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Signalling of the Ret receptor tyrosine kinase through the c-Jun NH_2-terminal protein kinases (JNKs): evidence for a divergence of the ERKs and JNKs pathways induced by Ret

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The *RET* proto-oncogene encodes a functional receptor tyrosine kinase (Ret) for the Glial cell line Derived Neurotrophic Factor (GDNF). *RET* is involved in several neoplastic and non-neoplastic human diseases. Oncogenic activation of *RET* is detected in human papillary thyroid tumours and in multiple endocrine neoplasia type 2 syndromes. Inactivating mutations of *RET* have been associated to the congenital megacolon, i.e. Hirschprung's disease. In order to identify pathways that are relevant for Ret signalling to the nucleus, we have investigated its ability to induce the c-Jun NH_2-terminal protein kinases (JNK). Here we show that triggering the endogenous Ret, expressed in PC12 cells, induces JNK activity; moreover, Ret is able to activate JNK either when transiently transfected in COS-1 cells or when stably expressed in NIH3T3 fibroblasts or in PC Cl 3 epithelial thyroid cells. JNK activation is dependent on the Ret kinase function, as a kinase-deficient *RET* mutant, associated with Hirschsprung's disease, fails to activate JNK. The pathway leading to the activation of JNK by *RET* is clearly divergent from that leading to the activation of ERK: substitution of the tyrosine 1062 of Ret, the Shc binding site, for phenylalanine abrogates ERK but not JNK activation. Experiments conducted with dominant negative mutants or with negative regulators demonstrate that JNK activation by Ret is mediated by Rho/Rac related small GTPases and, particularly, by Cdc42.

Keywords: tyrosine kinase; transformation; jun; MEN2; thyroid

Introduction

RET encodes a tyrosine kinase receptor (Ret) (Takahashi *et al*., 1988), which is expressed, during embryogenesis, in kidney and nervous system, including neurons of the enteric tract. Consistently, *RET*-deficient mice show lack of enteric neurons and renal dysgenesis (Schuchardt *et al*., 1994), and 'loss of function' *RET* mutations are associated with Hirschsprung's disease (Romeo *et al*., 1994; Edery *et al*., 1994; Pasini *et al*., 1995; Carlomagno *et al*., 1996), which is characterized by the congenital defect of intestinal innervation. Recently, GDNF (glial cell line derived neurotrophic factor) has been identified as one functional ligand for Ret. GDNF binds to GDNFR-α, a glycosyl phosphatidylinositol-linked cell surface receptor, which, in turn, mediates Ret activation (Jing *et al*., 1996; Treanor *et al*., 1996). Consistent with that, mice with targeted disruption of the *GDNF* gene show a phenotype similar to that of *RET* knock-out mice (Sanchez *et al*., 1996).

Deregulation of the *RET* function has been demonstrated to be oncogenic. *RET* activation by gene rearrangement is found in nearly 40% of sporadic thyroid papillary carcinomas. These rearrangements lead to the fusion of its tyrosine kinase encoding domain to heterologous genes, generating the chimeric *RET*/PTC oncogenes (Grieco *et al*., 1990). *RET*/PTC1, the one more frequently isolated, is generated by the fusion of *RET* to the 5′-terminal region of a gene designated *H4* (Grieco *et al*., 1990). In addition, germline *RET* point mutations, responsible for the inheritance of the MEN2A (multiple endocrine neoplasia type 2A), MEN2B, and FTMC (familial medullary thyroid carcinoma) syndromes (Donis-Keller *et al*., 1993; Mulligan *et al*., 1993; Carlson *et al*., 1994; Hofstra *et al*., 1994), also lead to an activation of the transforming potential of *RET* (Santoro *et al*., 1995; Asai *et al*., 1995). In most of MEN2A and FMTC cases substitution of extracellular cysteines leads to a constitutive dimerization of the receptor (Santoro *et al*., 1995; Asai *et al*., 1995); in contrast, in the majority of MEN2B cases, a M918T mutation causes a change of Ret substrate specificity (Santoro *et al*., 1995; Songyang *et al*., 1995).

Receptor tyrosine kinases' signals are, at least in part, transduced by intracellular serine/threonine kinases designated MAPK (mitogen activated protein kinases) (reviewed by Marshall, 1995). MAPK include extracellular signal-regulated protein kinase (p44 and p42 ERK1 and ERK2, respectively), c-Jun amino-terminal kinases (JNKs), also designated stress-activated protein kinase (SAPKs), and p38 kinase. MAPK are activated by upstream dual-specific kinases.

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MEK1 and MEK2 are responsible for the activation of the ERKs, MKK3 and MKK6 for p38, and MKK4 (also referred to as SEK or JNKK1) for JNKs (Minden *et al*., 1994; Sanchez *et al*., 1994; reviewed by Karin, 1995). ERKs, in turn, are able to regulate the activity of enzymes and of nuclear proteins such as the ternary complex factor Elk-1, involved in cell proliferation control (reviewed by Hill and Treisman, 1995). On the other side, the two principal isoforms of JNKs, JNK1 and JNK2, were identified by their ability to bind and phosphorylate the amino-terminal (serines 63 and 73) domain of c-Jun (Hibi *et al*., 1993; Minden *et al*., 1994; Kyriakis *et al*., 1994; Derijard *et al*., 1994). Moreover, activated JNKs are able to phosphorylate at least two other transcription factors, such as ATF-2 (Gupta *et al*., 1995) and Elk-1 (Whitmarsh *et al*., 1995). S63/73-phosphorylated c-Jun has potent AP-1 activity and can control the expression of a number of genes, including c-*jun* itself. Several evidences demonstrate the relevance of the JNK pathway for the establishment of the neoplastic phenotype. The use of dominant-negative Jun proteins (Lloyd *et al*., 1991) and of *jun*-null fibroblasts (Johnson *et al*., 1996) demonstrated that functional c-Jun is essential for Ha-*ras* transformation; moreover, the enhanced phosphorylation of c-Jun is required for its cooperation with Ha-*ras* (Smeal *et al*., 1991). Trivalent arsenic (As^{3+}) was demonstrated to exert its tumor promoting activity by activating JNK1 (Cavigelli *et al*., 1996).

The signalling cascade leading to ERK activation is relatively well understood. Ligand-stimulation of receptor tyrosine kinases leads to the binding of adaptor proteins, such as Grb2 and Shc (Pelicci *et al*., 1992), containing Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains (reviewed in Schlessinger and Ullrich, 1992; Pawson, 1995; Hill and Treisman, 1995). Such adapters, when bound to the phosphorylated receptor, recruit Sos to the plasma membrane, where it activates Ras and, thus, the Raf-MEK1/MEK2-ERKs cascade (reviewed by Marshall, 1995). Several evidences indicate that JNKs are activated by a signalling cascade different from that activating ERKs. Stimuli that activates JNKs are distinct from those that enhance ERKs activity and include ultraviolet irradiation, heat shock, changes in osmolarity, protein synthesis inhibitors, such as cycloheximide and anisomycin, and cytokines such as tumour necrosis factor-α (TNF-α), and interleukin-1. TPA (12-O-tetradecanoyl phorbol-13-acetate), which is a potent ERK activator, has little effect on JNK activity (Derijard *et al*., 1994; Kyriakis *et al*., 1994). Ras, which is a potent ERK activator, raises JNK activity only to a limited extent, and, on the other side, Rac1 and Cdc42 small GTPases efficiently stimulate the JNK pathway without affecting ERK activity (Coso *et al*., 1995a; Minden *et al*., 1994, 1995). Accordingly, exchange factors (GEF) for Rac1 and Cdc42 potently induce JNK activity and negative modulators (RhoGAPp190 and RhoGDI) or dominant negative mutants for Rac1 and Cdc42 (N17Rac1 and N17Cdc42) effectively reduce JNK stimulation by EGF (Coso *et al*., 1995a; Minden *et al*., 1995). Activated Rac1 and Cdc42 can initiate, similarly to the Ras/Raf/MEK/ERK pathway, a kinase cascade which stimulates MEKK1 which, in turn, activates MKK4 (also referred to as SEK or JNKK1), the JNK activator (Manser *et al*., 1994).

Activated *RET* isoforms are highly transforming for NIH3T3 cells (Grieco *et al*., 1990; Santoro *et al*., 1994; 1995; Asai *et al*., 1995) and are able to induce the expression of differentiation markers in PC12 pheochromocytoma cells (Califano *et al*., 1995, 1996; Pasini *et al*., 1995). Moreover, the *RET*/PTC1 oncogene is able to transform rat thyroid epithelial PC Cl 3 cells, causing thyrotropin-independent growth and a loss of the differentiated phenotype (Santoro *et al*., 1993), and is able to induce thyroid carcinomas in transgenic mice (Jhiang *et al*., 1996; Santoro *et al*., 1996). Events following Ret activation and leading to these transforming and differentiating cell responses are only partially known. Ret has been found to activate the γ isoenzyme of phospholypase C (Santoro *et al*., 1994; Borrello *et al*., 1996), Ras (Santoro *et al*., 1994), and ERKs (van Weering *et al*., 1995). Borrello and co-workers have reported that Ret protein binds to and phosphorylates Shc (Borrello *et al*., 1994; Asai *et al*., 1996) and other groups (Lorenzo *et al*., 1997; Arighi *et al*., 1997) have demonstrated that tyrosine residue 1062 of Ret is a binding site for the Shc-PTB domain and that its mutation abrogates Shc binding and reduces Ret transforming ability.

To further address the question of how Ret signals are delivered to the nucleus, we have investigated the ability of wild type and oncogenic Ret variants to induce JNK activity in different cell types. Here we show that Ret is able to activate JNKs through a pathway requiring Rho/Rac-like small GTPases and that this pathway is distinct from that leading to ERK activation.

Results

Ret activation induces an increase of JNK activity in NIH3T3 fibroblasts, in epithelial PC Cl 3 thyroid cells, and in neuroepithelial PC12 cells

We have evaluated the ability of different *RET* constructs to induce JNK activity. Figure 1 shows a schematic representation of the Ret structure and of the constructs used in this study. Firstly, we analysed the phosphorylation capability of endogenus JNKs on the glutathione S-transferase (GST)-c-Jun(79) specific

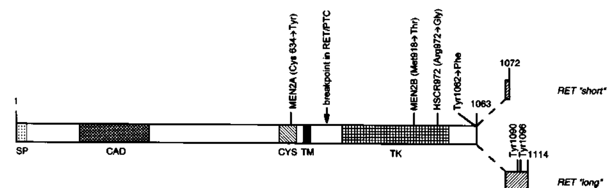

Figure 1 Schematic representation of *RET* structure. A scheme of Ret^{1072} and Ret^{1114} ('short' and 'long' isoforms); the mutations associated with HSCR (R972G), MEN2A (C634Y) and MEN2B (M918T), analysed in this study, and the position of the breakpoint originating *RET*/PTC oncogenes are shown. Tyrosine residue 1062, which is part of a Shc PTB binding site, and which was substituted with a phenylalanine in some experiments (Y1062F), is also shown. SP, signal peptide; CAD, cadherin homologous domain; CYS, cysteine-rich region; TM, transmembrane region; TK, tyrosine-kinase domain

substrate by a solid-phase kinse assay (Coso *et al*., 1995b). Since NIH3T3 cells do not express endogenous Ret, we analysed JNK activity in cells stably transfected with active *RET*/MEN2A and *RET*/MEN2B alleles (Santoro *et al*., 1995). A representative example of the assay and bar-graphs representing the average results from three independent experiments are reported in Figure 2a. Both activated forms of *RET* were able to stimulate JNK activity at levels (~20-fold) even higher than that caused by heat shock treatment (~tenfold) (lane +), used as positive control (Kyriakis *et al*., 1994). Also another activated form of *RET*, *RET*/PTC1 oncogene, was able to stimulate (~15-fold) JNK activity in NIH3T3 cells (data not shown).

Then, we evaluated whether wild type Ret was able to induce JNK activity when triggered by GDNF. Since it has been demonstrated that GDNF does not bind Ret directly but through the interaction with GDNFR-α, we stably transfected NIH3T3-*RET* cells (Santoro *et al*., 1995) with an expression vector for GDNFR-α (Jing *et al*., 1996). A marker selected mass population, expressing GDNFR-α, was obtained and GDNF-induced Ret phosphorylation was demonstrated (not shown). Untriggered NIH3T3-*RET*-GDNFR-α cells showed barely detectable JNK activity (Figure 2b). GDNF stimulation caused a rapid (after 10 min) JNK activation in NIH3T3-*RET*-GDNFR-α (Figure 2b) but not in untransfected NIH3T3 cells (data not shown).

In order to investigate Ret signalling to JNK in cells which are specific target for its oncogenic activation, we analysed Ret-dependent JNK activation in a thyroid epithelial cell line, the PC Cl 3 cell line. As shown in Figure 3a, untrasfected PC Cl 3 cells showed very low levels of JNK activity; this activity was promptly induced by heat shock treatment (lane +). PC Cl 3 cells expressing the *RET*/PTC1 oncogene (Santoro *et al*., 1993) showed a markedly enhanced (~tenfold) JNK activation when compared to untransfected PC Cl 3. Accordingly, the human TPC-1 cell line, a thyroid papillary carcinoma cell line spontaneously harbouring a *RET*/PTC1 oncogene, also showed a greatly enhanced JNK activity with respect to a normal human thyroid cell line (data not shown).

To evaluate Ret signalling to JNK in a cell system normally expressing Ret we selected PC12 cells, which derive from a rat pheochromocytoma (Greene and Tishler, 1976) and express endogenous Ret. We transfected PC12 with GDNFR-α and obtained a marker-selected mass population showing a readily detectable response (Ret phosphorylation and neurite outgrowth) to GDNF triggering (data not shown). Then, we evaluated JNK activation upon stimulation with GDNF, NGF (nerve growth factor), or EGF (epidermal growth factor). PC12 cells showed significant basal levels of JNK activity; GDNF triggering induced JNK activation at a similar extent as NGF and EGF, which are well-known JNK inducers (Coso *et al*., 1995a) (Figure 3b). Similar levels of JNK activation were induced in PC12 cells by heat shock treatment (not shown).

To confirm these data with an independent assay and to determine if the GST-c-Jun(79) phosphorylation, detected by the solid-phase kinase assay, was due to JNK kinases including JNK1, we measured JNK1 activity by an immunocomplex kinase assay in NIH3T3 and PC Cl 3 cells (Coso *et al*., 1995a). JNK1 was immunoprecipitated with anti-JNK1 antibodies and GST-c-Jun(79) was used as a substrate in an *in vitro* kinase assay. NIH3T3-*RET*/MEN2A showed a remarkable activation of JNK1 with respect to untransfected cells (~sevenfold) (Figure 4). Also, PC Cl 3-*RET*/PTC1 cells showed increased JNK1

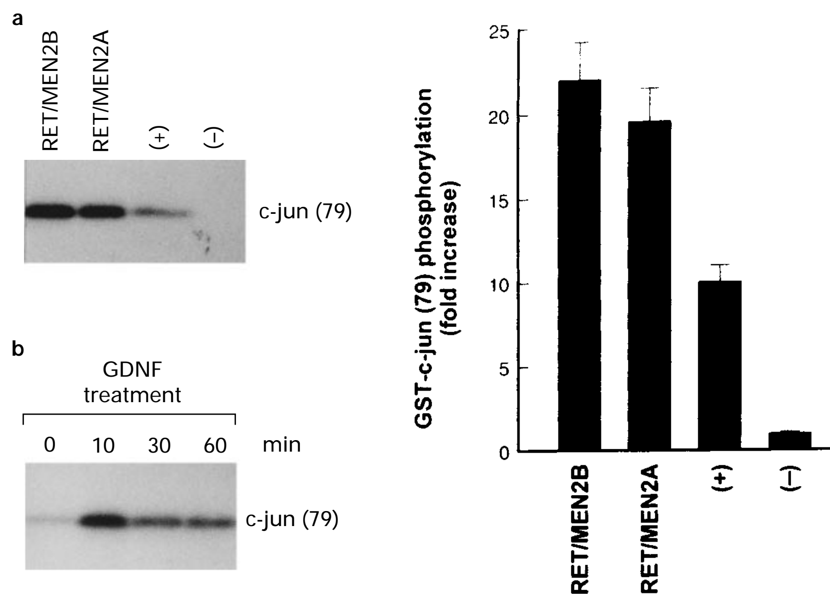

Figure 2 Ret activation of JNK in NIH3T3 cells. Solid phase kinase assay on GST-c-Jun(79) protein substrate. (**a**) five hundred μg of protein lysate from control NIH3T3 (−), NIH3T3 subjected to heat shock (42°C, 30 min) (+), NIH3T3 transfected with *RET*/MEN2A or *RET*/MEN2B, were precipitated with 1 μg of GST-c-Jun(79) and incubated with radiolabelled ATP. An example of one representative assay is reported on the left and the average results of three independent experiments, measured by phosphorimaging, are reported on the right. Error bars are reported. (**b**) NIH3T3-*RET*-GDNFR-α were serum-starved for 12 h and stimulated with GDNF for the indicated times. After harvesting, the GST-c-Jun(79) solid phase kinase assay was performed. The results were typical and representative of at least three independent experiments

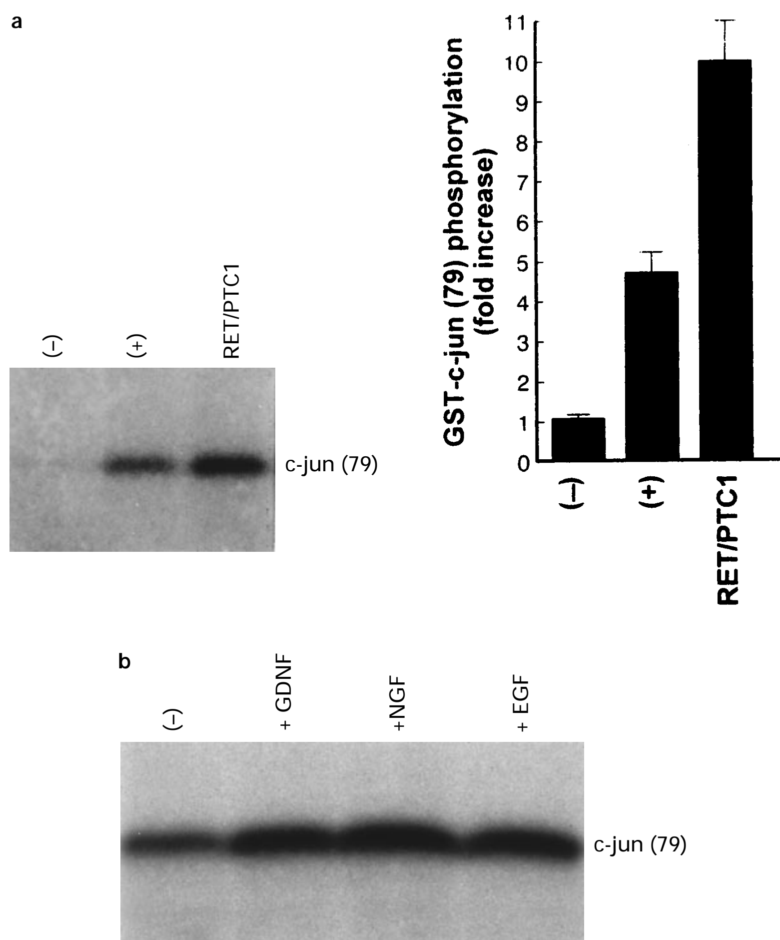

Figure 3 (**a**) Activation of JNK by *RET*/PTC1 oncogene in thyroid cells. Five hundred μg of protein lysate from control PC Cl 3 (−), PC Cl 3 subjected to heat shock (42°C, 30 min) (+), or PC Cl 3-*RET*/PTC1 were precipitated with 1 μg of GST-c-Jun(79) and incubated with radiolabelled ATP. An example of one representative assay is reported on the left and the average results of three independent experiments are reported on the right. Error bars are shown. (**b**) Activation of JNK by GDNF in PC12 cells. Solid phase kinase assay on GST-c-Jun(79) protein substrate. Five hundred μg of protein lysate from control PC12-GDNFR-α (−), or PC12-GDNFR-α treated, for 10 min, with 100 ng/ml of EGF, NGF or GDNF were precipitated with 1 μg of GST-c-Jun(79) and incubated with radiolabelled ATP. The results were typical and representative of at least three independent experiments

activity (~eightfold). Whereas in the case of NIH3T3-*RET*/MEN2A the extent of JNK activation, detected by the immunocomplex assay, was lower than that detected by the solid phase kinase assay (seven *vs* 20-fold), in the case of PC Cl 3 cells the extent of activation by the two methods was comparable, suggesting that, although in NIH3T3 cells Ret is able to activate multiple JNK isoforms, in thyroid cells one of the major targets of Ret signalling to JNKs is the JNK1 isoform. This can be explained by the observation that, at a variance from NIH3T3 cells transformed by activated *RET* alleles, PC Cl 3-*RET*/PTC1 cells showed increased levels of JNK1 protein (~threefold) (data not shown); thus, overexpression of JNK1 could be part of its activation mechanism by *RET*/PTC1 in thyroid cells.

Oncogenic forms of RET *activate epitope-tagged JNK1 in COS-1 cells*

Having ascertained that Ret is able to activate the JNK1 isoform, to study molecules involved in this process, we used a transient co-transfection assay in COS-1 cells. COS-1 lack Ret receptor (Carlomagno *et al*., 1996); thus, we co-transfected COS-1 with activated forms of *RET* (*RET*/MEN2A and *RET*/MEN2B) together with an HA epitope-tagged JNK1. After transfection, the transiently expressed HA-JNK1 was recovered by immunoprecipitation and its activity was determined by an immunocomplex kinase assay (Coso *et al*., 1995b). HA-JNK1 and *RET* constructs were efficiently expressed in COS-1 cells (data not shown, and see Figure 6). Figure 5 shows that both *RET*/MEN2A and *RET*/MEN2B induced an increase of JNK1 activity. JNK1 stimulation by *RET* was as efficient as that obtained by heat shock (lane +) or by cycloheximide (not shown), which are known inducers of JNK1 (Kyriakis *et al*., 1994; Coso *et al*., 1995a). Mutations associated with Hirschsprung's disease (HSCR) exert a 'loss of function' effect on Ret; specifically, a HSCR R972G substitution, in the TK domain of Ret, greatly impairs both *RET*/MEN2A kinase and transforming activities (Pasini *et al*., 1995; Carlomagno *et al*., 1996). As shown in Figure 5, JNK1 stimulation in COS-1 cells was significantly impaired when the HSCR R972G mutation was introduced in the *RET*/MEN2A construct. This result indicates that the kinase activity of Ret is required to activate JNK1

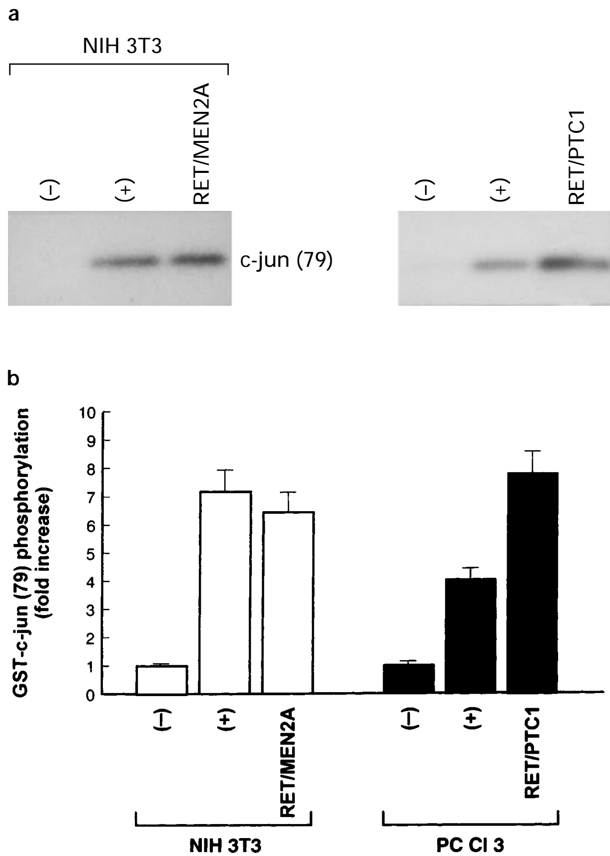

Figure 4 Immunocomplex JNK1 kinase assay in NIH3T3 and PC Cl 3 cells. Two mg of cell lysates from untransfected NIH3T3 stimulated (+) or not (−) with heat shock, and NIH3T3-*RET*/MEN2A, or from untransfected PC Cl 3 stimulated (+) or not (−) with heat shock and PC Cl 3-*RET*/PTC1 were immunoprecipitated with a JNK1-specific polyclonal antibody. Kinase reaction was performed on the immunoprecipitates using 1 μg of purified GST-c-Jun(79) as a substrate. An example of one representative assay (upper) and bar-graphs reporting the average results of three independent assays (lower) are reported

and that impairing JNK1 activation by *RET* is part of the inhibitory effects of *RET* mutations associated with Hirschsprung's disease.

The pathway leading to JNK1 activation by Ret diverges from that leading to ERK2 activation and involves Rho/Rac-related but not Ras small GTP-binding proteins

The recent observation that Rho-related GTPases (Rac1 and Cdc42) activate JNK (Coso *et al*., 1995a; Minden *et al*., 1995), prompted us to investigate if these GTPases participate to Ret signalling. As a tool to inhibit Rho-like GTPases, we used molecular constructs encoding either RhoGDI (guanine nucleotide dissociation inhibitor) or the catalytic domain of RhoGAPp190 (RhoGAPdp190) (Coso *et al*., 1995a, reviewed by Boguski and McCormik, 1993; Hall, 1994; Lamarche and Hall, 1994). These plasmids were transfected in COS-1 cells together with HA-JNK1 and *RET*/MEN2A plasmids. As shown in Figure 6a, both of them drastically reduced JNK1 activation by *RET*/MEN2A without affecting Ret/MEN2A and JNK1 expression. Then, we used an expression vector encoding a dominant negative mutant of Cdc42 (N17Cdc42) (Coso *et al*., 1995a). This dominant negative mutant, when transiently transfected in COS-1 cells, blocked JNK1 activation elicited by *RET*/MEN2A (Figure 6b). These results indicated that Ret signals to JNK1 are delivered through Rho-related GTPases and, among them, by Cdc42. We also examined whether Ras plays a key role in JNK1 activation by Ret, using a dominant negative mutant of Ras (N17Ras). JNK1 response to *RET*/MEN2A was not affected by expression N17Ras (Figure 6b). When we used, in the COS-1 transient expression assay, an HA-epitope tagged ERK2 Ret/MEN2A resulted to be a potent activator of ERK2, as evaluated by using the myelin basic protein (MBP) as a substrate (Figure 6c). However, at a variance from JNK1, ERK2 induction by *RET*/MEN2A was severely impaired by N17Ras and not by N17Cdc42 expression (Figure 6c). In Figure 6b and c it is also shown that the various constructs were efficiently expressed in the transfected COS-1 cells.

Ret tyrosine residue 1062 is part of a docking site for the Shc phosphotyrosine binding (PTB) domain. Mutation of tyrosine 1062 abrogates both Shc binding to Ret and Shc tyrosine phosphorylation induced by Ret (Asai *et al*., 1996; Lorenzo *et al*., 1997; Arighi *et al*., 1997; Melillo *et al*., in preparation). We constructed a Y1062F Ret/MEN2A protein mutant (*RET*/MEN2A^{Y1062F}) and we confirmed that, when expressed in COS-1 cells, it was correctly synthesized (Figure 7a, lower). Moreover, an immunoprecipitation with anti-Ret followed by blotting with anti-phosphotyrosine antibodies, indicated that, as reported (Asai *et al*., 1996; Lorenzo *et al*., 1997; Arighi *et al*., 1997), Y1062F mutation did not alter the intrinsic kinase activity of Ret/MEN2A (see for an example Figure 7b). As expected, Y1062F mutation abrogated *in vitro* binding of Ret/MEN2A to the PTB domain of Shc (Figure 7a, upper). Thus, we co-transfected COS-1 cells with *RET*/MEN2A or *RET*/MEN2A^{Y1062F} constructs together with HA epitope tagged JNK1 or ERK2. Kinase activity of immunoprecipitated JNK1 or ERK2 were then assayed on GST-c-Jun(79) or myelin basic protein (MBP) as substrates, respectively. ERK activation was almost completely abolished by the Y1062F mutation. In contrast, the activation of JNK1 by Ret/MEN2A was unaffected by the Y1062F mutation (Figure 7b).

A dominant negative mutant for SEK interferes with RET/*MEN2A signalling*

We have reported that activation of the pNGFI-A-CAT plasmid is part of the *RET* signalling in PC12 cells (Califano *et al*., 1996). NGFI-A is an immediate-early response gene, whose expression is rapidly stimulated upon growth factor treatment of PC12 cells (Janssen-Timmen *et al*., 1989). Thus, we have evaluated whether JNK activation is involved in *RET* signalling by using pNGFI-A-CAT as a target in transient transfection experiments in PC12 cells. To interfere with JNK activation, we have used a dominant negative construct for SEK (pEBG SEK KR). SEK, also referred to as MKK4 or JNKK1, is the kinase responsible for JNK activation, by acting downstream MLK3 and MEKK (Sanchez *et al*., 1994; Teramoto *et al*., 1996). In preliminary experiments we

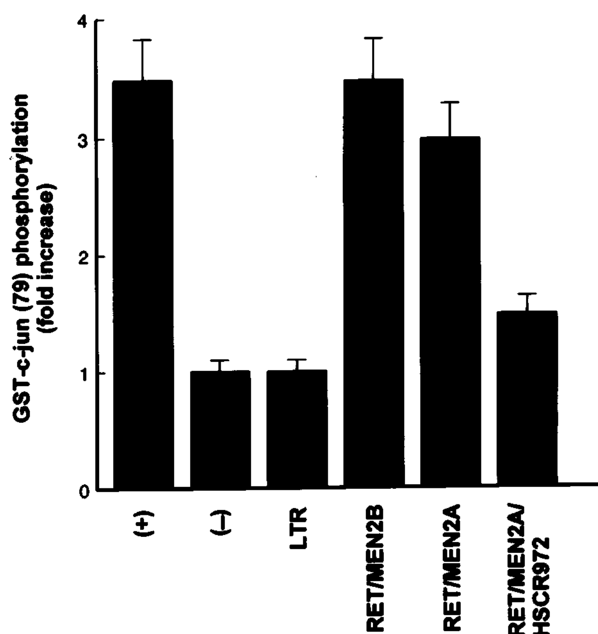

Figure 5 *RET* activation of JNK1 in transiently transfected COS-1 cells. Subconfluent COS-1 cells were transfected by electroporation with 1 μg of pcDNA3-HA-JNK1 and 2 μg of the indicated *RET* plasmids. Thirty-six hours after transfection, cells were harvested, 2 mg of cell lysates were immunoprecipitated with anti-HA antibody (12CA5), and an immunocomplex JNK1 assay was performed on GST-c-Jun(79) substrate. COS-1 transfected with HA-JNK1 alone (−), with HA-JNK1 and the empty vector (LTR), or with HA-JNK1 and then subjected to heat shock (42°C, 30 min) (+) were used as negative and positive controls, respectively. Average results of at least four independent transfections are reported. Parallel samples (500 μg) immunoprecipitated with anti-HA and blotted with anti-JNK1 demonstrated equal expression of the HA-JNK1 protein (not shown). Also Ret expression was demonstrated by immunoblot (not shown)

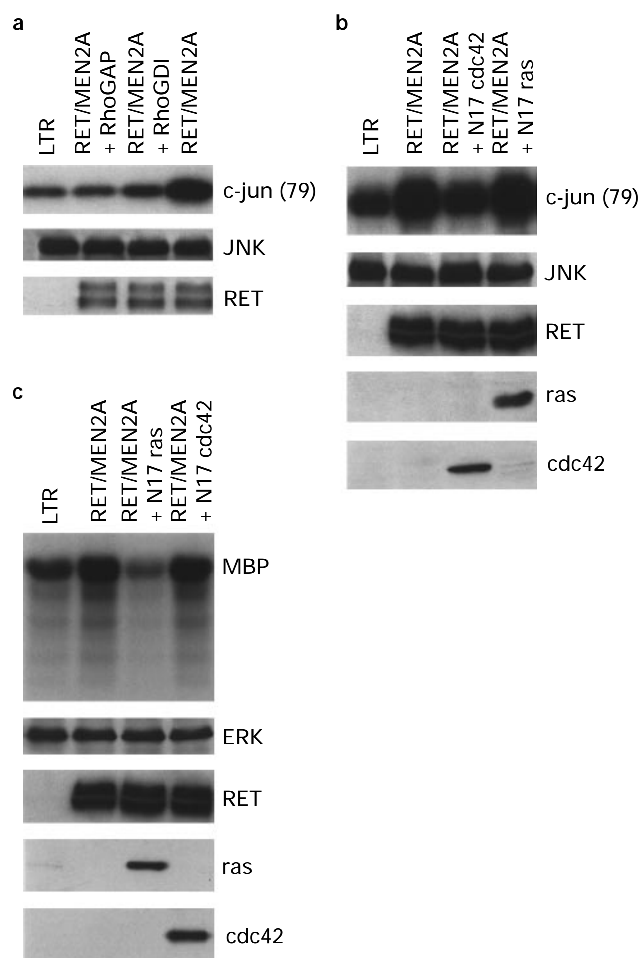

Figure 6 *RET* activation of JNK1 or of ERK2 in COS-1 cells is mediated by Rho-related or by Ras small GTPases, respectively. (**a**, **b**) Subconfluent COS-1 cells were transfected by electroporation with 1 μg pcDNA3-HA-JNK1, 2 μg of *RET*/MEN2A and, when indicated, with 2 μg of RhoGAPdp190, RhoGDI, N17Cdc42, or N17Ras plasmids. Thirty-six hours after transfection, cells were harvested and an immunocomplex JNK1 assay was performed on GST-c-Jun(79) substrate. Vector transfected (LTR) COS-1 were used as a negative control. Protein extracts (500 μg) immunoprecipitated with anti-HA and blotted with anti-JNK1, immunoprecipitated and blotted with anti-Ret, or blotted with anti-Ras or anti-Cdc42 antibodies demonstrated equal expression of the constructs among the different samples. (**c**) subconfluent COS-1 cells were transfected by electroporation with 1 μg pcDNA3-HA-MAPK, 2 μg of *RET*/MEN2A and, when indicated, with 2 μg of N17Cdc42 or N17Ras plasmids. Thirty-six hours after transfection, cells were harvested and an immunocomplex ERK2 assay was performed on myelin basic protein (MBP) as a substrate. A Western blot detected with anti-Ras or anti-Cdc42 antibodies demonstrated equal expression of the constructs among the different samples. The results were typical and representative of two independent experiments

have observed that pEBG SEK KR was able to cause a reduction in JNK1 activation by *RET*/MEN2A (Visconti R, unpublished). The average results of three independent transfections, each performed in duplicate, are reported in the bar graphs of the Figure 8b and a representative CAT assay is shown in Figure 8a. Transfection of *RET*/MEN2A resulted in a marked induction of CAT activity, as reported (Califano *et al*., 1996; Carlomagno *et al*., 1996). Co-expression of increasing amounts of pEBG SEK KR resulted in a reduction of pNGFI-A activation by *RET*/MEN2A.

Discussion

Here we show that Ret tyrosine kinase is able to signal through the JNK pathway in different cell lines. Several kinases belonging to the JNK family have been identified (Gupta *et al*., 1996). Immunoprecipitation with anti-JNK1 antibodies and transient expression of an epitope-tagged JNK1 construct demonstrated that JNK1 is at least one of the JNK-family members activated by Ret. That this ability may be relevant for the role played by Ret in human diseases is suggested by several lines of evidence. First, not only wild type Ret, when triggered by GDNF, but also chronically activated Ret variants associated with human tumoral diseases (*RET*/PTC1, *RET*/MEN2A, *RET*/MEN2B) activate JNK. Particularly, *RET*/MEN2B showed a slightly but reproducibly increased ability of activating JNKs with respect to *RET*/MEN2A. On the other side an Hirschsprung's mutation, which is known to impair Ret function, inhibited this action. JNK activation is exerted by activated *RET* constructs not only in heterologous cell systems such as NIH3T3 and COS-1 cells, but also in other cell types representing specific targets of Ret action *in vivo*, such as epithelial thyroid PC Cl 3 and PC12 cells. Moreover, at least in transiently transfected

PC12 cells, JNK activation seemed to be important for Ret signalling, since a dominant negative mutant for SEK impaired Ret ability of inducing an immediate-early gene promoter (pNGFI-A) stimulation. However, at a variance from NIH3T3 and PC Cl 3 cells, adoptive expression of chronically active *RET* mutants (*RET*/MEN2A and *RET*/MEN2B, Califano *et al*., 1996) was unable to induce JNK activity in PC12 cells (data not shown). We do not known why chronic stimulation of Ret is uneffective in activating JNK in PC12 cells. Expression of the JNK1 protein was unaffected by *RET*/MEN2A and *RET*/MEN2B, but it is possible that other components of the JNK activating machinery are down regulated upon the neuronal differentiation induced by chronic *RET* activation.

As in the case of other receptor tyrosine kinases (EGFR, as an example), Ret activation of JNK is mediated by GTPases of the Rho/Rac family (Coso *et al*., 1995a). In fact, negative regulators of these molecules (RhoGAP and RhoGDI) and a dominant negative construct for Cdc42 impaired JNK activation by Ret. Experiments conducted with other dominant negative constructs demonstrated that Rac1 is also involved in Ret activation of JNK1 and excluded that Rho could be another of such involved small GTPases (data not shown).

Several pieces of evidence indicate that ERKs and JNKs activities are differently regulated. Ha-*ras*, which is sufficient for full ERK activation, leads only to partial JNK activation and, on the other side, Rho/Rac-family proteins do not have a considerable effect on ERK2 activity (Coso *et al*., 1995a). Here, we report data indicating that these two pathways, in the case of

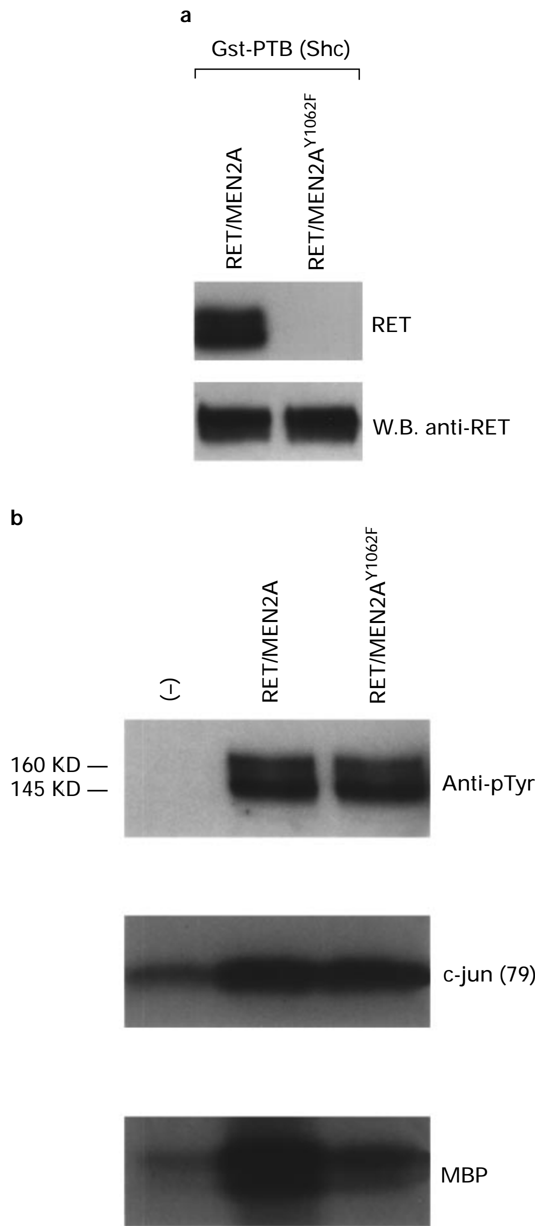

Figure 7 (**a**) *In vitro* binding of Ret/MEN2A proteins to the PTB domain of Shc. Subconfluent COS-1 cells were transfected with 5 μg of the indicated *RET* constructs and lysed 48 h after transfection. Two mg of protein lysate were incubated with 5 μg of Sepharose-bound GST-PTB (Shc) recombinant protein and subjected to SDS–PAGE. The resulting immunoblot was detected with anti-Ret antibodies (upper). One hundred μg of protein lysate was used for a Western blot to confirm the equal expression of the different Ret proteins (lower). (**b**) Activation of JNK1 or ERK2 by wild type or Y1062F *RET*/MEN2A constructs. Subconfluent COS-1 cells were transfected with 1 μg pcDNA3-HA-JNK1 or 1 μg of an epitope-tagged ERK2 (pcDNA3-HA-MAPK) constructs and 2 μg of the indicated *RET* constructs or the empty LTR vector (−). Thirty-six hours after transfection, cells were harvested, JNK1 activity was measured as above. ERK2 was immunoprecipitated from 2 mg of cell lysate with anti-HA antibody (12CA5) and incubated with labelled ATP and 20 μg of myelin basic protein (MBP). Parallel samples immunoprecipitated with anti-Ret and blotted with anti-phosphotyrosine monoclonal antibodies (4G10) were used as a control. These results are typical and representative of three independent experiments. Immunoblotting confirmed equal expression of JNK1 or ERK2 (not shown)

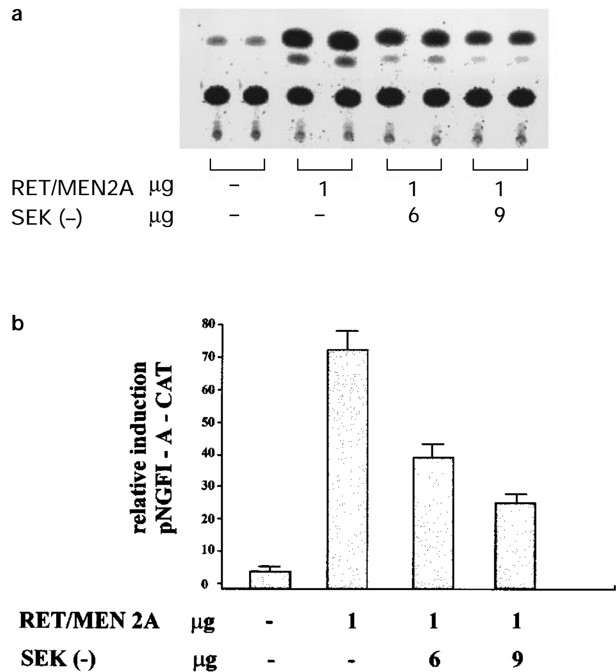

Figure 8 Inhibition of the *RET*-induced NGFI-A promoter activation by a dominant negative SEK mutant. PC12 cells were transfected with 0.5 μg of pNGFI-A-CAT, 1 μg of *RET*/MEN2A and increasing amounts of pEBG SEK KR [SEK (−)], as indicated. Sixty hours after transfection, total proteins were isolated and promoter induction determined by CAT assay. (**a**) One representative CAT assay is shown. (**b**) Bar-graphs of the relative induction are reported as fold increases above the basal activity of pNGFI-A-CAT reporter gene transfected alone. The results represent the average of three separate experiments performed in duplicate. Variation between experiments was less than 15% of the mean

RET signalling, are distinct. Ret is able to activate endogenous Ras and this pathway is relevant for its mitogenic (Grieco *et al*., 1995), as well as neuronal differentiating activities (Califano *et al*., 1995). *RET* encodes two alternatively spliced protein isoforms 1072 and 1114 amino acids long (Ret^{1072} and Ret^{1114}) both of which are able to activate Ras (Santoro *et al*., 1994; Grieco *et al*., 1995; van Weering *et al*., 1995; our unpublished observations). Only Ret^{1114} is able to bind Grb2 directly, probably through tyrosine residue 1096 (Borrello *et al*., 1994; Lorenzo *et al*., 1997; our unpublished observations). Conversely, both isoforms are able to bind and to induce the tyrosine phosphorylation of Shc (Borrello *et al*., 1994; Lorenzo *et al*., 1997). In particular, Ret tyrosine residue 1062, which is common to both Ret^{1072} and Ret^{1114}, is part of a docking site for the Shc phosphotyrosine binding (PTB) domain (Asai *et al*., 1996; Lorenzo *et al*., 1997; Arighi *et al*., 1997). Our preliminary results indicate that Y1062F mutation impairs Ret activation of endogenous Ras (Visconti *et al*., unpublished). Here, we have demonstrated that both a dominant negative construct for Ras or the substitution of Y1062 with phenylalanine (Y1062F) abrogates Ret^{1072}-mediated activation of ERK2, a *bona fide* downstream effector of Ras. Conversely, mutation of Y1062 did not alter Ret signalling to JNK. Together with the observation that a dominant negative construct for Ras (RasN17) did not significantly affect JNK activation by Ret, these data indicate that JNKs activation by Ret is largely independent on Ras. Thus, although a partial overlap in pathways leading to JNK and ERK activation was demonstrated in the case of EGFR (Coso *et al*., 1995a), in the case of Ret the two pathways seem to be completely divergent.

Indirectly, our data also indicate that Ret signalling involves small GTP-binding proteins other than Ras, i.e. Cdc42 and Rac1. Rac1 and Cdc42 proteins control formation of membrane ruffles (lamellipodia) and filopodia, respectively (reviewed by Bokoch and Der, 1993). There is also evidence that Rho/Rac-like proteins effects are not limited to cytoskeleton organization but are involved in the control of cell proliferation (Hill *et al*., 1995). For instance, products of the *vav* and *dbl* oncogenes are *bona fide* guanine nucleotide exchange factor for Rac1 (Crespo *et al*., 1997), and Cdc42 (Hart *et al*., 1991), and both oncogenes are efficient JNK inducers (Minden *et al*., 1995; Coso *et al*., 1995a; Crespo *et al*., 1997). Finally, activated Rac1 cooperates with Ras in oncogenic transformation (Qiu *et al*., 1995). Molecules involved in Ret signalling to Rho/Rac family members remain to be elucidated. One candidate could be the Src kinase which is activated by Ret (Melillo *et al*., in preparation). Indeed, ultraviolet irradiation, a known inducer of JNK, stimulates c-Src kinase, v-Src is an efficient JNK activator, and a partial inhibition of v-Src-mediated transformation is achieved by inactive forms of JNK1 (Minden *et al*., 1995; Liu *et al*., 1996). Another molecule that could be involved in this pathway is p190RhoGAP. In fact, it binds and is phosphorylated upon Ret triggering (Santoro *et al*., 1994), and it is a well known modulator of Rho/Rac activity (Boguski and McCormick, 1993). Finally, PI3 kinase has been described to mediate Ret effects on cytoskeleton (van Weering and Bos, 1997) and it acts upstream Rho-related GTPases in the case of PDGFR signalling (Hawkins *et al*., 1995). Moreover, both in the case of epidermal growth factor receptor (Logan *et al*., 1997) and of Met (Rodrigues *et al*., 1997), PI3 kinase has been demonstrated to be important for JNK activation. Whether this is also true for Ret remains to be established.

In conclusion, our findings suggest that signalling through these small GTPases is likely able to mediate the potent biological effects exerted by the end product of naturally occurring oncogenic *RET* genes in a variety of cell types.

Materials and methods

Expression plasmids and antibodies

All the *RET* constructs used in this study are cloned in the LTR vector and encode the Ret^{1072} protein isoform. LTR-*RET*, encoding wild type Ret, and LTR-*RET*/MEN2A and LTR-*RET*/MEN2B, encoding Ret mutants carrying C634Y and M918T mutations, respectively, are described elsewhere (Santoro *et al*., 1995). The LTR-*RET*/MEN2A/HSCR972 encodes a *RET*/MEN2A mutant carrying a Hirschsprung-associated mutation at codon 972 (R972G), and it has been previously described (Carlomagno *et al*., 1996). LTR-*RET*/MEN2A^{Y1062F} encodes a *RET*/MEN2A allele in which tyrosine 1062 has been replaced by phenylalanine. Briefly, to mutagenize Ret tyrosine 1062, PCR fragments containing the required mutation were generated by recombinant PCR (Higuchi, 1990) using LTR-*RET* as a template. Two primary reactions (a 'left' and a 'right' reaction) were performed, using standard PCR conditions (AmpliTaq, Perkin Elmer-Cetus Co). This yielded two products overlapping in the sequence corresponding to the reverse primer of the 'left' PCR and the forward primer of the 'right' PCR, and the mutation (TAT→TTT) was introduced as part of these overlapping PCR primers. Ten ng of the purified PCR products of the 'left' and the 'right' primary PCR reactions were annealed and elongated with five PCR cycles (95°C 1 min, 37°C 2 min, 72°C 1 min) followed by 15 cycles of a secondary PCR, using the 5′- and 3′-most oligonucleotides, as primers. The recombinant PCR products were cloned in the pT7Blue T vector (Novagen) and completely sequenced using the Sequenase Kit (USB). Finally, the fragment containing the mutation was excised by digestion with *Xho*I and *Mlu*I and cloned in the LTR-*RET*/MEN2A plasmid; this was sequenced in both strands of the region that underwent genetic manipulations. The primers used were the following (the mutated nucleotide is in brackets): Left PCR: 5′-GAGGAGACACCGCTGGTGGTGG-3′ (forward) and 5′-AATTCTGCCA(A)AGAGTTTGTT-3′ (reverse); Right PCR: 5′-AACAAACTCT(T)TGGCAGA-ATT-3′ (forward) and 5′-AAACGCGTACAGCGGTGC-TAGAATCTAGT-3′ (reverse). The expression vector for rat GDNFR-α (pSJA45-GDNFR-α) is reported elsewhere (Jing *et al*., 1996). Expression plasmids for the HA nonapeptide epitope-tagged JNK1 (pcDNA3-HA-JNK1) or ERK2 (pcDNA3-HA-MAPK), for the Cdc42 (pcDNA3-N17Cdc42, in which an asparagine residue, in position corresponding to codon 17 of Ras, was replaced by a threonine) and the Ras (N17Ras) dominant negative mutants, and for the two negative regulators of Rho-like small GTPases (pcDNA3-RhoGAPdp190 and pcDNA3-RhoGDI) have been already described (Coso *et al*., 1995a). The GST-PTB (Shc) construct was a generous gift of PG Pelicci.

Polyclonal antibodies against Ret were directed to the Ret tyrosine-kinase domain (Santoro *et al*., 1994). Anti-Ret

antibodies were used at 1 μg/ml for immunoprecipitation and at 0.1 μg/ml for Western blotting. Mouse monoclonal antibodies anti-HA epitope (clone 12CA5) (Wilson *et al*., 1984) were purchased from Boehringer Mannheim, Mannheim, Germany. Rabbit polyclonal anti-JNK antiserum was purchased from Santa Cruz Biotechnology, CA. The 4G10 anti-pTyr monoclonal antibody was purchased from Upstate Biotechnology, Lake Placid, NY. Anti-Ras and anti-Cdc42 antibodies were provided by Santa Cruz Biotechnology, CA.

Cell culture and transfection experiments

NIH3T3 cells were grown in DMEM supplemented with 10% foetal calf serum (FCS) (GIBCO-BRL). PC12 cells were grown in RPMI 1640 medium supplemented with 10% horse serum and 5% foetal calf serum (GIBCO-BRL). The PC Cl 3, thyroid epithelial cell line, was cultured in modified F12 medium, supplemented with 5% calf serum (GIBCO-BRL) and six growth factors (thyrotropin, hydrocortisone, insulin, transferrin, somatostatin and glycil-histidyl-lysine) (SIGMA Chemical Corporation) (Fusco *et al*., 1987). Stable transfectants of the thryoid (Santoro *et al*., 1993) and NIH3T3 cells (Santoro *et al*., 1995; Carlomagno *et al*., 1996) with the *RET* mutants have been described. To obtain NIH3T3 cells expressing both *RET* and GDNFR-α, NIH3T3-*RET* were transfected with pSJA45-GDNFR-α and a marker(G418)-selected mass population was obtained. This transfection was performed by using the lipofectamine reagent (GIBCO-BRL) following manufacturer's instructions. Briefly, 4×10^5 cells were plated in 60 mm dishes and incubated with 4 μg of plasmid and 12 μl of lipofectamine reagent in 1.2 ml OPTIMEM (GIBCO-BRL). After 4 h, 1.5 ml of DMEM supplemented with 20% fetal calf serum were added and after 24 h the incubation mixture was replaced with complete medium containing G418 for the marker selection. The resulting cells were demonstrated to express both Ret and GDNFR-α and to respond to GDNF-triggering with a readily detectable Ret tyrosine phosphorylation (not shown). When required, the cells were serum-deprived for 12 h and then stimulated with 100 ng/ml of human GDNF, purchased from Alomone Labs., Israel. PC12 cells express endogenous Ret. To obtain PC12-GDNFR-α, PC12 were transfected, as previously reported (Califano *et al*., 1996), with the pSJA45-GDNFR-α plasmid and a mass population was obtained by G418 selection. Ret tyrosine phosphorylation and neurite outgrowth, upon GDNF triggering, were demonstrated in the obtained cell population (not shown). For the JNK assay, PC12-GDNFR-α were stimulated, for 10 min, with 100 ng/ml GDNF, EGF or NGF, purchased from Upstate Biotechnology. COS-1 cells were maintained in DMEM supplemented with 10% FCS. Subconfluent COS-1 cells were transfected by electroporation (Bio-Rad Gene Pulser, 220 V; 250 μF) with 1 μg pcDNA3-HA-JNK1 or pcDNA3-HA-MAPK, 2 μg of the different *RET* mutants, and in some experiments 2 μg of RhoGAPdp190, RhoGDI, N17Cdc42, and N17Ras plasmids. Cells were incubated for 24 h in complete medium and for 12 h in serum-free medium.

Jun kinase assay

Cloning and purification of GST-c-Jun(79) substrate has been previously described (Coso *et al*., 1995b). This substrate contains the 79 amino-terminal amino acids of c-Jun, including the two serines, in position 63 and 73, that are specifically phosphorylated by the JNKs. For solid phase kinase assay, subconfluent plates of the different cell lines were incubated over-night in serum-free medium. When required the cells were subjected to heat shock (42°C, 30 min) before harvesting (Kyriakis *et al*., 1994). Cells were washed with ice-cold PBS and lysed at 4°C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1% Triton-X100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin and 20 μg/ml leupeptin. Cleared lysates (500 μg) were rocked for 3 h at 4°C in the presence of 1 μg of GST-cJun(79) bound to glutathione-agarose beads. Beads were washed three times with PBS containing 1% Nonidet P-40 and 2 mM sodium vanadate, once with 100 μM Tris (pH 7.5), 0.5 M LiCl, and once in kinase reaction buffer (25 mM HEPES, pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM sodium vanadate and 2 mM DTT). Samples were resuspended in 30 μl of kinase reaction buffer containing 1 μCi of [γ-^{32}P]ATP and 20 μM of unlabelled ATP. After 20 min at 30°C the reaction was terminated by adding 10 μl of 5×Laemmli Buffer. Samples were heated at 95°C for 5 min and analysed by SDS-gel electrophoresis on 12% acrylamide gels. Autoradiography was performed with the aid of an intensifying screen. The intensity of signals was analysed at the phosphorimager (Molecular Dynamics).

Immunocomplex JNK1 activity was determined upon immunoprecipitating cell lysates (2 mg) (from stable NIH3T3 or PC Cl 3 transfectants) with a JNK1-specific polyclonal antibody (Santa Cruz Biotechnology, CA), or cell lysates from pcDNA3-HA-JNK1 transfected COS-1 with anti-HA antibody 12CA5 (Boehringer Manneheim, Germany). Cleared lysates were incubated with 2 μl of antibody for 1 h at 4°C. Immunocomplexes were recovered with protein A-Sepharose (Pharmacia Biotech, Uppsala), and precipitates were washed six times in lysis buffer and then processed as above. Kinase reaction was performed using 1 μg of purified GST-cJun(79) substrate. Parallel samples immunoprecipitated with anti-HA and blotted with anti-JNK1 or immunoprecipitated and blotted with anti-Ret were used as a control.

ERK kinase assay

ERK activity was assayed in COS-1 cells, transiently transfected with epitope-tagged ERK2 (pcDNA3-HA-MAPK), by using 20 μg of myelin basic protein (MBP) as a substrate, as previously described (Coso *et al*., 1995b). Parallel samples immunoprecipitated with anti-Ret and blotted with anti-phosphotyrosine monoclonal antibodies (4G10, Upstate Biotechnology) were used as a control of Ret expression and phosphorylation.

Western blots and in vitro *binding experiments*

Lysates containing comparable amounts of total cellular proteins, estimated by a modified Bradford assay (Bio-Rad), were immunoprecipitated with the required antibody and analysed by SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose, and staining with the required primary antibody. Immunocomplexes were revealed by enhanced chemiluminescence detection kit (ECL, Amersham) using anti-rabbit or anti-mouse antiserum coupled to horseradish peroxidase. Bacterial cultures expressing recombinant pGEX (Pharmacia) containing the PTB domain of Shc were grown in LB and induced with 1 mM isopropyl β-D-thiogalactopyranoside for 3 h. The induced bacteria were lysed by sonication in PBS containing 10 μg/ml of aprotinin. The recombinant protein was purified using glutathione-sepharose (Pharmacia). Cells were lysed and the clarified lysates were incubated with 5 μg of immobilized GST-PTB protein for 60 min at 4°C. Protein complexes were resolved by SDS–PAGE, transferred to PVDF membranes and detected with anti-Ret antibodies.

pNGFI-A-CAT transient assay in PC12 cells

The pNGFI-A-CAT plasmid contains sequences from position −1150 to +200 of the NGFI-A promoter, fused to the chloramphenicol acetyl transferase (CAT) gene (Janssen-Timmen *et al*., 1989). For transient transfection assays, 3×10^5 PC12 cells were plated in 60 mm-diameter tissue culture dishes. Transfection was performed using the lipofectin reagent following the manufacturer's instructions (GIBCO-BRL). The pNGFI-A-CAT plasmid (Janssen-Timmen *et al*., 1989) is characterized by a very low basal level of activity in PC12 cells (lower than 0.5% of chloramphenicol conversion) (Califano *et al*., 1996). Transfections were carried out with 0.5 μg of reporter plasmid together with 1 μg of *RET*/MEN2A and increasing amounts (0, 6, 9 μg) of a dominant negative construct for SEK (pEBG SEK KR) (Sanchez *et al*., 1994). The same DNA concentration was reached by adding various amounts of the LTR control vector. Cell extracts were prepared 60 h after transfection and CAT activity was analysed by thin-layer chromatography with 95% chloroform-5% methanol, as previously described (Califano *et al*., 1996). Each experimental point was cut from the TLC plate and counted. For each experiment, the percentage of conversion to the acetylated form of chloramphenicol [^{14}C] was then calculated. The results were plotted as promoter induction relative to the induction exerted by the LTR vector alone.

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References

Arighi E, Alberti L, Torriti F, Ghizzoni S, Rizzetti MG, Pelicci G, Pasini B, Bongarzone I, Piutti C, Pierotti MA and Borrello MG. (1997). *Oncogene,* **14,** 773–782.
Asai N, Iwashita T and Takahashi M. (1995). *Mol. Cell. Biol.,* **15,** 1613–1619.
Asai N, Murakami H, Iwashita T, Matsuyama M and Takahashi M. (1996). *J. Biol. Chem.,* **271,** 17644–17649.
Boguski MS and McCormik F. (1993). *Nature,* **366,** 643–654.
Bokoch GM and Der CJ. (1993). *FASEB J.,* **7,** 750–759.
Borrello MG, Pelicci G, Arighi E, De Filippis L, Greco A, Bongarzone I, Rizzetti MG, Pelicci PG and Pierotti MA. (1994). *Oncogene,* **9,** 1661–1668.
Borrello MG, Alberti L, Arighi E, Bongarzone I, Battistini C, Bardelli A, Pasini B, Piutti C, Rizzetti MG, Mondellini P, Radice MT and Pierotti MA. (1996). *Mol. Cell. Biol.,* **16,** 2151–2163.
Califano D, Monaco C, De Vita G, D'Alessio A, Dathan NA, Possenti R, Vecchio G, Fusco A, Santoro M and de Franciscis V. (1995). *Oncogene,* **11,** 107–112.
Califano D, D'Alessio A, Colucci-D'Amato GL, De Vita G, Monaco C, Santelli G, Di Fiore PP, Vecchio G, Fusco A, Santoro M and de Franciscis V. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 7933–7937.
Carlomagno F, De Vita G, Berlingieri MT, de Franciscis V, Melillo RM, Colantuoni V, Kraus MH, Di Fiore PP, Fusco A and Santoro M. (1996). *EMBO J.,* **15,** 2717–2725.
Carlson KM, Dou S, Chi D, Scavarda N, Toshima K, Jackson CE, Wells SA Jr, Goodfellow P and Donis-Keller H. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 1579–1583.
Cavigelli M, Li WW, Lin A, Su B, Yoshioka K and Karin M. (1996). *EMBO J.,* **15,** 6269–6279.
Coso OA, Chiariello M, Yu J-C, Teramoto H, Crespo P, Xu N, Miki T and Gutkind JS. (1995a). *Cell,* **81,** 1137–1146.
Coso OA, Chiariello M, Kalinec G, Kyriakis JM, Woodgett J and Gutkind JS. (1995b). *J. Biol. Chem.,* **270,** 5620–5624.
Crespo P, Schuebel KE, Ostrom AA, Gutkind JS and Bustelo XR. (1997). *Nature,* **385,** 169–172.
Derijard B, Hibi M, Wu I-H, Barrett T, Su B, Deng T, Karin M and Davis RJ. (1994). *Cell,* **76,** 1025–1037.
Donis-Keller H, Dou S, Chi D, Carlson KM, Toshima K, Lairmore TC, Howe JR, Moley JF, Goodfellow P and Wells Jr, SA. (1993). *Hum. Mol. Gen.,* **2,** 851–856.
Edery P, Lyonnet S, Mulligan LM, Pelet A, Dow E, Abel L, Holder S, Nihoul-Fékété C, Ponder BAJ and Munnich A. (1994). *Nature,* **367,** 378–380.
Fusco A, Berlingieri MT, Di Fiore PP, Portella G, Grieco M and Vecchio G. (1987). *Mol. Cell. Biol.,* **7,** 3365–3370.
Green LA and Tishler AS. (1976). *Proc. Natl. Acad. Sci. USA,* **73,** 2424–2428.
Grieco M, Santoro M, Berlingieri MT, Melillo RM, Donghi R, Bongarzone I, Pierotti MA, Della Porta G, Fusco A and Vecchio G. (1990). *Cell,* **60,** 557–563.
Grieco D, Santoro M, Dathan NA and Fusco A. (1995). *Oncogene,* **11,** 113–117.
Gupta S, Campbell D, Derijard B and Davis RJ. (1995). *Science,* **267,** 389–393.
Gupta S, Barrett T, Whitmarsh AJ, Cavanagh J, Sluss HK, Derijard B and Davis RJ. (1996). *EMBO J.,* **15,** 2760–2770.
Hall H. (1994). *Science,* **264,** 1413–1414.
Hawkins PT, Eguinoa A, Qui R-G, Stokoe D, Cooke FT, Walters R, Wennstrom S, Claesson-Welsh L, Evans T, Symons M and Stephens L. (1995). *Curr. Biol.,* **5,** 393–403.
Hart MJ, Eva A, Evans T, Aaronson SA and Cerione RA. (1991). *Nature,* **354,** 311–314.
Hibi M, Lin A, Smeal T, Minden A and Karin M. (1993). *Genes Dev.,* **7,** 2135–2148.
Higuchi R. *Recombinant PCR*. (1990). In: *PCR protocols: A guide to methods and applications*. MA Innis, DH Gelfand, JJ Sninski and TJ White (ed). Academic Press, Inc., San Diego, pp. 177–183.
Hill CS and Treisman R. (1995). *Cell,* **80,** 199–211.
Hill CS, Wynne J and Treisman R. (1995). *Cell,* **81,** 1159–1170.
Hofstra RMW, Landsvater RM, Ceccherini I, Stulp RR, Stelwagen T, Luo Y, Pasini B, Hoppener JWM, van Amstel HK, Romeo G, Lips CJM and Buys CHCM. (1994). *Nature,* **367,** 375–376.
Janssen-Timmen U, Lemaire P, Mattéi MG, Revelant O and Charnay P. (1989). *Gene,* **80,** 325–336.
Jhiang SM, Sagartz JE, Tong Q, Parker-Thornburg J, Capen CC, Cho JY, Xing S and Ledent C. (1996). *Endocrinology,* **137,** 375–378.
Jing S, Wen D, Yu Y, Holst PL, Luo Y, Fang M, Tamir R, Antonio L, Hu Z, Cupples R, Louis JC, Hu S, Altrock BW and Fox GM. (1996). *Cell,* **85,** 1113–1124.
Johnson R, Spiegelman B, Hanahan D and Wisdom R. (1996). *Mol. Cell. Biol.,* **16,** 4504–4511.
Karin M. (1995). *J. Biol. Chem.,* **270,** 16483–16486.
Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J and Woodgett JR. (1994). *Nature,* **369,** 156–160.

Lamarche N and Hall A. (1994). *Trends Genet.*, **10,** 436 – 440.
Liu ZG, Baskaran R, Lea-Chou ET, Wood LD, Chen Y, Karin M and Wang JYJ. (1996). *Nature,* **384,** 273 – 276.
Lloyd A, Yancheva N and Wasylyk B. (1991). *Nature,* **352,** 635 – 638.
Logan SK, Falasca M, Hu P and Schlessinger J. (1997). *Mol. Cell. Biol.,* **17,** 5784 – 5790.
Lorenzo MJ, Gish GD, Houghton C, Stonehouse TJ, Pawson T, Ponder BAJ and Smith DP. (1997). *Oncogene,* **14,** 763 – 771.
Manser E, Leung T, Salihuddin H, Zhao ZS and Lim L. (1994). *Nature,* **367,** 40 – 46.
Marshall CJ. (1995). *Cell,* **80,** 179 – 185.
Minden A, Lin A, McMahon M, Lange-Carter C, Derijard B, Davis RJ, Johnson GL and Karin M. (1994). *Science,* **266,** 1719 – 1723.
Minden A, Lin A, Claret F-X, Abo A and Karin M. (1995). *Cell,* **81,** 1147 – 1157.
Mulligan LM, Kwok JBJ, Healey CS, Elsdon MJ, Eng C, Gardner E, Love DR, Mole SE, Moore JK, Papi L, Ponder MA, Telenius H, Tunnacliffe A and Ponder BAJ. (1993). *Nature,* **363,** 458 – 460.
Pasini B, Borrello MG, Greco A, Bongarzone I, Luo Y, Mondellini P, Alberti L, Miranda C, Arighi E, Bocciardi R, Seri M, Barone V, Radice MT, Romeo G and Pierotti M. (1995). *Nature Genet.,* **10,** 35 – 40.
Pawson T. (1995). *Nature,* **373,** 573 – 580.
Pelicci G, Lanfrancone L, Grignani F, McGlade J, Cavallo F, Forni G, Nicoletti I, Pawson T and Pelicci PG. (1992). *Cell,* **70,** 93 – 104.
Qiu RG, Chen J, Kirn D, McCormick F and Symons M. (1995). *Nature,* **374,** 457 – 459.
Rodrigues GA, Park M and Schlessinger J. (1997). *EMBO J.,* **16,** 2634 – 2645.
Romeo G, Ronchetto P, Luo Y, Barone V, Seri M, Ceccherini I, Pasini B, Bocciardi R, Lerone M, Kaariainen H and Martucciello G. (1994). *Nature,* **367,** 377 – 378.
Sanchez I, Hughes RT, Mayer BJ, Yee K, Woodgett JR, Avruch J, Kyriakis JM and Zon LI. (1994). *Nature,* **372,** 794 – 798.
Sanchez MP, Silos-Santiago I, Frisen J, He B, Lira SA and Barbacid M. (1996). *Nature,* **382,** 70 – 73.
Santoro M, Melillo RM, Grieco M, Berlingieri MT, Vecchio G and Fusco A. (1993). *Cell Growth Diff.,* **4,** 77 – 84.
Santoro M, Wong WT, Aroca P, Santos E, Matoskova B, Grieco M, Fusco A and Di Fiore PP. (1994). *Mol. Cell. Biol.,* **14,** 663 – 675.
Santoro M, Carlomagno F, Romano A, Bottaro DP, Dathan NA, Grieco M, Fusco A, Vecchio G, Matoskova B, Kraus MH and Di Fiore PP. (1995). *Science,* **267,** 381 – 383.
Santoro M, Chiappetta G, Cerrato A, Salvatore D, Zhang L, Manzo G, Picone A, Portella G, Santelli G, Vecchio G and Fusco A. (1996). *Oncogene,* **12,** 1821 – 1826.
Schlessinger J and Ullrich A. (1992). *Neuron,* **9,** 383 – 391.
Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F and Pachnis V. (1994). *Nature,* **367,** 380 – 383.
Smeal T, Binetruy B, Mercola DA, Birrer M and Karin M. (1991). *Nature,* **354,** 494 – 496.
Songyang Z, Carraway III KL, Eck MJ, Harrison SC, Feldman RA, Mohammadi M, Schlessinger J, Hubbard SR, Smith DP, Eng C, Lorenzo MJ, Ponder BAJ, Mayer BJ and Cantley LC. (1995). *Nature,* **373,** 536 – 540.
Takahashi M, Buma Y, Iwamoto T, Inaguma Y, Ikeda H and Hiai H. (1988). *Oncogene,* **3,** 571 – 578.
Teramoto H, Coso OA, Miyata H, Igishi T, Miki T and Gutkind JS. (1996). *J. Biol. Chem.,* **271,** 27225 – 27228.
Treanor JJS, Goodman L, de Sauvage F, Stone DM, Poulsen KT, Beck CD, Gray C, Armanini MP, Pollock RA, Hefti F, Phillips HS, Goddard A, Moore MW, Buj-Bello A, Davies AM, Asai N, Takahashi M, Vandlen R, Henderson CE and Rosenthal A. (1996). *Nature,* **382,** 80 – 83.
van Weering DH, Medema JP, van Puijenbroek A, Burgering BMT, Baas PD and Bos JL. (1995). *Oncogene,* **11,** 2207 – 2214.
van Weering DH and Bos JL. (1997). *J. Biol. Chem.,* **272,** 249 – 254.
Wilson IA, Niman HL, Houghten RA, Cherenson AR, Connolly ML and Lerner RA. (1984). *Cell,* **37,** 767 – 778.
Whitmarsh AJ, Shore P, Sharrocks AD and Davis RJ. (1995). *Science,* **269,** 403 – 407.