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Coordinated Activation of Autophosphorylation Sites in the RET Receptor Tyrosine Kinase

IMPORTANCE OF TYROSINE 1062 FOR GDNF MEDIATED NEURONAL DIFFERENTIATION AND SURVIVAL*

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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 (Tyr^{905}) and downstream signaling (Tyr^{1015}, Tyr^{1062}, and Tyr^{1096}) 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 Tyr^{905}, Tyr^{1015}, and Tyr^{1062} 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 activation of MAPKs ERK1 and ERK2 and the AKT kinase in response to GDNF and diminished GDNF-dependent neuronal differentiation and 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 Tyr^{1062} in mediating neuronal survival by GDNF.

The GDNF[1] ligand family, a group of polypeptides structurally related to the transforming growth factor-β superfamily, is involved in the control of neuronal survival and differentiation, kidney morphogenesis, and spermatogonial cell fate (1–4). Each of the four members of the GDNF family (*i.e.* GDNF, NTN, ART, and PSP) binds specifically to different members of a small family of glycosylphosphatidylinositol-anchored receptors, the GDNF family α-receptors, of which four different members (GFRα1–4) are currently known (1, 2, 5). Intracellular signaling is accomplished by the recruitment of a receptor tyrosine kinase, RET, to the GDNF·GFRα complex. Although all members of the GDNF ligand family utilize RET as a signaling receptor subunit, specificity is achieved by differential binding to individual GFRα molecules. GFRα receptors can mediate activation of RET when expressed on the surface of the same cell (activation in *cis*) or when presented in soluble form or immobilized on the cell matrix or neighboring cells (activation in *trans*) (6, 7). Upon ligand binding, RET is thought to form dimers and become phosphorylated at specific cytoplasmic tyrosine residues. Tyrosine autophosphorylation is required for the catalytic activity of RET and for downstream signaling. Thus, tyrosine autophosphorylation constitutes the first intracellular event of the RET signaling cascade activated by members of the GDNF ligand family.

Eighteen tyrosine residues, 2 in the juxtamembrane domain, 11 in the kinase domain, and 5 in the carboxyl-terminal tail, are present in the cytoplasmic domain of the long isoform of RET. Tyrosine 905 in the RET kinase domain corresponds to tyrosine 416 in the activation loop of the cytoplasmic tyrosine kinase Src, a conserved residue in many tyrosine kinases known to play a crucial role in kinase activation (8). Mutation of tyrosine 905 to phenylalanine (Y905F) impairs the kinase activity and abolishes the transforming activity of RET-MEN2A, an oncogenic, constitutively active form of RET identified in patients with multiple endocrine neoplasia type 2A (9). Tyrosine 905 is also involved in the binding of two adaptor proteins containing SH2 domains, Grb7 and Grb10, presumably involved in downstream signaling events (10–12). Six additional tyrosines (Tyr^{687} in the juxtamembrane domain; Tyr^{826} in the catalytic domain; and Tyr^{1015}, Tyr^{1029}, Tyr^{1062}, and Tyr^{1096} in the carboxyl-terminal tail) have been shown to be autophosphorylated in various oncogenic forms of RET by site-directed mutagenesis and phosphopeptide mapping experiments (13). The functional importance of the phosphorylation

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[1] The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; NTN, neurturin; ART, artemin; GFRα, GDNF family receptor-α; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; NGF, nerve growth factor; DRG, dorsal root ganglion/ganglia; SCG, superior cervical ganglion/ganglia; E, embryonic day; P, postnatal day; MAPKs, mitogen-activated protein kinases.

of Tyr^{687}, Tyr^{826}, 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 YL*X*L, 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 NK*X*Y, 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 oncogenic 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 phosphotyrosine-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). Despite its high degree of connectivity to multiple intracellular pathways, the biological function of this residue in the wild-type RET receptor has not been investigated. Finally, tyrosine 1096, located in the 51-residue carboxyl-terminal tail that is specific for the long isoform of RET, is part of the sequence PY*XN*X, a well known binding site for the Grb2 adaptor protein. Grb2 has been found to interact with the long isoform of RET/PTC2 and wild-type RET via this residue (17, 23).

In this work, we have investigated the phosphorylation of individual tyrosine residues in the cytoplasmic domain of RET in cell lines, cultured primary neurons, and *in vivo*. We have studied differences in the kinetics of phosphorylation and dephosphorylation of individual residues and whether different members of the GDNF ligand family may be capable of inducing distinct patterns of tyrosine phosphorylation in RET. For this purpose, we have generated antibodies recognizing the specific phosphorylation of tyrosines 905, 1015, 1062, and 1096 in this receptor. We have introduced several of these phosphotyrosine-specific antibodies into cell lines and primary neurons to investigate the functional roles played by individual tyrosine residues of RET in GDNF-mediated downstream signaling and neuronal survival.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—The MG87-α1/RET and MG87-α3/RET lines were derived from MG87 fibroblasts by stable transfection of GDNF receptor subunits. MG87-α1/RET cells express rat GFRα1 and the long isoform of human RET. MG87-α3/RET cells express mouse GFRα3 and the long isoform of human RET. Neuro2A-α1 cells were generated by stable transfection of the mouse neuroblastoma Neuro2A with rat GFRα1. MN1 is an immortalized mouse motor neuron cell line (24). Recombinant rat GDNF was produced in Sf21 insect cells and purified as previously described (25). Nerve growth factor (NGF) was purchased from Promega, NTN from PeproTech, and GFRα1-Fc from R&D Systems. Recombinant ART was a generously provided by Bob Gordon (Jannssen Research Foundation, Beerse, Belgium). The Chariot reagent used for protein transduction was from ActiveMotive (Rixensart, Belgium).

Generation of Antibodies—Phosphorylated 15-mer peptides corresponding to four predicted phosphorylation sites in the long isoform of the mouse RET receptor (see Table I) were synthesized, coupled to keyhole limpet hemocyanin, and used to immunize rabbits by standard procedures. Antisera were evaluated by enzyme-linked immunosorbent assays against phosphorylated and unphosphorylated versions of the peptides. Peptide synthesis, rabbit immunizations, and antibody collection was done by Research Genetics. Antisera showing high titer in enzyme-linked immunosorbent assays were then screened by immunoblotting with protein extracts from control and GDNF-stimulated MG87-α1/RET cells. Reactive antisera were purified by sequential affinity chromatography steps. Total immunoglobulins were first purified on a protein G column (POROS G, PerSeptive Biosystems) and eluted with 0.1 M glycine (pH 2.7). Antisera to $Tyr(P)^{905}$, $Tyr(P)^{1015}$, and $Tyr(P)^{1062}$ showed little or no cross-reactivity against unphosphorylated RET or irrelevant phosphotyrosines and were then affinity-purified over an Affi-Gel 15 affinity column (Bio-Rad) coupled with the corresponding phosphopeptides. Because the anti-$Tyr(P)^{1096}$ antisera demonstrated cross-reactivity with unphosphorylated RET as well as other phosphotyrosines, these antibodies were first applied to an Affi-Gel 15 affinity column coupled with the unphosphorylated Tyr^{1096} peptide, followed by chromatography on a phosphotyrosine affinity column (Sigma). The eluent from these two steps was then applied to an Affi-Gel affinity column coupled with the $Tyr(P)^{1096}$ phosphopeptide. Affinity-purified antibodies were eluted with 0.1 M glycine (pH 2.7), immediately neutralized with 1 M Tris-HCl (pH 9.0), and dialyzed against Tris-buffered saline.

Immunoprecipitation and Immunoblot Analysis—Cell line monolayers in 10-cm plates were changed to serum-free medium 2–4 h prior to stimulation with the indicated factors (10 min unless otherwise indicated). Cells were then lysed in a nonionic ice-cold detergent (lysis buffer: 10 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM EDTA, 10% glycerol, and 1% Nonidet P-40) containing a mixture of protease inhibitors (Roche Molecular Biochemicals) and phosphatase inhibitors (1 mM NaO_4Va. 20 mM NaF, and 10 mM β-glycerophosphate). Cell lysates were cleared by centrifugation at 1500 $\times$ *g* for 10 min and immunoprecipitated by overnight incubation at 4 °C with anti-RET antibodies and 40 μl of protein G-Sepharose beads (Amersham Biosciences, Inc.). The immunoprecipitates were washed three times with lysis buffer, solubilized in sample buffer, run on SDS-polyacrylamide gels, and blotted onto polyvinylidene difluoride membranes (Amersham Biosciences, Inc.). Blots were first probed with anti-phosphopeptide antibodies, followed by alkaline phosphatase-conjugated anti-IgG, and developed with the enhanced chemi fluorescence Western detection system (Amersham Biosciences, Inc.). All blots were scanned in a Storm 840 fluorimager (Molecular Dynamics, Inc.). For reprobing, blots were stripped for 90 min at room temperature in 0.1 M acetic acid and 0.15 M NaCl. Antibodies against phosphotyrosine (used at 1:1000 dilution) and the long (1:1000) and short (1:500) isoforms of human RET were from Santa Cruz Biotechnology. For peptide competition assays, phosphorylated and unphosphorylated peptides at 10 or 100 nM were preincubated with the antibodies for 30 min at room temperature, and the mixture was then used in immunoblotting.

For developmental analysis of RET phosphorylation, DRG were collected from C57 mice at embryonic day (E) 15, E17, postnatal day (P) 0, P9, P16, and the adult stage in ice-cold Tris-buffered saline containing 1 mM NaO_4Va. DRG from three mice were pooled for each embryonic time point, whereas DRG from one mouse were enough for postnatal stages. Tissues were lysed in 70 μl of 1% lysis buffer, cleared by centrifugation, submitted to SDS-PAGE, and immunoblotted onto polyvinylidene difluoride membranes as described above. Each blot was first probed with an anti-phosphopeptide antibody, stripped, and then reprobed with anti-RET antibodies.

SCG, DRG, and Nodose Ganglion Primary Neuronal Cultures and Survival Assays—P1 rat SCG were dissociated by incubation for 30 min at 37 °C in phosphate-buffered saline containing 0.025% trypsin (Invitrogen) and for an additional 30 min after addition of 5 mg/ml collagenase (Sigma), followed by mechanical trituration. Dissociated cultures from E17 mouse DRG and E9 chick nodose ganglia were prepared by incubation for 10 min at 37 °C in phosphate-buffered saline containing 0.025% trypsin, without collagenase and NGF treatments. Neurons were plated in polyornithine/laminin-coated dishes and maintained in neuronal medium (1:1 Dulbecco's modified Eagle's medium/nutrient mixture F-12, Invitrogen), 2 mM glutamine, and 1 mg/ml bovine serum albumin. SCG and nodose ganglion cultures were supplemented with 10 μM cytosine β-D-arabinofuranoside. SCG neurons were maintained in 20 ng/ml NGF. For biochemical analyses, neurons were maintained for 4 days before a 4-h starvation (*i.e.* without NGF in the case of SCG) and stimulation with GDNF (100 ng/ml) in the presence or absence of soluble GFRα1-Fc (100 ng/ml) for the indicated times. The cells were then lysed, and lysates were processed by SDS-PAGE and immunoblotting as described above.

For survival assays, SCG neurons were first maintained for 2 days in neuronal medium supplemented with NGF; washed; and changed to

TABLE I

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

Dots indicate identical positions in the alignment. Phosphorylated tyrosine residues are shown in boldface. Known downstream targets are indicated. PLCγ, phospholipase Cγ.

	Sequence	Downstream targets
Tyr(P)^{905}		
Mouse	DVYEEDS**Y**VKKSKGR	
Rat	.....	Grb7, Grb10
Chicken	.....R....	
Human	.....R.Q..	
Tyr(P)^{1015}		
Mouse	MMVKSRD**Y**LDLAAST	
Rat	.....	PLCγ
Chicken	.....	
Human	....R.....	
Tyr(P)^{1062}		
Mouse	TWIENKL**Y**GMSDPNW	
Rat	.....	Shc, FRS2, Dok4/5
Chicken	.....Y...	
Human	.....	
Tyr(P)^{1096}		
Mouse	RYANDSV**Y**ANWMVSP	
Rat	.....	Grb2
Chicken	.....	
Human	.....	

neuronal medium containing either NGF (100 ng/ml) or anti-NGF antibodies (Roche Molecular Biochemicals) together with GDNF (100 ng/ml) and, where indicated, soluble GFRα1-Fc (100 ng/ml). DRG neurons were plated directly with the indicated factors without NGF preincubation. Phase-bright, neurite-bearing neurons were counted 24 and 48 h after treatment.

Chariot-mediated Protein Transduction—MN1 cells and chick nodose ganglion neurons were cultured in 24-well plates. Protein transduction using the Chariot reagent was essentially performed according to the manufacturer's instructions. In our hands, the highest efficiency of protein transduction was obtained if performed in serum-free medium with cells still in suspension prior to plating. Because SCG neurons need to be treated for a few days with NGF to develop GDNF responsiveness, antibody transduction was performed in embryonic chick nodose ganglion neurons, which are readily responsive to GDNF immediately after extraction (25). Two μg of antibody was used together with 2 μl of Chariot reagent.

After Chariot-mediated transduction, MN1 cells were first allowed to adhere to the plastic plate for 4 h and then stimulated for 5 min with 100 ng/ml GDNF. The cells were lysed as described above, and 10 μg of protein lysate was processed by SDS-PAGE and immunoblotting with antibodies against phosphorylated ERK1/2 or phosphorylated AKT (Cell Signaling, New England Biolabs Inc.) at 1:2000 dilution. As a control for loading, the blot was stripped and reprobed with total anti-AKT antibodies (Cell Signaling, New England Biolabs Inc.) used at 1:1000 dilution or anti-tubulin antibodies. Neurite outgrowth assay of MN1 cells was performed as previously described (7). For survival assay, neurons were plated after Chariot-mediated transduction and maintained in 100 ng/ml GDNF for 48 h. Phase-bright neurons were counted in the entire well (between 100 and 400 neurons/well); the results presented are averages of three different wells.

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 I). 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. 1*A*). 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. 1*A*). 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. 1*B*), whereas replacement of non-cognate tyrosines had no effect (data not shown). Because mutation of Tyr^{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. 1*B*), but not with antibodies against Tyr(P)^{905} (Fig. 1*B*). 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. 1*C*), 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. 2*A*). RET phosphorylation was maximal between 10 and 15 min after ligand stimulation and could still be detected after 120 min (Fig. 2*A*). Dephosphorylation of the 4 tyrosines following maximal activation also proceeded with comparable kinetics in all cases (Fig. 2*A*). Similar results were obtained in MN1 cells, except that tyrosine phosphorylation decayed more rapidly in these cells compared with fibroblast cells (Fig. 2*B*). 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^{905}, Tyr^{1015}, and Tyr^{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-

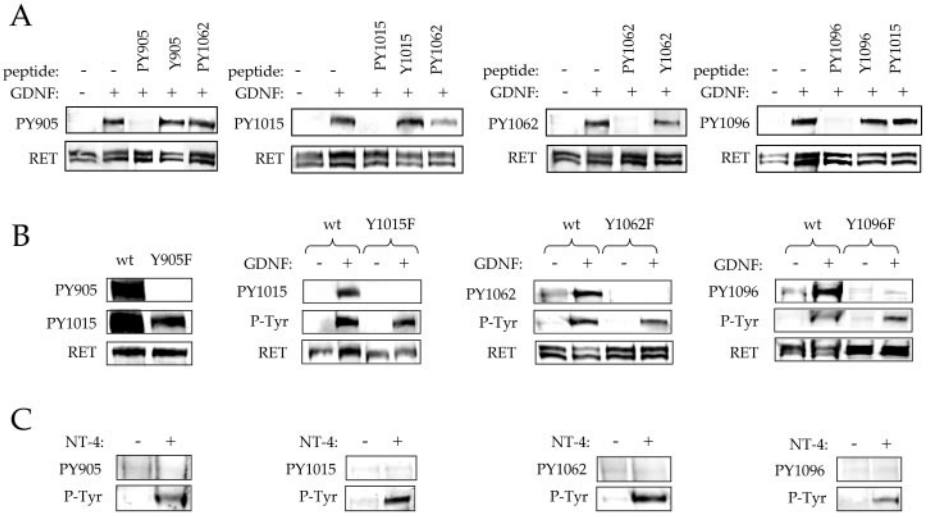

FIG. 1. **Characterization of antibody specificity.** *A*, competition with unphosphorylated or irrelevant phosphorylated peptides. Shown are immunoblots of RET immunoprecipitates from lysates of MG87-α1/RET cells stimulated with GDNF. Blots were probed with the indicated anti-phosphopeptide antibodies after preincubation with the indicated peptides and phosphopeptides (used at 100 nM) (*upper panels*). The blots were then reprobed with anti-RET antibodies (*lower panels*). *B*, immunoreactivity tested against RET molecules carrying specific tyrosine mutations. Shown are immunoblots of RET immunoprecipitates from lysates of cells transfected with wild-type (*wt*) or the indicated mutant human RET receptors (*upper panels*). Transiently transfected COS cells were used for the *first panel group* probed with anti-Tyr(P)^{905} antibodies. The other *three panel groups* show MG87-α1/RET cells (*wt*) or MG87-α1 cells stably transfected with the indicated RET mutants after stimulation with GDNF as indicated. The blots were reprobed with anti-RET (*lower panels*) and anti-Tyr(P) (*middle panels*) antibodies. *C*, lack of reactivity against phosphotyrosine (*PY*) epitopes in another receptor tyrosine kinase. Shown are immunoblots of TrkB immunoprecipitates from lysates of MG87-TrkB cells stimulated with neurotrophin-4 (*NT-4*) and probed with the indicated anti-phosphopeptide antibodies (*upper panels*). The blots were reprobed with total anti-phosphotyrosine (*P-Tyr*) antibodies (*lower panels*).

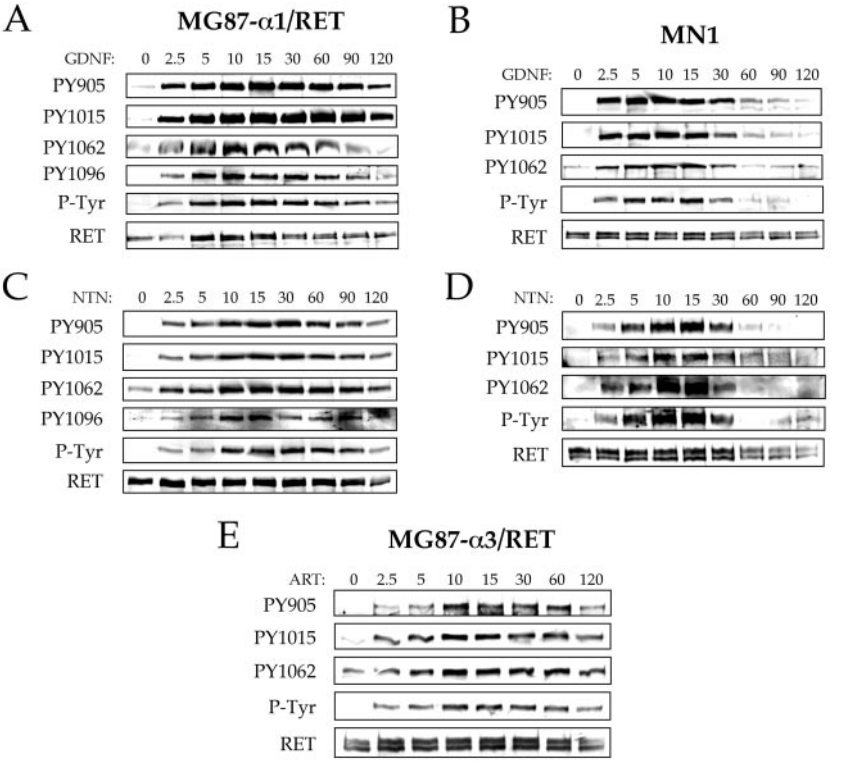

FIG. 2. **Synchronized phosphorylation of individual tyrosine residues following activation of RET in *cis*.** Shown are representative immunoblots of RET immunoprecipitates from lysates of MG87-α1/RET cells (*A* and C), MN1 cells (*B* and *D*), and MG87-α3/RET cells (*E*) after stimulation with the indicated ligands (GDNF (*A* and *B*), NTN (*C* and *D*), and ART (*E*)) for the indicated periods of time (in minutes). Indicated to the left are the antibodies used for immunoblotting in each case. Although small differences could be seen between individual tyrosine sites within a given experiment, these were not consistent from experiment to experiment, indicating that phosphorylation and dephosphorylation were coordinated in the four sites studied. *PY* and *P-Tyr*, phosphotyrosine.

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. 2*E*). Thus, we conclude that Tyr^{905}, Tyr^{1015}, Tyr^{1062}, and Tyr^{1096} 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 Trans 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

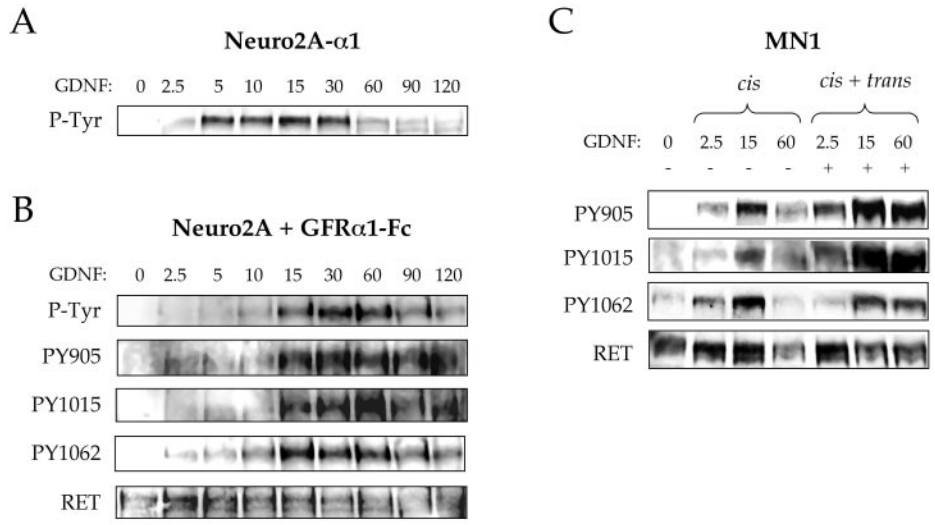

FIG. 3. **Synchronized phosphorylation of individual tyrosine residues following activation of RET in *trans* and increased phosphorylation upon combined in *cis/trans* activation.** *A*, RET phosphorylation in *cis*. Shown is an immunoblot of RET immunoprecipitates from lysates of Neuro2A-α1 cells stimulated 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 soluble 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* + in *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 in parental 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. 3*A*). 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. 3*B*). Similar to the results observed after stimulation in *cis*, phosphorylation of Tyr^{905}, Tyr^{1015}, and Tyr^{1062} was synchronized following stimulation of Neuro2A cells with GDNF plus soluble GFRα1 (Fig. 3*B*). 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. 3*B*). 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* + in *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 individual RET tyrosines compared with GDNF alone (Fig. 3*C*). The phosphorylation patterns of the individual residues were comparable, in support of a synchronized mode of activation after stimulation in *trans*. Phosphorylation of Tyr^{1096}, 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-$Tyr(P)^{1096}$ 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 Tyr^{905}, Tyr^{1015}, and Tyr^{1062} (Fig. 4*A*). Phosphorylation of Tyr^{1096} 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. 4*B*), 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. 4*B*). The increased phosphorylation observed in the presence of soluble GFRα1 correlated with enhanced survival in culture compared with GDNF alone (Fig. 4*C*).

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 Tyr^{905}, Tyr^{1015}, and Tyr^{1062} in DRG taken from E15 and E17 mouse embryos (Fig. 4*D*). Interestingly, phosphorylation of the 3 residues declined sharply shortly after birth to levels that were barely above detection at postnatal and adult stages (Fig. 4*D*). 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. 4*E*). As in SCG neurons, addition of soluble GFRα1-Fc potentiated the survival response of embryonic DRG neurons to GDNF (Fig. 4*E*).

Phosphorylation of Tyr^{1062} Is Required for RET Downstream Signaling and GDNF-mediated Survival of Sensory Neurons—Mutations of RET Tyr^{1062} 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

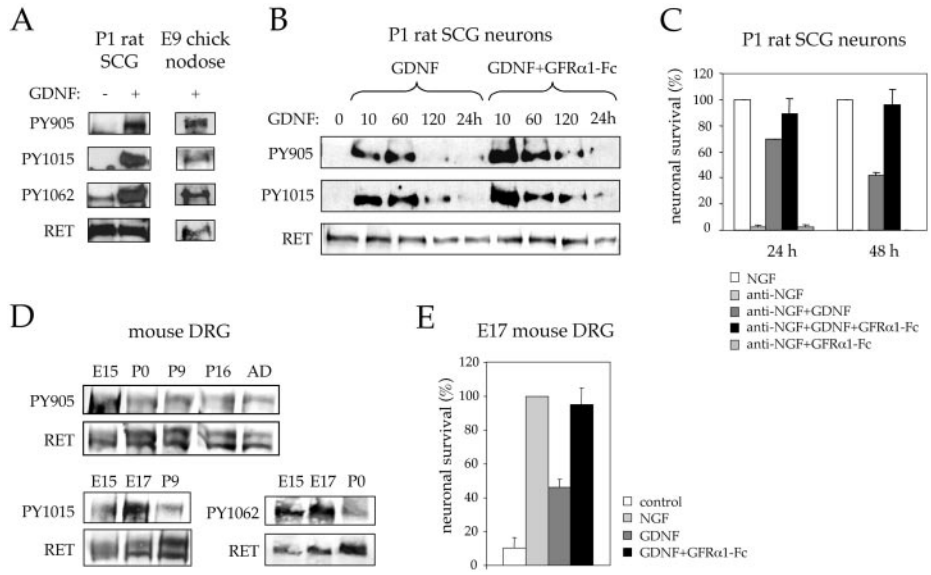

FIG. 4. **Phosphorylation of RET tyrosines 905, 1015, and 1062 in neurons *in vitro* and *in vivo*.** *A*, phosphorylation of individual RET tyrosines in cultures of P1 rat SCG neurons and E9 chick nodose neurons. The blots show direct probing with the indicated antibodies of lysates from cells treated with GDNF for 10 min as indicated. The *lower panels* show the final reprobing with anti-RET antibodies. *B*, kinetics of tyrosine phosphorylation in rat SCG neurons in *cis* and in *trans*. The blots show direct probing with the indicated antibodies of lysates from cells treated with GDNF or GDNF plus GFRα1-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 GFRα1-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 GFRα1-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. *PY*, phosphotyrosine; *AD*, adult.

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. 5*A*). No inhibitory effect on ERK phosphorylation could be seen when the Chariot reagent or antibodies were used separately (Fig. 5*A*). Because Tyr^{1062} is also linked with the PI3K 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. 5*A*), demonstrating that treatment with anti-Tyr(P)^{1062} antibodies likely affects all downstream signaling mediated by Tyr^{1062} 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 nodose ganglion, a neuronal subpopulation that is highly responsive to the survival-promoting effects of GDNF (25) (see also "Experimental Procedures"). Cotreatment with GDNF and soluble GFRα1 produces a robust morphological differentiation of MN1 cells plated in a collagen matrix (7). Cells that were transduced with anti-Tyr(P)^{1062} antibodies showed a greatly attenuated response to the differentiation effects of GDNF + GFRα1 (Fig. 5*B*). Under the same conditions, neither anti-Tyr(P)^{905} nor anti-Tyr(P)^{1015} antibodies had any effect on MN1 neurite outgrowth (Fig. 5*B*). As indicated above, all of our anti-phosphopeptide antibodies were capable of recognizing activated RET in chick sensory neurons (Fig. 4*A*), in agreement with the high conservation of the corresponding peptide sequences across different vertebrate species (Table I). GDNF promoted a robust survival response in E9 nodose ganglion neurons after 48 h in culture compared with controls (Fig. 5*C*). Importantly, treatment with the Chariot reagent alone or with an irrelevant antibody had no effect on GDNF-mediated survival of sensory neurons (Fig. 5*C*). In the presence of GDNF, transduction of anti-Tyr(P)^{1062} antibodies reduced neuronal survival to 50% at 48 h compared with GDNF alone (Fig. 5*C*). Without the Chariot reagent, anti-Tyr(P)^{1062} antibodies had no effect on GDNF-mediated neuronal survival (Fig. 5*C*). Interestingly, neither anti-Tyr(P)^{905} nor anti-Tyr(P)^{1015} antibodies affected neuronal survival in the presence of GDNF (Fig. 5*C*), indicating a specific role of RET Tyr^{1062} phosphorylation in GDNF-mediated survival of sensory neurons.

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: Tyr^{905} in the catalytic domain and Tyr^{1015}, Tyr^{1062}, and Tyr^{1096} 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 Tyr^{1062} is required for the mitogenic activities of the *RET/PTC1* oncogene in a carcinoma cell line. In this work, we have used antibodies against Tyr(P)^{905}, Tyr(P)^{1015}, Tyr(P)^{1062}, and Tyr(P)^{1096} 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

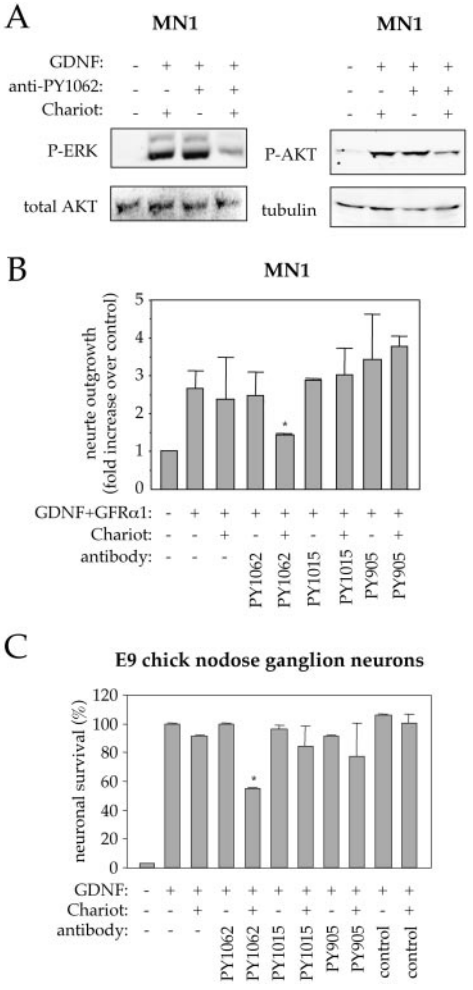

FIG. 5. **Phosphorylation of Tyr^{1062} is required for RET downstream signaling and GDNF-mediated survival of sensory neurons.** *A*, analysis of ERK and AKT phosphorylation in MN1 cells. The *upper panels* shows phosphorylated ERK (*P-ERK*) and phosphorylated AKT (*P-AKT*) immunoblots of MN1 cell lysates treated with GDNF and Chariot-antibody conjugates as indicated. The blots were reprobed with total anti-AKT antibodies (*lower left panel*) or anti-tubulin antibodies (*lower right panel*). *B*, neurite outgrowth induced by GDNF and GFRα1 in MN1 cells treated with different Chariot-antibody conjugate combinations. Cells with neurites longer than two-cell diameters were counted in different fields of independent wells. Results are expressed as -fold increase over control (set to 1) and represent the means ± S.E. of three independent experiments performed in triplicate. *, $p < 0.05$ *versus* GDNF + GFRα1. *C*, GDNF-mediated survival of E9 chick nodose ganglion neurons treated with different Chariot-antibody conjugate combinations. Guinea pig anti-goat Ig antibodies were used as a control. Results are expressed relative to the survival observed in GDNF alone (set to 100%) and represent the means ± S.E. of three independent experiments performed in triplicate. *, $p < 0.05$ *versus* GDNF. *PY*, phosphotyrosine.

the GDNF ligand family using different GFRα receptors acting in *cis* or in *trans*. Our results indicate that phosphorylation of individual tyrosines in RET is developmentally regulated in the DRG *in vivo*. Finally, using protein transduction into primary neuronal cultures, we demonstrated that phosphorylation of Tyr^{1062} is directly involved in GDNF-dependent neuronal survival.

Synchronized Phosphorylation of Individual RET Tyrosine Residues upon Ligand-mediated Activation—Based on crystallographic studies on the insulin β-receptor, Hubbard *et al.* (29) proposed that autophosphorylation of a specific tyrosine(s) in the activation loop is the initial event leading to receptor tyrosine kinase activation. In this model, autophosphorylation of tyrosine 1162 of the insulin β-receptor is proposed to induce a 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 Tyr^{490} 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 neurotrophic 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-α1/RET and MG87-α3/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 begun to accumulate (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-α1, 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 GFRα1 receptors. However, RET tyrosine phosphorylation was increased and prolonged in cells stimulated in *trans* with GDNF and soluble GFRα1 compared with GDNF alone. In addition, in cells lacking GFRα1 receptors, RET tyrosine phosphorylation was delayed after stimulation in *trans* compared with cells expressing endogenous GFRα1 stimulated in *cis* (*i.e.* Fig. 3, *A* and *B*). Thus, although RET is not able to discriminate among different ligands, a quantitative 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 GFRα1 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 GFRα 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 GFRα1-Fc. Finally, the fact that RET phosphorylation could be seen up to adult stages (only $Tyr(P)^{905}$ was analyzed here) indicates that cells in the mouse DRG are continuously exposed to GDNF family ligands *in vivo*. The functions of these factors in adult sensory neurons are only beginning to be understood and may involve acute regulation of physiological properties such as injury-induced plasticity of sodium channel subunits (43).

Role of Tyr^{1062} in GDNF-mediated Neuronal Differentiation and Survival—Our results using protein transduction of anti-phosphopeptide antibodies demonstrate for the first time a role for RET Tyr^{1062} in GDNF-mediated neuronal differentiation and survival. The high degree of connectivity of this residue with a number of major intracellular pathways, including the Ras/ERK and PI3K/AKT pathways, predicted an important role for Tyr^{1062} in the biological activities of the RET receptor. The fact that this residue constitutes a major (although not the only) route by which RET activates PI3K and AKT, a crucial pathway for GDNF-mediated survival of SCG neurons (17), is in agreement with its importance for the biological activity of this receptor in sensory neurons. On the other hand, the inability of anti-$Tyr(P)^{905}$ and anti-$Tyr(P)^{1015}$ antibodies to affect neuronal survival suggests that none of their targets are involved in this activity. Notably, the lack of effect of antibodies against $Tyr(P)^{905}$, a residue involved in the activation of the RET kinase, indicates that, once phosphorylated, this tyrosine does not participate in downstream events required for neuronal survival.

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