks, stress-activated protein kinases; and SEK1, appears to be a dual-specificity protein kinase (MAPKK), which activates the JNKs/SAPKs (Sanchez et al., 1994).

duction from extracellular signals. Injection of oncogenic Ras into quiescent fibroblasts stimulates DNA synthesis in the absence of mitogenic growth factors (Ferasmisco et al., 1984; Morris et al., 1989). Oncogenic Ras and Raf will also mimic the effect of nerve growth factor (NGF) in stimulating neurite outgrowth in PC12 cells (Bar-Sagi and Ferasmico, 1985; Noda et al., 1985; Wood et al., 1993). It now appears that Ras may have at least two effectors, Raf and PI3-kinase (Rodriguez-Viciana et al., 1994), and activated Ras would therefore be expected to be able to exert a multiplicity of effects. However, it is more surprising that gain-of-function mutants of MEK and ERK overcome receptor defects since they lie on a single signal transduction pathway downstream of Ras and Raf and would be expected to have more restricted effects. The conclusion that activation of the ERK pathway is sufficient is strengthened by the finding that expression of constitutively activated forms of MEK, generated by site-directed mutagenesis, induces mitogenesis and transformation in fibroblasts as well as differentiation of PC12 cells (Cowley et al., 1994; Mansour et al., 1994).

These results focus attention on the activation of the ERK family of mitogen-activated protein kinases (MAPKs) as a critical event in signal transduction from receptor tyrosine kinases. We will therefore consider some of the aspects of the control of this pathway and a model for how both differentiation and proliferation can be signaled by activation of this pathway.

The Ras/Raf/MEK/ERK Pathway

In the Ras/Raf/MEK/ERK pathway, a small guanine nucleotide-binding protein links receptor tyrosine kinase activation to a cytosolic protein kinase cascade. Central to the activation of this pathway is the activation of Ras to the GTP form through the promotion of guanine nucleotide exchange on Ras. This occurs through the complex of the exchange factor Sos and the adaptor protein Grb2 being recruited to tyrosine-phosphorylated receptors or through Shc–Grb2–Sos complexes (reviewed by Schlessinger, 1994).

The Ras/Raf/MEK/ERK pathway is one example of what are generically termed "MAPK" pathways. MAPK pathways have as their "core" a three-component protein kinase cascade consisting of a serine/threonine protein kinase (MAPKKK), which phosphorylates and activates a dual-specificity protein kinase (MAPKK), which in turn phosphorylates and activates another serine/threonine protein kinase (MAPK) (Figure 1). In the Ras/Raf/MEK/ERK pathway, Raf corresponds to MAPKKK, MEK corresponds to MAPKK, and ERK corresponds to MAPK. These pathways serve to link signals from the cell surface to cytoplasmic and nuclear events. In addition to the receptor tyrosine kinase–coupled Ras/Raf/MEK/ERK pathway, MAPK pathways mediate cell shape, osmotic integrity, and pheromone responses in yeasts (reviewed by Ammerer, 1994; Herskowitz, 1995 [this issue of *Cell*]), stress responses in mammalian cells (Han et al., 1994; Galcheva-

Gargova et al., 1994; Rouse et al., 1994), and cytokine signaling (Freshney et al., 1994) as well as the receptor tyrosine kinase–coupled Ras/Raf/MEK/ERK pathway. It is now emerging that there is a second MAPK pathway in mammalian cells that involves $p21^{Ras}$; this pathway mediates some of the signals that result in the N-terminal phosphorylation of Jun (Figure 1). It involves Ras, MEKK-1, a dual-specificity kinase (SEK1), and the MAPK Jun kinase (JNKs/SAPKs) (Hibi et al., 1993; Minden et al., 1994; Sanchez et al., 1994; Yan et al., 1994).

As far as is known at present from experimental data and sequence comparisons, the mechanisms of activation of the MAPKKs and MAPKs are likely to be identical or very similar in all MAPK pathways. Activation of MEK has been shown to result from phosphorylation by Raf of two serine residues that are four amino acids apart in kinase subdomain VIII (Alessi et al., 1994; Zheng and Guan, 1994). Two identically positioned serine residues or serine and threonine residues within a consensus motif, LID/NSXANS/T, are found in all members of the MAPKK family thus far sequenced (for a recent compilation of MAPKK sequences, see Banuett and Herskowitz, 1994). Substitution of the serine or threonine residues in this consensus with acidic amino acids to mimic phosphorylation leads to partial activation in MEK (Alessi et al., 1994; Mansour et al., 1994). The conservation of the putative phosphorylation sites means that it is very likely that all dual-specificity MAPKKs are regulated in the same way and that their activation can be mimicked by substituting negatively charged amino acids at these sites. All MAPKs contain a TXY motif in kinase subdomain VIII, the phosphorylation of which on threonine and tyrosine is essential for activity.

While identical phosphorylation events are likely to be responsible for the activation of MAPKKs and MAPKs, there appear to be multiple mechanisms for regulating MAPKKKs. For the Schizosaccharomyces pombe pheromone response pathway, there is an obligate requirement for direct interaction between active RasGTP and MAPKKK/byr2 (Van Aelst et al., 1993). However, in the Saccharomyces cerevisiae pheromone MAPK pathways, there does not appear to be a role for Ras in activating MAPKKK/STE11; instead, a kinase STE20 is involved (Leberer et al., 1992). Similarly, in the S. cerevisiae cell wall pathway, the MAPKKK/BCK1 is activated by a protein kinase C homolog, PKC1 (Lee et al., 1993).

The activation of Raf by receptor tyrosine kinases requires $p21^{Ras}$. Recent work demonstrates that the role of Ras is to recruit Raf to the plasma membrane (Leevers et al., 1994; Stokoe et al., 1994), where another tyrosine kinase–generated signal fully activates the membrane-bound Raf (Williams et al., 1992; Leevers et al., 1994; Fabian et al., 1994). Thus, tyrosine kinases generate two signals that interact to activate Raf; one signal is the formation of RasGTP, while the other is unknown. Why two signals should be required to fully activate Raf is a puzzle, but one rationalization is that two signals are used because Ras has multiple effectors. It is now clear that Ras is required for the Raf/Mek/ERK pathway, for PI3-kinase activation (Rodriguez-Viciana et al., 1994), and for a MAPK pathway involving MEKK-1 and the Jun kinases (Lange-Carter and Johnson, 1994; Minden et al., 1994). The second signal would then be needed to acheive specificity, otherwise every signal that activates Ras would activate all effector pathways.

Although there is much still to learn about the roles of each component in MAPK pathways, at the present time it seems that MAPK is essentially the effector of each pathway. The role of MAPKKK may be solely to phosphorylate and activate MAPKK, and the only role of MAPKK is to phosphorylate and activate MAPK. However, MAPK may have multiple substrates and therefore can set in motion a very wide range of events. In the case of the Ras/Raf/MEK/ERK pathway, the substrates of ERK1 and ERK2 include transcription factors and other kinases (reviewed by Blenis, 1993; Treisman, 1994; Hill and Treisman, 1995 [this issue of *Cell*]). Genetic evidence from D. melanogaster strongly suggests that transcription factors are the targets of receptor tyrosine kinase–mediated activation of ERKs. Phosphorylation of these transcription factors may relieve an inhibitory effect in the case of yan (Lai and Rubin, 1992) or lead to activation in the case of pointed (Brunner et al., 1994a).

In PC12 Cells, Cellular Responses Are Determined by the Duration of ERK Activation

The response of PC12 cells to receptor tyrosine kinase activation has been extensively used as an experimental system to study signal transduction and how activation of some receptors leads to differentiation while the activation of others leads to proliferation. Treatment of PC12 cells with fibroblast growth factor or NGF leads to outgrowth of neurites and eventual cessation of cell division (Greene and Tischler, 1976), whereas treatment with epidermal growth factor (EGF) leads to a proliferative signal (Huff et al., 1981). An expectation of these studies was the identification of differentiation-specific pathways. One example has been found in the NGF, but not the EGF, stimulation of tyrosine phosphorylation of an 80 kDa protein, SNT (Rabin et al., 1993). At the qualitative level, many signal transduction events seem to be shared between differentiation and proliferation in PC12 cells (Chao, 1992). However, notable quantitative differences are found between differentiation and proliferative signals. NGF stimulation results in a persistent elevation of RasGTP, whereas EGF produces only a short-lived rise in RasGTP (Muroya et al., 1992). ERK (MAPK) activation is sustained for several hours following NGF stimulation, but it is short lived after EGF stimulation (Heasley and Johnson, 1992; Traverse et al., 1992; Nguyen et al., 1993). In part, these differences may reflect the differences in receptor down-regulation of the EGF receptor and TrkA. Another difference between the two pathways is that EGF and NGF utilize different signal transduction pathways to stimulate RasGTP: the Grb2–Sos complex is directly bound to the EGF receptor, whereas TrkA stimulates Shc phosphorylation and the formation of Shc–Grb2–Sos complexes without direct binding of Grb2–Sos to TrkA (Buday and Downward, 1993; Obermeier et al., 1994; Stephens et al., 1994).

The association of prolonged ERK activation with NGF stimulation of PC12 cells has led to the idea that it is sus-

tained activation of this pathway that leads to differentiation. This is consistent with the findings that both oncogenic Ras and oncogenic Raf can stimulate neurite outgrowth (Noda et al., 1985; Wood et al., 1993), since both of these oncoproteins would be expected to produce prolonged activation of ERKs (Leevers and Marshall, 1992). Two different types of experiments lend support to the idea that there is no receptor-specific pathway of differentiation and that sustained activation of ERKs is sufficient for PC12 differentiation. In the first set of experiments, the effects of heterologous expression of receptors and alterations in endogenous receptor number have been examined. PC12 cells do not express the platelet-derived growth factor receptor, but transfection of platelet-derived growth factor receptor constructs leads to PDGF-dependent differentiation and sustained activation of ERKs (Heasley and Johnson, 1992). The endogenous insulin receptor of PC12 cells does not trigger RasGTP loading, ERK activation, or differentiation (Ohmichi et al., 1993); however, if it is overexpressed, ERK is activated and differentiation results (Dikic et al., 1994). While stimulation of the endogenous EGF receptor does not lead to differentiation, overexpression leads to EGF-dependent differentiation and sustained activation of ERKs (Traverse et al., 1994). Since the EGF receptor is more rapidly downregulated than the NGF receptor through internalization and phosphorylation (Countaway et al., 1992), these results suggest that it is the number of active cell surface receptors that determines whether ERK activation is sustained and differentiation results. An important corollary to receptor overexpression experiments comes from the converse approach. PC12 cell lines have been selected that do not respond to NGF as a differentiation signal. In these cells, the number of TrkA–NGF receptor molecules per cell is reduced, ERK activation is transient, and the response to NGF is proliferation (Schlessinger and Bar-Sagi, 1995). In every situation examined thus far, manipulating receptor numbers to produce differentation correlates with sustained activation of ERKs, whereas transient activation is not associated with differentiation.

The second set of experiments suggesting that ERK activation is the key event in PC12 differentiation is the introduction of MEK mutants. Appropriate mutations in the phosphorylation sites of MEK1 that are the sites of activation by Raf lead to activating and interfering mutants. The interfering mutants block ligand activation of ERK and block differentiation of PC12 cells, whereas the activating mutants induce neurite outgrowth in the absence of differentiating factors (Cowley et al., 1994). The effect of the activated MEK is direct rather than through the induction of growth factors that act back through cell surface receptors because induction of differentiation by activated MEK is not blocked by microinjection of Ras neutralizing antibodies, which block ligand-activated differentiation.

A Model

A model based on these experiments is one that shows cells can enact differentiation or proliferative responses to receptor tyrosine kinases purely on the basis of the duration of ERK activation. An attractive rationalization for

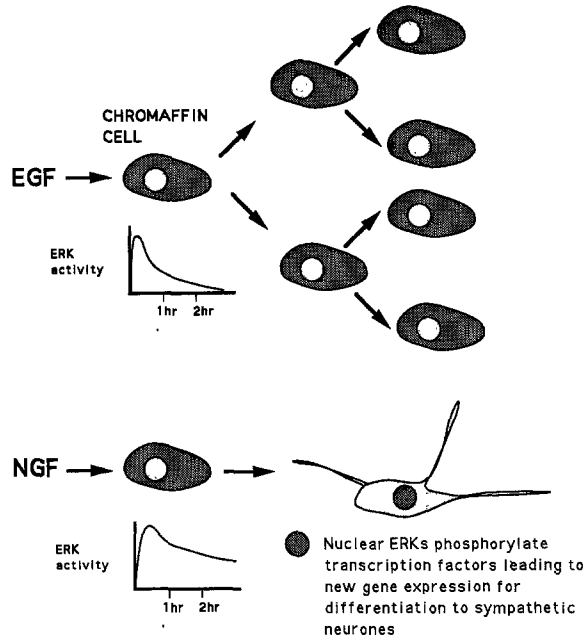

Figure 2. Sustained Activation Leads to Translocation of ERKs and the Induction of New Gene Expression

The relative amount of ERK in the cytoplasm and the nucleus is indicated by the level of stippling.

the manner by which sustained activation of ERKs can lead to a different cellular response than transient activation rests on the observation that ERKs can translocate to the nucleus upon activation (Chen et al., 1992). In every case examined thus far in PC12 cells, sustained ERK activation is associated with translocation of ERKs to the nucleus (Traverse et al., 1992, 1994; Nguyen et al., 1993; Dikic et al., 1994), whereas transient activation does not lead to nuclear translocation. Transient activation will therefore have very different consequences for gene expression compared with sustained activation because nuclear accumulation of active ERK will result in phosphorylation of transcription factors (Figure 2). In this way, the quantitative difference in ERK activation is translated into a qualitative difference in transcription factor activation. Implicit in this model is the idea that the cellular response is determined by which transcription factors are present in the cell. Thus, the activation of the receptor tyrosine kinase and the subsequent activation of ERKs is just the final switch that seals a fate determined by previous developmental events that set which ERK-responsive transcription factors are present in the cell. It is not a requirement of this model that sustained activation of ERKs invariably leads to differentiation, whereas transient activation always leads to proliferation. In other cell types, the converse may be true: it is clear that in fibroblasts, sustained activation of ERKs is associated with proliferation, not differentiation (Meloche et al., 1992; Mansour et al., 1994; Cowley et al., 1994). The important feature of the model is that cells can use transient and sustained activation of ERKs to determine different responses.

The idea of sustained versus transient ERK activation being critical for changes in gene expression can be

readily extended to threshold effects in development, in which small changes in ligand concentration resulting from developmental gradients produce qualitative differences in gene expression (Green et al., 1992). Small differences in ligand concentration could lead to sustained versus transient ERK activation and thereby could lead to nuclear translocation to alter gene expression (Dikic et al., 1994; Traverse et al., 1994).

There are potentially many different ways for receptors to signal transient versus sustained ERK activation. It has already been demonstrated that differences in receptor number markedly affect the duration of ERK activation. As discussed above, small changes in ligand concentration may have similar effects. The rate of internalization of receptors and whether they are down-regulated as a result of activation of the Ras/Raf/MEK/ERK pathway may also affect the duration of signaling. The EGF receptor may be more rapidly internalized than the NGF receptor and is subject to down-regulation through phosphorylation (Countway et al., 1992). In addition, different levels and kinetics of ERK activation could be generated by differential usage of signaling pathways downstream of receptors. The activation of Ras has multiple pathways, including direct receptor–Grb2–Sos complexes (Buday and Downward, 1993), receptor–Syp–Grb2–Sos complexes (Li et al., 1994), and Shc–Grb2–Sos complexes (Obermeier et al., 1994; Stephens et al., 1994). There may also be Ras- (Burgering et al., 1993; Howe et al., 1992) and Raf-independent (Lange-Carter and Johnson, 1994) routes to ERK activation. Each of these different signaling pathways could have different consequences for the level and duration of ERK activation and could be selectively used by different receptors to regulate the amplitude and duration of signaling.

A serious limitation in applying this model to other systems is that it is based wholly on experiments with PC12 cells. It is conceivable that these cells may represent a very abnormal system and that, in other cell types, there are qualitative differences in signaling events for proliferation versus differentiation. However, it is clear from studies in both C. elegans and D. melanogaster that the same components of receptor tyrosine kinase signaling that are involved in the decisions about cell fate are also involved in proliferation since defects in Ras, Raf, MEK, or ERK lead to reduced cell proliferation in embryos and are lethal (Dickson and Hafen, 1994; Tsuda et al., 1993; Perrimon, 1993).

Conclusions

The arguments put forward here propose that when cells make decisions about proliferation versus differentiation through receptor tyrosine kinase signaling, they do it by differences in the duration of ERK activation. Such considerations highlight the importance in development of restricted expression of receptors and especially their ligands (reviewed by Jessell and Melton, 1992). If the presence of growth factors and receptors was not limited in time and space, the activation of a pathway common to all receptor tyrosine kinases would be disastrous. In addition, prior developmental events limit which cells respond to ligand. At one level, this restriction could operate by limiting in which cells a transcription factor is expressed, but restrictions must also operate at other levels. In the D. melanogaster eye, only the R7 cells respond to activation of the sevenless receptor by the bride of sevenless ligand expressed on the R8 cell even though surrounding cells express the receptor and contact the ligand. Exactly where this restriction operates within the Ras/Raf/MEK/ERK pathway is an intriguing issue.

In the model presented here, receptor tyrosine kinases work in development as switches to complete a developmental program determined by previous developmental events. This means that the outcome of receptor tyrosine kinase signaling depends both on the duration of ERK activation and cell context. A critical aspect of cell context will be which ERK-responsive transcription factors are present.

This review has heavily emphasized the role of those receptor tyrosine kinase signaling events that result in ERK activation; the question then remains whether other signaling events contribute to differentiation or proliferation. For example, PI3-kinase activation by recruitment of p85 subunits to either the NGF receptor or the fibroblast growth factor receptor is not necessary for the differentiation response of PC12 cells and does not appear to affect ERK activation (Obermeier et al., 1994; Spivak-Kroizman et al., 1994). Signaling events that do not regulate ERK activation may mediate aspects of tyrosine kinase signaling such as ligand-stimulated cell survival, cytoskeletal rearrangements, cell migration, and chemotaxis (Ridley et al., 1992; Ridley and Hall, 1992). This is illustrated by the finding that activation of protein kinase C by the c-Kit receptor appears to promote cell motility rather than mitogenesis (Blume-Jensen et al., 1993). It is possible that much of the specificity in receptor signaling is for purposes other than controlling proliferation and differentiation.

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