The catalytic and signaling activities of RET, a tyrosine kinase receptor for glial cell line-derived neurotrophic factor (GDNF), are controlled by the autophosphorylation of several tyrosine residues in the RET cytoplasmic domain. To analyze the phosphorylation state of individual tyrosines, we generated antibodies recognizing specific phosphotyrosine sites involved in the catalytic (Tyr1062) and downstream signaling (Tyr1015, Tyr1029, and Tyr1062) activities of this receptor. Stimulation with GDNF induced coordinated phosphorylation of the 4 tyrosine residues in neuronal cell lines and in primary cultures of sympathetic neurons isolated from rat superior cervical ganglia. Neurturin and artemin, two other members of the GDNF ligand family, also induced synchronized phosphorylation of RET tyrosines with kinetics comparable to those observed with GDNF. Tyrosine phosphorylation was maximal 15 min after ligand stimulation, decaying thereafter with similar kinetics in all 4 residues. Co-stimulation with a soluble form of the GFRα1 co-receptor potentiated ligand-dependent phosphorylation of different intracellular tyrosines to a similar extent and increased the survival of superior cervical ganglion neurons compared with treatment with GDNF alone. In vivo, high levels of phosphorylated Tyr1062, Tyr1015, and Tyr1062 were detected in embryonic mouse dorsal root ganglia, with a sharp decline at early postnatal stages. Protein transduction of anti-Tyr(P)1062 antibodies into cultured cells reduced phosphorylation of different intracellular tyrosines to a similar extent and increased the survival of embryonic sensory neurons from the nodose ganglion. These results demonstrate synchronized utilization of individual RET tyrosine residues in neurons in vivo and reveal an important role for RET Tyr1062 in mediating neuronal survival by GDNF.
of Tyr^{687}, Tyr^{526}, and Tyr^{1029} is unknown. On the other hand, phosphorylation of Tyr^{1015}, Tyr^{1062}, and Tyr^{1096} has been linked to distinct downstream signaling events. Tyrosine 1015 is part of the motif YLXXL, a docking site for phospholipase Cγ and mutation of the corresponding residue in the RET/PTC2 oncogene impairs its ability to activate phospholipase Cγ and reduces drastically its oncogenic activity in NIH 3T3 cells (14). Tyrosine 1062 is part of the motif NKXY, which constitutes a docking site for the phosphotyrosine-binding domain of Shc and FRS2 adaptor proteins. Interaction between phosphorylated Tyr^{1062} and either of these two adaptors leads to activation of the Ras/ERK and PI3K/AKT pathways in oncoenic as well as ligand-activated RET (15–20). Interestingly, the splicing event that leads to the generation of the short and long isoforms of RET takes place precisely after Tyr^{1062} and places this tyrosine residue in a perfect context for binding to SH2 domains in the short (but not the long) RET isoform. Thus, both phosphory- 

tyrosine-binding-domain-containing and SH2 domain-containing target proteins may bind to this phosphorylated tyrosine in the short RET isoform. Tyrosine 1062 has also been implicated in the binding of Enigma to RET (12), although this interaction appears to be independent of tyrosine phosphorylation. Recently, a role for this tyrosine residue in the docking and activation of different members of the Dok family of adaptor molecules has also been demonstrated (21). Mutation of tyrosine 1062 (Y1062F) dramatically impairs the transforming activity of oncogenic RET-MEN2A and RET-MEN2B (22). Desine 1062 (Y1062F) dramatically impairs the transforming ac-

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Sequences of peptides used for immunization (based on mouse RET) aligned in the corresponding regions of rat, chicken, and human RET.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Downstream targets</th>
</tr>
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<tbody>
<tr>
<td>Tyr(P)905</td>
<td></td>
</tr>
<tr>
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<td>DVEYEDSYVKRSGR</td>
</tr>
<tr>
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<td>Human</td>
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<tr>
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<tr>
<td>Mouse</td>
<td>MNNKRSDYLDAAST</td>
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<td>Human</td>
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<td>Mouse</td>
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<tr>
<td>Human</td>
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Dots indicate identical positions in the alignment. Phosphorylated tyrosine residues are shown in boldface. Known downstream targets are indicated. PLCγ, phospholipase Cγ.

Activation of Individual Phosphotyrosine Residues in RET

RESULTS

Generation and Characterization of Antibodies to Individual Phosphotyrosine Sites in RET—To produce polyclonal antibodies directed against specific phosphotyrosine sites in RET, we immunized rabbits with 15-mer synthetic phosphopeptides corresponding to four distinct motifs in the cytoplasmic domain of mouse RET. The phosphotyrosine motifs targeted by this approach included Tyr(P)905, Tyr(P)1015, Tyr(P)1062, and Tyr(P)1096. These sequence motifs are highly conserved in RET from other vertebrate species, including rat, chicken, and human (Table 1). The antibodies were purified from rabbit sera by sequential affinity chromatography steps as described under “Experimental Procedures.”

In fibroblast cells stably expressing the GFRα1 co-receptor and the long isoform of human RET (MG87-α1/RET), antibodies against Tyr(P)905, Tyr(P)1015, Tyr(P)1062, and Tyr(P)1096 specifically recognized phosphorylated RET in cells treated with GDNF, but not in untreated cells (Fig. 1A). Similar results were obtained in a mouse motor neuron cell line (MN1) endogenously expressing GDNF receptors (data not shown). Competition experiments indicated that each of the antibodies was specific for the phosphorylated form of its cognate peptide, as only the corresponding phosphopeptide (but not the unphosphorylated peptide or other unrelated phosphopeptides) was able to block the detection of activated RET (Fig. 1A). The specificity of the antibodies was further tested in fibroblast cells stably expressing mutant forms of human RET carrying specific amino acid replacements of cytoplasmic tyrosines, namely Y1015F, Y1062F, and Y1096F. In each case, detection of ligand-activated RET was abolished by mutation of the corresponding tyrosine residue to phenylalanine (Fig. 1B), whereas replacement of non-cognate tyrosines had no effect (data not shown). Because mutation of Tyr(P)905 affects the kinase activity of the receptor, the specificity of the antibodies against Tyr(P)905 was tested in COS cells transiently overexpressing RET carrying the Y905F mutation. Overexpression in COS cells led to high levels of ligand-independent RET phosphorylation, even in the Y905F mutant, which could be detected with anti-phosphotyrosine antibodies (data not shown) or anti-Tyr(P)1015 antibodies (Fig. 1B), but not with antibodies against Tyr(P)905 (Fig. 1B). Because the peptides used for immunization contain sequence motifs that partially overlap with analogous sites in other tyrosine kinase receptors, we tested the ability of our antibodies to detect tyrosine phosphorylation in the neurotrophin-4 receptor TrkB, which, like RET, also contains phosphotyrosine docking sites for Shc, FRS2, and phospholipase Cγ. However, none of the four antibodies was able to recognize ligand-activated TrkB (Fig. 1C), indicating that they are indeed specific for the phosphorylation status of distinct tyrosine residues in RET.

Synchronized Phosphorylation of Individual Tyrosine Residues following Activation of RET in Cis—To study the kinetics of phosphorylation of individual tyrosine residues in RET after ligand stimulation, we used fibroblast cell lines expressing GFRα1 or GFRα3 together with the wild-type RET receptor (MG87-α1/RET and MG87-α3/RET, respectively) and MN1 cells expressing endogenous GFRα1, GFRα2, and RET receptors. GDNF stimulation elicited synchronized phosphorylation of tyrosines 905, 1015, 1062, and 1096 in MG87-α1/RET cells, corresponding to the pattern of total tyrosine phosphorylation detected by phosphotyrosine antibodies (Fig. 2A). RET phosphorylation was maximal between 10 and 15 min after ligand stimulation and could still be detected after 120 min (Fig. 2A). Dephosphorylation of the 4 tyrosines following maximal activation also proceeded with comparable kinetics in all cases (Fig. 2A). Similar results were obtained in MN1 cells, except that tyrosine phosphorylation decayed more rapidly in these cells compared with fibroblast cells (Fig. 2B). Differences in the kinetics of receptor phosphorylation in different cell types could be due to different levels of receptor expression, as shown for the NGF receptor TrkA (26), or to different complements of protein-tyrosine phosphatases. Phosphorylation of Tyr(P)905, Tyr(P)1015, and Tyr(P)1062 was also detected in the short isoform of RET after immunoprecipitation from MN1 cells (data not shown). No differences could be seen between the two RET isoforms regarding activation of individual phosphotyrosine residues.

We also investigated whether different ligands of the GDNF family could induce distinct patterns of phosphorylation of individual tyrosine residues in RET. Stimulation of MG87-α1/RET fibroblasts or MN1 cells with NTN resulted in a phospho-
rylation pattern very similar to that observed with GDNF (Fig. 2, C and D). Also, ART, signaling via GFRα3 in MG87-α3/RET cells, induced a pattern of tyrosine phosphorylation comparable to those of GDNF and NTN (Fig. 2E). Thus, we conclude that Tyr905, Tyr1015, Tyr1062, and Tyr1096 become phosphorylated and dephosphorylated in a synchronized manner after ligand stimulation and that different GDNF family ligands utilizing different GFRα receptors induce comparable patterns of tyrosine phosphorylation.

Synchronized Phosphorylation of Individual Tyrosine Residues following Activation of RET in cis and Increased Phosphorylation upon Combined in cis/Trans Activation—The effects of GDNF stimulation of RET in cis (i.e., GFRα1 expressed in the same cell) versus in trans (i.e., GFRα1 supplied exogenously) on the pattern of phosphorylation of individual tyrosines was examined in Neuro2A cells, a mouse neuroblastoma expressing endogenous RET, but little or no GFRα1. Treatment with GDNF alone produced no detectable RET
Fig. 3. Synchronized phosphorylation of individual tyrosine residues following activation of RET in cis and increased phosphorylation upon combined in cis/trans activation. A, RET phosphorylation in cis. Shown is an immunoblot of RET immunoprecipitates from lysates of Neuro2A-α1 cells stimulated with GDNF for different times (in minutes) and probed with total anti-phosphotyrosine (P-Tyr) antibodies. B, RET phosphorylation in trans. Shown are immunoblots of RET immunoprecipitates from lysates of parental Neuro2A cells stimulated with GDNF and a soluble form of GFRα1 (GFRα1-Fc) for different times (in minutes). Indicated to the left are the antibodies used for immunoblotting in each case. The lower panel shows the final reprobing with anti-RET antibodies. C, combined in cis/trans RET stimulation. Shown are immunoblots of RET immunoprecipitates from lysates of MN1 cells stimulated with GDNF (in cis) or a combination of GDNF and soluble GFRα1-Fc (in cis + trans) for different times (in minutes). Indicated to the left are the antibodies used for immunoblotting in each case. The lower panel shows the final reprobing with anti-RET antibodies. PY, phosphotyrosine.

Phosphorylation of RET in cis and trans activation in Neuro2A cells (data not shown). In Neuro2A cells stably transfected with GFRα1 (Neuro2A-α1), GDNF induced rapid and transient RET phosphorylation, which returned to basal levels 60 min after treatment (Fig. 3A). In contrast, stimulation of parental Neuro2A cells with GDNF and a soluble form of GFRα1 (GFRα1-Fc) resulted in a delayed but sustained phosphorylation of RET, which persisted for up to 120 min after treatment (Fig. 3B). Similar to the results observed after stimulation in cis, phosphorylation of Tyr905, Tyr1015, and Tyr1062 was synchronized following stimulation of Neuro2A cells with GDNF plus soluble GFRα1 (Fig. 3B). In agreement with the pattern of total RET tyrosine phosphorylation, phosphorylation of individual tyrosines was delayed until ~10 min and was sustained for up to 120 min (Fig. 3B). Finally, we compared the patterns of RET tyrosine phosphorylation in MN1 cells expressing RET and GFRα1 treated with GDNF alone (in cis) versus GDNF plus soluble GFRα1 (in cis + trans), a situation more likely to be encountered by neurons that express RET and GFRα1 in vivo. In this case, treatment with soluble GFRα1 both potentiated and prolonged phosphorylation of RET tyrosines compared with GDNF alone (Fig. 3C). The phosphorylation patterns of the individual residues were comparable, in support of a synchronized mode of activation after stimulation in trans. Phosphorylation of Tyr1062, present in the long RET isoform only, could not be detected in Neuro2A or MN1 cells (data not shown). We think this is due in part to the relatively lower sensitivity of this antibody and also to the fact that, in contrast to the MG87 cell lines used above stably transfected with the RET long isoform, both Neuro2A and MN1 cells express endogenous levels of the short and long isoforms of RET, of which only the latter can be recognized by the anti-Tyr(P)1062 antibody.

Phosphorylation of RET Tyrosines 905, 1015, and 1062 in Neurons in Vitro and in Vivo—We then used our antibodies to investigate the phosphorylation of individual RET tyrosine residues in neurons stimulated with GDNF in vitro. GDNF treatment of primary cultures of P1 rat SCG neurons or E9 chick nodose ganglion neurons induced robust phosphorylation of Tyr905, Tyr1015, and Tyr1062 (Fig. 4A). Phosphorylation of Tyr1062 could not be detected in primary neuronal cultures, probably due to the lower sensitivity of this antibody (see above). The individual tyrosines at positions 905 and 1015 also appeared to be phosphorylated in a coordinated way in primary neurons, as was observed in cell lines (Fig. 4B), with a peak at 10 min and lower levels at 120 min after GDNF stimulation. Similar to MN1 cells, addition of soluble GFRα1 potentiated and prolonged the phosphorylation of these sites, allowing its detection for up to 24 h of treatment (Fig. 4B). The increased phosphorylation observed in the presence of soluble GFRα1 correlated with enhanced survival in culture compared with GDNF alone (Fig. 4C).

The phosphorylation patterns of individual tyrosine residues in RET were investigated in mouse DRG developing in vivo. Although DRG sensory neurons express both RET and GFRα receptors during embryonic development, they have been found to respond in vitro to GDNF only at postnatal stages (27). We found robust phosphorylation of Tyr905, Tyr1015, and Tyr1062 in DRG taken from E15 and E17 mouse embryos (Fig. 4D). Interestingly, phosphorylation of the 3 residues declined sharply shortly after birth to levels that were barely above detection at postnatal and adult stages (Fig. 4D). In agreement with a role for RET and GFRα receptors in neuronal survival of embryonic mouse DRG neurons, GDNF promoted survival in vitro of dissociated E17 mouse DRG neurons (Fig. 4E). As in SCG neurons, addition of soluble GFRα1-Fc potentiated the survival response of embryonic DRG neurons to GDNF (Fig. 4E).

Phosphorylation of Tyr1062 Is Required for RET Downstream Signaling and GDNF-mediated Survival of Sensory Neurons—Mutations of RET Tyr1062 affect the ability of GDNF to induce activation of the Ras/ERK and PI3K/AKT pathways in transfected fibroblast cells (17). The ability of our anti-phosphopeptide antibodies to specifically recognize individual phosphorylated tyrosines in RET prompted us to test the importance of these residues for neuronal survival induced by GDNF. Purified antibodies were introduced into cells in culture using Chariot-mediated protein transduction (see “Experimental Procedures”). Control experiments using tetramethylrhodamine B isothiocyanate-labeled control antibodies demonstrated
that nearly 100% of the MN1 cells took up the antibodies only in the presence of the Chariot reagent and without any appreciable toxic effects (data not shown). In MN1 cells, transduction of anti-Tyr(P)1062 antibodies reduced activation of ERK1 and ERK2 in response to GDNF (Fig. 5A). No inhibitory effect on ERK phosphorylation could be seen when the Chariot reagent or antibodies were used separately (Fig. 5A). Because Tyr1062 is also linked with the P13K pathway and activation of the AKT kinase (17), we tested phosphorylation of AKT in MN1 cells after transduction with anti-Tyr(P)1062 antibodies. In the presence of the Chariot reagent, anti-Tyr(P)1062 antibodies diminished AKT phosphorylation in MN1 cells treated with GDNF (Fig. 5A), demonstrating that treatment with anti-Tyr(P)1062 antibodies likely affects all downstream signaling mediated by Tyr1062 in RET.

To evaluate the role of individual RET phosphotyrosine residues in GDNF-mediated biological activities, we used a neurite outgrowth assay in MN1 cells and a survival assay in sensory neurons isolated from the developing chick node ganglion, a neuronal subpopulation that is highly responsive to GDNF. Transduction of GDNF or GDNF plus GFRa1-Fc (both at 100 ng/ml) for the indicated periods of time (in minutes). The lower panel shows the final reprobing with anti-RET antibodies. C, survival of P1 rat SCG neurons with GDNF in the presence and absence of soluble GFRa1-Fc scored 24 and 48 h after treatment. Anti-NGF blocking antibodies were included as indicated. Results are expressed relative to the survival observed in NGF (set to 100%) and represent the means ± S.E. of three independent experiments performed in triplicate. D, phosphorylation of individual RET tyrosine residues in vivo in mouse DRG. Shown are immunoblots of equal amounts of protein from lysates of mouse DRG extracted at the indicated developmental stages and probed with the indicated phosphopeptide antibodies. The blots were reprobed with anti-RET antibodies (lower panels). E, survival of E17 mouse DRG neurons with GDNF in the presence and absence of soluble GFRa1-Fc scored 48 h after treatment. Results are expressed relative to the survival observed in NGF (set to 100%) and represent the means ± S.E. of three independent experiments performed in duplicate. F, phosphotyrosine; AD, adult.

**DISCUSSION**

Upon ligand stimulation, the RET tyrosine kinase receptor is autophosphorylated at a set of cytoplasmic tyrosine residues. This autophosphorylation allows the binding and activation of signaling molecules and therefore constitutes the first event of the intracellular signaling pathway of this receptor. To study the phosphorylation upon ligand stimulation of distinct tyrosine residues in RET, we have developed antibodies to 4 individual phosphotyrosines in this receptor: Tyr905, Tyr1015, Tyr1062, and Tyr1062 in the carboxyl-terminal tail. Using a similar strategy, Salvatore et al. (28) have recently reported that tyrosines 1015 and 1062 are indeed autophosphorylated in oncogenic forms of RET and that phosphorylation of Tyr1062 is required for the mitogenic activities of the RET/PTC1 oncogene in a carcinoma cell line. In this work, we have used antibodies against Tyr905, Tyr1015, Tyr1062, and Tyr1062 to demonstrate that these tyrosine residues are phosphorylated upon ligand stimulation in cell lines and in primary neurons expressing endogenous receptors in a synchronized way. Coordinated autophosphorylation of individual tyrosines was observed with different members of
Conformational change that exposes the ATP- and substrate-binding sites for catalysis. Furthermore, phosphorylation of this site was found to be completed before phosphorylation of other tyrosines began (30), suggesting a stepwise mode of phosphorylation of tyrosine residues during kinase activation. Moreover, in the TrkA receptor, the Shc binding at Tyr490 remained phosphorylated for a longer time than at tyrosines 674 and 675 in the activation loop (31). In contrast, our results indicate that, in the RET receptor, tyrosines located within the catalytic and signaling domains are phosphorylated and dephosphorylated in a coordinated way, suggesting that stepwise phosphorylation of activation loop tyrosines is not a general feature of the mechanism of receptor tyrosine kinase activation.

The somewhat unexpected discovery that all four members of the GDNF family signal through a common receptor tyrosine kinase raised the possibility that the different ligands could activate RET in different ways, perhaps through differential phosphorylation of cytoplasmic tyrosines or via differences in strength and duration of autophosphorylation events (32). In fact, several receptor tyrosine kinases appear to be capable of generating different responses to distinct ligands of otherwise comparable affinity. For example, the neurotrophin receptor TrkB binds both brain-derived neurotropic factor and neurotrophin-4, and both ligands stimulate activation of MAPKs in cortical neurons (33). However, only activation by neurotrophin-4 requires an intact Shc-binding site in the cytoplasmic domain of TrkB (33), indicating differences in the signaling mechanisms activated by these two neurotrophins via the TrkB receptor. It has also been shown that the epidermal growth factor receptor can be activated with different kinetics by its two ligands, epidermal growth factor and transforming growth factor-α (34) and, more recently, that the four ligands of the ErbB4 receptor (betacellulin and neuregulins 1–3) are able to elicit different patterns of tyrosine phosphorylation in this receptor (35). Although the limited biochemical evidence available suggests that GDNF and NTN may induce the activation of similar downstream pathways (36), more recent studies indicate that the two ligands may have different biological effects. For example, both GDNF and NTN are able to promote survival of midbrain dopaminergic neurons after a 6-hydroxydopamine lesion, although only GDNF is capable of stimulating neurite outgrowth from these neurons (37). In this study, we investigated the phosphorylation patterns of individual tyrosine residues in RET after stimulation with different ligands (e.g., GDNF, NTN, and ART) and in cells expressing a single type of GFRα co-receptor (e.g., MG87-a1/RET and MG87-a3/RET cells) or combinations of different GFRα receptors (e.g., MN1 cells and peripheral neurons). In none of these cases could we detect significant differences between the kinetics of phosphorylation and dephosphorylation of different tyrosine residues in RET. These observations indicate a robust mechanism of activation for the RET kinase and suggest that the RET receptor is unable to discriminate among different ligands or GFRα co-receptors. Differences in the biological activities elicited by different members of the GDNF family could still be explained by the existence of alternative, RET-independent signaling mechanisms, for which some evidence has recently been accumulated (38, 39).

**Increased and Prolonged Phosphorylation of Individual Tyrosine Residues after Activation of RET in cis plus in trans**—Comparison of tyrosine phosphorylation patterns in Neuro2A, Neuro2A-o1, and MN1 cells and SCG neurons after stimulation of RET in cis, in trans, or in cis + in trans revealed indistinguishable kinetics in the different phosphorylation sites investigated under the three stimulation regimes, indi-
cating no preferential activation of specific tyrosine sites after stimulation with soluble GFRA1 receptors. However, RET tyrosine phosphorylation was increased and prolonged in cells stimulated in trans with GDNF and soluble GFRA1 compared with GDNF alone. In addition, in cells lacking GFRA1 receptors, RET tyrosine phosphorylation was delayed after stimulation in trans compared with cells expressing endogenous GFRA1 stimulated in cis (i.e., Fig. 3, A and B). Thus, although RET is not able to discriminate among different ligands, a qualitative difference in the response of this receptor can be observed when the in cis and in trans stimulation regimes are directly compared. Potentiated and sustained RET tyrosine phosphorylation in SCG neurons after stimulation with GDNF and soluble GFRA1 correlated with enhanced neuronal survival, comparable to the effects of NGF. Together with recent observations made in developing enteric and sensory neurons (7, 40), these results demonstrate the generalized potential of exogenous GFRA1 receptors to potentiate the trophic activities of GDNF family ligands.

Robust Phosphorylation of Individual RET Tyrosine Residues during Embryonic Development of DRG Neurons in Vivo—As a first step toward elucidating patterns of RET activation in vivo, we investigated RET phosphorylation in freshly isolated mouse DRG from embryonic and postnatal stages. Our results showed robust phosphorylation of Tyr(905), Tyr(1015), and Tyr(1062) at embryonic stages (i.e., E15–17), with a pronounced decrease at early postnatal stages, suggesting that DRG neurons are exposed to high levels of GDNF family ligands during embryonic development. In agreement with this, ART expression has been detected in peripheral nerve roots at this developmental stage (41). This observation indicates a role for GDNF ligands in the control of neuronal survival and maturation and axonal growth of developing sensory neurons. Intriguingly, a recent developmental study indicated that cultures of dissociated mouse DRG neurons do not respond to the survival-promoting effects of GDNF family ligands until after birth (27). However, GDNF-null mutant mice display a 23% loss of DRG neurons already at birth (42), indicating the requirement of this factor for the survival of DRG neurons during (at least some) embryonic stages in vivo. In agreement with this, we found that GDNF can promote the survival of E17 mouse DRG neurons in vitro and that this activity can be further potentiated by soluble GFRA1-Fc. Finally, the fact that RET phosphorylation could be seen up to adult stages (only Tyr(P) 905 was correlated with enhanced neuronal survival. The high degree of connectivity of this residue for the survival of DRG neurons during (at least promoting effects of GDNF family ligands until after birth (27).

Tyr1062 at embryonic stages (41). This observation indicates a role for embryonic development. In agreement with this, ART expression is exposed to high levels of GDNF family ligands during

Acknowledgments—We thank Susanna Eketjall for the MG87–a/RE cell line; Rizaldy Scott and Valerie Besset for RET mutants and cell lines; Bob Gordon, Steve Masarwe, and Miroslav Cik for providing recombinant ART; Marie Pierre-Junier for critical reading of the manuscript; and Xiaoli Li for secretarial help.

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