The RET receptor tyrosine kinase is important for several different biological functions during development. The recruitment at the phosphorylated Tyr1062 site in RET of a number of different phosphorytosine binding (PTB) domain-containing adaptor proteins, including Shc and Frs2, plays a dominant role for the multiple different biological functions of the RET receptor during development, including stimulation of cell survival. Here, we demonstrate that a competitive recruitment of Shc as opposed to Frs2 mediates the survival signaling arising from RET activation. Based on results from a peptide array, we have genetically engineered the PTB domain binding site of RET to rewire its recruitment of the PTB proteins Shc and Frs2. An engineered RET that has a competitive interaction with Shc at the expense of Frs2, but not a RET receptor that only recruits Frs2, activates cell survival signaling pathways and is protective from cell death in neuronal SK-N-MC cells. Thus, cell type-specific functions involve a competitive recruitment of different PTB adaptor molecules by RET that activate selective signaling pathways.

The autophosphorylation of receptor tyrosine kinases (RTKs) creates binding sites for the SH2 or PTB domains of cytoplasmic signaling proteins, which typically recognize specific phosphorytosine-containing motifs within the activated receptor. Following their association with receptors, PTB domain proteins are themselves phosphorylated at tyrosine sites that selectively recruit the SH2 domains of downstream targets in a fashion that can be critical for cellular responses to RTKs (1–4). PTB domain proteins thereby serve as scaffolds to assemble specific signaling complexes and to stimulate intracellular pathways with the potential to modulate a range of different physiological processes, including cell proliferation, cell motility, and cell survival. PTB domains fold into an anti-parallel pseudo-β-sheet with a C-terminal α-helix, to create a binding site for peptides with a β-turn. These peptide motifs often have the sequence consensus NPXY, although the proline at the −2-position can be dispensable (5). In addition, the PTB domains of Shc, Frs2, IRS, and Dok family members require phosphorylation of the Tyr residue within the NXXY motif for high affinity binding (6). PTB domains can be rather versatile in their binding properties, and indeed the PTB domain of Frs2 engages an extended peptide sequence in the juxtamembrane region of the fibroblast growth factor receptor (FGFR1) in a different mode from NPXY motifs and in a fashion that is constitutive, although its activation is ligand-dependent (7, 8). In addition to binding a core NXXXY motif, the PTB domains of proteins, such as Shc and Frs2, discriminate between related binding sites on the basis of residues immediately amino-terminal to the NXXXY motif that influence the binding affinity. Thus, amino acid differences at positions −4 to −8 promote preferential binding to the Shc or Frs2 PTB domains (5, 9, 10). Consistent with this notion, the insulin receptor, which associates primarily with the PTB domain of IRS-1, but not Shc, can be re-engineered to selectively interact with ShcA by a single amino acid substitution at the −5-position in the sequence N-terminal to the core NXXXY PTB domain binding site (11).

The ret gene encodes an RTK (12, 13) that is essential for several cell populations during development (14). The glial cell line-derived neurotrophic factor (GDNF) family ligands, including GDNF, neurturin, persephin, and artemin, exert their physiological functions via activation of RET. Ligand-activated RET induces a number of different cellular responses that vary depending on the cell type and the developmental stage, including directed cell migration, proliferation, and control of cell survival/death in developing sympathetic (15), enteric (16–21), and parasympathetic (22) neurons, respectively. Gain- and loss-of-function mutations in ret can cause severe diseases in humans, including papillary thyroid carcinoma, multiple endocrine neoplasia (MEN) 2A, and 2B(III), familial medullary thyroid carcinoma, and Hirschsprung disease. More than 10 rearranged forms of RET have been cloned from sporadic and
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radiation-associated papillary thyroid carcinomas (23, 24), whereas germ line point mutations of RET have been identified in MEN2A, MEN2B, and familial medullary thyroid carcinoma. ret mutations in MEN2A or familial medullary thyroid carcinoma typically alter one of six cysteine residues in the RET extracellular cysteine-rich domain (25). These cysteine substitutions convert an intramolecular disulfide bond to an intermolecular linkage, resulting in permanent RET dimerization and ligand-independent activation (26, 27). In contrast, in most MEN2B cases, there is a methionine to threonine substitution at position 918 (M918T) of the kinase domain, which constitutively activates the kinase and may alter RET substrate specificity.

TyR1062 of RET, an in vivo autophosphorylation and multido-cking site, conforms to the NXXPY consensus for PTB domain recognition. Phosphorylation of TyR1062 appears functionally important, since substituting this tyrosine with phenylalanine impairs the transforming activity of the oncogenic MEN2A and MEN2B RET receptors (28), and targeted mutation of TyR1062 in mice causes a decrease in enteric neurons and induces renal hypoplasia (29), both of which resemble the phenotype of RET null mutant mice. Activated RET can potentially recruit a number of different docking proteins through the autophosphorylated TyR1062 site, including Shc (28, 30–31), Enigma (32), SNT/ Frs2 (33, 34), Dok (35, 36), and IRS-1 (37), accounting for the ability of the phosphorylated TyR1062 site to stimulate the Ras/ERK, phosphatidylinositol-3 kinase-3 kinase (PI3K)/Akt, p38 mitogen-activated protein kinase (MAPK), c-Jun amino-terminal kinase (JNK), and ERK5 pathways. Binding of the Shc PTB domain to the Tyr(P)-1062 site in RET facilitates RET-mediated phosphorylation of Shc at sites that consequently bind the Grb2 adaptor, which in turn recruits the Sos and Gabs1/2 proteins (38, 39), promoting activation of the Ras/ERK and PI3K/Akt signaling pathways. Frs2 also associates with Grb2, potentiates MAPK activation by RET, and increases cell proliferation (34, 41).

Unlike ShcA, Frs2 can also complex with the Shp-2 tyrosine phosphatase, which enhances stimulation of the Ras-MAPK pathway of RET (33). ShcA can also associate with proteins, such as Memo, that are not known to bind Frs2 (40). These results have led to the hypothesis that the balance of Shc and SNT/Frs2 binding to the Tyr(P)-1062 site may affect the nature of the intracellular signaling for cell proliferation, differentiation, and survival induced by activated RET (33). Because the binding of the PTB domain of one docking protein excludes the recruitment of another to the same site, different PTB domain proteins may compete for interaction with RET.

In this study, we have engineered the RET receptor to generate receptors that preferentially engage Shc, but not Frs2, and vice versa, designated RetShc+ and RetFrs+, respectively. We find that RetShc+ but not RetFrs+ activates signaling pathways resulting in cell survival. This difference in biological outcome supports the idea that one level of functional specificity in RTK signaling resides in the recruitment of different PTB domain proteins. The affinity profiles of a given RTK for distinct PTB domain proteins may therefore determine which of these docking proteins successfully compete for the activated receptor and thereby influence specific cellular functions. Our results may provide a better mechanistic insight into how different receptor tyrosine kinases instruct different cellular functions as a result of environmental signals.

EXPERIMENTAL PROCEDURES

DNA Constructs and Mutagenesis—The human cDNA for ret9 was originally obtained in the P717 vector as a gift from Marc Billaud (Université Claude Bernard). Site-directed mutagenesis was carried out using primer pairs according to QuikChange methods (Stratagene). Constructs were sequenced using BigDye version 1.1 kit (Applied Biosystems) with an ABI Prism 310 sequencer. RetFrs+ constructs were made through PCR cloning of the FGFR1 juxtamembrane segment (residues 410–426) with a C-terminal hemagglutinin epitope (the hemagglutinin epitope was not used for detection in this study) and subcloned in pCDNA3. Mammalian expression constructs for ShcA and Frs2α were gifts from R. Bradshaw (UCLA, Los Angeles, CA) and H. Berger (University of Birmingham). GST fusion constructs of ShcA- and Frs-PTB were made in pGEX-4T2 vector (GE Healthcare). Fluorescent markers used (green or red) were from the Enhanced FP range of Clontech.

Cell Culture and Transfection—COS-7 and HEK293T cells were grown in Dulbecco’s modified Eagle’s medium with 10% serum (fetal bovine serum) and 1 mM glutamine, whereas the neuroblastoma cell line SK-N-MC was maintained in Dulbec-
coc’s modified Eagle’s medium supplemented with 12% fetal bovine serum and 1 mM glutamine. All ligand stimulations were performed using 50 ng/ml recombinant human GDNF and 100 ng/ml of recombinant human GFRα1/FC chimera (RnD Sys-
tems). All transfections were performed using polyethylene-
mime at 0.8 μg/ml in PBS (25 kDa; Sigma) as described previ-
ously (41). Growth medium was replaced 5–7 h after transfection. Transfection efficiency was monitored by EGFP flu-
orescence was consistently >90%.

Antibodies and Reagents—Antibodies against RET9 and phosphotyrosine (PY99) were obtained from Santa Cruz Bio-
technology, Inc. (Santa Cruz, CA). Antibodies against hexahis-
tidine tags were from BD Biosciences. Anti-GST antibodies were from GE Healthcare. Antibodies for CREB, phospho-
CREB Ser133, Akt, phospho-Akt Ser473, p44/42 MAPK, phos-
pho-p44/42 MAPK, NFκB, phospho-NFκB, and cleaved caspase-3 Asp222 were from Cell Signaling. Cy2-conjugated secondary antibodies were obtained from Jackson Laboratories. GDNF and GFRα1 were purchased from R & D Biosystems.

Expression and Purification of GST Fusion Proteins—pGEX-
4-T2 plasmids containing the specific construct were trans-
fomed into the BL21 strain of Escherichia coli. Exponentially
growing cells were induced with 0.7 mM isopropyl-1-thio-
galactopyranoside for 4 h at 37 °C. The bacteria were cen-
trifuged, and the pellet was dissolved in cold PBS containing 1 mM phenylmethylsulfonyl fluoride and Complete EDTA-free pro-
tease inhibitor mixture tablets (Roche Applied Science). Cells were lysed by sonication on ice, after which PBS plus 2% Triton X-100 was added at 1:1. The suspension was incubated with glutathione-Sepharose beads (GE Healthcare) for 2 h at 4 °C. After repeated washings, captured protein was eluted by the addition of buffer containing 12 mM reduced glutathione (Sigma), 50 mM Tris, pH 7.4, and protease inhibitors (Roche...
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Applied Science). When appropriate, the GST tag was enzymatically cleaved off by incubation with Trombine (GE Healthcare). Purified proteins were dialyzed (Pierce Slide-Alyzer Bags) against 50% glycerol in PBS buffer and stored at −20 °C until use. Purity was checked by running samples on polyacrylamide gels followed by Comassie Blue staining of gels. Protein concentration was determined using the Bio-Rad protein assay.

Peptide Spot Arrays—Peptide arrays were prepared according to the spot synthesis method using standard Fmoc chemistry (42). Fmoc-protected and -activated amino acids (Intavis) were spotted on high density 24 × 18 arrays on 130 × 90-mm cellulose membranes with an AbiMed (Langfield, Germany) ASP422 robot. Membranes were blocked overnight in 10% skimmed milk, incubated with 1 μM purified GST fusion proteins in 0.1% Tween in Tris-buffered saline for 2 h at 4 °C, washed three times, and probed with rabbit polyclonal anti-GST antibody. Primary antibodies were detected by horseradish peroxidase-conjugated anti-rabbit antibody followed by enhanced chemiluminescence (Pierce).

**Immunoprecipitation, Pull-down Assays, and Protein Immunoblots—**Cells were lysed in modified radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 50 mM NaF, 1 mM EDTA, 10 mM 2-glycerolphosphate, 2 mM Na3VO4, 1% IGEPAL CA-630, 0.25% sodium deoxycholate, and 10% glycerol). For caspase-3 experiments, cells were lysed in CHAPS buffer (Amresco) and subjected to freeze-thaw cycles. Immunoprecipitations were carried out overnight at 4 °C using the appropriate antibodies immobilized on protein G-Sepharose. GST pull-down assays were done using the GST fusion protein baits immobilized on glutathione-Sepharose. In far Western experiments, membranes were incubated with GST-equipped purified peptides for 6 h at 4 °C. When competitive immunoprecipitation was performed, purified peptides equipped with a GST tag (100 pmol) replaced antibodies, and increasing amounts of the competitive peptide (without tag) were added. Precipitated protein were eluted by boiling in reducing Laemmli buffer. Proteins were fractionated on polyacrylamide gels and immobilized on polyvinylidene difluoride membranes. Equal RET expression levels were determined by RET Western blot on cell lysates. Western blot detection was carried out by the ECL method using a PhosphorImager (GE Healthcare). Protein bands were analyzed using ImageQuant software.

**Cell Survival Assays—**Cell survival response to GDNF was assessed using MTT reagent. Transfected SK-N-MC cells were stimulated with 50 ng/ml GDNF the day after transfection. One hour after ligand stimulation, cells were challenged with anisomycin (Sigma) at a final concentration of 50 μg/ml. Medium was replaced with anisomycin-free growth medium after 6 h, and cells were incubated for an additional 10 h. MTT reagent was then added and incubated for 4 h. Converted MTT and cells were incubated for an additional 10 h. MTT reagent was replaced with anisomycin-free growth medium after 6 h, and cells were then incubated with anisomycin (Sigma) at a final concentration of 50 μg/ml for 6 h. Cells were briefly rinsed with PBS, fixed in 3.8% formaldehyde, permeabilized with 0.5% bovine serum and ligand for 12 h, and the proliferative response was assayed using spectrometry of MTT conversion 36 h later.

**Apoptosis Assay—**SK-N-MC cells grown on glass coverslips were transfected and stimulated with GDNF the following day. Anisomycin treatment was done at a final concentration of 40 μg/ml for 6 h. Cells were briefly rinsed with PBS, fixed in 3.8% PFA, permeabilized in 0.2% Triton X-100 in PBS, and blocked with 5% goat serum. Fixed and permeabilized cells were then treated with antibodies against cleaved caspase-3 at 4 °C overnight. After washings in PBS, cells were incubated with Cy2-conjugated secondary antibodies for 1 h at room temperature. Cells were mounted in Vectashield H-1000 (Vector Laboratories), and positively stained cells were quantified under a fluorescence microscope (Zeiss Axiosoplan 2 with ×40/0.95 Korr objective) and normalized to total number of cells. Images were captured with a Jenoptik ProgRes-C14 camera using OpenLab image acquisition software.

**RESULTS**

**Distinction between ShcA and Frs2α PTB Domain Interaction with Ret—**Our first objective was to understand how residues flanking Tyr(P)-1062 of the RET receptor contribute to the strong binding of either ShcA or Frs2α PTB domains. We used spot synthesis technology to create an array of 12-mer peptides spanning residues 1054–1065 of the short isoform of RET (RET9). In these arrays, which are synthesized on a derivatized cellulose membranes, each residue in the RET peptide is changed, one at a time, to every other amino acid. We probed these RET 12-mer peptide arrays with the PTB domains of either ShcA or Frs2α, fused to the C terminus of GST. For each PTB domain, this approach reveals which amino acids are preferred or selected against at any given position of the RET peptide; this identifies the similarities and differences in the ligand-binding selectivity of the ShcA or Frs2α PTB domains, in the context of the RET Tyr(P)-1062 motif (Fig. 1A).

The spot array data confirmed the strict adherence to the PTB interaction consensus, NXXpY, such that substitution of either RET residue Asn-1059 or Tyr(P)-1062 abolished interaction with the PTB domains of both ShcA and Frs2α. In contrast, the replacement of intervening residues (Lys-1060 and Leu-1061) revealed differences between the binding specificities of the two PTB proteins. Selectivity at the −1 position (Leu-1061) was pronounced and showed minimal overlap between ShcA and Frs2α PTB domains. The peptide arrays also suggested different recognition specificities for ShcA and Frs2α extending beyond the canonical NXXpY binding consensus. Replacement of residues both upstream and downstream of the core NXXpY motif was tolerated differently by either PTB domain. For
example, Lys at any position within the 12-mer peptide, with the exception of Lys-1060, disfavored interaction with the ShcA PTB domain, which contrasted with the binding observed for Frs2α/PTB. Interestingly, the Frs2α/PTB domain interaction was notably compromised by replacement of Trp-1056, which conversely appeared to enhance ShcA-PTB binding (e.g., W1056A and W1056D).

**Rewiring RET Adaptor Binding**—Based on the preceding spot array data and published reports on relative affinities of ShcA and Frs2α to the receptors TrkA, TrkB, EGFR, IL4R, InsR, Erb2, and Erb3 and the polyoma middle T antigen (9–11, 46–49), we established point mutations surrounding Tyr-1062 in the RET9 isoform, with the goal of engineering mutant forms of RET that selectively bound one PTB domain protein but not the other. These mutations were initially established in the context of a constitutively active MEN2A (C634R mutation; abbreviated 2aRet) variant of RET in order to facilitate assessment of interaction with ShcA and Frs2α. The panel of RET point mutants were then co-expressed with either GST-tagged ShcA or His6-tagged Frs2α in both HEK293T and COS-7 cells and analyzed for co-precipitation. In agreement with the spot array data, the substitutions W1056A and W1056D augmented association with ShcA to the detriment of Frs2α (Fig. 1B). The mutation E1058D, which we introduced based on ShcA binding to EGFR, ErbB2, and ErbB3, did not affect binding to either scaffold proteins relative to wild-type RET but proved to be beneficial to ShcA binding at the expense of Frs2α in the double mutant W1056A/E1058D. The RET double mutant W1056A/E1058D, which from hereon we designate as Ret<sup>Shc</sup>+, showed a 1.8-fold enhancement of ShcA and a 3.3-fold reduction in Frs2α association as assessed by co-immunoprecipitation (Fig. 1, C–E, Table 1). None of the mutations made that were predicted to establish a bias for Frs2α association (S1054K, T1055K, I1057K, I1057M, and R1064M) compromised ShcA binding relative to wild-type Ret. This led us to rethink our strategy to establish a RET mutant specifically engineered for the recruitment of Frs2α, based on the observation that the PTB domain of Frs2 binds a region within the

**FIGURE 1. Rewiring Shc and Frs2 adaptor recruitment by engineering the PTB adaptor binding sequence in Ret.** A, peptide spot array interaction analysis. A 12-residue peptide representing the PTB binding domain of RET9 was sequentially mutated at single residues according to the figure. Purified Shc or Frs PTB domain was allowed to interact with the RET peptides, and binding was quantified. **Left membrane**, 2aShcA-PTB/RET interactions. **Right membrane**, 2aFrs2α-HIS-PTB/RET interactions. B, Western blot of RET precipitated by ShcA-GST or Frs2α-HIS PTB domains. RET mutants are as indicated.

**TABLE 1**

| RET mutants | GST-ShcA | HIS-Frs2α |
|-------------|----------|-----------|
| Wild type   | 1.0      | 1.0       |
| W56A        | 1.3      | 0.6       |
| E58D        | 0.95     | 1.1       |
| W56A,E58D   | 1.8      | 0.2       |
| W56A,E58D,I57F | 0.9   | 0.6      |
| W56D        | 1.1      | 0.65      |
| W56S        | 1.1      | 0.7       |
| S54K        | 1.3      | 1.1       |
| T55K        | 1.3      | 1.3       |
| I57K        | 1.5      | 1.0       |
| I57M        | 1.6      | 0.9       |
| R64M        | 1.8      | 1.3       |
| I57S,E58S   | 1.6      | 0.8       |
| I57A,E58G   | 1.0      | 0.6       |
| L61Q        | 0.95     | 1.0       |
| W56A,E58D,I57F,G63T | 1.1    | 0.6     |
| W56A,E58D,I57F,G63W | 1.2    | 0.4     |

**Rewiring RET Receptor Signaling**—Based on the preceding spot array data and published reports on relative affinities of ShcA and Frs2α to the receptors TrkA, TrkB, EGFR, IL4R, InsR, Erb2, and Erb3 and the polyoma middle T antigen (9–11, 46–49), we established point mutations surrounding Tyr-1062 in the RET9 isoform, with the goal of engineering mutant forms of RET that selectively bound one PTB domain protein but not the other.
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and the PTB domain proteins were direct. Extracts from COS-7 cells expressing 2aRetShc+, 2aRetFrs+, 2aRetWt, and 2aRetY1062F were immunoprecipitated for RET protein, and the precipitate was subjected to PAGE and transferred to membranes. Membranes were incubated with purified fusion proteins containing the PTB domains of ShcA or Frs2α, fused to GST, and bound peptides were identified by probing with anti-GST antibodies. Each sample was normalized to the levels of RET loaded in each condition. Both Shc PTB-GST and Frs2α PTB-GST were confirmed to interact directly with Ret. Shc PTB-GST interacted more strongly with 2aRetShc+ than 2aRetWt and significantly more weakly with both 2aRetFrs+ and the PTB-binding site mutant 2aRetY1062F. The Frs2α PTB domain displayed a reduced binding to the 2aRetShc+ and 2aRetY1062F but bound to 2aRetFrs+ to a level similar to that of 2aRetWt (Fig. 2).

Together, the co-precipitation and far Western experiments indicated that specific amino acid changes around the Tyr-1062 site in RET can alter the receptor's affinity for Shc and Frs2. Previous data have suggested that PTB proteins compete for binding to receptor tyrosine kinases (2, 5, 34). We assessed how ShcA and Frs2α might competitively interact with the rewired RET mutants in a GST pull-down assay. To this end, we overexpressed 2aRetShc+ and 2aRetFrs+ in COS-7 cells and assessed their ability to bind immobilized GST fusions containing the PTB domains of either ShcA or Frs2α in the presence of increasing concentrations of purified soluble PTB domains as competitors (Fig. 2, E and F). Thus, in this competitive immunoprecipitation, the GST-tagged ShcA or Frs2α PTB domains (“main peptide”) were competed with increasing concentrations of untagged Frs2α or ShcA-PTB domain at the indicated molar ratio. After pull-down using the GST tag, RET was detected by immunoblotting. The same lysate was used for both ShcA and Frs2α competition at each experimental setup (n = 6 with similar results) (one-way ANOVA; ***p < 0.001). WT, wild type.

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Conversely, purified ShcA-PTB domain was poor in competing...
out the interaction of the GST-Frs2α PTB domain with 2aRetFrs+ yet effectively released 2aRetShc+ from association with the GST-Frs2α PTB domain (Fig. 2F). This competitive binding analysis supports the proposition that it is possible to rewire RET signaling using these effector-biased mutants of Ret.

Selective Shc but Not Frs2 Adaptor Binding to RET Is Required for Cell Survival—Neuronal SK-N-MC cells expressing RetWT, RetFrs+, RetShc+, or RetY1062F (in an otherwise wild type background) were studied in cell survival and proliferation assays. SK-N-MC cells are attractive for the analysis of transfected ret mutants, since they do not express RET endogenously (43). Furthermore, SK-N-MC cells express Shc (44) and Frs2 (data not shown) endogenously. The ability of the different RET receptors to stimulate cell proliferation was first studied. SK-N-MC cells were cultured for 36 h with or without ligand (50 ng/ml GDNF and 100 ng/ml Gfrα1-Fc), and cell numbers were measured. During these culture conditions, there is little or no cell death, and cell numbers should therefore reflect cell proliferation. RetWT, RetFrs+, and RetShc+ elicited a similar ligand-dependent proliferative response. Ligand stimulation of RetY1062F led to a small but consistent increase, suggesting the participation also of Tyr-1062-independent signaling in RET-mediated cell proliferation (Fig. 3A). The various RET receptors were used to study ligand-induced survival from anisomycin-induced apoptosis. The rescue of SK-N-MC cells by activation of RetFrs+, RetShc+, or RetY1062F receptors was compared with that supported by RetWT receptors. RetWT prompted survival of nearly all cells (i.e. close to 100%). RetShc+ elicited a similar level of cell survival as seen with RetWT, whereas RetFrs+ and RetY1062F showed a markedly reduced ability to promote survival in response to ligand stimulation (Fig. 3).

The presence of cells containing activated/cleaved caspase-3 was examined 6 h after anisomycin treatment. Ligand stimulation of RetWT led to a reduced number of cells containing activated caspase-3. A similar reduction in the number of caspase-3-positive cells was seen in ligand-stimulated cells expressing RetShc+, whereas cells expressing RetFrs+ or RetY1062F were similar to RetWT without ligand stimulation (Fig. 3C). Quantitative data confirmed that GDNF reduced the number of cells expressing activated caspase-3 by activation of RetWT and RetShc+ but not RetFrs+ or RetY1062F receptors (Fig. 3D). The ability of the different RET mutants to block anisomycin- and starvation-induced

FIGURE 3. RET-mediated cell survival mediated by recruitment of Shc but not Frs2. A, proliferative response of SK-N-MC cells to ligand stimulation of the different RET receptors. Cells were cultured in the presence or absence of ligand for 36 h, and cell number was quantitated and presented as the percentage of wild-type RET without ligand stimulation (the presence (+) and absence (−) of ligand is indicated). No statistical significance was seen between RET mutants after ligand stimulation. B, induction of cell death by anisomycin in SK-N-MC cells and cell survival by GDNF-induced RetWT, RetShc+, RetFrs+, or RetY1062F activation. Shown is the percentage of rescue of cells following activation of the different RET receptors by the addition of RET ligand (+, presence of ligand; −, absence of ligand) normalized to non-anisomycin-treated cultures set as 100% (one-way ANOVA; ***, p < 0.001). C, micrographs of activated caspase-3 by immunohistochemistry of anisomycin-treated cells expressing RetWT, RetShc+, RetFrs+, or RetY1062F and stimulated with GDNF, as indicated. Green staining is caspase-3 immunoreactivity counterstained to see all cell nuclei with Hoechst 33342 (blue). D, quantification of cells immunoreactive for activated caspase-3/area (3.1 mm²) in anisomycin-treated cells with and without GDNF as indicated. Experiments were performed in triplicates (n = 4 experiments). RetWT values were set to 1, and values were compared with RetWT without ligand (one-way ANOVA; ***, p < 0.001). All cells were counterstained with Hoechst nuclear staining (blue). Scale bar, 40 μm. WT, wild type.
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Expression of Shc Is Required for RET-mediated Survival—To determine the specific role of Shc for the survival effects of Ret, siRNA against Shc was established. When transfected at 6 nM, expression of Shc protein was substantially attenuated as seen by Western blot analysis using an anti-pan-Shc antibody (Fig. 5A inset). In the cell survival assay using SK-N-MC cells, ligand stimulation of RetWT significantly increased survival at both 4 mg/ml and 6 μg/ml of anisomycin, although it was less efficient in rescuing cells at the higher concentration of anisomycin. siRNA against Shc abolished the ligand-induced RET-mediated survival at both concentrations of anisomycin (Fig. 5A). Increasing concentrations of siRNA (12 nM) led to a complete cell death of the SK-N-MC cells also in control cultures without anisomycin. RET activation was insufficient to increase survival in these cultures (data not shown).

PI3K/Akt and MAPK Signaling Pathways in Ret-mediated Cell Survival—To corroborate our results on the role of our rewired RET receptors for cell survival, we examined the intracellular signaling cascades required for Ret-mediated cell survival. SK-N-MC cells in cultures containing anisomycin were grown in the presence or absence of GDNF alone or in combination with PI3K and MAPK pathway inhibitors. Suppressing PI3K (LY294002) markedly attenuated ligand-induced cell survival, and although not as pronounced, a reduced survival was also seen when inhibiting the MAPK pathway (U0126) (Fig. 5B).

Rewiring Adaptor Recruitment Affects Downstream Signaling—We next investigated the overall effect of the mutations on downstream targets. Our biochemical results on the binding of Shc and Frs2 to the RetWT receptors indicated that alteration of downstream signaling may represent the consequence of improved Shc or Frs2 recruitment to Ret. The experiments were performed in both COS-7 cells and HEK293T cells with similar results. Cells expressing RetWT or the mutants were analyzed at different times after stimulation with RET ligands. RetWT receptors showed a clear ligand-induced increase of phosphorylated, active, AKT (P-AKT) over time. GDNF also induced an increase in p42 and p44 ERK phosphorylation by activation of RetWT in the presence of anisomycin with or without GDNF stimulation following anisomycin-induced (A) or serum withdrawal-induced (B) death. Note the pronounced reduction of activated caspase-3 by GDNF activation of RetWT and RetShc compared with ligand unstimulated cultures. The experiments were performed in both COS-7 cells and HEK293T cells with similar results. Cells expressing RetWT or the mutants were analyzed at different times after stimulation with RET ligands. RetWT receptors showed a clear ligand-induced increase of phosphorylated, active, AKT (P-AKT) over time. GDNF also induced an increase in p42 and p44 ERK phosphorylation by activation of RetWT receptors (Fig. 6).

Affects Downstream Signaling—We next investigated the overall effect of the mutations on downstream targets. Our biochemical results on the binding of Shc and Frs2 to the RetWT receptors indicated that alteration of downstream signaling may represent the consequence of improved Shc or Frs2 recruitment to Ret. The experiments were performed in both COS-7 cells and HEK293T cells with similar results. Cells expressing RetWT or the mutants were analyzed at different times after stimulation with RET ligands. RetWT receptors showed a clear ligand-induced increase of phosphorylated, active, AKT (P-AKT) over time. GDNF also induced an increase in p42 and p44 ERK phosphorylation by activation of RetWT receptors (Fig. 6).

FIGURE 4. Activation of caspase-3 in cells by anisomycin and starvation-induced death are prevented by GDNF activation of RetWT and RetShc but not RetFrS2 or RetY1062F. A and B, total protein was measured in lysates, and equal amounts were loaded on gel and subjected to PAGE. Shown are Western blots of activated caspase-3 in cells expressing RetWT, RetShc, RetFrS2, or RetY1062F with or without GDNF stimulation following anisomycin-induced (A) or serum withdrawal-induced (B) death. Note the pronounced reduction of activated caspase-3 by GDNF activation of RetWT and RetShc. C and D, quantification of the results presented as relative increase or decrease of caspase-3 levels by GDNF for anisomycin- or starvation-challenged cells, respectively (n = 4). WT, wild type.

FIGURE 5. Survival effects of RET activation are abolished by interrupting Shc-mediated signaling. A, cells overexpressing RetWT were transfected with siRNA against ShcA and ShcC. Survival was measured after anisomycin-induced apoptosis at the indicated concentrations. The data were normalized to non-anisomycin-treated cultures set as 100%. Inset, Western blot analysis probed using an anti-pan-Shc antibody (detecting ShcA, ShcB, and ShcC) with and without siRNA against Shc, showing the effective reduction of Shc by the siRNA strategy. B, cell survival of SK-N-MC cells expressing RetWT in the presence of anisomycin or without GDNF ligand together with inhibitors for the PI3K and MAPK signaling pathways, LY294002 and U0126, respectively, as indicated. 0, cultures grown without any inhibitor for signaling pathways (one-way ANOVA; *** p < 0.001; ** p < 0.01; * p < 0.05).
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DISCUSSION

The phosphorylated Tyr-1062, which has an N-terminal NXXY motif in the tail of the RET RTK, has the potential to recruit several different effectors through their PTB domains and is critical for the activation of intracellular signaling pathways that mediate the biological effects of RET. Here, we find that RET signaling can be artifically directed to specific effector molecules by introducing selected amino acid mutations in the PTB binding sequence N-terminal to Tyr-1062 of RET. For example, the RETWT mutant exhibits dominant signaling via Shc, whereas Frs2 only interacts very weakly with this receptor.

The FRS2 and Shc PTB domains have overlapping but distinct binding specificities. In phosphopeptide-binding assays, the PTB domain of Shc has distinct affinities for phosphorylated motifs derived from different receptor tyrosine kinases and shows the highest affinity for peptides containing a large aliphatic residue in position −5 (five residues N-terminal to the phosphotyrosine (9, 45), whereas IRS favors binding to sequences with some combination of hydrophobic amino acids at −6, −7, and −8 (5, 46). The insulin receptor binds preferentially to IRS-1 as compared with Shc. Substitution of Ser-960, 5 residues N-terminal to the autophosphorylated Tyr-960, with Ile results in a target specificity of the insulin receptor such that it stably associates with Shc (11). We find a systematic effect on FRS2 binding following a substitution of the −6 residue relative to Tyr-1062 (Trp-1056). Substitution to another aromatic (Phe or Tyr) or hydrophobic (Val, Ile, or Leu) amino acid was tolerated, whereas a substitution to a charged (Lys, Arg, His, Asp, or Glu) or to a small amino acid (Gly or Ala) led to a loss of binding. In contrast to FRS2 binding, Shc binding was not systematically influenced at this amino acid position. Whereas a charged amino acid (Asp) in this position did not significantly affect Shc binding, Ala led to increased Shc binding, showing that the latter single amino acid replacement leads to gain of function for Shc and loss of function for FRS2. Data from the peptide array suggested that the Glu to Asp substitution at −4 (E1058D) would further reduce FRS2 binding to RET. However, in the cell-based analyses, neither FRS2 nor Shc binding was found to be markedly affected by this mutation, but unexpectedly, the increased Shc binding in the RETWT mutant was enhanced by the E1058D mutation. The Glu to Asp mutation introduces an amino acid with one carbon atom shorter side chain but with maintained charge. Other strong Shc bind-

showed up to a 1.5-fold increased activation of AKT as compared with RETWT. Both RETshc+ and RETfrs+ increased activation of ERK more strongly than RETWT, with RETfrs+ being the strongest activator at all time points. RETY1062F did not significantly increase activation of ERK after GDNF application (Fig. 6).

RET has also been shown to activate the p65NFKB and CREB signaling pathways (38). p65NFKB and CREB activation was analyzed by measuring phosphorylated species of both signal transduction molecules (Fig. 6). Phosphorylated 65NFKB (P-p65NFKB) was only weakly activated in RETWT cells at 5 min after GDNF stimulation. RETshc+ showed an increased activation of NFKB at all time points longer than 10 min, whereas RETfrs− was a poor NFKB activator, similar to RETWT (Fig. 6). Phosphorylated CREB (P-CREB) was more robustly increased by GDNF activation of RETWT than p65NFKB, and the increase was seen at all time points after GDNF application. In RETshc+, P-CREB reached increased levels compared with RETWT, starting at 10 min. RETfrs− was the strongest CREB activator, showing increased levels compared with RETWT at several time points after ligand stimulation. Ligand activation of RETY1062F did not activate CREB (Fig. 6). The relation between PTB adaptor recruitment to RET, activation of signaling effectors, and functional outcome is summarized in Fig. 7.

FIGURE 6. Downstream signaling by RETshc+ and RETfrs+. A, cells overexpressing RETWT, RETshc+, RETfrs+, or RETY1062F were collected for analysis at the indicated time points after GDNF stimulation. Western blot analysis was performed for phospho-AKT, phospho-p65NFKB, phospho-p42 and p44 ERK, and phospho-CREB. The blots were then reprobed for AKT and RET to determine equal loading and expression of RET. B–E, quantification of the results from A compared with RETWT, which was set to 1 at the zero time points. WT, wild type; WB, Western blot.
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FIGURE 7. Schematic illustration summarizing the consequence of recruitment of different adaptors to RET for the activation of downstream signaling effectors and cell survival. WT, wild type.

ers, such as ErbB2 and ErbB3 receptors, have an Asp in the corresponding position (10). Predicted Shc loss of function mutations with retained Frs2 binding based on the spot blot array, including T1055K, I1057K, and I1057M, could not be confirmed in cell-based assays. A contributing factor to the low prediction of mutations may be that in the array we assay only for PTB domain binding of Shc, whereas in the cell-based assay (Fig. 1B), a full-length Shc, including an SH2 domain, is present. Although the SH2 domain may not be critical for RET recruitment and activation of Shc, it could add complexity to the binding that could not be measured in the peptide array.

The suggestion that the docking proteins Shc and Frs2 bind to RET in a competitive manner implies that a substitution in RET that biases binding to one of these PTB domain proteins would to some extent occur at the expense of the other. Using both pull-down and far Western experiments, we found that 2aRetShc+ bound at least a 4-fold increased level of Shc compared with Frs2. Furthermore, when purified soluble PTB domains were used in competition experiments, we found that the Frs2 PTB domain was ineffective in competing Shc binding to 2aRetShc+, even when present in a 10-fold molar excess, whereas the Shc PTB domain completely blocked Frs2 binding when present at the lowest tested concentrations. Thus, changes in relative binding of Shc and Frs2 to RET may be further amplified within cells as a result of competition to markedly affect the functional outcome of receptor engagement. The competitive binding of Shc and Frs2 to RET may also hold for other receptors, such as the TrkA receptor (47).

RET mediates an important role by promoting neuronal survival. Whereas the autophosphorylation of Tyr-981, which allows for the direct recruitment of Src, contributes to RET-mediated survival, the major survival signaling arises from Tyr-1062 (48). We examined the functional consequences of selectively recruiting Shc or Frs2 to RET. Our results show that Shc, but not Frs2, is important in signaling downstream of RET to promote cell survival in neuronal SK-N-MC cells. These results underscore the relative importance of Shc recruitment over Frs2 recruitment in the survival-promoting activity of activated RET, arguing that one level of specificity for a subset of receptor tyrosine kinases resides in whether Shc or Frs2 is complexed with the receptor. The different outputs resulting from recruitment of Shc, in contrast to Frs2, are presumably due to differences in the activation of intracellular signaling pathways by Shc or Frs2. Recruitment of either Shc or Frs2 to RET activates the ERK MAPK pathway via Grb2 (44, 49, 50), which depends on Tyr-1062.

Similarly, activation of PI3K and its downstream effector Akt by RET9 is also dependent on Tyr-1062 (39). Shc can recruit two signaling complexes to Ret, one leading to the Ras/ERK pathway by recruitment of Grb2/Sos and another to the PI3K/Akt pathway through recruitment of Grb2/Gab1, followed by p85PI3K (38, 39, 51). When normalized to RetWT at each time point, RetShc+ led to increased activation of ERK and Akt, as well as NFKb that is a downstream target of Akt (52) at 10 min and longer time points. Hence, an increased selectivity for Shc signaling by RET led primarily to a sustained activation of ERK, Akt, and NFKb compared with RetWT. Frs2 interacts with FGFR1 constitutively, independent of ligand activation, but is only able to recruit Grb2 upon fibroblast growth factor stimulation (7, 8). Frs2 bound to RET associates only with Grb2 but not Gab1/2, suggesting that it is mainly involved in the MAPK pathway and not the PI3K/Akt pathway (33). Ligand activation of RetFrs+ led to increased levels of p42 and p44 ERK MAPK as well as CREB. pAkt levels were similar in RetFrs and RetShc at time points later than 10 min after the addition of ligand. Therefore, association of Shc and Frs2 upon ligand binding to RET leads to signaling with different kinetics and, in part, through different intracellular pathways. The increased affinity for Shc and Akt activation by RetShc+ suggests that it could also have an elevated ability to promote survival compared with RetWT. However, we were unable to address this issue, since RetWT elicited close to 100% survival. The activation of Akt and stimulation of cell survival by RetShc+ but not RetFrs+ is consistent with the well-established role of Akt in antagonizing apoptosis (53, 54), which is mediated by inhibitory phosphorylation of the proapoptotic proteins BAD and caspase-9 and -3.
and also by activation of NFκB (55). Our data show that Frs2 recruitment is dispensable for Ret-mediated survival in SK-N-MC cells and suggest that ERK signaling plays a minor role. The results shows that Shc recruitment by RET is both required and sufficient for cell survival partly via activation of PI3K/Akt but possibly also via other Shc-activated signaling pathways, such as, for instance, NFκB.

The alternative splicing of the ret transcript results in two major forms of RET protein products, RET9 and RET51, whose C-terminal amino acid tails diverge 1 amino acid after Tyr-1062. RET9 has 9 amino acids, and RET51 has 51 amino acids C-terminal of Tyr-1062. We have studied the role of Frs2 and Shc recruitment to RET9 for its role in stimulating cell survival. RET9 is the most abundantly expressed isoform, and although the two isoforms behave similarly in several in vitro assays, they differ markedly in their ability to support embryonic development. By using gene targeting to establish monoisogetic ret mice, it was shown that RET9 is sufficient to support normal development, whereas mice expressing only RET51 have severe defects in the gut and renal development as a consequence of failure of ureteric bud initiation and branching (56), showing that RET9 is the major functional isoform in vivo. The RET9 and RET51 isoforms differ in their abilities to recruit Shc and Frs2. RET9 binds both Shc and Frs2 more strongly than does RET51 (33). The stronger interaction between Frs2 and RET9 appears to fully depend on the PTB domain of Frs2, since similar results are obtained using full-length and only the Frs2-PTB domain. The stronger binding of full-length Shc than the Shc-PTB domain to RET9, as compared with RET51 (39, 51, 57), indicates that whereas C-terminal amino acids that diverge between RET9 and RET51 contribute to Frs2-PTB domain binding to Ret, the increased Shc binding in RET9 as compared with RET51 occurs independently of the PTB domain interaction with Ret. Shc also has a SH2 domain interaction site in RET9 but not RET51 immediately C-terminal of Tyr-1062 that could account for a PTB domain-independent binding of Shc to RET9. Differences in PTB domain-dependent adaptor binding could, together with a shorter half-life of RET51 than RET9 due to increased ubiquitination (41), contribute to the functional differences in vivo.

The molecular mechanisms creating specificity in the versatile functions of RTKs in vivo for stimulating directed cell migration, proliferation, survival, and other cellular and molecular pathways are largely unknown. Our data suggest that the specific recruitment of adaptor proteins by RET is based on their competition for a single binding motif, and this can lead to both qualitative and quantitative features of signal transduction that are translated into a selective biological response. Therefore, the functional specificity of RTKs, such as RET, partly resides in the sequences of their PTB domain-binding sites, which result in distinctive recruitment profiles of PTB domain proteins. For the RET receptor, recruitment of Shc but not Frs2 is both necessary and sufficient to promote cell survival.

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