c-Kit-Kinase Induces a Cascade of Protein Tyrosine Phosphorylation in Normal Human Melanocytes in Response to Mast Cell Growth Factor and Stimulates Mitogen-Activated Protein Kinase But is Down-Regulated in Melanomas

Yoko Funasaka,* Teri Boulton,† Melanie Cobb,‡ Yosef Yarden,‡
Baoling Fan,* Stewart D. Lyman,§ Douglas E. Williams,§
Dirk M. Anderson,§ Rina Zakut,|| Yutaka Mishima,¶
and Ruth Halaban*

*Department of Dermatology, Yale University School of Medicine, New Haven, Connecticut 06510;

†Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas

75235; ‡Department of Chemical Immunology, The Weizmann Institute, Rehovot 76100, Israel;

§Immunex Research and Development Corporation, Seattle, Washington 98103; ||National Cancer

Institute, Surgery Branch, Bethesda, Maryland 20892; and ¶Kobe University School of Medicine,

Department of Dermatology, Chuo-ku, Kobe, Japan

Submitted to Cell Regulation August 29, 1991; Accepted October 17, 1991

The proto-oncogene c-Kit, a transmembrane receptor tyrosine kinase, is an important regulator of cell growth whose constitutively active oncogenic counterpart, *v-kit*, induces sarcomas in cats. Mutations in murine *c-kit* that reduce the receptor tyrosine kinase activity cause deficiencies in the migration and proliferation of melanoblasts, hematopoietic stem cells, and primordial germ cells. We therefore investigated whether c-Kit regulates normal human melanocyte proliferation and plays a role in melanomas. We show that normal human melanocytes respond to mast cell growth factor (MGF), the Kit-ligand that stimulates phosphorylation of tyrosyl residues in c-Kit and induces sequential phosphorylation of tyrosyl residues in several other proteins. One of the phosphorylated intermediates in the signal transduction pathway was identified as an early response kinase (mitogen-activated protein [MAP] kinase). Dephosphorylation of a prominent 180-kDa protein suggests that MGF also activates a phosphotyrosine phosphatase.

In contrast, MGF did not induce proliferation, the cascade of protein phosphorylations, or MAP kinase activation in the majority of cells cultured from primary nodular and metastatic melanomas that grow independently of exogenous factors. In the five out of eight human melanoma lines expressing *c-kit* mRNAs, c-Kit was not constitutively activated. Therefore, although c-Kit-kinase is a potent growth regulator of normal human melanocytes, its activity is not positively associated with malignant transformation.

INTRODUCTION

The mitogenic peptides identified thus far that support human melanocytes in vitro are several members from

the fibroblast growth factor (FGF)¹ family, i.e., basic FGF (bFGF) (Halaban *et al.*, 1987), (Halaban, 1988,

¹ Abbreviations used: bFGF, basic fibroblast growth factor; dbcAMP, dibutyryl cyclic adenosine monophosphate; EGF, epidermal growth

1989), K-FGF/Hst-1 (Halaban *et al.*, 1988), FGF-5 and FGF-6 (Halaban, 1989; Halaban *et al.*, 1991), and the unrelated hepatocyte growth factor (HGF) (Matsumoto *et al.*, 1991; Rubin *et al.*, 1991). All are known to stimulate receptors with tyrosine kinase activity (Coughlin *et al.*, 1988; Friesel *et al.*, 1989; Lee *et al.*, 1989; Ruta *et al.*, 1989; Dionne *et al.*, 1990; Johnson *et al.*, 1990; Mansukhani *et al.*, 1990; Partanen *et al.*, 1990; Reid *et al.*, 1990; Safran *et al.*, 1990; Bottaro *et al.*, 1991; Naldini *et al.*, 1991). These factors act as mitogens only in synergy with stimulators of protein kinase A (PKA) or protein kinase C (PKC) (Halaban *et al.*, 1987, 1988, 1991; Halaban, 1988, 1989, 1991; Matsumoto *et al.*, 1991; Rubin *et al.*, 1991). FGF and HGF also act in synergy with each other (Matsumoto *et al.*, 1991; Halaban, Rubin, Funasaka, Cobb, Boulton, Faletto, Rosen, Chan, Moellmann, Yoko, and Cook, unpublished results). Recently, evidence for the importance of yet another receptor-tyrosine kinase, c-Kit (Yarden *et al.*, 1987; Qiu *et al.*, 1988), in pigment cell viability was provided by genetic analyses of mice with mutations at the dominant white spotting (*W*) and steel (*Sl*) loci, encoding *kit* and its ligand mast cell growth factor (MGF), respectively (MGF is also known as Kit-ligand, stem cell growth factor, and steel factor) (Anderson *et al.*, 1990a; Copeland *et al.*, 1990; Flanagan and Leder, 1990; Huang *et al.*, 1990; Martin *et al.*, 1990; Williams *et al.*, 1990; Zsebo *et al.*, 1990a,b). This analysis showed that deficiency in the receptor-tyrosine kinase or its ligand cause partial failure of melanocytes to populate the skin, resulting in white spotting (Chabot *et al.*, 1988; Geissler *et al.*, 1988; Nocka *et al.*, 1989, 1990; Reith *et al.*, 1990; Tan *et al.*, 1990; Brannan *et al.*, 1991; Flanagan *et al.*, 1991). The mutations in *W* and *Sl* reduce, in addition, the proliferation of cells from the hematopoietic and germ cell lineages, causing anemia and sterility. More recently, a mutation in the tyrosine kinase domain of *c-kit*, which results in substitution of glycine with arginine at codon 664, was observed in a proband with classic autosomal dominant piebaldism, suggesting that human melanocytes are also dependent on c-Kit-kinase for normal development (Giebel and Spritz, 1992).

c-kit is the cellular homolog of the oncogene *v-kit* of the HZ4-feline sarcoma virus (Yarden *et al.*, 1987; Qiu *et al.*, 1988). The oncogenic mutation in the viral gene causes constitutive activation of the receptor kinase due to a deletion of the extracellular domain (Qiu *et al.*, 1988). A constitutively phosphorylated c-Kit has been

observed also in a murine mastocytoma cell line that was not expressing MGF, suggesting that the transformed cells harbored a mutation that caused constitutive activation of c-Kit (Rottapel *et al.*, 1991). Moreover, a chimeric molecule composed of the extracellular portion of the epidermal growth factor receptor (EGF-R) and the transmembrane and cytoplasmic domains of *c-kit* was shown to confer an EGF-dependent transformed phenotype on NIH 3T3 cells (Lev *et al.*, 1990). Therefore, elucidation of the biochemical events induced by MGF in melanocytes may provide information on c-Kit substrates controlling proliferation and malignant transformation in other cell types as well. As expected, MGF is a mitogen for normal human melanocytes, but only in the presence of synergistic factors. The ligand stimulated the phosphorylation of c-Kit and induced phosphorylation and dephosphorylation of other proteins. One of the downstream targets was identified as an early response kinase (ERK) (mitogen-activated protein [MAP] kinase). Most of the cultured melanoma cells, on the other hand, did not respond to MGF in the cell proliferation assay and the receptor-kinase was down-regulated, probably as part of the dedifferentiation occurring during malignant transformation to melanomas.

METHODS

Cell Culture and Proliferation Assays

Normal human melanocytes were established from foreskins in culture and maintained in Ham's F-10 medium supplemented with 2.5% newborn calf and 2.5% calf serum, 85 nM 12-O-tetradecanoyl phorbol-13-acetate (TPA), 0.1 mM IBMX, 1.0 mM insulin, 1.0 μ M Na₂VO₄, and 0.1 mM ascorbic acid as described (Halaban *et al.*, 1987, 1988). Melanocytes at passage 2–6 were used for these studies. Melanoma cells designated WW 94 and YU were established by us from primary nodular and metastatic melanomas, respectively (Halaban, 1990; Halaban *et al.*, 1991). Melanoma cell line MeWo (Bean *et al.*, 1975) was obtained from Dr. N. Lassam, University of Toronto, Toronto, Canada; WM 235A and WM 9 (Balaban *et al.*, 1984; Herlyn *et al.*, 1985) from Dr. M. Herlyn, The Wistar Institute, PA; and SW1614 (Pathak *et al.*, 1983) from Dr. T.C. Hsu, The University of Texas System Cancer Center, Houston, TX. Melanoma lines 501 mel and 624 mel were from Dr. R. Zakut, National Cancer Institute, Bethesda, MD. All melanomas were grown in culture in Ham's F-10 medium plus serum as described (Halaban *et al.*, 1989) without the other supplements listed above. These melanoma cells acquired aberrant autocrine mode of growth and express little or no melanocytic functions, such as melanin production. Primary normal human fibroblasts were established from foreskins and grown in the same medium as the melanoma cells. The fibroblasts were used at passage 2–4.

Stimulation of melanocyte growth was assayed by determining [³H]thymidine incorporation as a measure of DNA synthesis and by counting the number of cells. To determine [³H]thymidine incorporation, melanocytes were seeded in 24-well cluster plates (~15 000 cells/well) in chemically defined PC-1 medium (Ventrex Laboratories, Inc., Portland, ME), with MGF in the indicated concentrations, in the presence and absence of 1.0 mM dbcAMP (dibutyl cyclic adenosine monophosphate), without any other additives. [³H]thymidine was incorporated during the last 3 h of a 24-h incubation period as described (Halaban *et al.*, 1987, 1988). Data are means of duplicate wells. To determine cell proliferation, melanocytes were seeded in 12-well cluster plates in PC-1 medium. Experimental medium, consisting of PC-1 medium plus indicated additions, was added on the following day

factor; EGF-R, epidermal growth factor receptor; ERK, extracellular-signal-regulated kinase; FGF, fibroblast growth factor; GAP, GTPase activating protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; MAP2, microtubule-associated protein 2; MGF, mast cell growth factor; PDGF, platelet-derived growth factor; PKA, protein kinase A; PKC, protein kinase C; PLC- γ 1, phospholipase C- γ 1; PTPase, phosphotyrosine phosphatase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPA, 12-O-tetradecanoyl phorbol-13-acetate.

(day 1) and renewed 3 d later. Melanocytes were counted on day 1 and day 5 with a Coulter counter (Coulter Electronics, Hialeah, FL).

Antibodies, Immunoblotting, and Immunoprecipitation

We used a monoclonal antibody against phosphotyrosine (Upstate Biotechnology, Inc., Lake Placid, NY) for Western immunoblotting and monoclonal antibody PY20 (ICN Biomedical, Irvine, CA) for immunoprecipitation of phosphotyrosine-containing proteins. To identify c-Kit and intermediates in signal transduction, we used antiserum 212C directed against the C-terminus of c-Kit (Yarden *et al.*, 1987) and antisera 691 and 837 directed against two different peptides highly conserved in ERK1 and ERK2 (Boulton and Cobb, 1991). Monoclonal antibodies 2A7 and 2B12 against p125 and p120, respectively, were obtained from Drs. J.T. Parsons, S.B. Kanner, and A.B. Reynolds, Department of Microbiology and Cancer Center, University of Virginia Health Sciences, Charlottesville, VA. Anti-GAP antibodies were a gift from Dr. F. McCormick, Cetus Co., Emmerville, CA.

For Western blotting, melanocytes were incubated overnight in PC-1 medium in 8-cm² Petri dishes, with no other additions, then stimulated with 75 U/ml MGF at 37°C for the indicated times and harvested in boiling sample buffer. Lysates were kept at 100°C for 1 min, sonicated, electrophoresed, and transferred to immobilon PVDF transfer membrane (Millipore, Bedford, MA). Membranes were then incubated in blocking buffer (10 mM Tris(hydroxymethyl)aminomethane, pH 7.4, 0.9% NaCl, 0.01% NaN₃, 5% bovine serum albumin, 1% ovalbumin), immunoblotted with monoclonal anti-phosphotyrosine antibody, exposed to 1 μ Ci/ml [¹²⁵I]protein A (ICN Biomedical), and autoradiographed at -75°C for 7–16 h. Each lane contains ~50 μ g protein. Phosphotyrosine competed with the binding of the antibodies to the membrane.

For immunoprecipitation, growth factor-deprived normal human melanocytes were stimulated for 10 min with MGF as described before and then harvested in 0.5 ml modified lysis buffer (Margolis *et al.*, 1989) containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, 100 mM sodium fluoride, 10 mM pyrophosphate, 200 μ M Na₃VO₄, 0.01% NaN₃, 1 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (protease and phosphatase inhibitors from Sigma Chemicals, St. Louis, MO). In some experiments, the cultures were incubated in PC-1 medium plus 1 or 10 μ M Na₃VO₄ for 1 h before lysis. Lysates were kept on ice for 15 min and centrifuged for 5 min at 15 000 rpm in a microcentrifuge at 4°C. The supernatants (containing 150–1800 μ g of cell-extract protein, or 4 $\times$ 10⁷ cpm of ³⁵S, or 3 $\times$ 10⁶ cpm of ³²P in trichloroacetic acid precipitable material) were shaken for 2 h on ice with 5–30 μ l anti-c-Kit antiserum and IgG-sorb (washed twice in boiling sodium dodecyl sulfate [SDS] and 2-mercaptoethanol as described by Stern and Kamps, 1988). The reaction mixtures were then centrifuged and the immunoreacted IgG-sorb was washed twice (for immunoblotting) or 10 times over a 2-d period (³⁵S) or 3 times (³²P) in HNTG (20 mM HEPES, pH 7.5, 10% glycerol, 0.1% Triton X-100, 150 mM NaCl) buffer (Margolis *et al.*, 1989) containing 0.01% sodium azide. The immune complexes were eluted with 60 μ l SDS sample buffer for 5 min at 100°C, fractionated in SDS-polyacrylamide gel electrophoresis (PAGE), and subjected to Western blotting. X-ray film was exposed to the membranes at -75°C for 2 h to 3 d. For metabolic labeling with ³⁵S and ³²P, melanocytes were incubated in medium free of methionine and cysteine or phosphate, respectively, for 2–3 h before the addition of 500 μ Ci/ml Trans³⁵S-label (ICN Radiochemicals, Inc, Costa mesa, CA) or inorganic ³²P (Amersham Corporation, Arlington Heights, IL) for 3 h or 15 min (for pulse-chase experiments) and, where indicated, chased in nonradioactive medium containing unlabeled methionine and cysteine. In the case of ³²P labeling, dried gels were first autoradiographed overnight, then rehydrated in freshly prepared 66 g 85% KOH per liter H₂O, and incubated further in the same solution for 2 h at 55°C,

followed by 3 h incubation in 10% glacial acetic acid/10% isopropanol solution at room temperature with four changes as described (Cooper *et al.*, 1983).

Assay for MAP Kinase Activity in Melanocyte Extracts

Preparation of extracts and assay for MAP2 phosphorylation by ERKs was as described by Boulton *et al.* (1991b). In brief, melanocytes were incubated in PC-1-defined medium and stimulated with MGF for 5 min. The medium was removed, the cells rinsed, and resuspended in MAP2 kinase lysis buffer. The lysates were centrifuged at 97 000 $\times$ g for 60 min to obtain a supernatant. MAP2 phosphorylation was assayed for 10 min at 30°C in the presence of 50 μ M ATP (1–50 cpm/fmol) with 3 μ g MAP2 substrate and 10 μ g sample protein, in the presence or absence of staurosporine (1.0 and 0.1 μ M). Reactions were terminated by the addition of five-fold concentrated electrophoresis sample buffer and analyzed by SDS-PAGE. MAP2 bands were excised and counted by liquid scintillation.

RNAs Analysis

Total RNA was isolated by the guanidium isothiocyanate method with a CsCl centrifugation step (Chirgwin *et al.*, 1979; Chomczynski and Sacchi 1987). The RNAase protection assay was performed as described (Ausubel *et al.*, 1991). The c-kit probe used was a radiolabeled 115-mer fragment generated by linearizing human c-kit cDNA cloned in Bluescript M13- with Acc 1, followed by transcription with T3 polymerase in the presence of [α -³²P]UTP. This domain at the 3' end is highly specific to c-kit (Yarden *et al.*, 1987). Another c-kit probe, a 465-mer fragment spanning the HindIII and EcoRI sequences at the 5' end, encoding the extracellular domain of c-Kit gave similar results. The MGF probe was an ~610-mer fragment, generated by linearizing human MGF cDNA cloned in Bluescript with EcoRI followed by transcription with T3 polymerase. The protected hybridization products were fractionated in 5% polyacrylamide gels, followed by autoradiography with Kodak (Rochester, NY) XAR film at -75°C.

RESULTS

MGF is Mitogenic Toward Normal Human Melanocytes in the Presence of Synergists

Tests for the mitogenic response of human melanocytes to MGF were conducted in the presence or absence of potentially synergistic factors. The results show that in chemically defined medium suitable for the proliferation of fibroblasts and melanoma cells, MGF, like the other melanocyte mitogens listed above, required addition of dbcAMP or TPA to the growth medium (Figure 1, A and B). There was no stimulation of proliferation by MGF alone or in conjunction with bFGF or HGF. In addition, three cytokines, Il-3, Il-8, and melanoma growth stimulating activity (Richmond *et al.*, 1988) did not have any stimulating activity, either by themselves or in synergy with MGF or any other of the melanocyte growth factors mentioned above.

MGF Induces Tyrosine Phosphorylation of c-Kit and Phosphorylation and Dephosphorylation of Other Melanocyte Proteins

The initial steps in signal transduction by MGF in melanocytes did not appear to require dbcAMP or TPA. The results were identical whether synergistic factors

Figure 1. Mitogenic response of normal human melanocytes to MGF. (A) [³H]thymidine incorporation in the presence of increasing concentrations of MGF with or without 1.0 mM dbcAMP. (B) Cell number in the presence of dbcAMP or TPA, with (+) or without (-) MGF. Histograms represent number of cells expressed as percentage of first day count (4×10^4 /well). MGF, 75 U/ml (stock solution 50 μ g/ml, 20 U/ μ l); dbcAMP, 1.0 mM; TPA, 85 nM.

needed for the mitogenic response were present or not. MGF alone induced the phosphorylation of p145^{c-kit} and affected the phosphorylation of several other proteins (Figures 2 and 3). A time-course analysis by electrophoresis in a 6.5% polyacrylamide gel showed that within 1 min of MGF addition, proteins of 145 kDa (p145), 125 kDa (p125), and 95 kDa (p95) became phosphorylated on tyrosyl residues, followed minutes

later by the phosphorylation of proteins of 55 kDa (p55) and 40 kDa (p40) (Figure 2A). A phosphoprotein of 180 kDa (p180) disappeared within 1–5 min, possibly through dephosphorylation or degradation. Although p145 maintained its phosphorylated state for ≥ 30 min, the other PY-proteins had a shorter half-life and returned to basal levels within 10–20 min (Figure 2A). Additional PY-proteins, of 100, 38, and 35 kDa, and possibly others were resolved in an 8% polyacrylamide gel (Figure 2B). Such a complex pattern of tyrosine phosphorylation and dephosphorylation was not seen in ligand-stimulated NIH 3T3 fibroblasts engineered to express a chimeric EGF-R-*kit* kinase (Lev *et al.*, 1991) or in murine mast cells (Rottapel *et al.*, 1991).

Phosphorylated p145 was identified as c-Kit by immunoprecipitation with antibodies to c-Kit (Yarden *et al.*, 1987), followed by SDS-PAGE and Western blotting with anti-PY, as well as by immunoprecipitation of ³²P-labeled cell extracts (Figure 3). The bands in the immunoblots were compared with bands from ³⁵S-radio-labeled proteins immunoprecipitated with anti-c-Kit. Anti-PY immunoblots and ³²P-immunoprecipitates revealed phosphorylated p145^{c-kit} in MGF-stimulated melanocytes (Figure 3, A, B, and D), but unexpectedly, low levels of phosphorylated c-Kit were also present in

Figure 2. Time course of tyrosine phosphorylation on melanocyte proteins in response to MGF. (A) Autoradiogram of a Western blot with anti-phosphotyrosine monoclonal antibody showing the sequential patterns of phosphorylation of several proteins after addition of MGF. Cell extracts were electrophoresed in 6.5% polyacrylamide gel. (B) As in A, except that melanocytes were stimulated for only 10 min with MGF and cell extracts were electrophoresed in 8% polyacrylamide gel (containing SDS). Molecular size markers ($M_r \times 10^3$) from top to bottom: myosin, phosphorylase B, bovine serum albumin, ovalbumin, and carbonic anhydrase (Bethesda Research Laboratory, Gaithersburg, MD).

Figure 3. Phosphorylation of c-Kit in response to MGF. Western blots of anti-PY-reactive proteins (^{125}I); fluorographs of [^{35}S]radiolabeled proteins (^{35}S); and autoradiograph of ^{32}P -labeled proteins (^{32}P), immunoprecipitated (ip) from extracts of deprived (–) or MGF-stimulated (75 U/ml, 10 min) (+) melanocytes with antibodies to c-Kit ($\alpha\text{c-Kit}$), or control rabbit anti-mouse IgG (IgG). The arrows point to p145^{c-kit} and p125^{c-kit}. Immunoprecipitated proteins were resolved on 5% (A) or 7% (B), 8% (C) or 7.5% (D) polyacrylamide gels. In pulse-chase experiments, melanocytes were radiolabeled with ^{35}S for 15 min (p) or for 15 min and then incubated for 1 h in nonradioactive medium (c) (A). In B, C, and D, the period of labelling was 3 h. Autoradiography of ^{125}I -labeled immunoblots was overnight, of ^{32}P -labeled immune complexes, 7 d, as described in METH-ODS. The ^{35}S -labeled immune complexes were fluorographed for 2 d at -75°C .

^{32}P -labeled nonstimulated melanocytes as a band of 125 kDa, and to a much lesser extent, 145 kDa (Figure 3D). In addition, higher molecular weight forms of anti-c-Kit reactive PY-proteins, resistant to reducing and denaturing agents, appeared as a smear, extending from the top of the gel down to p145^{c-kit} and, at diminished intensity, to the level of p125 kDa (Figure 3, A, B, and D). In contrast, this distinct smear was not present in c-Kit immunoprecipitates from ^{35}S -labeled, MGF-stimulated melanocytes, where only a slight increase over background radioactivity in unstimulated cells was present (Figure 3C, compare minus to plus MGF). Phosphorylated p145^{c-kit} comigrated with mature fully processed ^{35}S -labeled c-Kit; phosphorylated p125 comigrated with newly synthesized, partially processed ^{35}S -labeled c-Kit, as revealed by pulse-chase experiment (Figure 3A) and by the 3-h labeling experiment (Figure 3B), suggesting that p125 is a c-Kit precursor that becomes phosphorylated by an as yet unknown mechanism. However, p125^{c-kit} was not the major 125-kDa protein phosphorylated in response to MGF because most of the tyrosyl-phosphorylated p125 immunoprecipitated with anti-PY after depletion of the extract of c-Kit with antibodies.

Because phosphorylated unstimulated c-Kit was not detected in anti-PY-immunoblots even in overexposed films, the constitutive phosphorylation of c-Kit is probably on serine and/or threonine residues. This will be confirmed in the future by phosphoamino acid analysis. So far, constitutively tyrosyl-phosphorylated immature and mature forms of c-Kit were observed only in P815 murine mastocytoma cells (Rottapel *et al.*, 1991).

Identification of One of the Intermediates of Kit-Kinase Signal Transduction as an ERK, a MAP Kinase

Serine/threonine kinases, known as MAP kinases or ERKs, are closely related kinases that are common in

intermediates in signal transduction pathways utilized by other receptors, such as the insulin-R, platelet-derived growth factor (PDGF)-R, epidermal growth factor (EGF)-R, FGF-R, thrombin-R, and PKC (Ahn and Krebs, 1990; Anderson *et al.*, 1990b; Boulton and Cobb, 1991; Boulton *et al.*, 1991a,b; Kawakami *et al.*, 1991; L'Allemain *et al.*, 1991). Three cDNA clones belonging to the ERK family and sharing regions of high identity have been isolated, and multiple isoforms of ERKs are recognized in mammalian cells by antisera raised against two separate conserved regions of ERK1 (designated antiserum 691 and 837) (Boulton and Cobb, 1991; Boulton *et al.*, 1991a). The three major immunoreactive proteins migrate in polyacrylamide gels as bands of 40–42 kDa (ERK2), 42–43 kDa (ERK1), and 45 kDa (ERK4) (Boulton and Cobb, 1991). To determine whether one or more of the ERKs are also intermediates in the c-Kit-regulated pathways in melanocytes, we checked whether melanocytic phosphoproteins in the range of 40–42 kDa comigrate with proteins that interact with anti-ERK antibody 691 and whether MGF stimulates MAP kinase activity.

Melanocytes expressed proteins of 40 and 42 kDa that were immunoreactive with anti-ERK 691 antibodies (Figure 4A, αERK). The two comigrating proteins became phosphorylated on tyrosine in response to MGF (Figure 4A, αPY). The 42-kDa anti-ERK-reactive protein possessed constitutive basal levels of phosphotyrosine. These results suggest that the 42- and 40-kDa phosphoproteins are ERK1 and 2, respectively, known to have similar electrophoretic mobility and to become phosphorylated on tyrosine in response to various mitogens (Boulton and Cobb, 1991; Boulton *et al.*, 1991b).

The stimulation of MAP2 kinase activity in response to MGF provided further support that ERKs function in the MGF signal transduction in melanocytes (Figure 4B). We measured activation of the enzyme in extracts of MGF-stimulated cultures in the presence of TPA or

Figure 4. The identification of p40-42 as ERKs. (A) MGF-deprived (–) and stimulated (+) melanocytes (as in Figure 2) were subjected to Western immunoblotting first with anti-PY, and after rinsing with 1% Triton X containing buffer, with anti-ERK 691 (Boulton *et al.*, 1991). (B) MAP kinase activity. Melanocytes were incubated in PC-1 medium overnight and treated with 75 U/ml MGF, 1.0 mM dbcAMP (cAMP), or 85 nM TPA, alone or in combination, for 5 min. MAP kinase in cell lysates was assayed in the presence or absence of 0.1 μ M staurosporine, as described by Boulton *et al.* (1991).

dbcAMP. Greatest MAP kinase activity was obtained by simultaneous addition of MGF and TPA (Figure 4B). To determine whether this activity was due to ERKs or perhaps to PKA or PKC, both of which can phosphorylate MAP2 as well, the extracts were assayed in the presence of staurosporine, a potent inhibitor of PKC and PKA but not MAP kinase (Boulton *et al.*, 1991b). Staurosporine strongly inhibited PKA-mediated phosphorylation of MAP2 (Figure 4B, cAMP and MGF+cAMP), suggesting that dbcAMP, which stimulates PKA, did not cause substantial stimulation of ERKs. On the other hand, staurosporine inhibited the TPA-stimulated MAP kinase activity only partially, consistent with findings in other systems in which TPA alone activated MAP kinase (Kawakami *et al.*, 1991). Most importantly, the drug inhibited relatively little of the stimulation of MAP2 phosphorylation seen in cells treated with MGF plus TPA (Figure 4B), indicating that ERK1 and/or ERK2 are activated by MGF with or without TPA. In agreement with these results is our observation that TPA alone, but not dbcAMP, stimulated the tyrosine-phosphorylation of p40 in melanocytes. The findings indicate that TPA potentiates the increase in MAP kinase activity by MGF. Because dbcAMP promotes MGF-induced mitogenesis but does not potentiate the effect of MGF on MAP kinase activity, its effect on mitogenesis must be mediated through a complementary component of the signal transduction pathway.

Melanocytic p125 is not the Known p120, p125, or GTPase-activating Protein (GAP)

Several known phosphoproteins of 120–125 kDa are substrates for tyrosine kinases. These are as follows: p125, a substrate for the transforming oncogenes *v-src*, *v-yes* and *v-crck* (Kanner *et al.*, 1990); p120 (Kanner *et al.*, 1990), phosphorylated in avian cells expressing middle T antigen, or in rodent cells in response to EGF, PDGF, and CSF-1 (Downing and Reynolds, 1991; Kanner *et al.*, 1991); and *ras* GAP (McCormick, 1989), phosphorylated in response to EGF and PDGF and coimmunoprecipitated with the EGF or PDGF receptors after ligand binding (reviewed in Cantley *et al.*, 1991). As shown by our data, the major melanocytic MGF-responsive p125 was none of the above (Figure 5). Immunoprecipitation with 2A7 (Kanner *et al.*, 1990), a monoclonal antibody to p125, showed that melanocytes expressed the antigen but that it was constitutively phosphorylated and did not serve as substrate for MGF-stimulated c-Kit-kinase (Figure 5A, α p125). The 125-kDa PY-2A7 antigen appeared as a fainter band than the MGF-stimulated p125. In addition, the MGF-responsive p125 remained in the supernatant after immunoprecipitation with the 2A7 antibodies (Figure 5B,

Figure 5. Lack of immunoreactivity of MGF-responsive p125 with antibodies to other known candidate 125-kDa proteins. (A) Immunoprecipitation (ip) with either anti-phosphotyrosine (α PY, 2 μ l, monoclonal antibodies PY-20, ICN for 2 h) or anti-p125 monoclonal antibody 2A7 (10 μ l of α p125, 16 hrs), followed by Western blotting (ib) with anti-PY. (B) Immunoprecipitation with anti-p125 (α p125) followed by immunoprecipitation with anti-PY (α PY). Western blotting with anti-PY. (C) Immunoprecipitation with anti-GAP antibodies (α GAP), followed by Western blotting with anti-PY antibodies (α PY), and after rinsing with 1% Triton X containing buffer, with α GAP (α GAP).

Figure 6. RNAase protection analysis of *c-kit* and MGF. Fifteen micrograms of total RNA from normal and malignant melanocytes were used per assay employing *c-kit* (A) or MGF (B) [α^{32} P]UTP-labeled riboprobes. Transfer RNA was used as a negative control. Arrows point at protected probes. The RNAase protection assays gave consistently similar results in three independent experiments derived from RNAs extracted at different times.

α PY). Similar experiments with monoclonal antibody 2B12, reacting with p120 (Kanner *et al.*, 1990, 1991; Downing and Reynolds, 1991), showed that this antigen, likewise, was not phosphorylated in response to *c-Kit* activation, although melanocytes expressed an unphosphorylated form of p120. Furthermore, although melanocytes expressed high levels of GAP, as revealed by immunoprecipitation with anti-GAP antibodies followed by Western blotting with anti-GAP antibodies, GAP was not phosphorylated on tyrosine in response to MGF (Figure 5C). The latter results are in agreement with observations obtained with murine mast cells (Rottapel *et al.*, 1991).

Down-Regulation of *c-Kit*-Kinase in Melanomas

A common characteristic of melanocytes from advanced primary (nodular) and metastatic melanomas is the ability to grow in vitro independent of growth factors required for their normal counterparts (reviewed in Halaban, 1991; Halaban *et al.*, 1991). This autonomy is

acquired in large part by the aberrant production of bFGF (Halaban *et al.*, 1988). However, expression of bFGF by itself is not sufficient to transform melanocytes to the tumorigenic phenotype (Dotto *et al.*, 1989), suggesting the presence of cooperating factors. A cooperating factor could be a constitutively active receptor kinase not from the bFGF-receptor family, such as *c-Kit*. We therefore tested the expression and biological activity of *Kit*-kinase in nodular primary and metastatic melanomas growing in vitro.

RNAase protection assay showed that three out of eight melanomas expressed detectable levels of *c-kit* gene transcripts (Figure 6), and anti-PY immunoblots showed that MGF induced *c-Kit* phosphorylation at normal or slightly lower levels in only 2 out of 11 cell lines, 501 mel and 624 mel (Figure 7, A and B), known also to express high levels of *c-kit* transcripts (Zakut, Perlis, Eliaho, Yarden, Halaban, and Rosenberg, unpublished data). Two other melanomas, MeWo and YU ZAZ6, in which faint immunoblot bands of phosphorylated *c-Kit* were detected only after overexposure of

Figure 7. Functional c-Kit is absent in the majority of melanomas. Anti-phosphotyrosine Western blots of protein from whole-cell extracts (A) or anti-c-Kit immunoprecipitates (B). Cells were deprived of (–) or stimulated with (+) MGF for 10 min, as in Figures 2–4. (A) Cell extracts (~60 µg protein) from normal melanocytes (N), nodular primary melanoma (WW 94), and metastatic melanomas (YU DAN3, YU SAC2, YU PAC7, 501 mel, and 624 mel), respectively, were loaded in each well. Autoradiography was for 16 h at –75°C. Notice the presence of phosphorylated c-Kit- and MGF-responsive phosphoproteins in only the melanoma cell lines 501 mel and 624 mel. Absence of MGF signal transduction was observed in four other metastatic melanoma cell lines. Bars on the right hand side indicate molecular weight markers of 106, 80, 49.5, and 32.5 kDa. (B) Cell extracts from normal (N) and malignant melanocytes (containing up to 1.8 mg protein) were subjected to immunoprecipitation with anti-c-Kit antibodies and immunoblotting with anti-PY-antibodies. The membrane was autoradiographed for 2 h (top) and then again for 24 h to detect faint bands (bottom) at –75°C.

the X-ray film (Figure 7B), expressed relatively high levels of *c-kit* mRNA (Figure 6A, and Lassam and Bickford, 1991); melanoma YU ZAZ6 also bound radiolabeled MGF at levels similar to those in normal melanocytes. None of the melanoma cell lines expressing high levels of *c-kit* mRNA possessed constitutively phosphorylated c-Kit receptors.

In all melanoma cell lines, MGF failed to stimulate an increase in DNA synthesis, and in one of the melanoma lines expressing normal levels of phosphorylated c-Kit, 501 mel, there was a 50% reduction in [³H] thymidine incorporation after 1 or 2 d incubation with MGF (3.8×10^4 and 1.9×10^4 cpm/well/3 h after 1 d without and with MGF, respectively). MGF also did not enhance MAP kinase activity in melanoma YU ZAZ6, expressing c-Kit transcripts, despite the small increase in activity in response to TPA or dbcAMP (Figure 8). In all of these cells the amounts of ERK1 and ERK2 expressed were similar as determined by Western blotting of whole cell extracts with anti-ERK antibodies.

A distinct characteristic of all the melanomas tested was constitutive expression of several tyrosyl-phosphorylated proteins of 130–135, 125, and 65–75 kDa (Figure 7A). The 180-kDa phosphoprotein that disap-

peared in response to MGF was expressed in melanomas in the tyrosyl-phosphorylated form, and the 40-kDa protein, probably ERK2, was not constitutively phosphorylated in melanomas (Figure 7A) but became so in response to TPA, corroborating the MAP kinase activity data (Figure 8). Importantly, however, basal MAP kinase activity was approximately twice as high in the melanoma cells as in normal melanocytes, measured in the absence or presence of staurosporine (compare results from Figures 4 and 8). Because these melanoma cells express bFGF (Halaban *et al.*, 1988, 1991; Rodeck *et al.*, 1991), it is possible that the phosphorylated state of the proteins was the result of constitutively stimulated bFGF-R.

The lack of response to exogenous MGF was not due to constitutive production of MGF, because RNAase protection assay with MGF riboprobe failed to reveal MGF transcripts in cell lines expressing *c-kit* mRNA (Figure 6, YU PAC7, WW 94, and SW 1614), and, as mentioned before, constitutively tyrosyl-phosphorylated c-Kit was not detected in this or any other melanoma cell strain (Figure 7A). Taken together, the results show that c-Kit activity cannot account for autonomous proliferation of most melanomas.

Figure 8. MAP kinase activity in melanoma cells. Metastatic melanoma cells YU ZAZ6 were incubated in PC-1 medium overnight and treated with 75 U/ml MGF, 1.0 mM dbcAMP (cA), or 85 nM TPA, alone or in combination, for 5 min. MAP kinase in cell lysates was assayed in the presence or absence of 0.1 μ M staurosporine as described in METHODS (see also Boulton *et al.*, 1991a).

DISCUSSION

MGF

Our results show that MGF is a mitogen for normal human melanocytes but only in the presence of synergistic factors. The requirement for a synergist is reminiscent of results obtained with murine and human hematopoietic cells in which MGF had colony-stimulating activity only in the presence of other cytokines (Anderson *et al.*, 1990a; Migliaccio *et al.*, 1991; Ratajczak *et al.*, 1991), as well as those observed in mouse primordial germ cell cultures, in which MGF promoted the viability of the cells but for proliferation required the presence of a putative factor present in a mouse embryonic fibroblast line STO (Dolci *et al.*, 1991; Godin *et al.*, 1991). MGF-promoted biological activity is probably not restricted to proliferation but also includes induction of differentiation. In human melanocytes, MGF increases melanin production, (Longley, Ding, Cuono, Lyman, Anderson, Williams, and Halaban, unpublished data) and in the mouse, MGF may have been the unidentified factor in the extracellular matrix of normal but not *Sl^d* fetal skin that enhanced the differentiation

of neural crest cells into melanocytes (Morrison-Graham *et al.*, 1990). MGF also was shown to stimulate the differentiation of murine mast cells (Tsai *et al.*, 1991) and colony-forming cells (Migliaccio *et al.*, 1991).

Phosphorylation (and Dephosphorylation) Cascades in Response to MGF

As predicted, the mitogenic stimulation by MGF of human melanocytes is mediated through autophosphorylation of c-Kit. We show here a complex pattern of protein phosphorylation and dephosphorylation induced by activated c-Kit that was not observed in ligand-stimulated mast cells or NIH 3T3 fibroblasts engineered to express the EGF-receptor-Kit hybrid molecule (Lev *et al.*, 1991; Rottapel *et al.*, 1991). In addition, we have identified new intermediates, one or more of the kinases known as ERKs.

One of the most prominent proteins that became phosphorylated in response to MGF was a p125 that was not GAP or two other known proteins of similar size that become phosphorylated in response to ligands. This protein is also probably not the unprocessed p125^{c-kit}. In addition, a prominent p125 phosphoprotein was present in all melanoma cells tested even in the absence of c-kit gene transcripts, suggesting that it is activated through an internal loop constitutively active in melanomas and is essential for proliferation.

The disappearance of PY-p180 in response to MGF, probably through dephosphorylation, suggests that activation of a phosphotyrosine phosphatase (PTPase) might be important in the transduction of MGF signals in pigment cells toward differentiation rather than proliferation. Phosphorylated p180 was constitutively present in all the melanoma cells tested, all of which were amelanotic. The identity of p180 is not yet known. It is neither the PDGF-R nor the EGF-R, both phosphoproteins of similar size, because normal human melanocytes do not express these receptors and do not respond to the respective ligands (Real *et al.*, 1986; Westermarck *et al.*, 1986; Halaban, 1988). Ligand activation of a PTPase, possibly through tyrosine phosphorylation, was demonstrated in the case of CD45, a hematopoietic cell-surface antigen essential for T-cell receptor activation (Stover *et al.*, 1991). Also, the proto-oncogene pp60^{c-src} (reviewed in Cantley *et al.*, 1991) and the protein kinase p34^{cdc2}, a key enzyme leading to the mitotic phase in diverse eukaryotic cells (reviewed in Nurse, 1990), are activated by dephosphorylation of a regulatory tyrosine residue.

MAP Kinases

MGF activates ERK in the melanocyte system. This serine/threonine kinase requires tyrosyl and threonyl phosphorylation for activity, suggesting that it integrates signals from different phosphorylation pathways (Anderson *et al.*, 1990b). In the melanocyte system, it is also

activated by the two other mitogenic peptides, bFGF and HGF (Halaban, Rubin, Funasaka, Cobb, Boulton, Faletto, Rosen, Chan, Moellmann, Yoko, and Cook, unpublished data). MAP kinase is thus a common intermediate shared by three types of growth factors activating distinct receptors, suggesting that its activity is needed for melanocyte proliferation. So far, the known ERKs substrates are ribosomal protein S6 kinase II, which phosphorylates S6, a component of the 40S ribosomal subunits (Ahn *et al.*, 1990; Gregory *et al.*, 1989; Sturgill *et al.*, 1988), Raf-1 (Anderson *et al.*, 1991; Lee *et al.*, 1992), and c-Jun (Pulverer *et al.*, 1991). It is not yet clear whether activation of all three is needed for stimulation of mitogenesis. Interestingly, ERK basal activity was higher in melanomas compared with normal melanocytes, suggesting that the aberrant expression of bFGF in these cells may cause an increase in the activities of these enzymes. MAP kinases may affect also melanocyte dendricity because MAP2 expression is required for neurite extension (Dinsmore and Solomon, 1991).

Other known substrates for activated c-Kit that become also associated with the receptor are phospholipase C- γ 1 (PLC- γ 1), phosphatidylinositol 3'-kinase, and the serine/threonine kinases Raf-1 (Lev *et al.*, 1991; Rottapel *et al.*, 1991). Immunoprecipitation experiments using antibodies to PLC- γ 1 and Raf1, followed by immunoblotting with anti-phosphotyrosine antibodies, failed in our hands to demonstrate conclusively that these two are also phosphorylated in response to MGF in melanocytes.

Melanomas

Melanomas are highly variable with regard to chromosomal abnormalities, loss or retention of specific DNA markers, expression of specific antigens and oncogenes, and expression of specific growth factor receptors or growth factors (reviewed in Halaban and Moellmann, 1991). A common characteristic is, however, the aberrant expression of bFGF, which contributes to the autonomous mode of melanoma cell growth (Halaban *et al.*, 1988, 1991). The absence of functional c-Kit might be another characteristic of the transformed phenotype of advanced melanomas. In a recent study, employing Northern and Southern blot analysis, *c-kit* mRNAs were detected in only 2 of 14 melanomas, whereas the *c-kit* gene seemed to be intact (Lassam and Bickford, 1992). In contrast, in another study employing also Northern blot analysis, *c-kit* gene transcripts were detected in ~50% of the 14 metastatic melanoma lines tested (Zakut, Perlis, Eliaho, Yarden, Halaban, and Rosenberg, unpublished data). It is not clear whether *c-kit* down-regulation in melanomas is the result of malignant transformation commonly observed for other genes controlling differentiated functions, such as melanin production (Halaban *et al.*, 1983), or whether it

contributes to the malignant state. In murine mast cells, expression of *c-kit* mRNA is down-regulated by cytokines, such as IL-3, granulocyte-macrophage stimulating factor (GM-CSF), and erythropoietin (Welham and Schrader, 1991). Melanoma cells are known to produce cytokines, such as PDGF, IL-1 α and IL-3, GM-CSF, and others (reviewed in Köck *et al.*, 1991). Therefore, it is possible that these factors suppress the expression of *c-kit* in melanomas. This does not rule out, however, the possibility that absence of functional c-Kit in melanomas contributes to their malignant dedifferentiated phenotype indirectly, such as by elimination of a protein tyrosine phosphatase activity, suggested by our studies to be one of the intermediates activated by MGF.

ACKNOWLEDGMENTS

We thank Dr. F. McCormick for the anti-GAP antibodies; Drs. J.T. Parsons, S.B. Kanner, and A.B. Reynolds for the 2A7 and 2B12 monoclonal antibodies; Drs. Wayne White, Antonio Buzaid, and Stephen Aryan for providing the melanoma specimens; Dr. D. Stern for advice; Dr. G. Moellmann for discussing and editing the manuscript; Dr. Jack Longley for useful comments; and Jack Schreiber, Christopher Cook, and Colleen Vanderbilt for technical help. Supported in part by NIH grants 1R01-AR39848 and 5-R29-CA44542 and DK 34128 to M.H.C.

REFERENCES

- Ahn, N.G., and Krebs, E.G. (1990). Evidence for an epidermal growth factor-stimulated protein kinase cascade in Swiss 3T3 cells. *J. Biol. Chem.* 265, 11495–11501.
- Anderson, N.G., Li, P., Marsden, L.A., Williams, N., Roberts, T.M., and Sturgill, T.W. (1991). Raf-1 is a potential substrate for mitogen-activated protein kinase in vivo. *Biochem. J.* 277, 573–576.
- Anderson, D.M., Lyman, S.D., Baird, A., Wignall, J.M., Eisenman, J., Rauch, C., March, C.J., Boswell, H.S., Gimpel, S.D., Cosman, D., and Williams, D.D. (1990a). Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell* 63, 235–243.
- Anderson, N.G., Maller, J.L., Tonks, N.K., and Sturgill, T.W. (1990b). Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature* 343, 651–653.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.) (1991). *Current Protocols in Molecular Biology*, vol. 1, New York: John Wiley & Sons.
- Balaban, G., Herlyn, M., Guerry IV, D., Bartolo, R., Koprowski, H., Clark, W.C., and Nowell, P.C. (1984). Cytogenetics of human malignant melanoma and premalignant lesions. *Cancer Genet. Cytogenet.* 11, 429–439.
- Bean, M.A., Bloom, B.R., Herberman, R.B., Old, L.J., Oettgen, H.F., Klein, G., and Terry, W.D. (1975). Cell-mediated cytotoxicity for bladder carcinoma: evaluation of a workshop. *Cancer Res.* 35, 2902–2913.
- Bottaro, D.P., Rubin, J.S., Faletto, D.L., Chan, A.M.-L., Kmiecik, T.E., Vande Woude, G.P., and Aaronson, S.A. (1991). Identification of the HGF receptor as the *c-met* protooncogene product. *Science* 251, 802–804.
- Boulton, T.G., and Cobb, M.H. (1991). Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies. *Cell Regul.* 2, 357–371.

- Boulton, T.G., Gregory, J.S., and Cobb, M.H. (1991a). Purification and properties of extracellular signal-regulated kinase 1, an insulin-stimulated microtubule-associated protein 2 kinase. *Biochemistry* 30, 278-286.
- Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., DePinho, R.A., Panayotatos, N., Cobb, M.H., and Yancopoulos, G.D. (1991b). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* 65, 663-675.
- Brannan, C.I., Lyman, S.D., Williams, D.E., Eisenman, J., Anderson, D., Cosman, D., Bedell, M.A., Jenkins, N.A., and Copeland, N.G. (1991). Steel-Dickie mutation encodes a c-Kit ligand lacking transmembrane and cytoplasmic domains. *Proc. Natl. Acad. Sci. USA* 88, 4671-4674.
- Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991). Oncogenes and signal transduction. *Cell* 64, 281-302.
- Chabot, B., Stephenson, D.A., Chapman, V.M., Besmer, P., and Bernstein, A. (1988). The proto-oncogene *c-kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse *W* locus. *Nature* 335, 88-89.
- Chirgwin, J.J., Przbyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18, 5294.
- Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156-159.
- Cooper, J.A., Sefton, B.M., and Hunter, T. (1983). Detection and quantification of phosphotyrosine in proteins. *Methods Enzymol.* 99, 387-405.
- Copeland, N., Gilbert, D.J., Cho, B.C., Donovan, P.J., Jenkins, N.A., Cosman, D., Anderson, D., Lyman, S.D., and Williams, D.E. (1990). Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. *Cell* 63, 175-183.
- Coughlin, S.R., Barr, P.J., Cousens, L.S., Fretto, L.J., and Williams, L.T. (1988). Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity in vivo. *J. Biol. Chem.* 263, 988-993.
- Dinsmore, J., and Solomon, F. (1991). Inhibition of MAP2 expression affects both morphological and cell division phenotypes of neuronal differentiation. *Cell* 64, 817-826.
- Dionne, C.A., Crumley, G., Bellot, F., Kaplow, J.M., Searfoss, G., Ruta, M., Burgess, W.H., Jaye, M., and Schlessinger, J. (1990). Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. *EMBO J.* 9, 2685-2692.
- Dolci, S., Williams, D.E., Ernst, M.K., Resnick, J.L., Brannan, C.I., Boswell, S.B., and Donovan, P.J. (1991). Requirement for mast cell growth factor for primordial germ cell survival in culture. *Nature* 352, 809-811.
- Dotto, P.G., Moellmann, G., Ghosh, S., Edwards, M., and Halaban, R. (1989). Transformation of murine melanocytes with basic fibroblast growth factor cDNA and oncogenes and selective suppression of the transformed phenotype in a reconstituted cutaneous environment. *J. Cell Biol.* 109, 3115-3128.
- Downing, J.R., and Reynolds, A.B. (1991). PDGF, CSF-1, and EGF induce tyrosine phosphorylation of p120, a pp60^{src} transformation-associated substrate. *Oncogene* 6, 607-613.
- Flanagan, J.G., Chan, D.G., and Leder, P. (1991). Transmembrane form of the *kit* ligand growth factor is determined by alternative splicing and is missing in the *Sl^d* mutant. *Cell* 64, 1025-1035.
- Flanagan, J.G., and Leder, P. (1990). The *kit* ligand: a cell surface molecule altered in steel mutant fibroblasts. *Cell* 63, 185-194.
- Friesel, R., Burgess, W.H., and Maciag, T. (1989). Heparin-binding growth factor 1 stimulates tyrosine phosphorylation in NIH 3T3 cells. *Mol. Cell. Biol.* 9, 1857-1865.
- Geissler, E.N., Ryan, M.A., and Housman, D.E. (1988). The dominant-white spotting (*W*) locus of the mouse encodes the *c-kit* proto-oncogene. *Cell* 55, 185-192.
- Giebel, L.B., and Spritz, R.A. (1992). Mutation of the *c-kit* (mast/stem cell growth factor receptor) proto-oncogene in human piebaldism. *Proc. Natl. Acad. Sci. USA* 88, 8696-8699.
- Gregory, J., Boulton, T., Sang, B.-C., and Cobb, J. (1989). An insulin-stimulated ribosomal protein S6 kinase from rabbit liver. *J. Biol. Chem.* 264, 18377-18401.
- Halaban, R. (1988). Responses of cultured melanocytes to defined growth factors. *Pigm. Cell Res.* 1, 18-26.
- Halaban, R. (1989). Growth regulation in normal and malignant melanocytes. In: *Human Melanoma: Immunology, Diagnostics, and Therapy*, ed. S. Ferrone, New York: Springer-Verlag, 3-14.
- Halaban, R. (1991). Growth factors regulating normal and malignant melanocytes. In: *Melanoma Research: Genetics, Growth Factors, Metastases, and Antigens*, ed. L. Nathanson, Boston, Dordrecht, London: Kluwer Academic Publishers, 19-40.
- Halaban, R., Funasaka, Y., Lee, P., Rubin, J., Ron, D., and Birnbaum, D. (1991). Fibroblast growth factors in normal and malignant melanocytes. *Ann. NY Acad. Sci.* 638, 232-243.
- Halaban, R., Ghosh, S., and Baird, A. (1987). bFGF is the putative natural growth factor for human melanocytes. *In Vitro Cell. & Dev. Biol.* 23, 47-52.
- Halaban, R., Kwon, B.S., Ghosh, S., Delli Bovi, P., and Baird, A. (1988). bFGF as an autocrine growth factor for human melanomas. *Oncogene Res.* 3, 177-186.
- Halaban, R., and Moellmann, G. (1991). Proliferation and malignant transformation of melanocytes. *Crit. Rev. Oncogenesis* 2, 247-258.
- Halaban, R., Pomerantz, S.H., Marshall, S., Lambert, D.T., and Lerner, A.B. (1983). Regulation of tyrosinase in human melanocytes grown in culture. *J. Cell Biol.* 97, 480-488.
- Herlyn, M., Thurin, J., Balaban, G., Bannicelli, J.L., Herlyn, D., Elder, D.E., Bondi, E., Guerry, D., Nowell, P., Clark, W.H., and Koprowski, H. (1985). Characteristics of cultured human melanocytes isolated from different stages of tumor progression. *Cancer Res.* 45, 5670-5676.
- Huang, E., Nocka, K., Beier, D.R., Chu, T.-Y., Buck, J., Lahm, H.-W., Wellner, D., Leder, P., and Besmer, P. (1990). The hematopoietic growth factor KL is encoded at the *Sl* locus and is the ligand of the *c-kit* receptor, the gene product of the *W* locus. *Cell* 63, 225-233.
- Johnson, D.E., Lee, P.L., Lu, J., and Williams, L.T. (1990). Diverse forms of a receptor for acidic and basic fibroblast growth factors. *Mol. Cell. Biol.* 10, 4728-4736.
- Kanner, S.B., Reynolds, A.B., and Parsons, J.T. (1991). Tyrosine phosphorylation of a 120-kilodalton pp60^{src} substrate upon epidermal growth factor and platelet-derived growth factor receptor stimulation and in polyomavirus middle-T-antigen-transformed cells. *Mol. Cell. Biol.* 11, 713-720.
- Kanner, S.B., Reynolds, A.B., Vines, R.R., and Parsons, J.T. (1990). Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc. Natl. Acad. Sci. USA* 87, 3328-3332.
- Kawakami, M., Nishida, E., Tobe, K., Hoshi, M., Kadowaki, T., Kasuga, M., and Sakai, H. (1991). Activation of MAP kinase and enhanced phosphorylation of the 350-kDa protein by mitogenic stimuli in quiescent Balb/3 3T3 cells. *Exp. Cell Res.* 193, 120-126.

- Köck, A., Schwarz, T., Micksche, M., and Luger, T.A. (1991). Cytokines and human malignant melanoma immuno- and growth-regulatory peptides in melanoma biology. In: *Melanoma Research: Genetics, Growth Factors, Metastases, and Antigens*, ed. L. Nathanson, Kluwer Academic Publishers, 41–66.
- L'Allemain, G., Pouyssegur, J., and Weber, M.J. (1991). p42/mitogen-activated protein kinase as a converging target for different growth factor signaling pathways: Use of pertussis toxin as a discrimination factor. *Cell Regul.* 2, 675–684.
- Lassam, N., and Bickford, S. (1992). Loss of *c-kit* expression in cultured melanoma cells. *Oncogene* 7, 51–56.
- Lee, P.L., Johnson, D.E., Coussens, L.S., Fried, V.A., and Williams, L.T. (1989). Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. *Science* 245, 57–60.
- Lee, R.-M., Cobb, M.H., and Blakeshear, P.J. (1992). Evidence that extracellular signal-regulated kinases (ERKs) are the insulin-activated Raf-1 kinases. *J. Biol. Chem. (in press)*.
- Lev, S., Yarden, Y., and Givol, D. (1990). Receptor functions and ligand-dependent transforming potential of a chimeric *kit* proto-oncogene. *Mol. Cell. Biol.* 10, 6064–6068.
- Lev, S., Yarden, Y., and Givol, D. (1991). A specific combination of substrates is involved in signal transduction by the *kit*-encoded receptor. *EMBO J.* 10, 647–654.
- Mansukhani, A., Moscatelli, D., Talarico, D., Levytska, V., and Basilio, C. (1990). A murine fibroblast growth factor (FGF) receptor expressed in CHO cells is activated by basic FGF and Kaposi FGF. *Proc. Natl. Acad. Sci. USA* 87, 4378–4382.
- Margolis, B., Rhee, S.G., Felder, S., Mervic, M., Lyall, A., Levitzki, A., Ullrich, A., Zilberstein, A., and Schlessinger, J. (1989). EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. *Cell* 57, 1101–1107.
- Martin, F.H., Sugs, S.V., Langley, K.E., Lu, H.S., Ting, J., Okino, K.H., Morris, C.F., McNiece, I.K., Jacobsen, F.W., Mendiaz, E.A., Birkett, N.C., Smith, K.A., Johnson, M.J., Parker, V.P., Flores, J.C., Patel, A.C., Fisher, E., Erjavec, H.O., Herrera, C.H., Wypych, J., Sachdev, R.K., Pope, J.A., Leslie, I., Wen, E., Lin, C.-H., Cupples, R.L., and Zsebo, K. (1990). Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell* 63, 203–211.
- Matsumoto, K., Tajima, H., and Nakamura, T. (1991). Hepatocyte growth factor is a potent stimulator of human melanocyte DNA synthesis and growth. *Biochem. Biophys. Res. Commun.* 176, 45–51.
- McCormick, F. (1989). *ras* GTPase activating protein: signal transmitter and signal terminator. *Cell* 56, 5–8.
- Migliaccio, G., Migliaccio, A.R., Valinski, J., Langley, K., Zsebo, K., Visser, W.M., and Adamson, J.W. (1991). Stem cell factor induces proliferation and differentiation of highly enriched murine hematopoietic cells. *Proc. Natl. Acad. Sci. USA* 88, 7420–7424.
- Morrison-Graham, K., West-Johnsrd, L., and Weston, J.A. (1990). Extracellular matrix from normal but not *Steel* mutant mice enhances melanogenesis in cultured mouse neural crest cells. *Dev. Biol.* 139, 299–307.
- Naldini, L., Vigna, E., Narsimhan, R.P., Gaudino, G., Zarnegar, R., Michaelopoulos, G.K., and Comoglio, P.M. (1991). Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene *c-Met*. *Oncogene* 6, 501–504.
- Nocka, K., Majumder, S., Chabot, B., Ray, P., Cervone, M., Bernstein, A., and Besmer, P. (1989). Expression of *c-kit* gene products in known cellular targets of *W* mutations in normal and *W* mutant mice—evidence for an impaired *c-kit* kinase in mutant mice. *Genes & Dev.* 3, 816–826.
- Nocka, K., Tan, J.C., Chiu, E., Chu, T.Y., Ray, P., Traktman, P., and Besmer, P. (1990). Molecular bases of dominant negative and loss of function mutations at the murine *c-kit*/white spotting locus: *W³⁷*, *W^v*, *W⁴¹* and *W*. *EMBO J.* 9, 1805–1813.
- Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. *Nature* 344, 503–508.
- Pathak, S., Drwinga, H.L., and Hsu, T.C. (1983). Involvement of chromosome 6 in rearrangements in human malignant melanoma cell lines. *Cytogenet. Cell Genet.* 36, 573–579.
- Partanen, J., Mäkelä, T.P., Alitalo, R., Lehtväslaiho, H., and Alitalo, K. (1990). Putative tyrosinase kinases expressed in K-562 human leukemia cells. *Proc. Natl. Acad. Sci. USA* 87, 8913–8917.
- Pulverer, B.J., Kyriakis, J.M., Avruch, J., Nikolakaki, E., and Woodgett, J.R. (1991). Phosphorylation of *c-jun* mediated by MAP kinases. *Nature* 353, 670–674.
- Qiu, F., Ray, P., Brown, K., Barker, P.E., Jhanwar, S., Ruddle, F.H., and Besmer, P. (1988). Primary structure of *c-kit*: relationship with the CSF-1/PDGF receptor kinase family—oncogenic activation of *v-kit* involves deletion of extracellular domain and c-terminus. *EMBO J.* 7, 1003–1011, 1988.
- Ratajczak, M., Shen, Y.M., and Gewitz, A. (1991). Role of c-Kit proto-oncogene in human in vitro hematopoiesis. *Clin. Res.* 39, 190A.
- Real, F.X., Rettig, W.J., Chesa, P.G., Melamed, M.R., Old, L.J., and Mendelsohn, J. (1986). Expression of epidermal growth factor receptor in human cultured cells and tissues: relationship to cell lineage and stage of differentiation. *Cancer Res.* 46, 4726–4731.
- Reid, H.H., Wilks, A.F., and Bernard, O. (1990). Two forms of the basic fibroblast growth factor receptor-like mRNA are expressed in the developing mouse brain. *Proc. Natl. Acad. Sci. USA* 87, 1596–1600.
- Reith, A.D., Rottapel, R., Giddens, E., Brady, C., Forrester, L., and Bernstein, A. (1990). *W* mutant mice with mild or severe developmental defects contain distinct point mutations in the kinase domain of the *c-kit* transmembrane receptor. *Genes & Dev.* 4, 390–400.
- Richmond, A., Balentien, E.D., Thomas, H.G., Glass, G., Barton, D.E., Spiess, J., Bordoni, R., Francke, U., and Derynck, R. (1988). Molecular characterization and chromosomal mapping of melanoma growth stimulatory activity, a growth factor structurally related to b-thromboglobulin. *EMBO J.* 7, 2025.
- Rodeck, U., Melber, K., Kath, R., Menssen, H.-D., Varello, M., Atkinson, B., and Herlyn, M. (1991). Constitutive expression of multiple growth factor genes by melanoma cells but not normal melanocytes. *J. Invest. Dermatol.* 97, 20–26.
- Rottapel, R., Reedijk, M., Williams, D.E., Lyman, S., Anderson, D.M., Pawson, T., and Bernstein, A. (1991). The *Steel/W* signal transduction pathway: Kit autophosphorylation and its association with a unique subset of cytoplasmic signaling protein is induced by the steel factor. *Mol. Cell. Biol.* 11, 3043–3051.
- Rubin, J.S., Chan, A.M., Bottaro, D.P., Burgess, W.H., Taylor, W.G., Cech, A.C., Hirschfeld, D.W., Wong, J., Miki, T., Finch, P.W., and Aaronson, S.A. (1991). A broad spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. *Proc. Natl. Acad. Sci. USA* 88, 415–419.
- Ruta, M., Burgess, W., Givol, D., Epstein, J., Neiger, N., Kaplow, J., Crumley, G., Dionne, C., Jaye, M., and Schlessinger, J. (1989). Receptor for acidic fibroblast growth factor is related to the tyrosine kinase encoded by the *fms*-like gene (FLG). *Proc. Natl. Acad. Sci. USA* 86, 8722–8726.
- Safran, A., Avivi, A., Orr-Urtreger, A., Neufeld, G., Lonai, P., Givol, D., and Yarden, Y. (1990). The murine *flg* gene encodes a receptor for fibroblast growth factor. *Oncogene* 5, 635–643.
- Stern, D.F., and Kamps, M.P. (1988). EGF-stimulated tyrosine phosphorylation of p185^{neu}: a potential model for receptor interaction. *EMBO J.* 7, 995–1001.

- Stover, D.R., Charbonneau, H., Tonks, N.K., and Walsh, K.A. (1991). Protein-tyrosine-phosphatase CD45 is phosphorylated transiently on tyrosine upon activation of Jurkat T cells. *Proc. Natl. Acad. Sci. USA* 88, 7704–7707.
- Sturgill, T.W., Ray, B., Erikson, E., and Maller, J.L. (1988). Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature* 334, 715–718.
- Tan, J.C., Nocka, K., Ray, P., Traktman, P., and Besmer, P. (1990). The dominant *W⁴² spotting* phenotype results from a missense mutation in the *c-kit* receptor kinase. *Science* 247, 209–212.
- Tsai, M., Takeishi, T., Thompson, H., Langley, K.E., Zsebo, K.M., Metcalfe, D.D., Geissler, E.N., and Galli, S.J. (1991). Induction of mast cell proliferation, maturation, and heparin synthesis by the rat *c-kit* ligand, stem cell factor. *Proc. Natl. Acad. Sci. USA* 88, 6382–6386.
- Welham, M.J., and Schrader, J.W. (1991). Modulation of *c-kit* mRNA and protein by hematopoietic growth factors. *Mol. Cell. Biol.* 11, 2901–2904.
- Westermarck, B., Johnsson, A., Paulsson, Y., Betsholtz, C., Heldin, C.-H., Herlyn, M., Rodeck, U., and Koprowski, H. (1986). Human melanoma cell lines of primary and metastatic origin express the genes encoding the chains of platelet-derived growth factor (PDGF) and produce a PDGF-like growth factor. *Proc. Natl. Acad. Sci. USA* 83, 7197–7200.
- Williams, D.E., Eisenman, J., Baird, A., Rauch, C., Van Ness, K., March, C.J., Park, L.S., Martin, U., Mochizuki, D.Y., Boswell, H.S., Burgess, G.M., Cosman, D., and Lyman, S.D. (1990). Identification of a ligand for the *c-kit* proto-oncogene. *Cell* 63, 167–174.
- Yarden, Y., Kuang, W.-J., Yang-Feng, T., Coussens, L., Munemitsu, S., Dull, T.J., Chen, E., Schlessinger, J., Francke, U., and Ullrich, A. (1987). Human proto-oncogene *c-kit*: a new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J.* 6, 3341–3351.
- Zsebo, K., Williams, D.A., Geissler, E.N., Broudy, V.C., Martin, F.H., Atkins, H.L., Hsu, R.-Y., Birkett, N.C., Okino, K.H., Murdock, D.C., Jacobsen, F.W., Langley, K.E., Smith, K.A., Takeishi, T., Cattanch, B.M., Galli, S.J., and Suggs, S. (1990a). Stem cell factor is encoded at the *Sl* locus of the mouse and is the ligand for the *c-kit* tyrosine kinase receptor. *Cell* 63, 213–224.
- Zsebo, K.M., Wypych, J., McNiece, I.K., Lu, H.S., Smith, K.A., Karkare, S.B., Sadchev, R.K., Yuschenko, V.N., Birkett, N.C., Williams, L.R., Satyagal, V.N., Tung, W., Bosselman, R.A., Mendiaz, E.A., and Langley, K.E. (1990b). Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. *Cell* 63, 195–201.