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*Advances in Brief*

# Tyrosine 1062 of RET-MEN2A Mediates Activation of Akt (Protein Kinase B) and Mitogen-activated Protein Kinase Pathways Leading to PC12 Cell Survival[1]

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## Abstract

**The RET tyrosine kinase is a functional receptor for neurotrophic ligands of the glial cell line-derived neurotrophic factor (GDNF) family. Loss of function of RET is associated with congenital megacolon or Hirschsprung's disease, whereas germ-line point mutations causing RET activation are responsible for multiple endocrine neoplasia type 2 (MEN2A, MEN2B, and familial medullary thyroid carcinoma) syndromes. Here we show that the expression of a constitutively active *RET-MEN2A* oncogene promotes survival of rat pheochromocytoma PC12 cells upon growth factor withdrawal. Moreover, we show that the RET-MEN2A-mediated survival depends on signals transduced by the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades. Thus, in PC12 cells, RET-MEN2A associates with the PI3K regulatory subunit p85 and promotes activation of Akt (also referred to as protein kinase B) in a PI3K-dependent fashion; in addition, RET-MEN2A promotes MAPK activation. PI3K recruitment and Akt activation as well as MAPK activation depend on RET-MEN2A tyrosine residue 1062. As a result, tyrosine 1062 of RET-MEN2A is essential for RET-MEN2A-mediated survival of PC12 cells cultured in growth factor-depleted media.**

## Introduction

Growth factors of the GDNF[3] family (GDNF, neurturin, persephin, and artemin) are potent survival factors for several neuronal populations (1). GDNF family ligands interact with glycosylphosphatidylinositol-anchored membrane receptors, called GFR$\alpha$s, that, in turn, induce dimerization and activation of the RET receptor tyrosine kinase (1). Signals from GDNF growth factors can be transduced intracellularly via RET-dependent and RET-independent mechanisms. Indeed, it has been reported recently that GDNF can stimulate neuronal cell survival in a RET-independent fashion, possibly via GFR$\alpha$-dependent pathways (2). Nevertheless, the analysis of *null* mice indicates that also RET signaling is critical for survival. Accordingly, enteric neural crest cells were demonstrated to undergo apoptosis in the foregut of embryos lacking the RET receptor (3).

HD consists of a defective development of the enteric nervous system that leads to congenital megacolon. MEN2 syndromes (MEN2A, MEN2B, and familial medullary thyroid carcinoma) predispose to medullary thyroid carcinomas, pheochromocytomas, and other neoplasms. Germ-line mutations in the RET receptor tyrosine kinase are associated with both HD and MEN2 diseases. The majority of HD mutations causes the loss of RET function, whereas MEN2 mutations induce constitutive activation of the kinase and of the oncogenic potential of RET (4).

The cytoplasmic domain of RET contains 14 tyrosine residues and a longer form (1114 residues long), which arises because of alternative splicing, contains two additional ones. Phosphorylated tyrosines 905 and 1015 act as docking sites for the Grb7/Grb10 adaptors (5, 6) and for phospholipase C$\gamma$ (7), respectively. Tyrosine 1062 is essential for the binding of Shc (8–10) and Enigma (11) to RET. Shc is a docking protein involved in the coupling of several receptors to the Ras/MAPK pathway (12). Enigma is a PDZ and LIM domain-containing protein that, by associating with RET through the second LIM domain and to the plasma membrane through its PDZ domain, can be involved in recruitment and clustering of RET protein products at the membrane level (11). Mutations of tyrosine 1062 impair neoplastic transformation mediated by RET-derived oncogenes (8). RET activates the Ras/MAPK (13) and PI3K/Akt (14) pathways. Class I PI3Ks are composed of p85 regulatory and p110 catalytic subunits. The p85 subunit possesses two SH2 domains which mediate, directly or indirectly (through docking proteins), PI3K recruitment to receptor tyrosine kinases (15). The PI3K-produced D3 phosphorylated phosphoinositides, in turn, modulate the activity of effectors, including the serine-threonine kinase Akt (protein kinase B; Ref. 16). Both the MAPK pathway (17) and the PI3K/Akt pathway inhibit apoptosis after activation (16, 18).

Here we show that a ligand-independent, constitutively active RET oncogene (*RET-MEN2A*) promotes PI3K/Akt- and MAPK-dependent survival of PC12 cells, and that tyrosine 1062 of RET-MEN2A is essential for activation of both pathways.

## Materials and Methods

**Plasmids and Antibodies.** The RET expression vectors used in this study encode the "short" (1072 residues long) RET isoform. Expression vectors for RET(C634Y), *i.e.*, RET-MEN2A, and for the Y1062F mutant of RET-MEN2A were cloned in long-terminal repeat vector, as described previously (19). The dominant interfering Akt construct is described elsewhere (20); it contains the kinase inactive Akt K179 M mutant, modified by the addition of the HA epitope. The pCDNA3-MEK-A plasmid containing a dominant interfering form of MEK that does not bind to ATP (K97A) was a kind gift of Dr. J. S. Gutkind. Anti-RET polyclonal antibodies were described previously (19). Anti-Akt, anti-phosphoAkt (Ser473), anti-MAPK, and anti-phospho-MAPK antibodies were purchased from New England Biolabs (Beverly, MA). Anti-phosphotyrosine antibodies (4G10) and the GST p85/N-SH2 protein were from Upstate Biotechnologies, Inc. (Lake Placid, NY).

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[3] The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; HD, Hirschsprung disease; MEN2, multiple endocrine neoplasia type 2; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; HA, hemagglutinin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

**Cells and Transfections.** PC12 cells were grown in RPMI 1640 supplemented with 10% horse serum and 5% FCS (Life Technologies, Inc., Paisley, PA). Subconfluent cells were transfected by using the lipofectin reagent following the manufacturer's instructions (Life Technologies, Inc.). Mass populations of transfected cells were marker selected with the addition of mycophenolic acid, as described previously (19).

**Protein Analysis.** Protein extractions and Western blots were performed according to standard procedures. Immune complexes were detected with the enhanced chemiluminescence kit (Amersham Corp., Buckinghamshire, United Kingdom). For the "pull-down" experiments, clarified cell lysates (2 mg) were incubated with 5 $\mu$g of immobilized GST p85/N-SH2 fusion protein for 60 min at 4°C; protein complexes were analyzed by immunoblot. The Akt immune-complex kinase assay was performed on 500 $\mu$g of protein lysates as described (20); histone 2B was used as substrate (Boehringer Mannheim, Mannheim, Germany). MAPK activity was assayed by immune-complex kinase assay on 500 $\mu$g of protein lysates using myelin basic protein as a substrate, as described elsewhere (21).

**DNA Fragmentation Analysis and TUNEL Assay.** For the extraction of fragmented DNA, $2 \times 10^6$ cells/sample were lysed in a buffer containing 0.5% Triton X-100, 5 mM Tris buffer (pH 7.4), 20 mM EDTA. Intact nuclei were removed by centrifugation, and soluble DNA was purified by phenol extraction and ethanol precipitation. Soluble DNA was analyzed by electrophoresis on a 1.2% agarose gel (22). An equal number ($5 \times 10^3$) of cells was subjected to TUNEL assay (22) following the manufacturer's instructions (Boehringer). Apoptosis was evaluated by Fast Red (Dako Co., Carpinteria, CA) staining.

## Results

**RET-MEN2A Survival Effect Is Abrogated by the Y1062F Substitution.** Rat pheochromocytoma PC12 rapidly undergo apoptosis upon withdrawal of serum (22). To study whether activated RET can transduce survival signals in a neuronal cell setting, we transfected PC12 cells with the RET(C634Y) (RET-MEN2A) construct. The C634Y MEN2A-associated RET mutation causes constitutive dimerization mediated by disulfide bonds and activation of the RET kinase (19). In parallel, PC12 cells were also transfected with a RET-MEN2A construct carrying the tyrosine 1062 to phenylalanine (Y1062F) mutation. Then, starvation-induced apoptosis was analyzed by the TUNEL assay in parental and in MEN2A- and MEN2A(Y1062F)-expressing PC12 cells. Representative microscopic fields are shown in Fig. 1*A*, and the average results of three independent experiments are reported in Fig. 1*B*. Apoptotic nuclei were very scarce in the three cell lines in the presence of complete medium. Upon starvation (12 h), 30 ± 5% nuclei scored apoptotic in parental PC12 cells, whereas <2% of RET-MEN2A cells scored apoptotic. In marked contrast, 40 ± 8% nuclei resulted apoptotic in serum-starved RET-MEN2A(Y1062F) cells. To confirm these findings, internucleosomal DNA fragmentation, a hallmark of apoptotic cell death, was assessed in the three cell lines. Upon starvation, the characteristic ladder-like electrophoretic pattern of apoptotic DNA was observed in parental and RET-MEN2A(Y1062F)-expressing cells but not in RET-MEN2A cells (Fig. 1*C*). The expression and phosphorylation levels of RET-MEN2A(Y1062F) and RET-MEN2A proteins were comparable, as demonstrated by immunoprecipitating RET and staining the immunoblot with anti-RET or anti-phosphotyrosine antibodies (Fig. 1*D*). Thus, tyrosine 1062 is required for RET-MEN2A antiapoptotic effects in PC12 cells.

**The PI3K/Akt Pathway Is Involved in RET-MEN2A-mediated PC12 Cell Survival.** Because the serine-threonine Akt (protein kinase B) protein kinase is a nodal regulator of cell survival (16, 18), we examined whether RET-MEN2A expression activates Akt in PC12 cells. To this aim, we used antibodies that detect phosphorylation of Ser-473, a marker of Akt activation. We found that RET-MEN2A expression induced a constitutive phosphorylation of Akt (Fig. 2*A*, *left*). This activation was PI3K dependent, because it was abrogated by treatment with wortmannin (50 nM) or LY294002 (10 $\mu$M), two pharmacological inhibitors of PI3K (Fig. 2*A*, *right*). Then, to prove that the PI3K/Akt pathway was involved in RET-MEN2A-mediated cell survival, RET-MEN2A-expressing cells were serum starved for 12 h in the presence of wortmannin or LY294002, and apoptosis was evaluated by DNA laddering. RET-MEN2A expressing cells underwent apoptosis after PI3K inhibition (Fig. 2*B*). Finally, to directly assess the role of Akt in the transduction of RET-MEN2A-induced

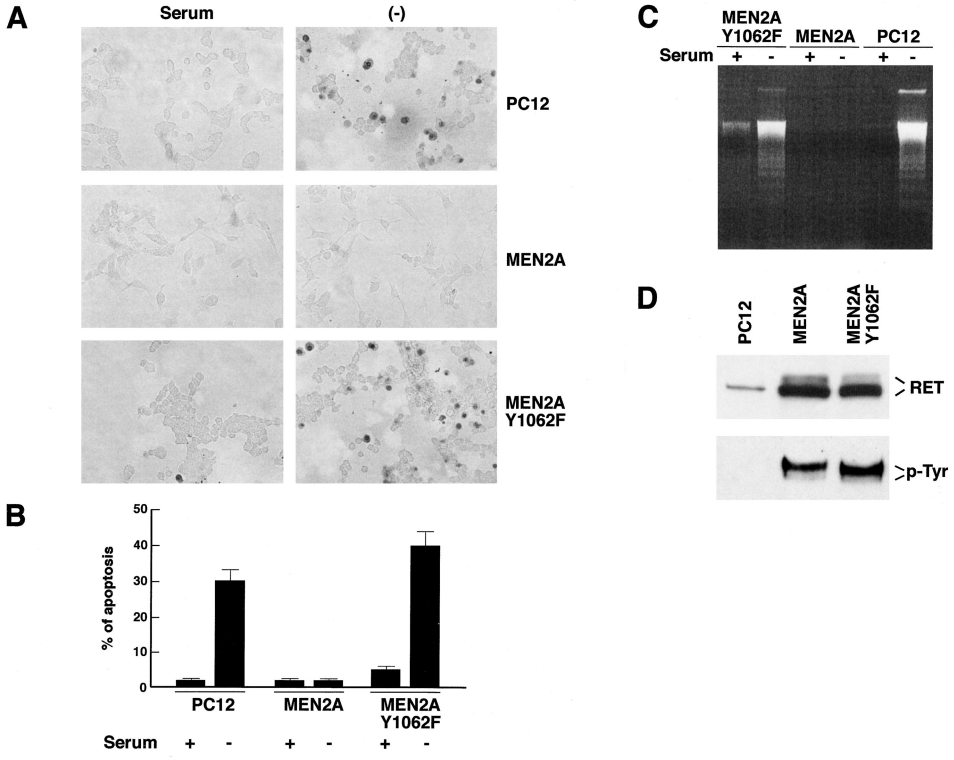


Fig. 1. *A*, the indicated PC12 cell lines were maintained for 12 h in the presence (serum) or absence (−) of serum and subjected to the TUNEL reaction. Representative fields are shown; *dark cells* are the apoptotic TUNEL-positive ones. *B*, the percentages of apoptotic nuclei were calculated by counting a minimum of 200 cells in 10 randomly selected fields of each specimen; the average results of three independent experiments are reported. Variations between single experiments were <20% of the mean; *bars*, SD. *C*, soluble DNA was extracted from cells ($2 \times 10^6$) starved for 12 h and run on a 1.5% agarose gel; the experiment shown here is representative of three independent assays. *D*, 500 $\mu$g of protein lysates were immunoprecipitated with anti-RET, and the immunoblot was stained with anti-phosphotyrosine (anti-pTyr) or anti-RET antibodies.

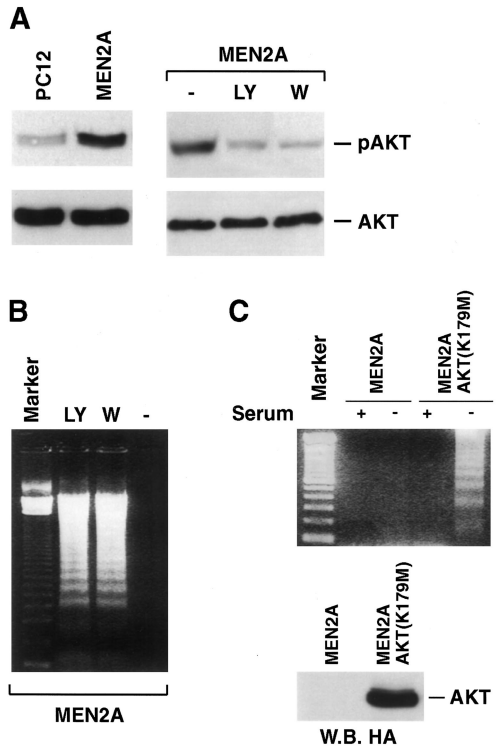


Fig. 2. *A*, protein lysates (50 $\mu$g) from the indicated cell lines, maintained for 12 h (when indicated) in the presence of 50 nM wortmannin (*W*) or 10 $\mu$M LY294002 (*LY*), were immunoblotted with anti-phospho-Akt (*upper panel*) or anti-Akt (*lower panel*) antibodies. *B*, PC12 MEN2A cells were maintained for 12 h in serum-free medium in the presence of 50 nM wortmannin (*W*) or 10 $\mu$M LY294002 (*LY*), as indicated. Soluble DNA was analyzed by agarose gel electrophoresis. *C*, PC12 MEN2A cells were transiently transfected with HA-AKT(K179 M) or with the empty vector; after 24 h, cells were kept for an additional 18 h in the presence or absence of serum, and DNA laddering was analyzed by electrophoresis. Western blot with anti-HA antibodies (100 $\mu$g of protein lysate) confirmed the expression of the Akt(K179 M) protein.

survival, we transiently transfected PC12-RET-MEN2A cells with the dominant-negative HA-Akt(K179 M) expression construct or with the empty vector, as a control. Expression of Akt(K179 M) was confirmed by probing the Western blot of transfected cell lysates with the anti-HA antibody (Fig. 2*C*, *bottom*). Apoptosis was monitored by DNA fragmentation. Fig. 2*C* (*upper*) shows that transient expression of HA-Akt(K179 M) caused DNA fragmentation of PC12-MEN2A cells in response to serum deprivation.

Tyrosine 1062 of RET-MEN2A could be necessary for RET-MEN2A survival signaling by mediating Akt activation. To test this hypothesis, we investigated whether the Y1062F mutation affected RET-MEN2A coupling to the PI3K/Akt pathway. PI3K activation is mediated by recruitment of the p85 regulatory subunit by receptor tyrosine kinases (15). Thus, we analyzed the association of RET-MEN2A and RET-MEN2A(Y1062F) proteins with p85. Protein lysates of PC12, PC12-MEN2A, and PC12-MEN2A(Y1062F) cells were immunoprecipitated with anti-p85 antibodies; the immunoblot was stained with anti-RET and anti-phosphotyrosine antibodies. RET-MEN2A proteins coimmunoprecipitated with p85; the Y1062F mutation strongly reduced this coimmunoprecipitation (Fig. 3*A*, *upper*). This reduced coimmunoprecipitation was paralleled by reduced tyrosine phosphorylation levels of p85 in MEN2A(Y1062F) with respect to MEN2A-expressing cells (Fig. 3*A*, *middle*). The interaction of p85 to tyrosine phosphorylated receptors is mediated by the p85 SH2 domains. Thus, PC12-MEN2A and PC12-MEN2A(Y1062F) cell lysates were subjected to an *in vitro* "pull-down" experiment with a GST p85/N-SH2 fusion protein; bound RET-MEN2A molecules were detected by Western blot. The results showed that the *in vitro* binding of the N-SH2 domain of p85 to RET-MEN2A requires tyrosine 1062 (Fig. 3*B*). By impairing PI3K recruitment, the Y1062F mutation could reduce Akt activation by RET-MEN2A. To test this possibility Akt activation was analyzed in parental, MEN2A, and MEN2A(Y1062F) cells by an *in vitro* kinase assay. Akt was immunoprecipitated from cell lysates, and the immunocomplexes were incubated with labeled ATP and histone 2B, a substrate for the Akt kinase. As shown above, RET-MEN2A expression induced constitutive activation of Akt; cells expressing RET-MEN2A with the Y1062F mutation exhibited virtually no activation of Akt (Fig. 3*C*, *upper*). These results were confirmed by a Western blot with anti-phosphoAkt antibodies (Fig. 3*C*, *middle*).

**The MAPK Pathway Is Involved in RET-MEN2A-mediated PC12 Cell Survival.** Shc is involved in Ras activation by receptor tyrosine kinases by recruiting Grb2-Sos complexes (12). Activated Ras plays a major role in controlling several downstream signaling pathways, including the Raf/MEK/MAPK pathway (23). The MAPK pathway, in turn, promotes cell survival (17). By mediating Shc recruitment, Y1062 could be essential for RET-MEN2A-induced MAPK activation. Thus, MAPK phosphorylation and MAPK activity in MEN2A- and MEN2A(Y1062F)-expressing cells were examined by Western blot and *in vitro* kinase assays. RET-MEN2A expression induced constitutive activation of MAPK; conversely, cells expressing RET-MEN2A with the Y1062F mutation exhibited minimal activation of MAPK (Fig. 4*A*).

If the MAPK activity, along with the Akt activity, contributes to the transduction of RET-MEN2A survival signals, a defect in MAPK activation could contribute to the inability of RET-MEN2A(Y1062F) to promote survival. To test this hypothesis, RET-MEN2A-expressing

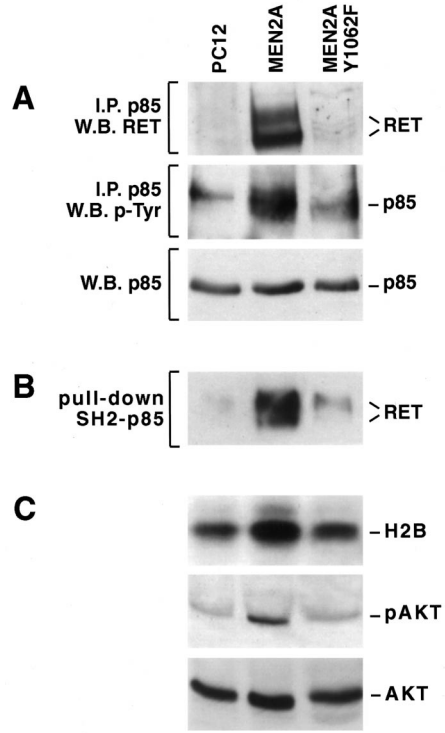


Fig. 3. *A*, proteins (2 mg) from the indicated cell lines were immunoprecipitated with anti-p85. The immunoblot was cut at the level of $M_r$ 100,000 molecular weight marker: the upper part was stained with anti-RET and the lower part with anti-pTyr antibodies. A direct immunoblot (50 $\mu$g of protein lysate) was stained with anti-p85 antibodies for normalization. *I.P.*, immunoprecipitation. *B*, 3 mg of proteins were subjected to a "pull-down" assay with GST/p85-N-SH2. The blot was probed with anti-RET antibodies. *C*, anti-Akt immunoprecipitates were incubated with histone 2B and labeled ATP; the reaction products were resolved by SDS-PAGE. Fifty $\mu$g of total lysates were immunoblotted with anti-Akt or anti-phosphoAkt (pSer473) antibodies.

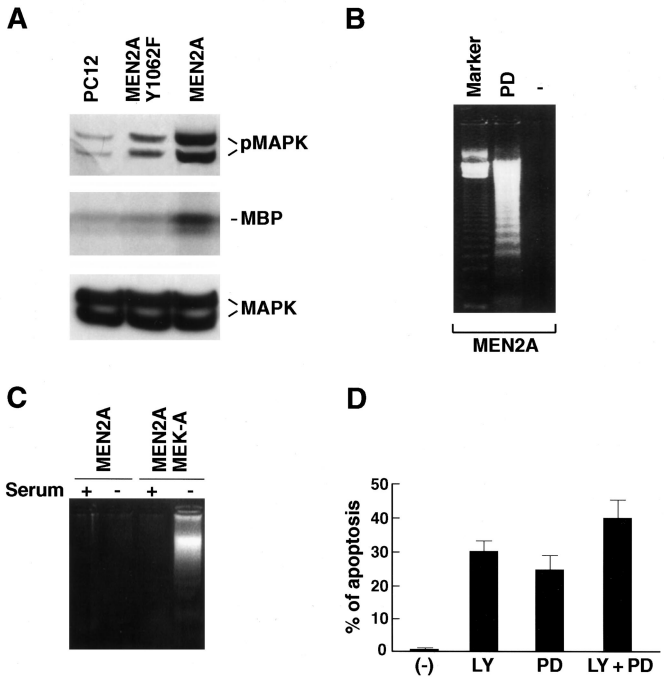


Fig. 4. *A*, MAPK was immunoprecipitated from 500 $\mu$g of total lysate of the indicated cell lines. The immunoprecipitates were incubated with myelin basic protein and labeled ATP; the reaction products were resolved by SDS-PAGE (*middle*). Fifty $\mu$g of total lysates were immunoblotted with anti-MAPK (*lower*) or anti-phosphoMAPK (*upper*) specific antibodies. *B*, PC12 MEN2A cells were grown in complete medium and then washed and maintained for 12 h in serum-free medium in the presence of 30 $\mu$M PD098059 (*PD*), as indicated. Soluble DNA was analyzed by electrophoresis. *C*, PC12 MEN2A cells were transiently transfected with MEK-A or with the empty vector; after 24 h, cells were kept for an additional 18 h in the presence or absence of serum, and DNA laddering was analyzed by electrophoresis. *D*, PC12 MEN2A cells were maintained for 12 h in serum-free medium in the presence of 10 $\mu$M LY294002 (*LY*), 30 $\mu$M PD098059 (*PD*), or a combination of the two inhibitors (*LY* + *PD*) and subjected to the TUNEL reaction. The average results of three independent experiments are reported.

cells were serum starved for 12 h in the presence of PD098059, a pharmacological agent that blocks MEK activity. Induction of apoptosis was monitored by DNA laddering. Fig. 4*B* shows that PD098059 treatment, indeed, diminished RET-MEN2A-mediated PC12 cell survival, along with the inhibition of MAPK activity (not shown). To confirm the role of MAPK in the transduction of RET-MEN2A-induced survival, we transiently transfected PC12-MEN2A cells with a dominant interfering form of the MAPK kinase (MEK-A) or with the empty vector. Expression of MEK-A was confirmed by Western blot (not shown), and apoptosis was monitored by DNA fragmentation. Fig. 4*C* shows that transient expression of MEK-A caused DNA fragmentation of PC12-MEN2A cells in response to serum deprivation.

Finally, to compare the relative importance of the PI3K/Akt and the MAPK pathways in RET-MEN2A-mediated cell survival, RET-MEN2A-expressing cells were serum starved for 12 h in the presence of LY294002, PD098059, or both, and apoptosis was analyzed by the TUNEL assay. The average results of three independent experiments are reported in Fig. 4*D*. Similar percentages of RET-MEN2A-expressing cells underwent apoptosis after PI3K or MAPK inhibition (30 $\pm$ 5% and 24 $\pm$ 5%, respectively), confirming that both pathways are essential for RET-MEN2A survival signaling. Addition of both pharmacological inhibitors had a partially additive effect, causing apoptosis of 40 $\pm$ 8% RET-MEN2A cells.

## Discussion

Here we show that RET-MEN2A promotes survival of PC12 cells. Moreover, we show that tyrosine 1062 is necessary for efficient activation of the MAPK and PI3K/Akt pathways by RET-MEN2A and that both pathways are essential for RET-MEN2A-mediated survival. It is likely that the PI3K and MAPK pathways are only partially redundant and are both essential for the survival signaling of RET-MEN2A, and that for this reason the apoptotic effect of the combination of LY294002 and PD098059 does not reach the sum of the apoptotic effects of each inhibitor.

Tyrosine 1062 of RET is the docking site for Shc (8–10), and it is well established that, by recruiting Grb2-Sos complexes, Shc contributes to activation of the Ras/MAPK pathway by receptor tyrosine kinases (13). Thus, the impaired coupling to Shc can explain the reduction of MAPK activation caused by the Y1062F mutation. In addition, here we show that RET-MEN2A associates with the PI3K regulatory subunit p85 and that tyrosine 1062 is essential for the RET-MEN2A/p85 binding. "Pull-down" experiments have shown that at least *in vitro* the $NH_2$-terminal SH2 domain of p85 participates to p85 binding to RET-MEN2A. Binding of p85 to receptor tyrosine kinases, through its SH2 domains, can be either direct or mediated by several docking proteins, such as Gab1, c-cbl, or proteins of the IRS family (15). Both the COOH- and the $NH_2$-terminal SH2 domain of p85 bind phosphorylated tyrosines, followed by a methionine at position +3 (24). Because neither Y1062 nor the other autophosphorylated RET-MEN2A tyrosines (25) satisfy this requirement, it is reasonable that the association between p85 and RET-MEN2A is indirect, and it is mediated by docking proteins. Gab1 is phosphorylated by RET-MEN2A and could be a mediator of the p85/RET-MEN2A interaction (26). To test this possibility, it should be investigated whether Gab1 recruitment to RET is mediated by tyrosine 1062. Whatever the mechanism, it is noteworthy that one single tyrosine residue of RET-MEN2A mediates the activation of two pathways that are both essential for promotion of cell survival. Similarly, it has been shown that tyrosine 490 of TRK, the prototype neurotrophic receptor, is involved in Shc recruitment and in the activation of PI3K/Akt and MAPK pathways (27). It is possible that such a pivotal role of one single docking site allows the integration of different signals that are necessary for mediating cell survival.

Here we show that RET-MEN2A mediates cell survival by activating both Akt and MAPK pathways. GDNF is able to signal survival also in the absence of RET through a pathway that is not associated to Akt activation and, thus, which is mechanistically different from that mediated by RET (2). It is conceivable that RET-dependent and RET-independent pathways may cooperate to mediate the very efficient antiapoptotic effects exerted by GDNF neurotrophic factors. In turn, lack of survival signaling may be responsible for the apoptosis of enteric neural crest cells in RET-*null* embryos (3) and can be one of the major consequences of RET inactivation in congenital megacolon (HD). Accordingly, it is interesting to observe that two recently identified HD mutations, delN1059 and L1061P, map in close proximity of Y1062, and that *RET-MEN2A* alleles carrying these mutations are defective in binding to Shc (28) and in the stimulation of Akt and MAPK.[4] On the other hand, prolonged survival caused by activating RET mutations may lead to oncogenesis, as supported by the fact that such mutations cause the neoplasms associated with the MEN2 syndromes.

Data presented in this report show that tyrosine 1062 is the RET docking site of either p85 or other proteins that facilitate p85 binding to the receptor. In either case, this site appears to be essential for the activation of the PI3K/Akt pathway. Studies in this report also show that this site is essential for the transduction of RET-generated survival signals that require activation of both PI3K/Akt and MAPK

[4] R. M. Melillo *et al.*, unpublished observations.

pathways. Earlier studies had linked Y1062 with Shc phosphorylation and the transduction of proliferative signals. Very recently, Segouffin-Cariou and Billaud (29) have shown that activation of the PI3K/Akt pathway is essential for the transforming activity of the *RET-MEN2A* oncogene. Studies now in progress will address the relative contribution of such pathways in the biological effects mediated by RET.

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