UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

Dottorato di Ricerca in Patologia e Fisiopatologia Molecolare

Tesi Sperimentale di Dottorato

"JNK and ERK8 as downstream effectors of receptor tyrosine kinases"

Coordinatore
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Candidato
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UNIVERSITA' DEGLI STUDI DI NAPOLI
"FEDERICO II"

Dipartimento di Biologia e Patologia Cellulare e Molecolare
"L. Califano"

Tesi di Dottorato in Patologia e Fisiopatologia Molecolare
XVII Ciclo

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Candidato: Dott. Carlo Iavarone

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UNIVERSITA' DEGLI STUDI DI NAPOLI "FEDERICO II"

JNK and ERK8 as downstream effectors of receptor tyrosine kinases

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Introduction

A key question in developmental biology is how cells perceive and respond properly to their enviroment. Cells must not only sense and distinguish between stimuli, but also transduce the signal accurately, to activate the appropiate responses. Signal transduction is the process by which extracellular signals are detected and converted into intracellular signals, which, in turn, generate specific cellular responses. Signal transduction systems are typically arranged as networks of sequential protein kinases. In such signalling cascades, MAP kinases (mitogen-activated protein kinases) carry out a crucial role.

MAP kinases are a super-family of serine-threonine protein kinases expressed in all eukaryotic cells. The basic assembly of MAP kinase pathways is a three-component module conserved from yeast to humans. This module includes three kinases that estabilish a sequential activation pathway comprising a MAP kinase kinase kinase (MKKK), a MAP kinase kinase (MKK), and MAP kinase (MK) (Widman et al., 1999) (Fig.1).

Receptor tyrosine kinases.

The MAP kinase transduction system is particularly important in growth factors signalling. Growth factors control cell growth, proliferation, differentiation, survival and migration by activating receptor tyrosine kinase (RTK) family members (Blume-Jensen and Hunter, 2001). Signalling by RTKs requires ligand-induced receptor oligomerization, but evidences indicate that RTKs oligomerization per se is not always sufficient for kinase activation. There seems to be an additional requirement for ligand-induced conformational switches, ensuring that the catalytic domains are juxtaposed in a proper configuration to enable phosphorylation (Schlessinger, 2000; Jiang and Hunter, 1999). Anyway, upon ligand binding, cytoplasmic tyrosine residues of RTKs becomes autophosphorylated and thus provide docking sites for a variety of phosphotyrosine-binding proteins. The specific recruitment of these proteins, which harbour various, catalytic and scaffolding domains, determines the signalling output (Blume-Jensen and Hunter, 2001).

Many RTKs, among which epidermial growth factor (EGFR) (Liebman, 2001), platelet-derived growth factor (PDGF) (Satoh et al., 1993; Nanberg and Westmark, 1993) and RET (Chiariello e al., 1998) stimulate, through the small GTP-binding Ras, different MAP kinase pathways.

MAP Kinases.

Pathways involving MAP kinases are activated in response to an extraordinary diverse array of stimuli. These stimuli vary from growth factors and cytokines to irradiation, osmolarity, and shear stress of fluid flowing over a cell. These stimuli induce a specific dual phosphorylation on a conserved motif, Thr-Xaa-Tyr, present in all MAP kinases (Fig.1). The best characterized substrates for MAP kinases are transcription factors. However, MAP kinases have the ability to phosphorylate many other proteins including other kinases, phospholipases, and cytoskeleton-associated proteins.

In mammals, there are many MAP kinases with different biological functions, grouped in distinctly regulated groups, of which the best known are ERK1/2 (extracellular signal related kinase, ERK), JNKs (jun amino terminal kinase, JNK) and p38, which are involved in many cellular events such as proliferation, differentiation, apoptosis and stress (Chang and Karin, 2001) (Fig.2). All MAP kinases recognize similiar phosphoacceptor sites composed of serine or threonine followed by a proline, and the amino acids that surround these sites further increase the specifity of recognition by the catalitic pocket of the enzyme. Full specificity is ensured through the interaction mediated by another site on the kinase that recognizes a distinct site on the substrate (docking site). Moerover, spatial localization of signalling molecules further auguments specificity in signal transduction (Roux and Bleins, 2004). Finally, cross-talk by scaffolding proteins regulate MAP kinase signaling beyond simple tethering (Chang and Karin, 2001; Qi and Elion, 2005).

ERK 1/2.

The MAP kinases can be activated by a wide variety of different stimuli, but in general, ERK1 and ERK2 are preferentially activated in response to growth factors and

phorbol esters. The mammalian ERK1/2 module, also known as the classical mitogen kinase cascade, consists of the MAPKKKs A-Raf, B-Raf, and Raf-1, the MAPKKs MEK1 and MEK2, and the MAPKs ERK1 and ERK2. ERK1 and ERK2 have 83% amino acid identity and are expressed to various extents in all tissues (Chen et al., 2001). Tipically, cell surface receptors such as tyrosine kinases (RTK) and G protein-coupled receptors transmit activating signals to the Raf/MEK/ERK cascade through different isoforms of the small GTP-binding protein Ras. Activated Raf binds to and phosphorylates the dual specificity kinases MEK1 and MEK2, which in turn phosphorylate ERK1 and ERK2 within a conserved Thr-Glu-Tyr (TEY) motif in their activation loop. (Hallberg et al., 1994). ERK1/2 are distribuited throughout quiescent cells, but upon stimulation, a significant population of ERK1/2 accumulates in the nucleus (Chen et al., 1992). While the mechanisms involved in nuclear accumulation of ERK1/2 remain elusive, nuclear retention, dimerization, phosphorylation, and release from cytoplasmic anchors have been shown to play a role (Pouyssegur et al., 2002). Activated ERK1 and ERK2 phosphorylate numerous substrates in all cellular compartments including various membrane proteins, such as the tyrosine kinase Syk, nuclear substrates, such as MEF2, c-Fos, c-Myc and STAT3, and cytoskeletal proteins, such as paxillin (Chen et al., 2001). ERK1/2 signaling has been implicated as a key regulator of cell growth and differentiation, as a consequence of their effects on cellular proliferation, inhibitors of the ERK pathway are entering clinical trials as potential anticancer agents (Kohno and Pouyssegur, 2003). Only the knockout of ERK1 has been described. *Erk1*$^{-/-}$ mice are viable and appear normal and with a modest defect in T-cell devolopment. It is likely that most ERK1 functions are equally served by ERK2 (Pages et al., 1999). A similar but more marked defect is present in transgenic mice expressing a dominant-negative MAPK kinase MEK1 in thymocytes. Indeed *Mek1*$^{-/-}$ mice die *in utero*, exhibiting defective placental vascularization (Giroux et al., 1999).

JNKs

The Jun kinases (JNK) were originally identified by their ability to phosphorylate c-Jun in response to UV-irradiation (Hibi et al., 1993). Three loci *Jnk* heve been identified. The respective proteins JNK1, JNK2 and JNK3, exist in 10 different spliced forms and are ubiquitously expressed, although JNK3 is present primarily in the brain. The JNKs are strongly activated in response to cytokines, UV irradiation, growth factor deprivation, DNA-damaging agents and, to a lesser extent,

some G protein-coupled receptors, serum, and growth factors (Kyriakis and Avruch, 2001).

Like ERK1/2, JNK activation requires dual phosphorylation on tyrosine and threonine residues within a conserved Thr-Pro-Tyr (TPY) motif. The MAPK kinases that catalyze this reaction are known as MEK4 and MEK7, and are themselves phosphorylated and activated by several MAPKK kinases, including MEKK1-4, MLK2 and MLK3 (Kyriakis and Avruch, 2001).

As for ERKs, JNKs may relocalize from the cytoplasm to the nucleus following stimulation (Mizukami et al., 1997). A well-known substrate for JNKs is the transcription factor c-Jun. Phosphorylationof c-Jun on Ser63 and Ser73 by JNK leads to increased c-Jun-dependent transcription (Weston and Davis, 2002). Several other transcription factors have been shown to be phosphorylated by the JNKs, such as ATF2 ans STAT3 (Kyriakis and Avruch, 2001).

All three *J*nk loci have been knocked out. None of the mutations results in lethality or obvoius defects. However, *J*nk*1*$^{-/-}$ *J*nk*2*$^{-/-}$ double mutants die at mid-gestation (E11), exhibiting defective neural-tube closure (Sabapathy et al., 1999). Thus, JNK functions needed for development and viability are not isoform specific. Unespectedly, deletion of the MAPKK kinase MKK4 results in a more severe phenotype than the combined loss of JNK1/2: mid-gestetional lethality caused by abnormal liver development (Ganiatsas et al., 1998). The same phenotype is caused by complete loss of c-Jun (Su et al., 1994).

p38s.

p38 is the archetypal member of the third MAP kinase-related pathway in mammalian cells (Han et al., 1994). The p38 module consists of several MAPKK kinases, including MEK kinases 1 to 4 (MEKK1-4), MLK2 and MLK3, ASK1 and Cot, the MAPK kinases MEK3 and MEK6 (MEKK3 and MEKK6), and the four known p38 isoforms, α, β, γ and δ (Kyriakis et al., 2001). In mammalian cells, the p38 isoforms are strongly activated by environmental stresses (oxidative stresses, UV irradiation, hypoxia, ischemia) and inflammatory cytokines (interleukin-1, IL-1, tumor necrosis factor alpha, TNF-α) but not appreciably by mitogenic stimuli. Most stimuli that activate p38 also activate JNKs, but only p38 is inhibited by the anti-inflammatory drug

SB203580, which has been extremely useful in delineating the function of p38 (Lee et al., 1994; Chen et al., 2001).

Activation of the p38 isoforms results from MEK3/6 catalyzed phosphorylation of a conserved Thr-Gly-Tyr (TGY) motif in their activation loop (Enslen et al., 2000). p38 was shown to be present in both the nucleus and cytoplasm of quiescent cells. Some evidences suggests that, following activation, p38 translocates from the cytoplasm to the nucleus (Raingeaud et al., 1995), but other data indicate that activated p38 is also present in the cytoplasm of stimulated cells (Ben-Levy et al., 1998).

A large body of evidence indicates that p38 activity is critical for normal immune and inflammatory responses (Ono and Han, 2000), p38 is indeed activated in macrophages, neutrophilis, and T cells by numerous extracellular mediators of inflammation, including chemoattractans, cytokines, chemokines, and bacterial lipolysaccharide (Ono and Han, 2000). p38 partecipates in macrophage and neutrophil functional responses, including respiratoty burst activity, chemotaxis, granular exocicytosis, adherence, and apoptosis, and also mediates T-cell differentiation and apoptosis by regulating gamma-interferon production (Ono and Han, 2000). Moreover, using SB203580 and constitutively active forms of p38 and MEK3/6, it has been shown that p38 regulates the expression of many cytokines, transcription factors, and cell surface receptors (Ono and Han, 2000). While the exact mechanisms involved in p38 immune functions are starting to emerge, activated p38 has been shown to phosphorylate several cellular targets, including cytosolic phospholipase A2, the microtubule-associated protein Tau, and the transcription factors ATF-1 and –2, MEF2A, NF-κB, Ets-1, Elk-1 and p53 (Ono and Han, 2000).

The only p38 isozyme whose *in vivo* function has been examined genetically is p38α. Inactivation of p38α results in embryonic lethality (Tamura et al., 2000). It is no clear whether the lack of compensation by other isoforms is indicative of distinct biochemical functions or a marked difference in expression patterns .

Big "atypical" MAP kinases.

The recently identified ERK5, ERK7 and ERK8 are significantly larger than the originally identified ERK1 and ERK2 due to an extended C-terminal domain. ERK5, also known as big mitogen-activated kinase 1 (BMK1) (Lee et al., 1995), is a 110 kDa protein, while ERK7 is 61 kDa protein and ERK8 is 60 kDa protein. All these MAP

kinases are activated by dual phosphorylation on Thr-Xaa-Tyr motif. Recent information indicates that the C-terminal regions of ERK5 and ERK7 have important regulatory functions. The C-terminal region of ERK5 appears to regulate negatively its kinase activity (Zhou et al., 1995) and contains a putative bipartite nuclear translocation signal for ERK5 that functions *in vivo* following activation (Yan et al., 2001). The C-terminal region of ERK5 also contains a myocyte enancher-binding factor 2-interacting region and a potent transcriptional activation domain (Kasler et al., 2000). Disruption of the gene encoding ERK5 led to angiogenic defects and embryonic lethality in mice (Yan et al., 2003)

ERK7 is activated by autophosphorylation, which is regulated through its C-terminal domain (Abe et al., 2001). Moreover, the C-terminal region is required for the ability of ERK7 to localize to the nucleus and inhibit growth (Abe et al., 1999).

ERK8 is the last identified member of the MAP kinase family (Abe et al., 2002). ERK8 represents the human orthologue of the rat ERK7 and is present in brain, kidney and lung. The overall amino acid identity of the human ERK8 and rat ERK7 sequences is 69%. Comparison of the kinase domains reveals a sequence identity of about 82%, whereas the amino acid sequence identity of the C-terminal regions is only 53% (Abe et al., 2002). By contrast, sequence identity between other ERK orthologues is significantly higher.

The possible physiological roles of ERK8 remain the less studied. The failure of ERK8 to phosphorylate many of tested substrates, c-jun, c-myc, histone H1, Ets-1, Elk-1 and paxillin has not elucitated its function. Its activation following stimulation by c-Src or cell exposure to serum hints at a function in response to mitogenic factors (Abe et al., 2002). Obviously, many possibilities remain to be explored when describing the function of ERK8.

The *objective* of the present work is to determine the relevant MAP kinase family members involved in the signals from tyrosine kinase receptors to the nucleus. In particular, we examined the role of JNK in *c-myc* expression induced by PDGF and the activation c-Abl mediates RET/PTC3-dependent of the novel ERK8 MAP kinase.

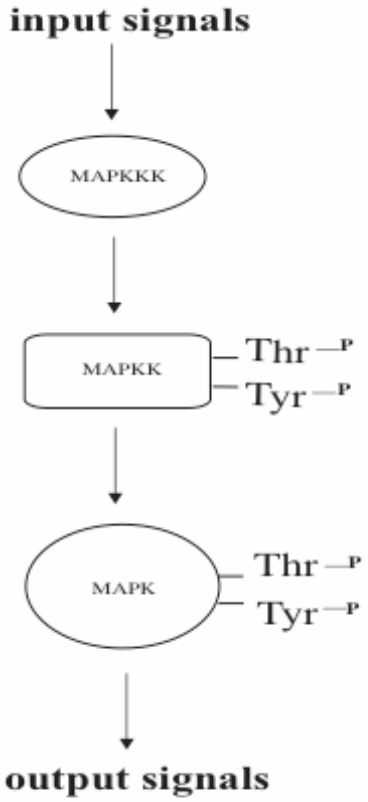


Fig.1. MAPK pathway module.
Core module of a mitogen-activaed protein kinase (MAPK) pathway is composed of three kinases, MAP kinase kinase kinase (MKKK), MAP kinase kinase (MKKK), MAP kinase (MAPK), that are sequentially activated by phosphorylation. MAPKs are activated by dual phosphorylation of conserved thronine and tyrosine residues within the activation loop (TXY).

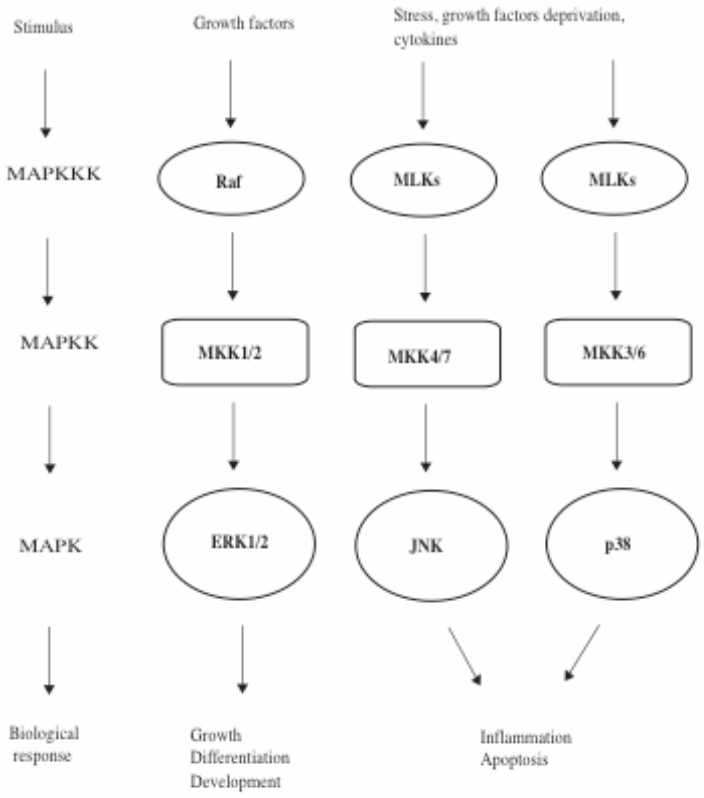


Fig.2. Signaling cascade of the MAPK pathways.
Schematic representation of signaling cascades leading to activation of MAP kinases and to respective biological functions.

Materials and Methods

Expression vectors.

pcDNAIII/GS-Myc-V5 was purchased from Invitrogen. Expression vectors for Rac12V and the corresponding effector domain mutants Rac12V/33N, Rac12V/37L, Rac12V/40H were kindly provided by C.J. Der (Westwick et al., 1997). The bacterial expression vector pGEX 4T3 GST-ATF2, and expression vectors for MEKK1 and MLK3 were described previously (Chiariello et al., 2000; Teramoto et al., 1996). PCDNAIII-Sis was generated by cloning the *sis* (PDGF BB) oncogene in the EcoRI and NotI restriction sites. The pmycAP1 luc reporter vector was obtained by cloning two mouse AP-1 elements in the pGL3 reporter vector (Promega). PCR amplifications of the c-Fos and c-Jun cDNAs were cloned in the pCEFL AU5 and pCEFL AU1 expression vectors, respectively. The JunDBD-SID expression vector was prepared cloning in pCEFL HA the DNA binding domain of c-Jun and the Sin3-binding domain of Mad. The Gal4-driven luciferase reporter plasmid pGal4 Luc was constructed by inserting six copies of a Gal4 responsive element and a TATA oligonucleotide to replace the simian virus 40 minimal promoter in the pGL3 vector (Promega). The Gal4-VP16 expression vector was prepared cloning the transactivation domain of the VP16 transcription factor in frame with the DNA-binding domain of Gal4, into the pCDNA III vector.

The expression vectors pCEFLP-SrcYF (constitutively active) and pCEFLP-SrcYF KM (dominant negative) were obtained by sub-cloning the corresponding cDNA obtained from pSM-SrcYF and pSM-SrcYF KM, kindly provided by H. Varmus (Chiariello et al., 2001). The HA-tagged form of Erk8 was generated by cloning the corresponding cDNA, kindly provided by M. Abe (Abe et al., 2002), in the pCEFL-HA vector. The expression vector for the dominant negative Erk8 KR molecule was also provided by M. Abe (Abe et al., 2002). To generate the pCEFL-HA-Erk8□ expression vector, we amplified by PCR the corresponding cDNA using an “expressed sequence tag” (est) obtained from ResGen (Clone ID 5742965). This sequence data has been submitted to the GenBank database under accession number AY994058. The pCDNA3-Ptc3 expression plasmid has been previously described (Melillo et al., 2001). The $Ptc3^{Y981}$, $Ptc3^{Y1015}$, $Ptc3^{Y1062}$, Ptc3 Kin^{dead} and $Ptc3^{V804}$ expression plasmid were generated by the QuikChangeTM Site-Directed Mutagenesis Kit (Stratagene), using

pCDNA3-PTC3 as a template. Expression vectors for c-Abl and its oncogenic form, Bcr/Abl p210 (Bcr/Abl), have been previously described (Lobo et al., 2005; Sanchez-Prieto et al., 2002). The dominant negative c-Abl (Abl-KD) expression vector was obtained by mutating a critical lysine in the kinase domain of c-Abl, contained in the pCEFL-AU5 vector. The c-myc and c-jun promoter reporter plasmids, pMyc-Luc and pJun-Luc, respectively, and the pCDNAIII-□-galactosidase (□-gal) expression vector have been previously described (Chiariello et al., 2000; Chiariello et al., 2001).

Reagents.

Human recombinant PDGF-BB (Intergen, NY) was used at a final concentration of 12.5 ng ml^{-1}. The selective JNK inhibitor SP600125 (Biomol, PA) was added to the cells 30 min before stimulation, at the indicated concentrations. The PP1 inhibitor was purchased from Biomol. All other chemicals were purchased from Sigma.

Cell culture and transfections.

293T cells and thyroid ARO cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine, and 100U/ml penicillin-streptomycin (Invitrogen). NIH3T3 fibroblasts were maintained in DMEM supplemented with 10% calf bovine serum (Bio Whittaker), 2mM L-glutamine, and 100U/ml penicillin-streptomycin (Invitrogen). 293T and NIH3T3 cells were transfected by the LipofectAMINE reagent (Invitrogen), while ARO cells were transfected by the Lipofectamine 2000 reagent (Invitrogen), respectively, in accordance with the manufacturer's instructions. For transfections, 200 ng of HA-Erk8 and HA-Erk8δ and 100 ng of SrcYF, Abl Act., Bcr/Abl and of the different Ptc3 expression vectors were used, unless otherwise indicated.

Antibodies.

As primary antibodies rabbit polyclonal antibodies against JNK1 (C-17), Rac1 (C-14), c-Jun (H-79), JunD (329), JunB (N-17), ATF2 (C-19) (Santa Cruz); Phospho c-Jun (Ser63) and Phospho c-Jun (Ser73) (Cell Signaling Technology); Erk2 (C-14) and c-Src (N-16) (Santa Cruz), phospho-MAPK (p42/p44) (Cell Signaling), RET and phospho-RET (phospho-Tyr905) (Carlomagno et al., 2004); mouse monoclonal antibodies against AU5, EGFP and haemagglutinin (HA) epitopes (HA.11; Berkley

Antibody Company, CA); JNK1 (PharMingen); c-Abl (BD Pharmingen) and to phospho-tyrosine, PY (Santa Cruz and Upstate Biotechnology).

EMSA, western blots, immunoprecipitations and ChIP analysis were performed using rabbit polyclonal antibodies against JNK1 (C-17), Rac1 (C-14), c-Jun (H-79), JunD (329), JunB (N-17), ATF2 (C-19) (Santa Cruz); Phospho c-Jun (Ser63) and Phospho c-Jun (Ser73) (Cell Signaling Technology); mouse monoclonal antibodies against haemagglutinin (HA) epitope (HA.11; Berkley Antibody Company, CA); JNK1 (PharMingen).

Western blot analysis.

Lysates of total cellular proteins or immunoprecipitates were analyzed by protein immunoblotting after SDS-PAGE with specific rabbit antisera or mouse monoclonal antibodies. Immunocomplexes were visualized by enhanced chemiluminescence detection (ECL or ECL Plus, Amersham-Pharmacia) with the use of goat antiserum to rabbit or mouse immunoglobulin G, coupled to horseradish peroxidase (Amersham-Pharmacia).

Reporter gene assays.

For each well, cells were transfected by the "LipofectAMINE Reagent" with different expression plasmids, together with 50 ng of the indicated reporter plasmid and 10 ng of pRL-null (a plasmid expressing the enzyme *Renilla* luciferase from *Renilla reniformis*) as an internal control. In all cases, the total amount of plasmid DNA was adjusted with empty vector.

NIH 3T3 cells were transfected with different expression plasmids together with 100 ng of the pMyc Luc reporter plasmid. ARO cells were transfected with different expression plasmids together with 20 ng of the pJLuc reporter plasmid. After 24 h incubation in serum-free media, the cells were lysed using reporter lysis buffer (Promega). Luciferase activity present in cellular lysates was assayed using D-luciferin and ATP as substrates, and light emission was quantitated using the $20^n/20^n$ luminometer as specified by the manufacturer (Turner BioSystems).

Northern blot analysis.

After 24-hrs starvation, NIH 3T3 cells were washed with cold PBS and total RNA was extracted by homogenization with Trizol (Invitrogen), in accordance with manufacture's specifications. Total RNA (10 μg) was fractionated in 2% formaldehyde-agarose gels, transferred to Hybond-XL nylon membranes (Amersham-Pharmacia Biotech) and hybridized with ^{32}P-labelled DNA probes prepared with the Prime-a-Gene Labelling System (Promega). As a probe, we used a 450-bp PstI DNA fragment from the human c-*myc* gene (pcDNAIII/GS-Myc-V5). The RNA membranes were pre-hybridized for more than 2 hrs in hybridization solution (ExpressHyb; Clontech) at 70^{o}C. The ^{32}P-labeled probe was added to the blots and hybridized for another 16 hrs at 60^{o}C. The blots were washed twice for 30 min each in 2X SSC-0.1% SDS at room temperature and then washed twice for 30 min each in 0.2X SSC-0.1% SDS at 60^{o}C. Accuracy of RNA loading and transfer was confirmed by fluorescence under ultraviolet light after staining with ethidium bromide.

Electrophoretic mobility shift assays (EMSA).

Nuclear extracts were obtained from NIH 3T3 cells plated in 10-cm plates and grown to 70% confluency, starved overnight and then stimulated with PDGF, when needed. Cells were washed in cold PBS and lysed in 400 μl of buffer A (10 mM HEPES pH=7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF). After 15 min on ice, 25 μl of 10% NP-40 was added and vigorously vortexed for 10 sec. Homogenates were centrifuged for 30 sec. Nuclear pellets were resuspended in 50 μl of ice-cold hypotonic buffer C (20 mM HEPES pH=7.9, 0.42 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and rocked at 4^{o}C for 15 min. Homogenates were centrifugated for 5 min and the supernatants (nuclear extracts) aliquoted and stored at –70^{o}C. After determining protein concentrations using Bio-Rad protein assay (Bio-Rad Laboratories), 2 μg of proteins were incubated at room temperature with 1 μg of poly-[dI-dC] and 0.1 μg of salmon sperm DNA in 20 μl binding buffer (12 mM HEPES pH=7.8, 60 mM KCl, 2 mM $MgCl_2$, 0.12 mM EDTA, 0.3 mM DTT, 0.3 mM PMSF, 12% glycerol) for 15 min. Complementary synthetic oligonucleotides containing the AP-1 responsive element plus adjacent sequences from the mouse c-*myc* promoter (AP1F, 5'-ATACCTGTGACTCATTCATTT-3' and AP1R, 5'-AAATGAATGAGTCACAGGTAT-3') were obtained from MWG Biotech and labeled with γ^{32}P-ATP using T4 polynucleotide kinase (Invitrogen). Labeled oligos were

purified using G25 columns (Amersham Pharmacia Biotech) and used as probes (20,000 cpm/reaction) added to the reactions for additional 15 min. Complexes were analyzed on non-denaturing (4.5%) polyacrylamide gels in TGE buffer (40 mM Tris, 270 mM Glycine, 2 mM EDTA=pH 8.0), run at 13V/cm at 4°C. For super-shift assays, 1 μg of the indicated antisera were added to the binding reaction.

Chromatin Immunoprecipitation (ChIP).

ChIP assays were performed using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology, NY), in accordance with the manufacturer's instructions. Briefly, chromatin from NIH 3T3 cells has been fixed by directly adding formaldehyde (1% final) to the cell culture media. Nuclear extracts have been isolated from the cells and then sonicated to obtain mechanical sharing of the fixed chromatin. Transcription factors-bound chromatin has been immunoprecipitated with specific antibodies, cross-linking has been reversed and the isolated genomic DNA has been amplified by PCR, using specific primers encompassing the murine c-*myc* promoter: forward AP66 (5'-ATACCTGTGACTATTCATTT-3'); reverse AP67 (5'-GATGCTTCCTTGCCTAAGAC-3'). The PCR products were separated on a 2% agarose gel. Primers used as a control for the ChIP analysis amplify an unrelated DNA sequence located on murine chromosome 5.

***In vitro* kinase assay.**

Confluent plates of transfected NIH3T3 were kept two hours (JNK assay) or overnight (MAPK assay) in serum-free medium. Cells were then washed with cold phosphate-buffered saline, and lysed at 4° C in a buffer containg 20 mM Hepes, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 1% IGEPAL, 2.5 mM $MgCl_2$, 1mM dithiothreitol, 2 mM sodium vanadate, 1mM phenylmathylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Lysates were clarified by centrifugation at 12,000 x g for 20 min at 4° C, and supernatants were incubated with 1 μg monoclonal antibody against JNK (PharMingen) or with 1 μg polyclonal antibody against Erk2 (C-14) (Santa Cruz), for 1 h at 4° C. Immunocomplexes were recovered with the aid of protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Pecipitates were washed three times with phosphate-buffered saline which contained 1% IGEPAL and 1mM vanadate, once with 100 mM Tris pH 7.5, 0.5 M LiCl, and once in kinase reaction buffer (12.5 mM MOPS,

pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5mM EGTA, 0.5 mM sodium fluoride, 0.5 mM vanadate).Assays were performed in a reaction buffer containing 1 μCi of [γ-^{32}P]ATP, 20 μM ATP, 3mM dithiothreitol and 1 μg GST-ATF2 and myelin basic protein (MBP, Sigma). After 30 min at 30°C, reactions were terminated by addition of 5X Laemli buffer. Samplers were heated at 95°C for 5 min and analyzed by SDS-gel electrophoresis on 12% acrylamide gels. Autoradiography was performed with the aid of an intensifying screen.

Study I

The PDGF controls *c-myc* expression through a JNK- and AP-1-dependent signaling pathway.

A wide range of growth factors, cytokines and mitogens is able to induce the expression of the c-*myc* proto-oncogene (Kelly et al., 1983; Roussel et al., 1991). In turn, c-*myc* is necessary for cellular proliferation induced by different oncogenic tyrosine kinases (Barone and Courtneidge, 1995) . In normal cells as well as in tumors, the ability of c-*myc* to control cellular proliferation has been mostly correlated to changes in its mRNA levels, through transcriptional and post-transcriptional mechanisms. In fact, most of the oncogenic alterations that target c-*myc* result in the increase of its messenger RNA and, in turn, of its protein (Grandori et al., 2000). Indeed, overexpression or gene amplification and translocations of *c-myc* are frequent causes of numerous solid and blood human tumors (Dang et al., 1999). In line with its ability to promote cell cycle progression, in quiescent fibroblasts *c-myc* expression is virtually undetectable. However, upon stimulation with growth factors such as the platelet-derived growth factor (PDGF), its mRNA and then protein levels are rapidly induced until cells progress through the G_1/S boundary of the cell cycle (Chiariello et al., 2000; Chiariello et al., 2001). Still, the mechanism by which growth factors promote the expression of c-*myc* is poorly understood. In this regard, we have recently described a Rac-dependent signaling pathway initiated by PDGF, controlling the expression of the c-*myc* proto-oncogene (Chiariello et al., 2001).

Rac effector domain mutants differentially impair endogenus c-myc expression.

To investigate the signaling pathways activated by Rac, impinging on the regulation of c-*myc* expression, we used specific constitutively active Rac effector domain mutants that are differentially impaired in their downstream signaling activities (Westwick et al., 1997; Joyce et al., 1999). We therefore compared the ability to stimulate c-*myc* expression of a constitutively active Rac12V mutant with that of Rac alleles harboring additional mutations in their effector domain (Rac12V/33N, Rac12V/37L and Rac12V/40H). We first explored, by northern blot analysis, the ability of Rac12V to induce c-*myc* expression, using PDGF as a positive control. As expected, the activated Rac12V mutant significantly induced c-*myc* expression, as evidenced by

an increase in the level of c-*myc* mRNA (Fig. 3A). Next, we investigated the effect of the double mutants. As shown in fig. 3B, both the Rac12V/37L and Rac12V/40H mutated proteins were ineffective in stimulating the expression of c-*myc*, while the Rac12V/33N protein was fully competent to induce the transcription of the c-*myc* proto-oncogene.

Recent work has established an impairment of JNK activation as a consequence of the transfection of the Rac12V/37L and Rac12V/40H effector domain mutants (Westwick et al., 1997). In line with these studies, data in fig. 3C show that both Rac12V/37L and Rac12V/40H not only were unable to stimulate c-*myc* expression, but they were also incapable to stimulate the activity of JNK, therefore suggesting the involvement of this kinase in Rac-induced c-*myc* expression. Conversely, the Rac12V/33N mutated protein activated JNK at a level similar to the positive control, Rac12V (Fig. 3C). All the Rac mutants were expressed at comparable levels (Fig. 3D). Together, these results strongly suggest that the JNK pathway is involved in the regulation of c-*myc* expression.

JNK activity is necessary for PDGF induction of c-myc expression.

The possibility that JNK participates in the regulation of c-*myc* expression prompted us to test whether the constitutive activation of this signal transduction pathway could stimulate the expression of the endogenous c-*myc* proto-oncogene. We therefore transfected NIH 3T3 cells with vectors expressing two upstream activators of the JNK cascade, the MEKK1 and MLK3 MAP kinase kinase kinases (MAPKKKs) (Teramoto et al., 1996; Joyce et al., 1999). As shown in fig. 4A, both proteins induced the transcription of the endogenous c-*myc* gene, at levels comparable to the positive control, Rac12V, indicating that JNK activation is sufficient to trigger the expression of c-*myc*.

Although JNKs have been isolated and characterized as stress-activated kinases, on the basis of their strong response to environmental stresses and inflammation stimuli, different growth factors are also able to stimulate their activity (Davis, 2000). Moreover, they have recently been involved in mediating the proliferative effects of some oncogenes, including the product of the *bcr-abl* oncogene (Hess et al., 2002). Based on our data, we next explored the participation of JNK in the regulation of c-*myc* expression induced by PDGF. We began exploring the ability of this mitogen to activate JNK. As shown in fig. 4B, exposure of NIH 3T3 fibroblasts to

PDGF induced activation of JNK, which peaked 30 minutes after stimulation. As an approach to examine the involvement of JNK in PDGF-induced c-*myc* expression, we took advantage of the availability of a synthetic compound, SP600125, a reversible ATP-competitive inhibitor that blocks JNK without significantly affecting other related kinases (Bennet et al., 2002). We first confirmed the ability of the drug to inhibit JNK-dependent pathways, in our experimental model. Indeed, SP600125 abolished PDGF-induced phosphorylation of the endogenous c-Jun protein in a dose dependent manner, as scored by western blot analysis using a mix of anti-phospho-Ser63 and -Ser73 c-Jun antibodies (Fig. 4C). Conversely, identical concentrations of the drug had no effect on PDGF-induced Erk1/2 activation and on Erk-dependent c-Fos phosphorylation (data not shown), indicating the specificity of the SP600125 for the JNK pathway, as compared to other highly related MAP kinase-signaling pathways. To test the involvement of JNK in PDGF-induced c-*myc* expression, we performed northern blot analysis on NIH 3T3 cells pretreated with increasing concentrations of the JNK inhibitor and then stimulated with PDGF for 1 hour. As a result, the drug strongly inhibited PDGF-induced c-*myc* expression, even at the lowest concentration of the drug (Fig. 4D). Remarkably, the kinetic of inhibition of c-*myc* expression was coincident with the results obtained for the inhibition of PDGF-induced JNK activation by SP600125 (Fig. 4C). Thus, the emerging picture from these data is that activation of the JNK pathway may regulate c-*myc* expression and, in turn, PDGF exploits JNK as a key molecule to promote c-*myc* expression, possibly through phosphorylation and activation of nuclear transcription factors.

A typical AP-1 responsive element in the c-myc promoter.

Two principal promoters, P1 and P2, drive the transcription of the human c-*myc* gene (Spencer and Groudine, 1991). Despite the extraordinary complexity in the regulation of c-*myc* expression, the rate of transcription from these two promoters is mainly governed by composite negative and positive regulatory elements comprised within a 2.3 kb domain located upstream of the promoters (Hay et al., 1987). Among these elements, E2F, Stat-3, NF-□B and TCF-4 binding sites have been identified (Kiuchi et al., 1999; He et al., 1998; Wong et al., 1995; Ji et al., 1994). In search for additional responsive elements that could mediate the JNK-dependent regulation of the c-*myc* gene, we performed computer-assisted analysis of its promoter region by the TRANSFAC database (Heinemeyer et al., 1998). Surprisingly, we could identify, 1.3 kb

upstream the human c-*myc* transcription start site, a TGAGTCA motif perfectly matching the canonical AP-1 responsive element (Shaulian and Karin, 2001) (Fig. 5A). Interestingly, a similar analysis found conserved responsive elements also in the promoters of murine and even drosophila c-*myc* genes (Fig. 5A). The sequences of the respective responsive elements were highly related to each other (Fig. 5A, boxed nucleotides) as opposed to their immediate flanking regions, suggesting that a strong selective pressure was exerted to maintain these sites intact during evolution.

Several short sequences similar to known response elements are frequently found in promoter regions of a variety of genes. However, the arrangement of these sites in relation to neighboring sequences often determines the functionality of the predicted binding site. Thus, we first studied the ability of an oligonucleotide containing the murine c-*myc* AP-1 responsive element plus adjacent sequences, to form DNA/proteins complexes by means of electrophoretic mobility shift assays (EMSA). As shown in fig. 5B, left panel, proteins from NIH 3T3 nuclear extracts recognized and strongly bound the c-*myc* AP-1 responsive element, as evidenced by the presence of a shifted complex that was more prominent 4 hours after PDGF addition, as a consequence of the accumulation of AP-1 proteins in the stimulated NIH 3T3 cells (Lallemand et al., 1997). The binding was specific, as it was efficiently competed by adding an excess of unlabeled c-*myc* AP-1 oligonucleotide (Fig. 5B, right panel). To further investigate the nature of the transcription factors bound to the described c-*myc* AP-1 element, we next performed super-shift experiments by incubating the binding reactions in the presence of specific antibodies against Jun family members. These proteins have been in fact described as substrates of the JNK signaling pathway and they could therefore possibly mediate the effect of this kinase on the c-*myc* promoter. As shown in fig. 5C, both c-Jun and JunD antibodies strongly decreased the electrophoretic mobility of the complexes derived from NIH 3T3 cells stimulated 30 minutes with PDGF, whereas the JunB antibody had a much lower effect. As an additional control, no ATF2 was detected in the complexes (Fig. 5C), in line with the fact that Jun:ATF2 heterodimers bind more efficiently atypical 8-bp, TGACGTCA sites (Van Dam et al., 1998). Conversely, among Fos proteins, only Fra2 was detected as part of the complexes (data not shown). On the basis of the binding observed *in vitro*, we next examined by Chromatin Immunoprecipitation (ChIP) analysis whether members of the Jun family could actually bind, *in vivo*, the endogenous c-*myc* promoter. In NIH 3T3 cells, ChIP assays clearly demonstrated the binding of both c-Jun and JunD to the

endogenous c-*myc* promoter, 30 minutes after PDGF addition (Fig. 5D), coincidently with the time-point at which PDGF induces maximal JNK stimulation (see fig. 4B). Conversely, we did not observe any *in vivo* binding of JunB to the promoter (Fig. 5D). As expected (see above), we could not detect ATF2 bound to the c-*myc* promoter (Fig. 5D), whereas it was able to bind the c-*jun* promoter, which harbors an atypical 8-bp, TGACATCA element (data not shown). The same analysis, performed on untreated, quiescent NIH 3T3 cells, gave similar results (data not shown), confirming that Jun family members are pre-bound to their responsive elements (Lallemand et al., 1997) and can be rapidly trans-activated by phosphorylation in response to external stimuli (Mechta-Grigoriou et al., 2001). As an additional control, no amplification was observed from the same immunoprecipitates when using primers recognizing DNA sequences unrelated to the c-*myc* promoter (Fig. 5D, lower panel). At this point it is important to notice that, while ChIP analysis was not able to detect binding of JunB to the c-*myc* promoter, EMSA experiments showed a small amount of this protein bound to the c-*myc* AP-1-containing EMSA probes. We have attributed this apparent difference to the *in vitro* nature of the EMSA and its limitations to precisely recapitulate the binding of the transcription factors at the level of the endogenous promoters. At the same time, this situation underscores the importance of the results from the ChIP assay, showing *in vivo* binding of c-Jun and JunD to the promoter. Altogether, these results indicate that proteins of the AP-1 family, specifically c-Jun and JunD, are able to recognize and bind, *in vivo*, the AP-1 element present in the c-*myc* promoter, therefore suggesting this element as a potential mediator of JNK-dependent regulation of c-*myc* expression induced by PDGF.

The AP-1 element controls PDGF stimulation of c-myc expression.

We next investigated whether the c-*myc* AP-1 element was able to mediate PDGF-induced stimulation of c-*myc* expression. The control of histone acetylation is a key step in the general regulation of cellular transcriptional events (Grunstein, 1997). In turn, a model has been recently proposed in which the trans-activation potential of c-Jun and, possibly, of its related proteins, is constitutively repressed by a histone deacetylases (HDACs)-containing complex, which physically interacts with c-Jun itself and can be released upon JNK-dependent phosphorylation of the protein (Weiss et al., 2003). We therefore reasoned that an artificial molecule specifically targeting HDACs to AP-1 elements could recapitulate HDAC-dependent negative regulation of AP-1

containing promoters, but in a dominant repressive fashion (unable to be relieved by upstream stimuli). We therefore engineered a molecule in which the DNA Binding Domain (DBD) of c-Jun has been fused to the Sin3-binding domain of Mad (SID). The resulting protein (JunDBD-SID) is able to bind Sin3 and, through this, recruit HDACs (Ayer et al., 1996). We expect this repressor to be able to specifically inhibit transcription by targeting, through the c-Jun DBD, AP-1 elements that are present in the endogenous promoters. To control the specificity of the repressor, we first engineered a reporter plasmid carrying the *luciferase* gene expressed under the control of a tandemly repeated AP-1 element from the murine c-*myc* gene (pmycAP1 luc). Such construct behaves as a typical AP-1 reporter, its activity being readily induced by the c-Jun and c-Fos members of the AP-1 family (Fig. 6A) and by upstream stimulators of the JNK pathway, MEKK1 and MLK3 (Teramoto et al., 1996; Minden et al., 1994) (Fig. 6B). We hypothesized that the expression of *luciferase* from this construct should be strongly inhibited by the JunDBD-SID repressor, through specific targeting to the c-*myc* AP-1 and recruitment of HDACs. As expected, very low amounts of the JunDBD-SID repressor were sufficient to completely abolish the activity of the pmycAP1 Luc reporter induced by *sis*, the oncogenic form of the *PDGF* oncogene (Fig. 6C), while not affecting the luciferase activity induced by a Gal4-VP16 (Fig. 5D) or a p53 molecule (data not shown), on their respective reporter vectors. These data therefore confirmed the effectiveness and specificity of the engineered protein and the dependency of its activity upon the presence of functional AP-1 elements. It is also important to notice that such experiments not only control the specificity of our approach, but also contribute to establish that the c-*myc* AP-1 is a fully functional element that can be stimulated by PDGF and the JNK pathway. This further supports the hypothesis that JNK plays a key role in the regulation of c-*myc* expression, possibly induced by PDGF activation of its cognate receptors.

Finally, to prove the ability of the AP-1 element to regulate the transcription of the c-*myc* promoter, we analyzed by northern blot the RNAs produced by PDGF-treated cells expressing the JunDBD-SID protein. Strikingly, the AP-1 repressor clearly inhibited PDGF-induced accumulation of c-*myc* mRNA (Fig. 6E). Altogether, these findings strongly support the idea that the AP-1 sequence identified in the c-*myc* promoter is functional, being able to control the c-*myc* expression induced by PDGF, through the recruitment of members of the AP-1 family of transcription factors. In all, these findings also contribute to understand some of the molecular mechanisms by

which c-Jun acts as a positive regulator of the cell cycle (Shaulian and Karin, 2001; Mechta-Grigoriou et al., 2001) as only very few c-Jun targets involved in the control of the cell cycle, have been yet identified. This study, in fact, add c-*myc* to the short list of prototypes genes, such as *cyclin D1* and *p53* (Albanese et al., 1995; Schreiber et al., 1999), that are regulated by c-Jun and involved in cellular proliferation. Our finding also show that JunD is bound to the c-*myc* promoter and can regulates c-*myc* expression, which may help to explain the role of this protein as mediator of cellular survival (Weitzman et al., 2000; Lamb et al., 2003). Interestingly, although several studies have described a pro-apoptotic effect for JNK (Davis, 2000), more recent evidences show that downstream of this kinase, JunD acts as a sensor that transmit survival or apoptotic signals depending on the state of others transcription factors (Lamb et al., 2003). As c-*myc* itself has been involved in both pro- and anti-apoptotic responses, the mechanism by which regulation of c-*myc* expression by JNK-c-Jun/JunD relates to these two opposite responses will warrant further investigation.

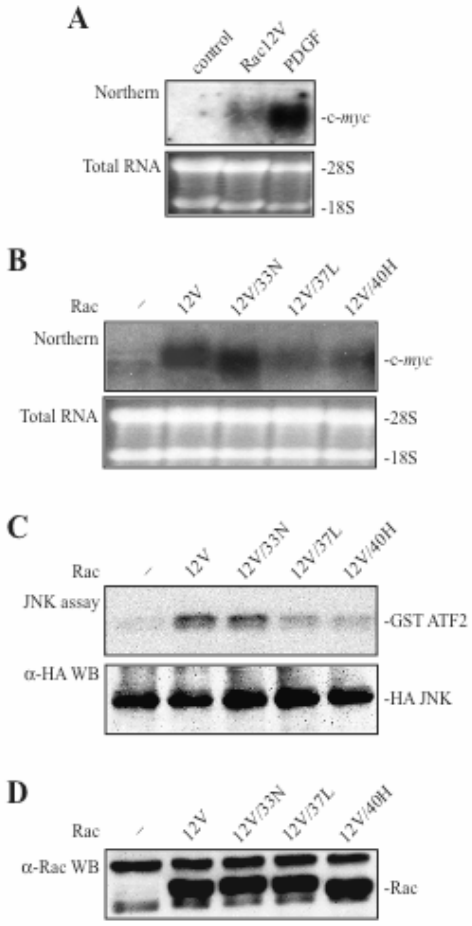


Fig.3 Effects of Rac effector domain in mutants on the expression of the endogenous *c-myc* gene.
(A), analysis of c-myc mRNA expression in NIH 3T3 cells stimulated 1 hour with PDGF or transiently expressing the activated form of Rac1, Rac12V (2 μg). (B), analysis of c-myc mRNA in NIH 3T3 cells transiently transfected with constitutively active Rac effector domain mutants (2 μg), as indicated. (C), stimulation of HA-JNK (1 μg) activity in transiently transfected NIH 3T3 cells expressing specific constitutively active Rac effector domain mutants (2 μg). (D), analysis of the expression of the constitutively active Rac effector domain mutants (2 μg). -, no treatment; α, antibody against; WB, western blot.

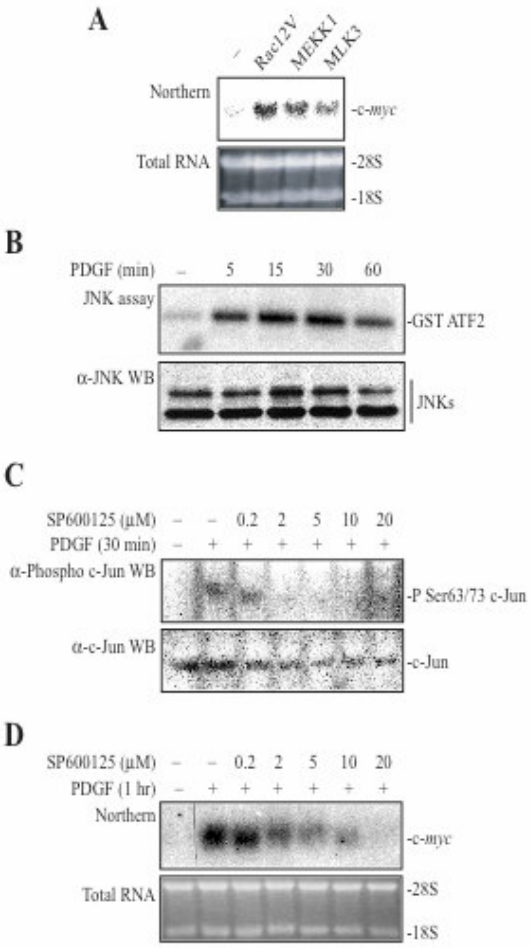


Fig.4 A role for JNK in PDGF induction of *c-myc* expression.
(A), Analysis of c-myc mRNA expression in NIH 3T3 cells transfected with expression vectors for Rac12V, MEKK1 and MLK3 (2 μg each). (B), Stimulation of endogenous JNK activity in NIH 3T3 cells treated with PDGF for the indicated durations. (C), Analysis of serine-63 and serine-73 phosphorylation of the endogenous c-Jun in NIH 3T3 cells pre-treated with increasing concentrations of the specific JNK inhibitor, SP600125, and then stimulated for 30 minutes with PDGF. (D), Analysis of c-myc mRNA in NIH 3T3 cells pre-treated with increasing concentrations of the specific JNK inhibitor, SP600125, and then stimulated for 1 hour with PDGF. -, no treatment; α, antibody against; WB, western blot.

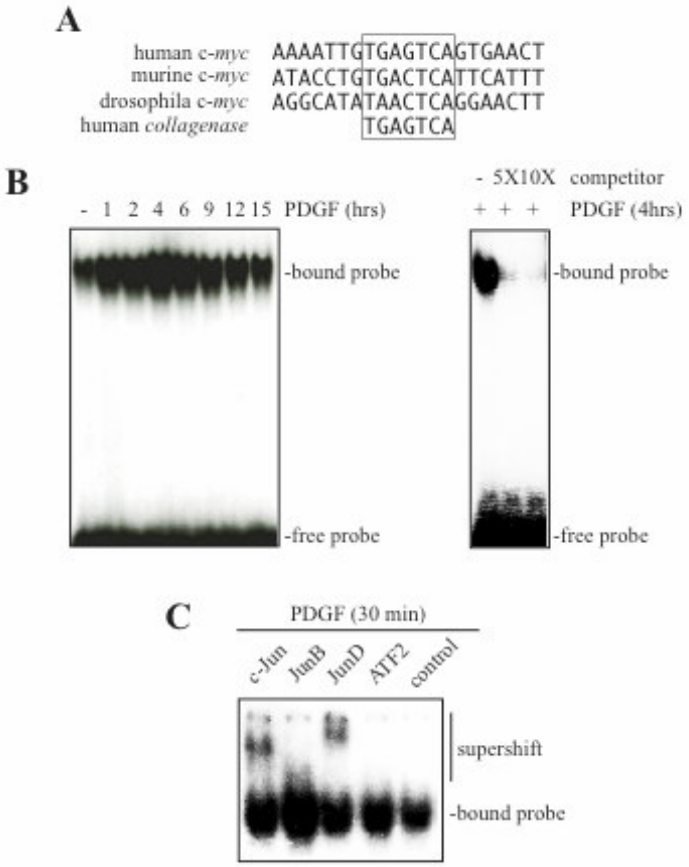
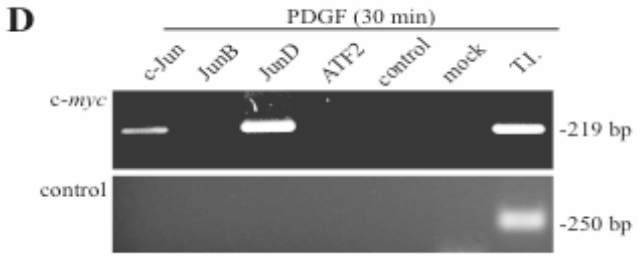


Fig.5 A typical AP-1 element in the *c-myc* promoter, recognized by the c-Jun and JunD AP-1 family members. (A), Sequences of predicted AP-1 responsive elements from humand mouse and drosophila c-myc promoters, obtained by the TRANSFAC database. The well-established AP-1 element of the human collagenase gene is used as a comparison. (B), Left panel, binding of nuclear proteins to the AP-1 responsive element from the mouse c-myc promoter, determined by electrophoresis mobility shift assay (EMSA), upon PDGF stimulation of NIH 3T3 cells. Right panel, control of the specificity of the EMSA analysis on the AP-1 element of the mouse c-myc promoter using the unlabeled oligonucleotides as competitors in concentration of five and ten fold-molar excess versus the probe. (C), Super-shift analysis of nuclear factors bound to the AP-1 element of the mouse c-myc promoter.



(D), ChIP analysis of the murine c-myc promoter from NIH 3T3 cells treated 30 minutes with PDGF. Immunoprecipitates from each sample were analyzed by PCR using primers for the mouse c-myc promoter, as specified in the "Experimental Procedures" section. As a control, a sample representing linear amplification of the total input chromatin was included in the PCR (T.I.). Additional controls included an immunoprecipitation lacking specific antibody (control) and amplification of a sample without chromatin (mock). In the lower panel, samples from the same immunoprecipitates were analyzed by PCR, using primers recognizing an unrelated DNA sequence present on murine chromosome 5. Nuclear extracts were obtained from NIH 3T3 cells treated 30 minutes with PDGF. -, no treatment.

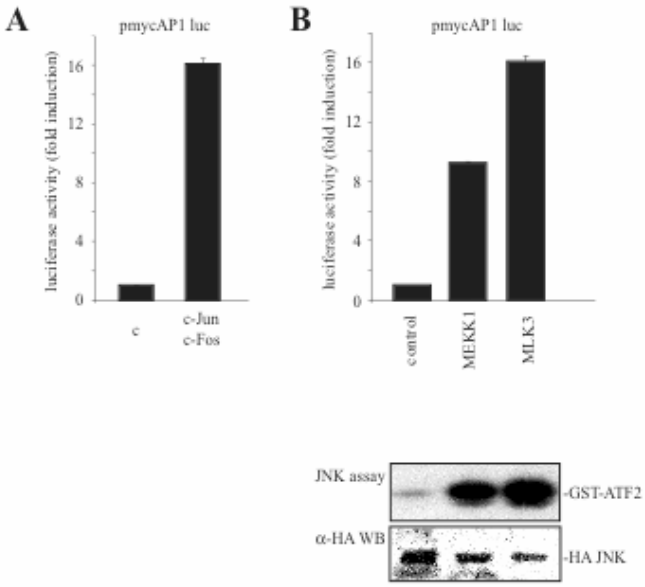
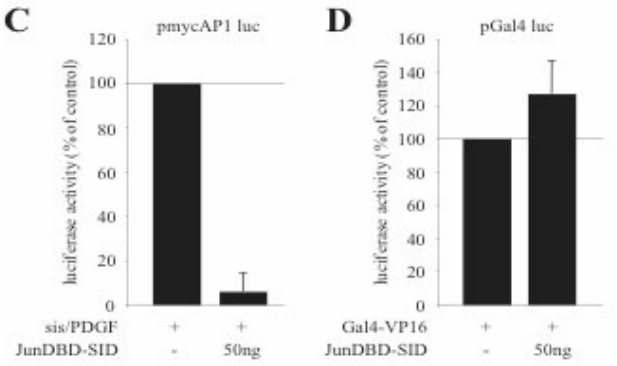
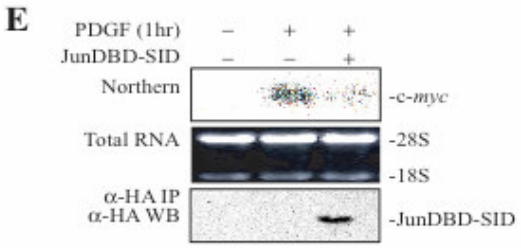


Fig. 6 PDGF stimulation of *c-myc* expression is controlled by the AP-1 responsive element.
(A), Stimulation of the c-myc AP-1 in NIH 3T3 cells cotransfected with the pmyc AP1 luc reporter (50 ng) and expression vectors for c-Jun and c-Fos (0.5 µg each). (B), Stimulation of the c-myc AP1 responsive element in NIH 3T3 cells cotransfected with pmyc AP1 luc (50 ng) and expression vectors for MEKK1 and MLK3 (0.5 µg each). As a control, NIH 3T3 cells co-transfected with an epitope-tagged HA-JNK (0.5 µg) and expression vectors for MEKK1 and MLK3 (0.5 µg each) were also subjected to JNK *in vitro* kinase assay (lower panels).





(C), Analysis of the stimulation of the c-myc AP1 element by the PDGF/Sis oncogene in NIH 3T3 cells cotransfected with pmyc AP1 luc (50 ng), with expression vector for the PDGF/Sis oncogene (0.5 µg) and with the AP-1 repressor, JunDBD-SID (50 ng), as indicated. (D), Analysis of the stimulation of the Gal4 responsive element by the Gal4-VP16 chimerical transcription factor, in NIH 3T3 cells cotransfected with pGal4 luc (100 ng), with expression vector for the Gal4-VP16 molecule (0.5 µg) and with the AP-1 repressor (50 ng), JunDBD-SID, as indicated. (E), Analysis of c-myc mRNA expression in NIH 3T3 cells transfected with an expression vector for the JunDBD-SID molecule (2 µg) and then stimulated with PDGF for 1 hour. Equal loading of RNA was confirmed. Expression of the HA-epitope tagged Jun DBD-SID protein was confirmed by anti-HA immunoblot performed after anti-HA immunoprecipitation of the samples.
-, no treatment; α-, antibody against; c, control; WB, western blot; IP, immunoprecipitation.

Study II

Activation of the ERK8 MAP kinase by RET/PTC3, a constitutively active form of the RET proto-oncogene.

RET is a typical trans-membrane receptor tyrosine-kinase (RTK), essential for the development of the sympathetic, parasympathetic and enteric nervous system and of the kidney (Schuchardt et al., 1994). In complex with four glycosylphosphatidylinositol (GPI)-anchored coreceptors, GFR-□ 1–4, the RET protein binds growth factors of the glial-derived neurotrophic factor (GDNF) family, mediating their intracellular signaling (Airaksinen & Saarma, 2002). As for other RTKs, ligand interaction triggers autophosphorylation of different RET intracellular tyrosine residues that work as docking sites for several adaptor and effector signaling molecules (Santoro et al., 2004). Among such tyrosines, while Tyr^{981} is a binding site for c-Src, Tyr^{1062} has been shown to mediate the interactions with most of RET effectors and to be responsible for activation of the Ras/Erk, PI3K/Akt, Jnk, p38 and Erk5 signaling pathways (Kurokawa et al., 2003). Finally, Tyr^{1015} is a recognized docking site for PLCγ (Borrello et al., 1996).

Gain-of-function mutations of *RET* have been repeatedly described in several human tumors (Pasini et al., 1996). *RET* germline point mutations are in fact responsible for the three clinical subtypes of the Multiple Endocrine Neoplasias type 2 (MEN2) syndrome, MEN2A, MEN2B and Familial Medullary Thyroid Carcinoma (FMTC) (Santoro et al., 2004). In addition, fusion of the intracellular kinase domain of *RET* with heterologous genes, caused by chromosomal inversions or translocations, generates the *RET/PTC* oncogenes, which represent the genetic hallmark of papillary thyroid carcinomas (PTC), accounting for more than 80-90% of all thyroid carcinomas (Sherman, 2003). Among the at least ten different *RET/PTC* rearrangements, *RET/PTC1* and *RET/PTC3*, generated by the fusion with the *H4* and *RFG* genes, respectively, are the most common types, accounting for more than 90% of all rearrangements (Nikiforov, 2002).

Erk8 is activated by RET-dependent signaling pathway.

We performed an *in silico* analysis of *Erk8* gene expression in mouse tissues, through the public Mouse Gene Prediction Database resource (http://mgpd.med.utoronto.ca/) (Zhang et al., 2004). Among other tissues, *Erk8* was

expressed at very high levels in the thyroid, therefore suggesting a role for this kinase in signaling pathways involved in the homeostasis and/or pathology of this organ. As the RET/PTC oncogenes are frequently involved in human papillary thyroid carcinomas (Santoro et al., 2004), we decided to investigate their ability to modulate Erk8 activation. In particular, we investigated the role of RET/PTC3, a chimeric oncogene generated by the fusion of *RET* with the *RFG* gene (Fig. 7A) (Santoro et al., 1994).

As an approach to score Erk8 activation, we used an anti-phospho-MAPK (Erk2) antibody that recognizes phosphorylation in the conserved MAP kinase TEY motif. We performed western blot analysis of 293T cells transfected with an HA epitope-tagged form of the Erk8 kinase, as previously described (Abe et al., 2002), and then distinguished the transfected HA-Erk8 and the endogenous Erk2 by their different molecular weights, ~60 kDa and ~45 kDa, respectively. As shown in figure 8B, RET/PTC3 overexpression readily induced Erk8 activation, at a level comparable to an activated form of c-Src (Src YF), used as a positive control (Abe et al., 2002). Of note, no signal in the ~60 kDa range was detected in the absence of HA-Erk8 transfection (Fig. 7B), indicating that the anti-phospho-MAPK antisera specifically recognized the Erk8 protein. As an additional control for the activity of RET/PTC3 and Src YF, both proteins activated the Erk2 MAP kinase (Fig. 7B), also scored by anti-phospho-MAPK western blot. Altogether, these results indicate that RET/PTC3 stimulates Erk8 activity.

The Erk8 carboxy-terminal modulates activation of the MAP kinsase by RET/PTC3.

While classical MAP kinases such as Erks, Jnks and p38s are only slightly larger than their minimum Ser/Thr kinase core, the atypical Erk5, Erk7 and Erk8 MAP kinases all contain long C-terminal domains whose functions are largely unknown. Yet, recent experiments performed on Erk5 (Buschbeck & Ullrich, 2005) and Erk7 (Abe et al., 2001) have demonstrated a role for their C-terminal tail in the regulation of kinase intracellular localization and activity. Thus, we set up to investigate a role for the Erk8 C-terminal domain in RET/PTC3-dependent activation of the kinase.

The genomic organization of the *Erk8* gene has been previously described (Abe et al., 2002). By *in silico* analysis of available "expressed sequence tags" (est) clones we identified an *Erk8* cDNA whose corresponding protein, when expressed, presented a molecular weight shorter (~35 kDa) than the described Erk8 protein (~60 kDa) (Fig. 8A). We named this protein Erk8□ (accession # AY994058). Comparative analysis of the sequences for *Erk8*, *Erk8*δ and the *Erk8* gene

(http://www.ncbi.nlm.nih.gov/genome/guide/human/) revealed that *Erk8*δ corresponded to an alternatively spliced form of *Erk8* in which an alternative exon 8 (exon 8a) contained a "stop" codon (Fig. 8B), therefore determining a 254-aminoacid long protein, lacking the Erk8 C-terminal domain (Fig. 8C). Thus, we took advantage of the availability of this naturally occurring C-terminally truncated protein, to evaluate the role of this domain in RET/PTC3-dependent Erk8 activation. As shown in figure 8D, RET/PTC3 was not able to induce Erk8δ activation while, as a control, it strongly activated Erk8. In the same experimental condition, Src YF, a described activator of Erk8 (Abe et al., 2002), also failed to stimulate Erk8δ activation (Fig. 8D), therefore establishing a key role for the C-terminal domain of Erk8 in the activation of this MAP kinase by various upstream stimuli.

Tyrosine 981 of RET/PTC3 is necessary for Erk8 activation.

Tyrosine phosphorylated residues in the kinase domain of RET, as well as of its derivate oncogenes, usually represent docking sites for adaptor proteins and enzymes that are able to propagate the signal to the intracellular environment (Santoro et al., 2004). We therefore used RET/PTC3 molecules in which different tyrosine phosphorylation sites have been inactivated by mutating them to phenylalanines, to ascertain the dependency of RET/PTC3-induced Erk8 activation on the presence of these specific residues. Also, as these tyrosines have already been linked to the activation of different specific signaling pathways (Santoro et al., 2004), this approach could grant us the possibility to suggest the participation of some of these effectors in the modulation of Erk8 activity. In particular, tyrosine^{981} binds c-Src (Encinas et al., 2004), tyrosine^{1015} is a docking site for PLCγ (Borrello et al., 1996) and tyrosine^{1062} is a multiple docking site that mediates most of RET signaling pathways (Kurokawa et al., 2003), including Erk2 activation (Chiariello et al., 1998). 293T cells were transiently transfected with the HA-Erk8 molecule, together with RET/PTC3, RET/PTC3^{Y981}, RET/PTC3^{Y1015} or RET/PTC3^{Y1062}, respectively (numbers indicating RET/PTC3 tyrosine residues correspond to their position in the wild-type RET receptor). Surprisingly, based on the observation that tyrosine^{1062} mediates most of RET signaling pathways (Kurokawa et al., 2003), the RET/PTC3^{Y1062} mutant activated Erk8 at an extent comparable to the RET/PTC3 molecule while, as expected (Chiariello et al., 1998), this mutation strongly affected Erk2 activation (Fig. 9). The tyrosine^{1015} mutation, involving a known binding site for PLCγ (Borrello et al., 1996), also did not

affect Erk8 activation by RET/PTC3 (Fig. 9). Conversely, tyrosine^{981} mutation determined a dramatic reduction in RET/PTC3-dependent Erk8 activation, although resulting irrelevant to Erk2 activation (Fig. 10). As a control, RET/PTC3 Kin^{dead}, a kinase-inactive form of RET/PTC3 containing a mutation in the ATP-binding catalytic lysine (Lys^{758}), was unable to activate both Erk8 and Erk2 (Fig. 9). These results therefore imply tyrosine^{981} of RET/PTC3 as a major site recognized by signaling molecules mediating RET/PTC3-dependent Erk8 activation. In addition, as tyrosine^{981} has been previously recognized as a key residue for the binding of c-Src to RET (Encinas et al., 2004), they also suggest a role for c-Src in mediating RET/PTC3-initiated signals impinging on Erk8 activation.

Src activity is dispensable for RET/PTC3-dependent Erk8 activation

Based on the above information and on the observation that c-Src activates Erk8 (Abe et al., 2002), we next sought to investigate if c-Src was able to mediate RET/PTC3-dependent Erk8 activation. A classical approach to establish a role for Src kinases in cellular processes takes advantage of a pyrazolo-pyrimidine compound, PP1, which binds the ATP-binding pocket of these kinases therefore blocking their enzymatic activity (Hanke et al., 1996) and biological functions (Chiariello et al., 2001). Although PP1 has been described to affect RET kinase activity (*in vitro* IC_{50}=100 nM) (Carlomagno et al., 2002), a specific mutation in valine^{804} in the RET kinase domain confers resistance (>50-fold increase of the IC_{50}) to the compound (Carlomagno et al., 2004). We, therefore, introduced such mutation in the RET/PTC3 kinase domain (RET/PTC3^{V804}) rendering its activity significantly resistant to PP1, as scored by RET/PTC3^{V804} auto-phosphorylation and activation of Erk2 (Fig. 10A). As expected, kinase activity of the parental RET/PTC3 molecule was completely abolished at comparable concentrations (compare the 5-10 μM PP1 lanes) as evidenced by both RET/PTC3 auto-phosphorylation and activation of Erk2 (Fig. 10B). Surprisingly, while strongly inhibiting Src (data not shown), PP1 treatment of RET/PTC3^{V804}-transfected cells only slightly affected Erk8 activity even at the highest doses tested (10 μM) (Fig. 10C) and after extensive times of treatment (up to 10 hrs treatment, at 5 μM concentration) (Fig. 4D), thus excluding a role for c-Src and its related kinases (Hanke et al., 1996) in the control of RET/PTC3-induced Erk8 activation. As a complementary approach to ascertain the role of Src kinases in RET/PTC3 activation of Erk8, we also used a dominant negative form of c-Src, Src YF KM (Chiariello et al.,

2001). As shown in figure 10E, overexpression of the dominant negative molecule did not affect Erk8 activation while it effectively inhibited PDGF-induced activation of the c-*myc* promoter (Fig. 10F) (Chiariello et al., 2001). Altogether, these data clearly indicate that RET/PTC3 can use a Src-independent pathway to activate the Erk8 MAP kinase.

c-Abl mediates RET/PTC3-dependent Erk8 activation.

c-Abl, the cellular homologue of the Abelson murine leukemia virus, has been implicated in different cellular processes ranging from cell growth to survival, cellular stress, DNA-damage response and cell migration (Hantschel & Superti-Furga, 2004). From the structural point of view, c-Abl contains SH3, SH2 and tyrosine kinase domains whose arrangement and sequence very much resemble that of c-Src (Hantschel & Superti-Furga, 2004). These observations prompted us to investigate whether, similarly to Src, an activated form of c-Abl could induce Erk8 activation and, in turn, whether c-Abl could mediate RET/PTC3 activation of Erk8. As shown in figure 11A, an oncogenic, activated form of c-Abl, the Bcr/Abl fusion protein, readily induced Erk8 activation, at a level comparable to an activated form of c-Src (Src YF), used as a positive control (Abe et al., 2002). Thus, we decided to investigate whether c-Abl is able to act as a link between RET/PTC3 and the stimulation of Erk8. As an approach, we used a kinase-defective, dominant negative form of c-Abl, Abl-KD. This dominant negative molecule strongly inhibited the RET/PTC3-dependent activation of Erk8 (Fig. 11B), thus suggesting that c-Abl is a likely mediator in the pathway connecting RET/PTC3 to the activation of the Erk8 MAP kinase.

To control a vast range of cellular processes, c-Abl interacts with a large variety of cellular proteins, including phosphatases, kinases, signaling adaptors, transcription factors, cytoskeletal proteins and cell cycle regulators (Hantschel & Superti-Furga, 2004). To determine whether c-Abl can interact with Erk8 *in vivo*, 293T cells were transfected with HA-Erk8 and either wild-type c-Abl or the control vector, immunoprecipitated with an anti-HA antibody and then analized by western blot with an anti-abl antisera. As shown in figure 11C, c-Abl clearly co-immunoprecipitated with Erk8, therefore suggesting a role for physical interaction in the control of Erk8 activation by c-Abl.

We have previously shown that tyrosine^{981} in RET/PTC3 mediates RET/PTC3-dependent Erk8 activation (Fig. 9), representing a major site recognized by signaling molecules intervening in such process. We therefore investigated whether the tyrosine^{981} residue was also able to mediate RET/PTC3 activation of c-Abl. Taking advantage of the

observation that tyrosine phosphorylation of c-Abl correlates with its activation (Plattner et al., 1999), we cotransfected an autophosphorylation-impaired, AU5-tagged, c-Abl molecule, together with RET/PTC3, RET/PTC3^{Y981}, RET/PTC3^{Y1015} or RET/PTC3^{Y1062}, respectively, immunoprecipitated these samples by anti-AU5 antibodies and analyzed them by anti-phospho-tyrosine western blot. As show in figure 11D, RET/PTC3 clearly induced Abl phosphorylation. Importantly, RET/PTC3^{Y981} was strongly impaired in its ability to induce phosphorylation of the c-Abl protein, as compared to RET/PTC3 (Fig. 11D). In the same experiment, RET/PTC3^{Y1062} and RET/PTC3^{Y1015} exerted more limited or no effects, as compared to RET/PTC3 (Fig. 11D). Ultimately, the RET/PTC3 Kin^{dead} was unable to induce c-Abl phosphorylation, establishing a requirement for RET/PTC3 kinase activity in c-Abl activation (Fig. 11D). Together, these results clearly indicate that RET/PTC3, through its tyrosine^{981}, can utilize an Abl-dependent pathway to stimulate Erk8 activation.

A kinase-defective mutant for Erk8 interferes with RET/PTC3 signaling.

The expression of the c-*jun* proto-oncogene is rapidly and transiently induced by different growth factors and cellular oncogenes (Marinissen et al., 1999). Among them, an oncogenic rearrangement of the RET proto-oncogene is able to strongly induce c-*jun* expression (Ishizaka et al., 1991), therefore establishing this gene as part of RET signaling pathway. To investigate whether the RET/PTC3 oncogene was able to stimulate the activity of the c-*jun* promoter, we took advantage of the availability of a reporter plasmid carrying the *luciferase* gene under the control of the murine c-*jun* promoter (Chiariello et al., 2000; Marinissen et al., 1999). Cotransfection of thyroid ARO cells with this reporter plasmid and increasing concentrations of the RET/PTC3 cDNA revealed that this oncogene could strongly induce the activity of the c-*jun* promoter (Fig. 12A). To evaluate whether Erk8 activation is involved in RET/PTC3 signaling to the c-*jun* promoter, we next used a dominant negative, kinase defective (data not shown) Erk8 molecule. For these experiments, we therefore cotransfected RET/PTC3 with the c-*jun* reporter plasmid and increasing amounts of the Erk8 KR expression vector. As shown in figure 12B, the dominant negative Erk8 molecule caused a strong, although incomplete inhibition of RET/PTC3-dependent c-*jun* promoter stimulation, suggesting the existence of both Erk8-dependent and -independent pathways linking RET/PTC3 to the expression of the c-*jun* proto-oncogene.

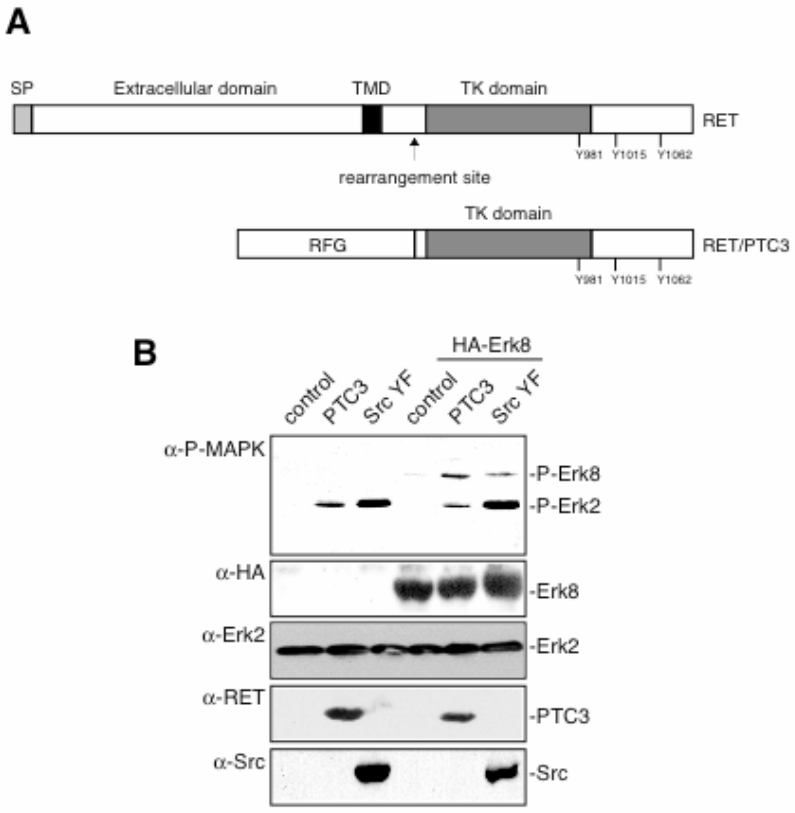


Fig. 7 RET/PTC3 induces ERK8 activation.
(A) Schematic representation of the wild-type RET protein and of its activated form, the RET/PTC3 oncogene. The position of three tyrosine autophosphorylation sites and of important protein domains are also showed. SP, signal peptide; TMD, trans-membrane domain; TK, tyrosine kinase; Y, tyrosine; RFG, RET Fused Gene. Numbers indicating RET/PTC3 tyrosine residues correspond to their position in the wild-type RET receptor. (B) RET/PTC3 induces ERK8 activation. Analysis of HA-Erk8 activation in 293T cells cotransfected with the RET/PTC3 (PTC3) and Src YF expression vectors and analyzed by western blot with anti-phospho MAPK antisera. Activation of endogenous Erk2 by RET/PTC3 and Src YF was used as an additional control for the activity of the two oncogenes. The expression of HA-Erk8 (a-HA panel), Erk2(α-Erk2 panel), Src YF (α-Src panel) and RET/PTC3 (α-RET panel) was also confirmed.
Control: cells transfected with β -galatosidase; α: anti.

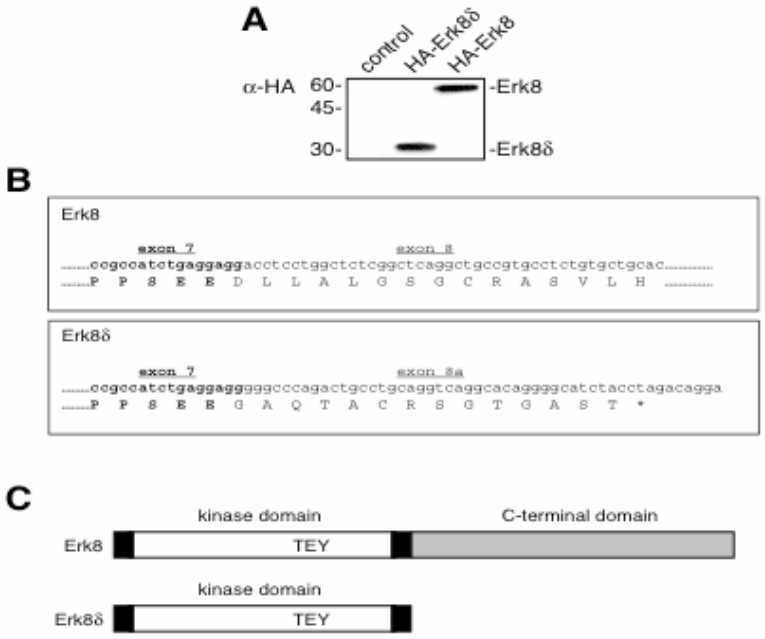
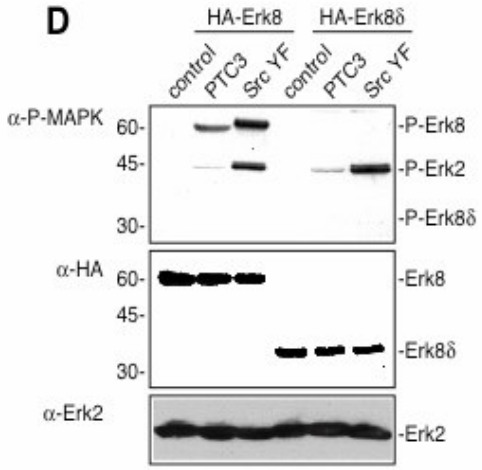


Fig. 8 The Erk8 carboxy-terminal domain mediates the acivation of the MAP kinase by RET/PTC3. (A), Erk8 and Erk8δ expression in transiently transfected 293T cells. (B), Comparison of Erk8 and Erk8δ nucleotide and protein sequences in the region of the alternative splicing. (C), Schematic representation of Erk8 and Erk8δ protein structures. The relative position of important residues and protein domains is indicated.



(D), Analysis of Erk8 and Erk8δ activation in 293T cells cotransfected with the RET/PTC3 (PTC3) and Src YF expression vectors. Activation of endogenous Erk2 by RET/PTC3 and Src YF was used as an additional control for the activity of the two oncogenes. The expression of HA-Erk8, HA-Erk8δ (α-HA panel) and Erk2 (α-Erk2 panel) was also confirmed.
Control: cells transfected with b-galatosidase; α: anti.

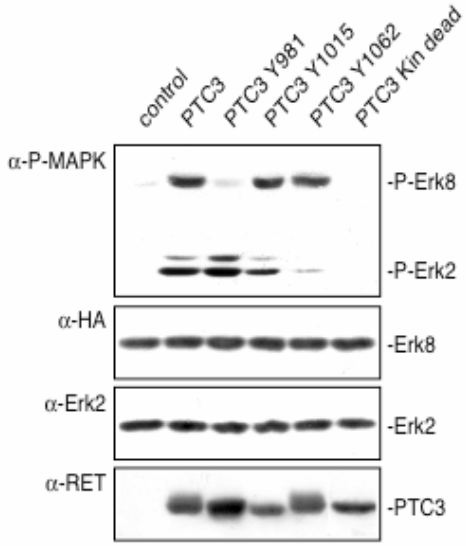
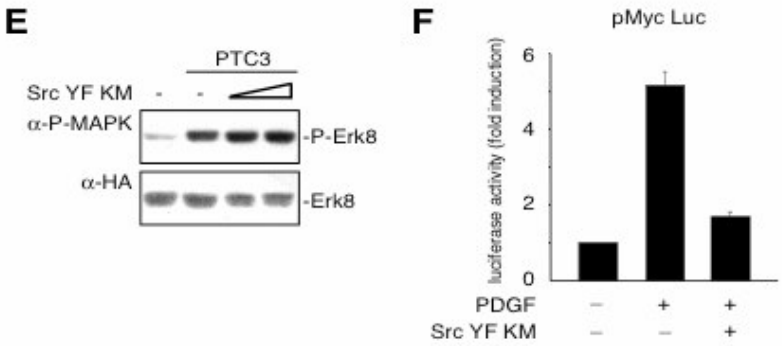


Fig.9 RET/PTC3 requires tyrosine 981 to activate ERK8.
Stimulation of Erk8 activation by RET/PTC3 (PTC3) and by tyrosine mutated forms of the oncogene, in 293T cells co-transfected with an expression vector for HA-Erk8 (α-P-MAPK panel). Activation of endogenous Erk2 by RET/PTC3 and its different mutated forms wa s used as a parallel control for the activity of the oncogenes (α-P-MAPK panel). The expression of HA-Erk8 (α-HA panel), Erk2 (α-Erk2 panel) and RET/PTC3 wild-type and mutants (α-RET panel) was also confirmed.
Control: cells transfected with β-galatosidase; α: anti.



(E), Analysis of Erk8 activation (upper α-P-MAPK panel) in 293T cells transfected with HA-Erk8, RET/PTC3 and increasing concentrations (250 and 500 ng) of the dominant negative Src YF KM. The expression of HA-Erk8 (α-HA panel) was also confirmed. Control: cells transfected with b-galatosidase; a: anti. (F), Inhibition of PDGF-induced c-myc promoter activity by Src YF KM. Stimulation of c-myc promoter transcriptional activity in NIH 3T3 ells transfected with the c-myc promoter reporter plasmid (pMyc-Luc) and a Src dominant negative expressing vectors (Src YFKM) and then stimulated for 4 h with PDGF (12.5 ng ml-1). Results represent luciferase activity in each sample, normalized for the corresponding efficiency of transfection and they are the average ± standard errors of triplicate samples from a typical experiment.

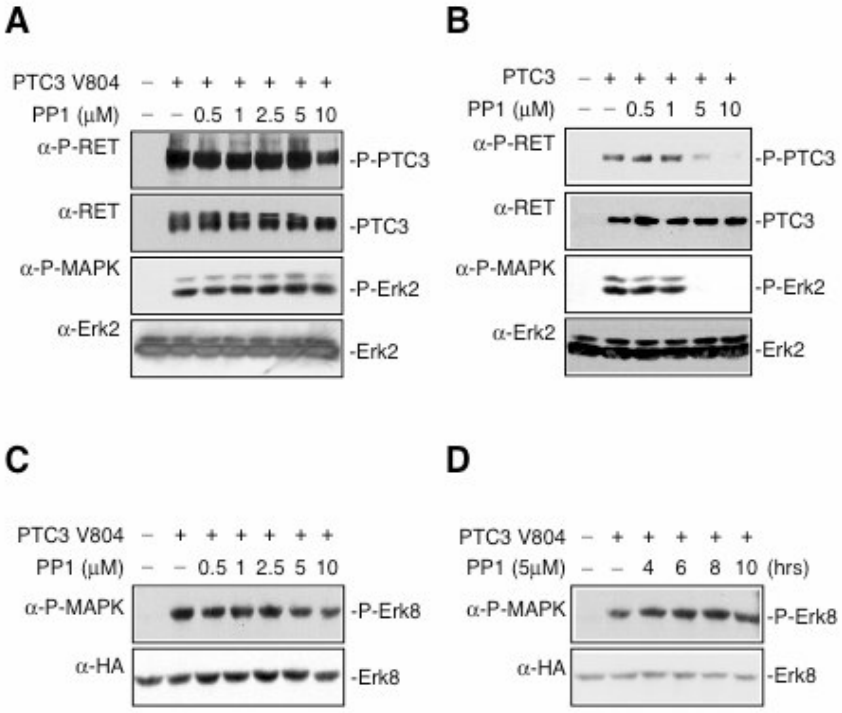
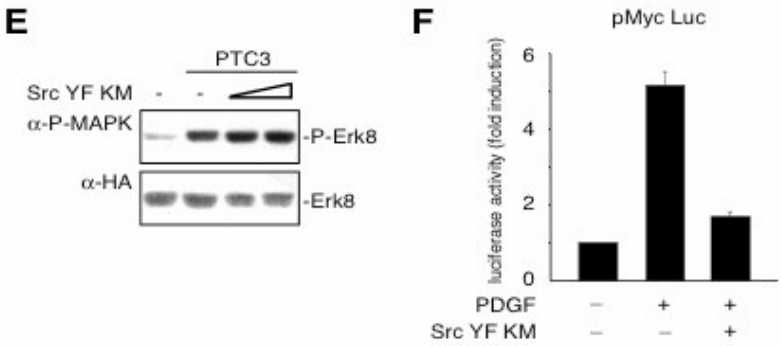


Fig.10 Erk8 activation by RET/PTC3 does not depend on c-Src.
(A), Effect of PP1 on RET/PTC3V804 auto-phosphorylation (α-P-RET panel), in 293T cells transfected as indicated, pre-treated three hours with different concentrations of the inhibitor and then analyzed by an anti-phospho RET specific antisera. As a control, activation of endogenous Erk2 was also scored by western blot with anti-phospho MAPK antisera (α-P-MAPK panel). Expression of RET/PTC3V804 and Erk2 was confirmed by western blot performed with anti-RET and anti-Erk2 antisera, respectively (α-RET and a-Erk2 panels). (B), Same as in A, using RET/PTC3 to activate Erk8. (C), Same as in A, analyzing RET/PTC3V804-dependent Erk8 activation. (D), Effect of PP1 on RET/PTC3V804-dependent Erk8 activation (α-P-MAPK panel), in 293T cells transfected as indicated and pre-treated with PP1 (5 μM) for increasing times. Erk8 expression was also confirmed (α-HA panel).



(E), Analysis of Erk8 activation (upper α-P-MAPK panel) in 293T cells transfected with HA-Erk8, RET/PTC3 and increasing concentrations (250 and 500 ng) of the dominant negative Src YF KM. The expression of HA-Erk8 (α-HA panel) was also confirmed. Control: cells transfected with b-galatosidase; a: anti. (F), Inhibition of PDGF-induced c-myc promoter activity by Src YF KM. Stimulation of c-myc promoter transcriptional activity in NIH 3T3 ells transfected with the c-myc promoter reporter plasmid (pMyc-Luc) and a Src dominant negative expressing vectors (Src YFKM) and then stimulated for 4 h with PDGF (12.5 ng ml-1). Results represent luciferase activity in each sample, normalized for the corresponding efficiency of transfection and they are the average ± standard errors of triplicate samples from a typical experiment.

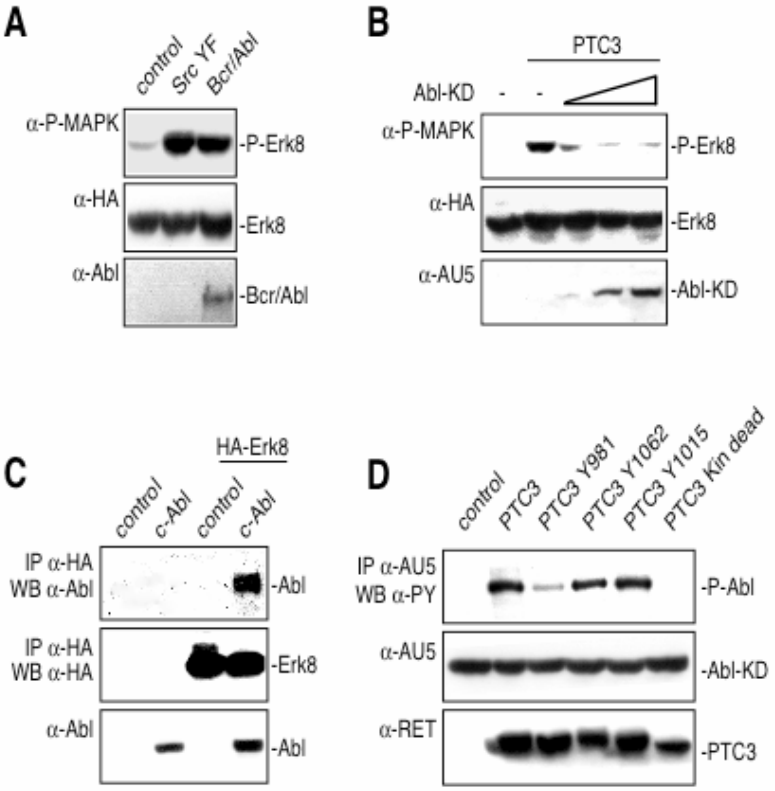


Fig. 11 c-Abl mediates RET/PTC3-dependent ERK8 activation.
(A), Analysis of HA-Erk8 activation in 293T cells cotransfected with Src YF and the oncogenic form of c-Abl, Bcr/Abl. Samples were analyzed by western blot with anti-phospho MAPK antisera. The expression of HA-Erk8 (α-HA panel) and Bcr/Abl (α-Abl panel) were confirmed. (B), Analysis of Erk8 activation (α-P-MAPK panel) in 293T cells transfected with HA-Erk8, RET/PTC3 and increasing concentrations (100, 250 and 500 ng) of the dominant negative Abl-KD. The expression of HA-Erk8 (α-HA panel) and of Abl-KD (α-AU5 panel) were also confirmed. (C), In vivo interaction of Erk8 with c-Abl. 293T cells were co-transfected with an expression vector for c-Abl (500 ng) together with plasmids for HA-Erk8 (500 ng) or control vector (β-galactosidase). Samples were next immunoprecipitated by anti-HA antibodies and then analyzed by anti-Abl antibodies. The expression of HA-Erk8 (middle panel) and RET/PTC3 wild-type and mutants (lower panel) was also confirmed. (D), Stimulation of c-Abl phosphorylation by RET/PTC3 (PTC3) and by tyrosine mutated forms of the oncogene. 293T cells were co-transfected with an expression vector for AU5-Abl-KD together with plasmids for RET/PTC3 and its tyrosine mutants. Samples were next immunoprecipitated by anti-AU5 antibodies and then analyzed by anti-phospho-tyrosine antibodies (α-PY). The expression of AU5-Abl-KD (α-AU5 panel) and RET/PTC3 wild-type and mutants (α-RET panel) was also confirmed.
Control: cells transfected with β-galatosidase; α: anti.

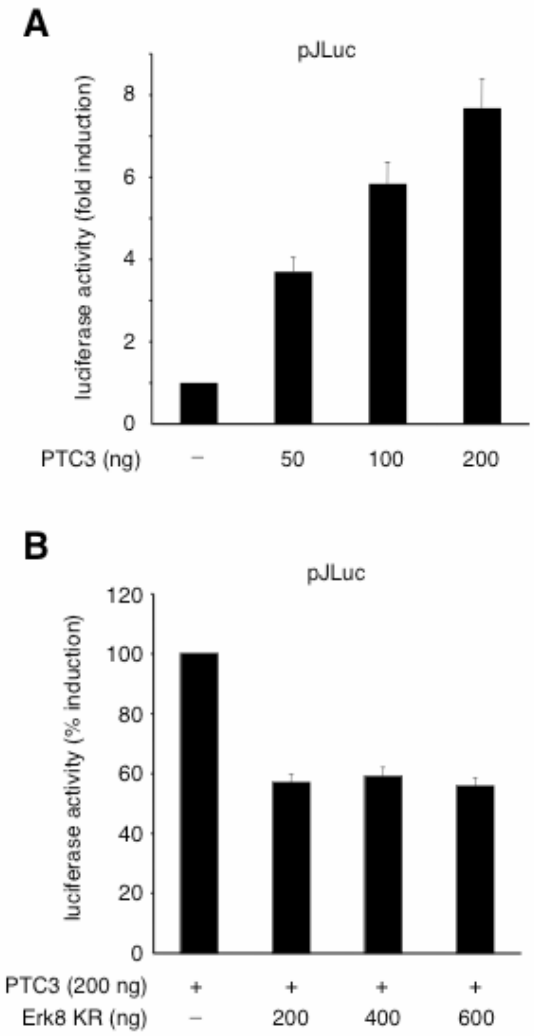


Fig.12 Inibition of c-jun promoter activation by a dominant negative Erk8 mutant. (A), Stimulation of c-jun promoter transcriptional activity in ARO cells transfected with the c-jun promoter reporter plasmid (pJLuc) and increasing concentrations of a RET/PTC3 expressing vectors. (B), Inhibition of RET/PTC3-induced c-jun promoter activity by Src YF KM. Stimulation of c-myc promoter transcriptional activity in NIH 3T3 cells co-transfected with pJLuc, RET/PTC3 and a Erk8 dominant negative expressing vector (Erk8 KR). Results represent luciferase activity in each sample, normalized for the corresponding efficiency of transfection and they are the average ± standard errors of triplicate samples from a typical experiment.

Discussion

PDGF induces c-*myc* expression through the Src-dependent activation of the Vav2 exchange factor, acting on the small GTPase Rac (Chiariello et al., 2001). By studying the downstream components of the Rac pathway, in the first study, we show that JNK and two AP-1 family members, c-Jun and JunD, are essential components of the signaling cascade that mediates PDGF stimulation of c-*myc* expression (Fig. 13), which, significantly, establishes a new functional connection between Jun proteins and the c-*myc* proto-oncogene. The proposed pathway also suggests a mechanism by which both JNK and Jun proteins might exert their proliferative or apoptotic potential, through the expression of the c-*myc* proto-oncogene. Further work will be required to establish the contribution of the "JNK-Jun pathway" to the biological responses of tyrosine kinase receptors such as the PDGF receptors as well as other membrane receptors that use the c-Myc protein to signal cellular proliferation (Iavarone et al., 2003).

The complexity of the mechanisms mediating intracellular signaling by RET and its activated forms, the RET/PTC and MEN2 oncogenes, has just begun to be appreciated. Indeed, the biological functions of these proteins result from the coordinated activity of multiple kinase cascades, whose integrated signals control renal development, histogenesis of the enteric nervous system and, possibly, tumor formation (Nikiforov, 2002; Pasini et al., 1996; Santoro et al., 2004). In the second study, finding that RET/PTC3 activates Erk8 raises the possibility of a novel Erk8-dependent signaling pathway controlling RET biological functions. Interestingly, we have shown that Erk8 activation depends on the integrity of tyrosine^{981}, while tyrosine^{1062} mutation does not affect RET/PTC3-dependent activation of the kinase. This result clearly differentiates Erk8 from other MAP kinases already involved in RET signaling whose activation, on the contrary, strictly depends on RET tyrosine^{1062} (Hayashi et al., 2000).

Although RET tyrosine^{981} has been previously recognized as a docking site for c-Src (Encinas et al., 2004) and this kinase modulates Erk8 activation (Abe et al., 2002), surprisingly, RET/PTC3 activation of Erk8 does not depend on c-Src. This result therefore suggests that additional molecules interact with tyrosine^{981} of RET/PTC3 and are responsible for the control of Erk8 activity. Indeed, in this report we present evidences that c-Abl controls RET/PTC3-dependent Erk8 activating phosphorylation (Fig. 14). As a corollary to this finding, for the first time we show that c-Abl is able to mediate RET-dependent signaling pathways. Not only RET/PTC3 induces c-Abl phosphorylation but

such phenomenon also seems to be mediated by tyrosine^{981}, in line with our observation that this tyrosine mediates Erk8 activation. These findings strongly support each other, especially considering that, up to now, the only known signaling molecule downstream of this tyrosine was c-Src, while most of the other RET effectors depended on the integrity of tyrosine^{1062}.

c-Abl as well as c-Src contain well characterized SH3 domains, with an high degree of conservation in terms of sequence identity and structure (Hantschel & Superti-Furga, 2004). In c-Abl, this domain is important both for interaction with different proteins and for participation to an intramolecular regulatory mechanism (Wang, 2004). On the other hand, Erk8 contains two putative SH3-binding sites in its C-terminal tail (Abe et al., 2002). As the c-Src SH3 domain interacts *in vitro* with Erk8 (Abe et al., 2002) and we have demonstrated that c-Abl interacts *in vivo* with this MAP kinase, it is possible that this interaction is mediated by the c-Abl SH3 domain. This hypothesis is currently under investigation.

The more recently identified Erk5, Erk7 and Erk8 molecules differentiate from classical MAP kinases (Erks, Jnks and p38s) in that they present long carboxy-terminal domains with no strong homology to other mammalian proteins. By using a naturally occurring Erk8 splice variant, Erk8δ, lacking the long carboxy-terminal domain, we show a key role for this domain in RET/PTC3-dependent activation. It is intriguing the possibility that distinct stimuli differently activate the Erk8 and Erk8δ proteins and, conversely, that Erk8δ may represent a modulator of Erk8 activation.

Upon activation of different MAP kinases, a large number of transcription factors appears to control the expression of several growth promoting genes, such as c-*jun* and c-*fos*, and, through these, control a vast variety of cellular functions. Specifically, the c-*jun* promoter has already been show to represent a key site for the integration of signals coming from both cellular oncogenes (Chiariello et al., 2000) and extracellular ligands (Marinissen et al., 1999). It is therefore not surprising our observation that a dominant negative Erk8 molecule only partially inhibits the activation of the c-*jun* promoter. Indeed, we have previously demonstrated that signaling from RET impinges on the activation of at least another MAP kinase, Jnk (Chiariello et al., 1998), which is able to control the activity of the c-*jun* promoter (Marinissen et al., 1999). We can therefore expect Erk8 to be part of the complex network of kinases, whose activation ultimately

determines the specific biological response to the activation of RET and its related oncogenes in different cellular environments.

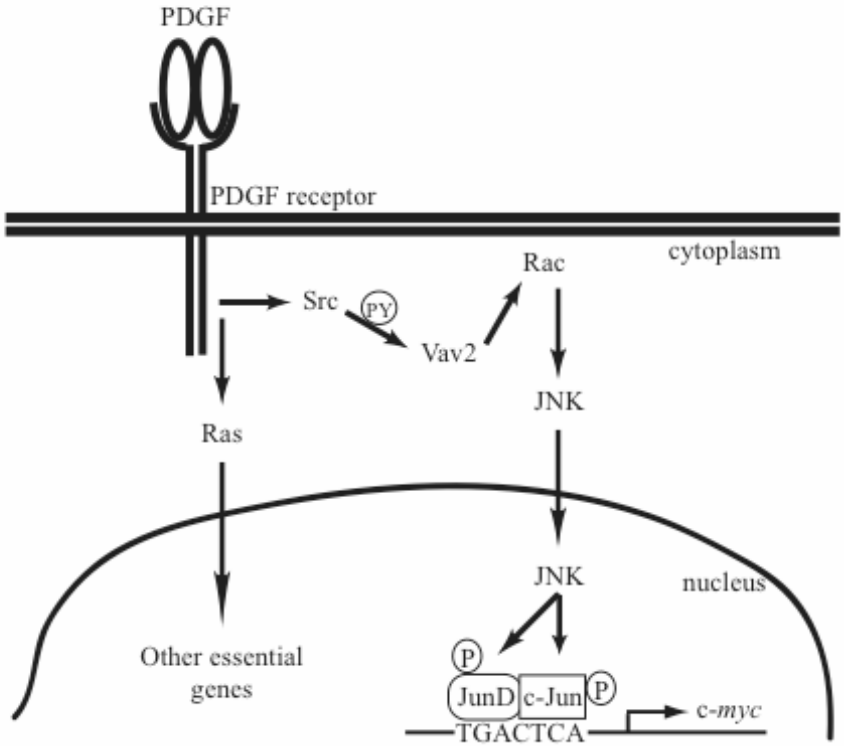


Fig. 13 Schematic representation of the pathway connecting PDGF receptors to the stimulation of *c-myc*. Schematic representation of the pathway connecting PDGF receptors to the stimulation of c-myc expression, through the Src-dependent activation of Vav2 and Rac and, in turn, stimulation of JNK activity, impinging on the regulation of the activity of AP-1 transcription factors bound to the promoter of the c-myc proto-oncogene.

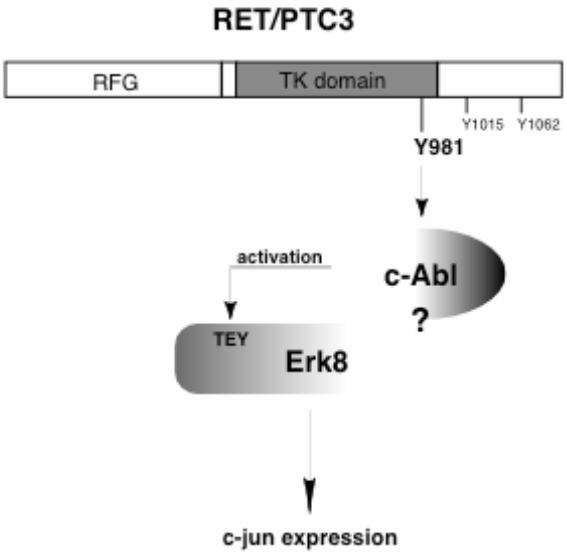


Fig. 14 Shematic representation f the pathway connecting RET/PTC3 to the stimulation of Erk8 activation, through tyrosine 981-dependent activation of c-Abl.
TK, tyrosine kinase; RFG, RET Fused Gene. Numbers indicating RET/PTC3 tyrosine residues correspond to their position in the wild-type RET receptor.

Conclusions

In mammals, MAP kinases signaling cascades regulate important cellular processes including gene expression, cell proliferation, cell motility, cell survival and cell death. The continual characterization of MAP kinases signaling complexes and the identification of novel substrates should reveal overlapping and unique biological functions for the various MAP kinases (Roux and Bleins, 2004). Less than a decade ago the kinases constituting mammalian MAPK pathway were identified through intense efforts in attempt to understand the molecular events underlying cellular responses to extracellular signals. During this decade the kinases constituting MAPK pathways have come to be appreciated as key cellular signal transducers and thus attractive targets for drug development. Successful drug development has required the demonstration that a large gene family with highly conserved catalytic core could be targeted with specific and potent small-molecule inhibitors. These efforts are now beginning to be useful with initiation of clinical trials in multiple human diseases (English and Cobb, 2002).

In conclusion, the data shown in this work illustrate the role of two relevant MAP kinase family members involved in the activation of nuclear signals primarly elicited by PDGF and Ret receptors: the former, JNK on transcriptional acivation of *c-myc*; the latter, ERK8 on the signaling c-Abl mediates RET/PTC3. Further work will be required to estabilish how these signals integrate and regulate the transcription of target genes.

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