THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 4, Issue of January 24, pp. 2199–2206, 1997

Printed in U.S.A.

Functional Characterization of an Epidermal Growth Factor Receptor/RET Chimera*

(Received for publication, June 26, 1996, and in revised form, October 28, 1996)

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The *RET* (recombined in transfection) gene encodes a receptor tyrosine kinase homolog involved in innervation of the gut and renal development. A chimeric epidermal growth factor receptor (EGFR)/RET receptor was constructed which contained the extracellular and transmembrane domains of the EGF receptor fused to the intracellular domain of *RET*. This construct was expressed in NIH 3T3 cells, and the functional properties of the receptor were characterized and compared with those of the wild type EGF receptor. Whereas the EGF receptor exhibited both high and low affinity binding sites for ^{125}I-EGF, the EGFR/RET chimera exhibited only low affinity binding of ^{125}I-EGF. The chimera was able to internalize EGF more rapidly than the wild type EGF receptor and recycled to the cell surface at twice the rate of the EGF receptor. Pulse-chase experiments indicated that EGF stimulated the degradation of the wild type EGF receptor but had no effect on the rate of degradation of the EGFR/RET receptor. The combination of increased recycling and decreased degradation resulted in the relatively inefficient down-regulation of the EGFR/RET chimera. Incubation of cells expressing the wild type EGF receptor with phorbol 12-myristate 13-acetate led to a reduction in ^{125}I-EGF binding and a loss in EGF-stimulated tyrosine phosphorylation. However, phorbol 12-myristate 13-acetate treatment had only a limited effect on EGF binding and EGF-stimulated tyrosine kinase activity in cells expressing EGFR/RET chimeras. These findings suggest that the ret tyrosine kinase is not regulated by many of the common mechanisms used to terminate signaling via growth factor receptors. Such persistent activation of the Ret tyrosine kinase may be relevant to the physiological function of Ret in cells that normally express this growth factor receptor.

The *RET* gene encodes a receptor tyrosine kinase homolog (1, 2). *RET* was first identified in DNA transfection studies in which the gene had been recombined in transfection to generate a fusion protein that induced the transformation of NIH 3T3 cells (3). Subsequent studies have shown that activating mutations in the *RET* gene are responsible for several dominantly inherited human neoplasias including multiple endocrine neoplasia type 2A, multiple endocrine neoplasia type 2B, and familial medullary thyroid carcinoma (4–8). These conditions are all characterized by medullary thyroid carcinoma with variable involvement of other endocrine tissues such as the adrenal gland and the parathyroid gland.

Inactivating mutations in *RET* result in Hirschsprung disease (9–11). Hirschsprung disease is characterized by megacolon resulting from a failure to innervate the gut. *RET* knockout mice also exhibit megacolon, confirming the involvement of *RET* in this symptom, but their most remarkable phenotype is renal agenesis (12). The association of mutations in *RET* in neoplasia as well as Hirschsprung disease suggests that this tyrosine kinase is important in cell proliferation and development.

Because the ligand for Ret has only recently been identified (13–17), chimeric receptors containing the extracellular domain of the EGF[1] receptor and the intracellular domain of Ret have been used to characterize the ligand-activated form of the Ret tyrosine kinase. Santoro *et al.* (18) expressed an EGFR/RET chimera in NIH 3T3 cells and reported that EGF stimulated the tyrosine kinase activity of EGFR/RET and enhanced the exchange of GDP for GTP on Ras in these cells. However, the chimeric receptor failed to stimulate MAP kinase activity or phosphatidylinositol 3-kinase activity. van Weering *et al.* (19) expressed a similar EGFR/RET chimera in SK-N-MC cells, a neuroectodermal cell line. In this system, EGF stimulated the tyrosine kinase activity of the EGFR/RET chimera and also led to the sustained activation of MAP kinase. The authors suggested that the differences observed between their cell lines and those of Santoro *et al.* (18) indicated a cell type-specific ability of Ret to stimulate MAP kinase activation. Despite the fact that EGF failed to stimulate MAP kinase in 3T3 cells expressing the EGFR/RET chimera, this growth factor did induce both proliferation and transformation of these cells (18), suggesting that activation of MAP kinase may not be required for Ret to promote proliferation of 3T3 cells.

In this report, we functionally characterize a chimeric EGFR/RET receptor expressed in NIH 3T3 cells with respect to its ligand binding and internalization properties. In addition, regulation of the EGFR/RET chimera via receptor down-regulation and protein kinase C-dependent mechanisms was investigated. The findings suggest that many of the common mechanisms used to terminate signaling via growth factor receptors do not function in the Ret tyrosine kinase system. The absence of such termination events suggests that prolonged activation of Ret and/or a maintenance of sensitivity to Ret ligand may be physiologically important for cells that normally express this growth factor receptor.

* This work was supported in part by National Institutes of Health Grant P01CA5352 (to H. D.-K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; MAP, mitogen-activated protein; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; PMA, phorbol 12-myristate 13-acetate; GDNF, glial cell-derived neurotrophic factor.

EXPERIMENTAL PROCEDURES

Materials

NIH 3T3 cells expressing wild type EGF receptors were as described previously (20). Antibodies to Ret and Ras-GAP were purchased from Santa Cruz Biotechnology, Inc. Anti-phosphotyrosine antibodies (PY-20) were from Transduction Laboratories. [^{35}S]Methionine was from Amersham. Polyclonal antibodies against the EGF receptor (DB1) and ^{125}I-EGF were prepared as described previously (21). Western blots were developed using enhanced chemiluminescence reagents from Amersham. All other reagents were from Sigma Chemical Co.

Methods

Construction of EGFR/RET Chimera—The extracellular and transmembrane domains of EGF receptor (nucleotides 1–2225) (22) were fused in-frame with the intracellular domain of short form of Ret (RET9, nucleotides 2122–end) (23). The fusion was done by generating an in-frame *Sal*I site as described previously by Santoro *et al.* (18). In addition, the carboxyl terminus of the construct was epitope tagged with the 9 amino acids of influenza hemagglutinin tag (HA tag).

An *Xba*I-*Bam*HI fragment containing the first 1347 nucleotides of the EGF receptor was ligated with an 873-base pair *Bam*HI-*Sal*I fragment to generate the entire extracellular and transmembrane domains of EGF receptor (plasmid pSDP10) with an engineered *Sal*I site at the 3′ end. The 873-base pair *Bam*HI-*Sal*I fragment was generated by polymerase chain reaction using primers corresponding to nucleotides 1331–1350 of the EGF receptor and a primer sequence (5′-CCTCTCCTGCAGCAGTCGACGCAGCGTGCGC-3′) to create the *Sal*I site (underlined) at nucleotide 2219 (amino acid 657) of the EGF receptor sequence.

A *Hin*dIII-*Nsi*I fragment containing full-length Ret was obtained by restriction digestion of pSV2c-ret (24). This fragment was ligated to a 250-base pair *Nsi*I-*Not*I fragment containing the HA tag generated by polymerase chain reaction using pSV2c-ret DNA. The HA tag fragment was generated using primer 4623 (5′-TCCCATGCATTTACTAGATTCTACCCTTACGATGTACCGGATTACGCATAGCACCGCTGTCCCCTCTGC-3′), which contains an *Nsi*I site (underlined) and inserts the nine amino acids of the HA tag (underlined) immediately before the stop codon of RET9, and primer 4646 (5′-CCGGGCGGCCGCCCAACTCGCGAGGGGATCGAG-3′), which introduces an *Not*I site (underlined) and is in the 5′ end of the neomycin gene of pSV2c-ret. This construct was placed in pSK1 Bluescript to generate the full-length epitope-tagged form of RET.

To generate an *Sal*I site in Ret at the junction point, polymerase chain reaction primers 4499 (5′-ATGTCGACACTGCTACCACAAGTTTGCC-3′, nucleotides 2122–2142 of RET) and 4500 (antisense nucleotides 2318–2298) were used to amplify a ~200-base pair fragment that contained an engineered *Sal*I site. The polymerase chain reaction product was digested with *Sal*I and *Bam*HI. This linker fragment was then ligated to a *Bam*HI-*Not*I fragment (from epitope-tagged RET) and *Sal*I-*Not*I-digested pSK1 Bluescript in a three-fragment ligation to generate plasmid pSDP18.

The final chimeric EGFR/RET construct was then generated by ligating an *Sac*II-*Sal*I fragment from pSDP10 (5′ end of the EGF receptor) with the *Sal*I-*Not*I fragment from pSDP18 (3′ end of epitope-tagged RET) and *Sac*II-*Not*I-digested pM2-HA vector (25) in a three-fragment ligation to generate plasmid pSDP24.

Cell Culture and Transfection—NIH 3T3 cells were grown in DMEM containing 10% calf serum and transfected with the appropriate plasmids using a calcium phosphate precipitation method (26). After 2 days, cells were trypsinized and replated in media containing 400 μg/ml G418. Individual colonies were selected after 2–3 weeks of growth in G418-containing medium.

^{125}I-EGF Binding—For Scatchard analyses, cells in six-well dishes were incubated for 2 h at 4 °C in DMEM containing 40 mM HEPES, pH 7.4, 0.1% bovine serum albumin (HEPES-binding medium) plus 50 pM ^{125}I-EGF and increasing concentrations of unlabeled EGF. At the end of the incubation, cells were washed three times in HBSS and were dissolved in 1 ml of 1 M NaOH. The solubilized material was counted for ^{125}I in a γ counter. Data were analyzed using the LIGAND computer program (27).

For ^{125}I-EGF internalization, cells in six-well dishes were washed twice with warmed HBSS and then incubated for the indicated period of time with 1 nM ^{125}I-EGF at 37 °C in HEPES-binding medium. Nonspecific binding was determined in duplicate wells containing 100 nM unlabeled EGF. At each time point, half of the cultures were washed three times in HBSS prior to dissolution in 1 N NaOH and γ counting. This provided a measure of total cell-associated ^{125}I-EGF. The other half of the cultures were washed twice for 2 min in acid wash buffer (50 mM glycine, 100 mM NaCl, pH 3.0) prior to dissolution and γ counting. This quantitated the internalized radioligand. For In/Sur plots, the surface ^{125}I-EGF was calculated by subtracting the internalized ^{125}I-EGF from the total cell-associated ^{125}I-EGF.

Down-regulation—Cells were incubated with 2 or 25 nM EGF for the indicated time at 37 °C. Cultures were then washed twice at 4 °C with cold HBSS, twice for 2 min with acid wash buffer, and twice with HBSS. Monolayers were then incubated for 2 h at 4 °C with 50 pM ^{125}I-EGF. Total ^{125}I-EGF binding was determined as indicated above following three washes with ice-cold HBSS. Nonspecific binding was determined in duplicate wells containing 100 nM unlabeled EGF and was subtracted from total binding to yield specific ^{125}I-EGF binding at each time point.

Cell Lysates—Cells in 60-mm dishes were incubated in DMEM containing 0.1% bovine serum albumin for 1 h at 37 °C prior to use. EGF (50 nM) was added directly to the incubation buffer, and cells were incubated for the indicated times at 37 °C. At the end of the incubation, cells were washed once in ice-cold HBSS and lysed by scraping into 300 μl of RIPA buffer (10 mM Tris, pH 7.2, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 500 μM sodium orthovanadate, and 10 mM *p*-nitrophenylphosphate. Lysates were incubated on ice for 10 min with periodic vortexing and then clarified by centrifugation at 12,000 × *g* for 10 min.

Recycling and Degradation of ^{125}I-EGF—Cells were plated in six-well dishes and were preloaded with ^{125}I-EGF by incubation for 20 min at 37 °C in the presence of 1 nM ^{125}I-EGF. Monolayers were subsequently washed twice in HBSS, twice in acid wash buffer, and twice in HBSS. All washes were carried out at 4 °C. At time zero prewarmed DMEM containing 0.1% bovine serum albumin was added to the cultures, and the cells were shifted to 37 °C. At the indicated times following the shift to 37 °C, the culture medium was removed. Half was counted for ^{125}I to determine the total amount of radiolabel released from the cells. The other half of the medium was precipitated with 10% trichloroacetic acid to determine the fraction of released ^{125}I which represented intact, precipitable ^{125}I-EGF.

Western Blot Analysis—Aliquots of cell lysates containing 100 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis on 10% gels and then electrophoretically transferred to nitrocellulose. The nitrocellulose was blocked using 10% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20. Nitrocellulose membranes were incubated with primary antibody for 1 h at room temperature, washed, and bands visualized using enhanced chemiluminescence according to the manufacturer's instructions.

[^{35}S]Methionine Metabolic Labeling—Cells were plated in D60 plates and grown for 3 days in the presence of standard growth medium containing 100 μCi of [^{35}S]methionine. At time zero the labeling medium was removed and replaced with DMEM containing 0.1% bovine serum albumin in the absence or presence of 50 nM EGF. At the indicated times cultures were extracted with RIPA buffer and the lysates frozen in liquid nitrogen. When all cultures had been processed the lysates were thawed and assayed for protein. Aliquots of cell lysate containing 350 μg of protein were then immunoprecipitated with an anti-EGF receptor antibody or an anti-RET antibody. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Bands corresponding to the EGF receptor or the EGFR/RET chimera were excised from the gel and counted for ^{35}S.

RESULTS

Ligand Binding and Tyrosine Phosphorylation—Constructs encoding the wild type EGF receptor or the EGFR/RET chimera were expressed in NIH 3T3 cells, and stable clones were selected as described under "Experimental Procedures." Fig. 1 shows Scatchard plots for the binding of ^{125}I-EGF to cells expressing either the wild type EGF receptor or the EGFR/RET chimera. Binding of ^{125}I-EGF to the wild type EGF receptor resulted in a curvilinear Scatchard plot that could be resolved into two classes of binding sites. The high affinity site accounted for 15–20% of the total sites and exhibited a K_d of 0.2 nM. The low affinity site exhibited a K_d of approximately 4 nM. By contrast, binding of ^{125}I-EGF to cells expressing the EGFR/RET chimera generated a linear Scatchard plot indicative of a single class of sites with a K_d essentially identical to that of the low affinity site seen for the wild type EGF receptors. The total number of cell surface receptors was similar in the clones

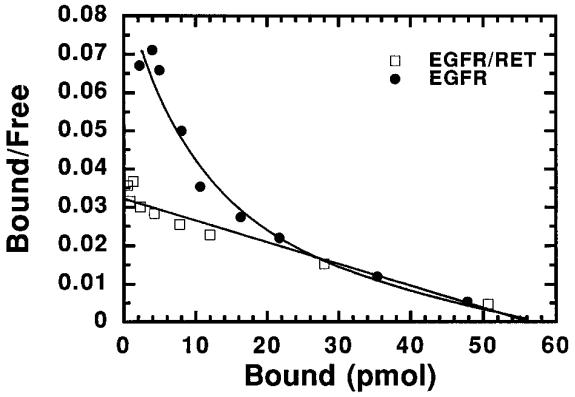

FIG. 1. **Scatchard plot for the binding of ^{125}I-EGF to cells expressing wild type EGF receptors or EGFR/RET chimeras.** Cells were plated in 35-mm dishes and cultured for 2 days prior to use. ^{125}I-EGF binding and Scatchard analyses were carried out as described under "Experimental Procedures." *Points* represent the mean of triplicate determinations.

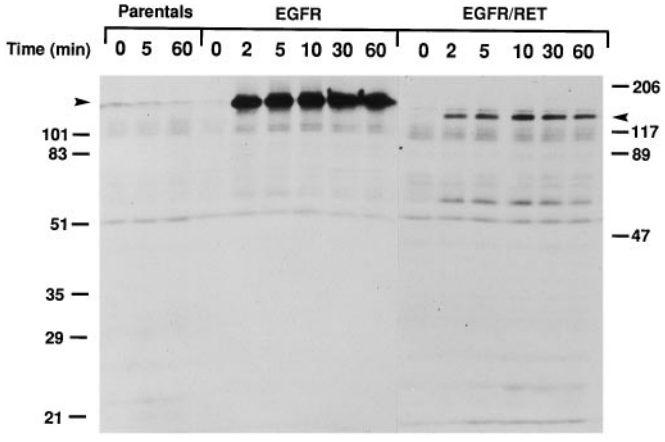

FIG. 2. **EGF-stimulated tyrosine phosphorylation in cells expressing wild type EGF receptors or EGFR/RET chimeras.** Parental NIH 3T3 cells or NIH 3T3 cells expressing the wild type EGF receptor or the EGFR/RET chimera were treated with 50 nM EGF for the indicated times. Lysates were prepared in RIPA buffer and analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blotting using an anti-phosphotyrosine antibody. *Arrowheads* indicate the location of the EGF receptor (*left arrowhead*) or the EGFR/RET chimera (*right arrowhead*). Molecular masses of marker proteins (in kDa) are indicated on the *right* and *left* of the *panel*.

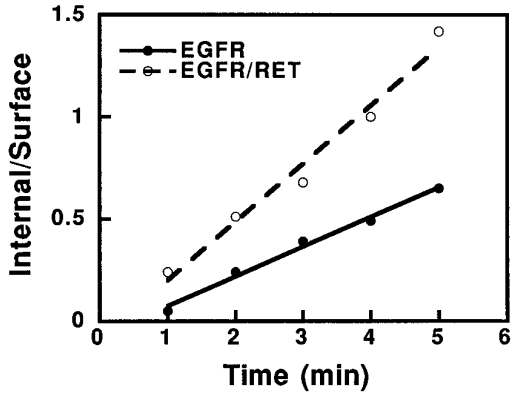

FIG. 3. **Internalization of ^{125}I-EGF by the wild type EGF receptor or the EGFR/RET chimera.** Cells were plated in 35-mm dishes and cultured for 2 days prior to use. Monolayers were washed twice in warm HBSS and transferred to warm HEPES-binding medium containing 1 nM ^{125}I-EGF. Cells were incubated at 37 °C for the indicated times. Total cell-associated ^{125}I-EGF and internalized ^{125}I-EGF were then determined as described under "Experimental Procedures." Cell surface counts were calculated by subtracting the counts internalized from the total cell-associated counts. The results are reported as the ratio of the internalized counts to the counts present on the cell surface at the indicated time. *Points* represent the mean of triplicate determinations.

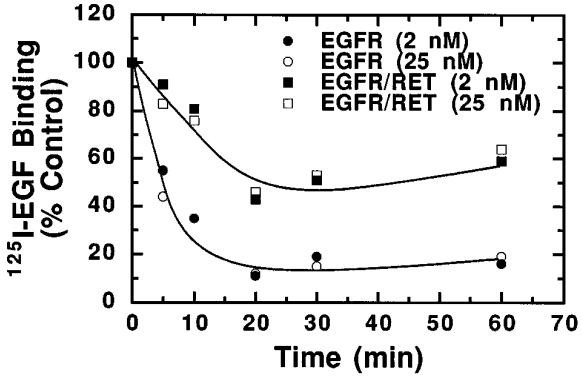

FIG. 4. **Short term down-regulation of the EGF receptor and the EGFR/RET chimeric receptor.** Cells grown on 35-mm plates were treated with 2 or 25 nM EGF at 37 °C for the indicated time. Monolayers were subsequently washed with cold HBSS and acid wash buffer to remove cell surface EGF. Binding of 50 pM ^{125}I-EGF was then determined as outlined under "Experimental Procedures." Results represent the mean of triplicate determinations.

expressing the wild type EGF receptor and the EGFR/RET chimera and was approximately 100,000 receptors/cell.

Treatment with EGF led to the enhancement of tyrosine phosphorylation in cells expressing either the wild type EGF receptor or the EGFR/RET chimera. As shown in Fig. 2, the EGF receptor and the EGFR/RET chimera were the principal targets of tyrosine phosphorylation in the cells. Despite the fact that the two cell lines express similar numbers of their respective receptors, the level of autophosphorylation of the EGFR/RET chimeras was substantially lower than the level of phosphorylation of the wild type EGF receptors. Nonetheless, the EGF-stimulated phosphorylation of other cellular proteins was largely the same in the two cell lines. In some experiments, a protein with a molecular weight of ~65,000 was phosphorylated more strongly in cells expressing the EGFR/RET chimera than in cells expressing the EGF receptor (see Fig. 2). This protein was identified as the 62-kDa Ras-GAP-associated protein by immunoprecipitation (data not shown).

Internalization and Down-regulation—The ability of wild type EGF receptors and EGFR/RET chimeras to mediate internalization of ^{125}I-EGF was examined next. Cells were incubated in the presence of 1 nM ^{125}I-EGF at 37 °C for increasing lengths of time, and the internalized ^{125}I-EGF was measured as described under "Experimental Procedures." The data were plotted as the ratio of the internalized ligand to the surface bound ligand (Fig. 3) (28). The data demonstrate that both the EGF receptor and the EGFR/RET chimera readily internalized ^{125}I-EGF. However, the rate of internalization of ligand was 2-fold higher for the chimeric receptor than for the wild type EGF receptor (0.28 min^{-1} *versus* 0.14 min^{-1} for the chimera and the EGF receptor, respectively).

The internalization of ligand-receptor complexes leads to receptor down-regulation if the internalized receptors are targeted to the lysosomes for degradation. To determine whether ligand internalization led to the down-regulation of the EGFR/RET chimera, cells expressing wild type EGF receptors or EGFR/RET chimeras were treated with 2 or 25 nM EGF for increasing periods of time at 37 °C. Following a wash with low pH buffer to remove surface-bound EGF, residual cell surface receptors were detected by ^{125}I-EGF binding (Fig. 4). Pretreatment of cells expressing the wild type EGF receptor led to the

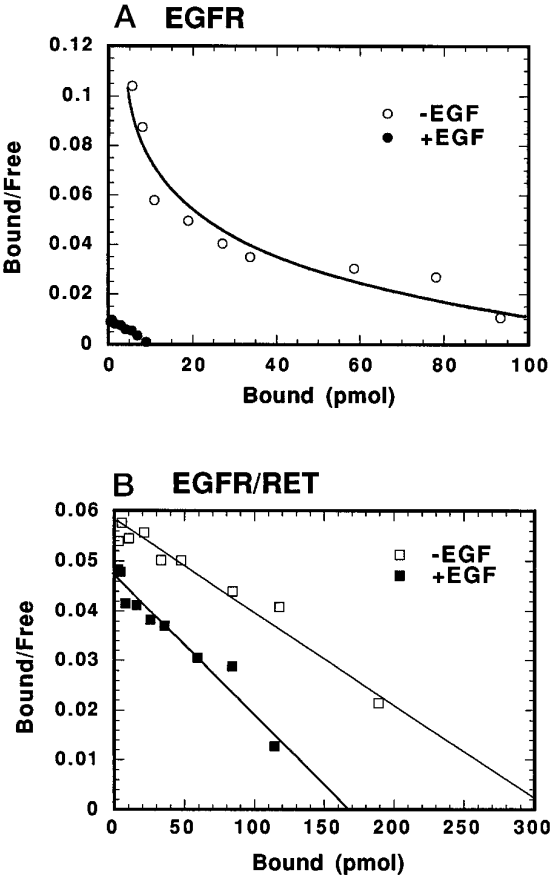

FIG. 5. **Scatchard analysis of cells expressing EGF receptors or EGFR/RET chimeric receptors after a 24-h incubation with EGF.** Cells expressing wild type EGF receptors or EGFR/RET chimeras were plated on 35-mm plates and grown to confluence. Cultures were then incubated for 24 h in the absence (*open symbols*) or presence (*closed symbols*) of 50 nM EGF. Monolayers were washed with cold HBSS and acid wash buffer to remove cell surface EGF. ^{125}I-EGF binding and Scatchard analyses were then performed as outlined under "Experimental Procedures." Values represent the mean of triplicate determinations and have been corrected for the ~30% increase in cell number which occurred in each cell line in the presence of EGF. Cells expressing wild type EGF receptors grew to a density of 4×10^5 cells/well in the presence of EGF. Cells expressing EGFR/RET chimeras grew to a density of 9×10^5 cells/well in the presence of EGF.

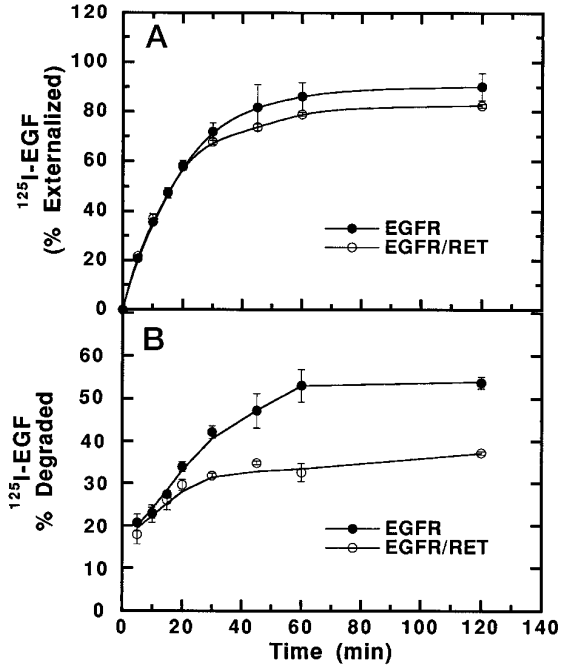

FIG. 6. **Recycling and degradation of ^{125}I-EGF.** Cells expressing wild type EGF receptors or EGFR/RET chimeric receptors were preloaded with ^{125}I-EGF as described under "Experimental Procedures." At the indicated times after shift of the cultures to 37 °C, the culture medium was collected and an aliquot counted for ^{125}I to determine the fraction of internalized ^{125}I which had been released into the culture medium (*panel A*). A second aliquot was subjected to precipitation with 10% trichloroacetic acid, and the trichloroacetic acid-soluble supernatant was counted to determine the fraction of released ^{125}I which represented degraded forms of ^{125}I-EGF (*panel B*). Points represent the mean ± S.D. of triplicate determinations.

rapid loss of 80–90% of the cell surface ^{125}I-EGF binding. The EGFR/RET chimera also underwent down-regulation under these conditions. However, the maximal extent of down-regulation was only half that seen in cells expressing the wild type EGF receptor. This difference was not the result of differences in the affinity of the two receptors for EGF as similar results were seen when subsaturating (2 nM) and saturating (25 nM) concentrations of EGF were used to down-regulate the receptor.

Longer incubations of cells with EGF produced similar results. Treatment of cells with 50 nM EGF for times up to 30 h led to a progressive loss in binding of ^{125}I-EGF to cell surface receptors. Scatchard analyses of cells expressing wild type EGF receptors indicated that treatment with EGF for 24 h led to the loss of approximately 90% of the cell surface EGF receptors, including all of the high affinity receptors (Fig. 5*A*). Similar analyses of cells expressing the EGFR/RET chimeras demonstrated that only about 50% of the cell surface receptors were lost after 24 h of EGF treatment (Fig. 5*B*).

The inefficient down-regulation of the EGFR/RET chimera could be the result of either enhanced recycling of the chimera to the cell surface or decreased degradation of the receptor or both. To examine the first possibility, cells were preloaded with ^{125}I-EGF by incubation with radioligand for 20 min at 37 °C. Residual cell surface ^{125}I-EGF was then removed by washing with a low pH buffer at 4 °C. Cells were shifted to 37 °C, and the externalization of ^{125}I was followed. As shown in Fig. 6*A*, the rate of release of ^{125}I into the culture medium was similar in cells expressing the wild type EGF receptor and the EGFR/RET chimera. However, in cells expressing the wild type EGF receptor, approximately 60% of the released material represented degraded forms of ^{125}I-EGF, whereas for cells expressing the EGFR/RET chimera, only ~30% of the externalized material was degraded (Fig. 6*B*). These data indicate that ^{125}I-EGF is mainly recycled intact by the EGFR/RET chimera, presumably due to recycling of the receptor to the cell surface and release of bound ligand.

To determine whether decreased receptor degradation also contributed to the inefficient down-regulation of the EGFR/RET chimera, the rates of degradation of wild type EGF receptor and EGFR/RET chimeric receptor were compared in an [^{35}S]methionine pulse-chase experiment. As shown in Fig. 7, wild type EGF receptors exhibited a half-life of approximately 11 h in the absence of EGF. This decreased to about 4.5 h in the presence of EGF. Thus, ligand binding enhanced the rate of degradation of the EGF receptor. By contrast, the addition of ligand had no effect on the rate of degradation of the EGFR/RET chimera, which exhibited a half-life of 16 h in the absence or presence of EGF.

Western blot analyses of total cellular EGF receptors were consistent with these data. When cells expressing wild type EGF receptors were incubated with 50 nM EGF for periods of time ranging from 0 to 24 h, there was a consistent decline in receptor levels throughout the time course (Fig. 8*A*). Receptor

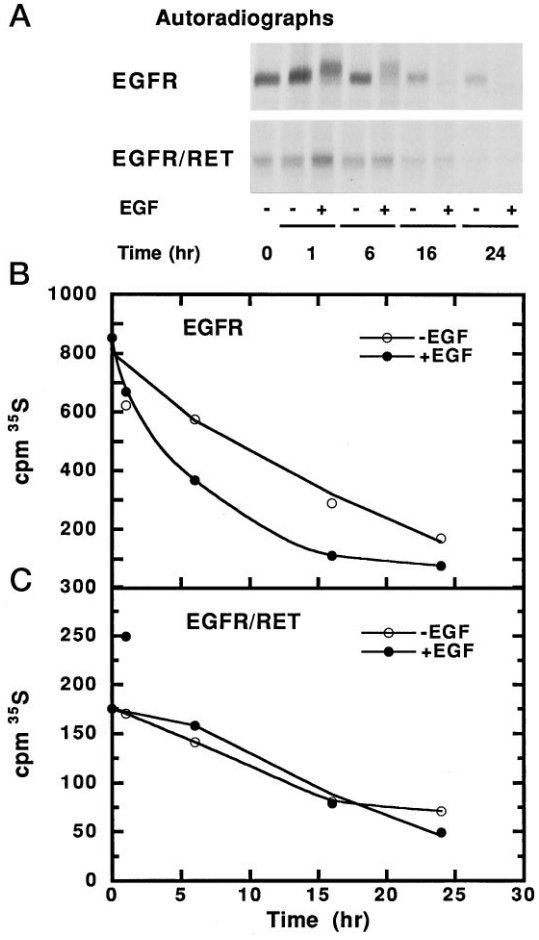

FIG. 7. **Degradation of EGF receptors and EGFR/RET chimeric receptors.** Cells expressing wild type EGF receptors or EGFR/RET chimeric receptors were metabolically labeled with [^{35}S]methionine. At time zero the labeling medium was replaced with standard culture medium containing vehicle (*open circles*) or 50 nM EGF (*closed circles*). At the indicated times, cells were lysed in RIPA buffer and immunoprecipitated using and anti-EGFR antibody or an anti-ret antibody. Immunoprecipitates were run on 10% SDS-polyacrylamide gels and subjected to autoradiography. *Panel A*, autoradiographs of the SDS gel. *Panel B*, bands representing the EGF receptor or the EGFR/RET chimeric receptor were excised from the gel and counted for ^{35}S.

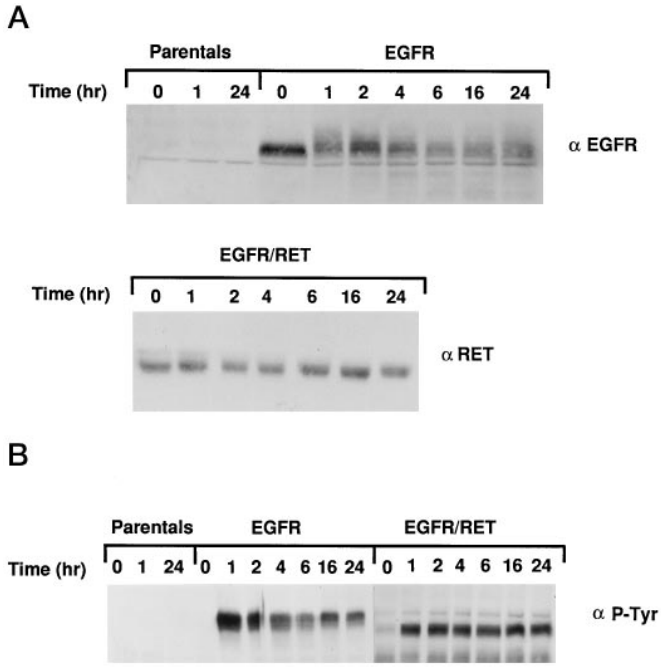

FIG. 8. **Receptor levels and autophosphorylation during long term treatment with EGF.** Parental cells or cells expressing wild type EGF receptors or EGFR/RET chimeric receptors were grown for the indicated times in the presence of 50 nM EGF. Lysates were then prepared and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose and analyzed by Western blotting using an anti-EGF receptor or anti-Ret antibody (*panel A*) or an anti-phosphotyrosine antibody (*panel B*).

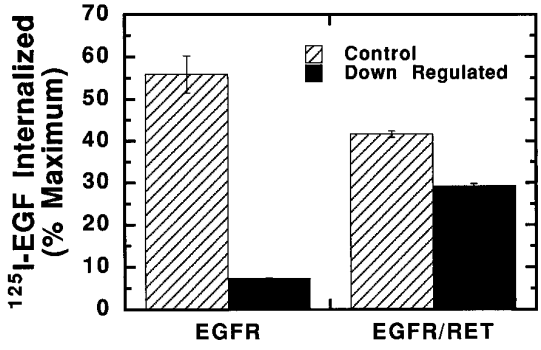

FIG. 9. **Down-regulation of ^{125}I-EGF internalization by wild type EGF receptors and EGFR/RET chimeric receptors.** Cells expressing EGF receptors or EGFR/RET chimeras were incubated in the absence (*Control*) or presence (*Down Regulated*) of 25 nM EGF for 20 min at 37 °C. Monolayers were washed with cold HBSS and acid wash buffer to remove cell surface EGF. Cells were then incubated in HEPES-binding medium at 37 °C for 5 min in the presence of 1 nM ^{125}I-EGF. Total cell-associated ^{125}I-EGF and internalized ^{125}I-EGF were determined as described under "Experimental Procedures." Results represent the mean ± S.D. of triplicate determinations.

autophosphorylation paralleled total receptor levels with wild type EGF receptors exhibiting a slow decline in tyrosine phosphorylation over the 24-h time period (Fig. 8*B*). Western blots using anti-Ret antibodies revealed that the total cellular levels of the EGFR/RET chimera varied little throughout the 24-h incubation with EGF, confirming the lack of effect of EGF on the rate of chimeric receptor degradation. Surprisingly, the EGFR/RET chimeras remained fully autophosphorylated throughout the entire time course, indicating that the internalized receptors were active and capable of signaling.

Desensitization—Prolonged treatment with agonist often leads not only to receptor down-regulation but also to receptor desensitization. Receptor desensitization refers to the situation wherein receptors, although present on the cell surface, are not functional and are unable to transduce a signal. To determine whether the residual EGFR/RET chimeric receptors were still functional, cells expressing wild type EGF receptors or EGFR/RET chimeric receptors were treated with 25 nM EGF for 20 min to induce down-regulation of cell surface receptors. After a wash with a low pH buffer, ^{125}I-EGF was added to the cells, and the internalization of this ligand was assessed after 5 min at 37 °C. As shown in Fig. 9, pretreatment with EGF of cells expressing the wild type EGF receptor led to a 90% loss in the ability of the receptor to internalize ^{125}I-EGF, presumably due to the internalization and down-regulation of the bulk of the cell surface receptors. Although the cells expressing the EGFR/RET chimera exhibited approximately a 40–50% loss in cell surface receptor number (Fig. 4), their ability to internalize ^{125}I-EGF subsequently was reduced by only ~25% compared with controls. These results indicate that the residual cell surface EGFR/RET chimeras remain functional after down-regulation has occurred.

Regulation by PMA—PMA is a tumor-promoting phorbol ester that stimulates the activity of protein kinase C and leads to the phosphorylation of threonine residues within the EGF receptor (29). Treatment of cells with PMA results in the loss of

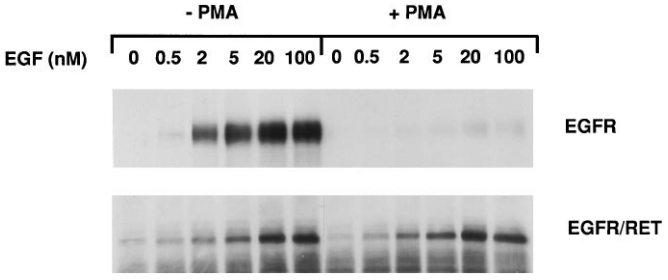

FIG. 10. **Effect of PMA on receptor autophosphorylation.** Cells expressing wild type EGF receptors or EGFR/RET chimeric receptors were treated with 100 nM PMA for 30 min at 37 °C. Cultures were then incubated with increasing concentrations of EGF for 5 min at 37 °C, and lysates were prepared. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Tyrosine phosphorylation was visualized by Western blotting with an anti-phosphotyrosine antibody.

cell surface EGF receptors and an inhibition of EGF-stimulated tyrosine kinase activity (30–33). To determine whether the Ret tyrosine kinase was also subject to regulation by a protein kinase C-mediated pathway, cells expressing wild type EGF receptors or EGFR/RET chimeras were treated for 30 min at 37 °C in the absence or presence of 100 nM PMA and analyzed for ^{125}I-EGF binding. Treatment with PMA resulted in the loss of 90% of ^{125}I-EGF binding to the wild type EGF receptor. Scatchard analyses indicated that the decrease in binding was due to the loss of all high affinity and most low affinity sites as well a reduction in the affinity of the low affinity sites for EGF from 3.8 ± 0.2 nM to approximately 15 ± 3 nM (n = 2). The effect of PMA on the binding of ^{125}I-EGF to cells expressing EGFR/RET chimeras was limited and consisted solely of a small decrease in the affinity of the single class of binding sites for EGF from 5 ± 1 nM to 8 ± 2 nM (n = 2) (data not shown).

Fig. 10 shows the dose-response curves to EGF for the stimulation of receptor autophosphorylation in cells treated with or without 100 nM PMA. In cells expressing the wild type EGF receptor, prior treatment with PMA significantly blocked EGF-stimulated autophosphorylation at every level of EGF examined. By contrast, pretreatment with PMA had little effect on the ability of EGF to stimulate autophosphorylation in cells expressing the EGFR/RET chimera. Thus, the activity of the ret tyrosine kinase does not appear to be modulated via a protein kinase C-dependent mechanism.

DISCUSSION

Previous studies of EGFR/RET chimeras have demonstrated that the chimeras respond to EGF and mediate the activation of a variety of downstream signaling pathways including the activation of phospholipase C, Ras, and MAP kinase (18, 19). The studies reported here extend these findings by providing insight into the function and regulation of the EGFR/RET chimera.

Many growth factor receptor kinases, including the EGF receptor, exhibit high and low affinity binding sites for their ligands. Although the presence of two classes of EGF binding sites was readily demonstrable in cells transfected with the wild type EGF receptor, cells expressing the EGFR/RET chimera consistently showed only a single class of low affinity binding sites for EGF. In the case of the EGF receptor, the presence of two classes of binding sites has been attributed to the ability of the receptor to dimerize (34–36). Dimer formation occurs even in the isolated, soluble extracellular domain of the EGF receptor, suggesting that this domain alone is capable of inducing receptor dimerization (37). The fact that the EGFR/RET chimera that contains the extracellular and transmembrane domains of the EGF receptor fails to exhibit high affinity ligand binding sites implicates the cytosolic domain in some aspect of high affinity ligand binding. Experiments demonstrating a loss of high affinity binding in EGF receptors with mutations in the intracellular domain are consistent with a role for the cytosolic portion of the receptor in high affinity ligand binding (38). It is possible that high affinity ligand binding is stabilized by interactions between the intracellular domains of EGF receptor monomers that are lacking in the Ret tyrosine kinase. Glial cell-derived neurotrophic factor (GDNF), the Ret ligand, binds with only low affinity to the Ret tyrosine kinase. High affinity GDNF binding requires the presence of an auxiliary protein, termed the GDNF-α receptor (15, 16). The GDNF-α receptor is a glycosylphosphatidylinositol-linked protein with no intracellular domain. Thus, it is unlikely that it interacts directly with the tyrosine kinase domain of Ret. However, it is possible, that the GDNF-α receptor aids dimer formation, promoting interaction of the cytosolic domains of Ret and stabilizing high affinity binding.

Despite the fact that the EGFR/RET chimera exhibited only low affinity EGF binding, the receptor was able to transduce a signal in NIH 3T3 cells. In particular, EGF stimulated the autophosphorylation of EGFR/RET. However, although binding studies indicated that the clones used in these experiments expressed similar numbers of EGF receptors or EGFR/RET chimeras, the level of autophosphorylation of the EGFR/RET chimera was substantially lower than that of the EGF receptor. This difference in autophosphorylation could be due to the presence of fewer sites of autophosphorylation in Ret than in the EGF receptor or could be the result of a lower extent of phosphorylation overall. Liu *et al.* (39) recently reported findings that suggest that Ret contains five or six sites of autophosphorylation, a number similar to that found in the EGF receptor. Thus, the lower level of autophosphorylation observed in the EGFR/RET chimera is likely to be due to a reduced overall level of phosphorylation of the chimera. This does not appear to be the result of a decrease in the intrinsic tyrosine kinase activity of EGFR/RET, as the phosphorylation of other cellular proteins was similar to that seen in cells expressing the wild type EGF receptor.

EGFR/RET internalized ^{125}I-EGF at a rate that was approximately 2-fold higher than the rate of ligand internalization by wild type EGF receptors. Internalization of receptors appears to be mediated by cytoplasmic sequences that promote clustering of receptors in coated pits and interaction with clathrin adapter proteins (40, 41). Sequences containing an Asn-Pro-Xaa-Tyr motif have been shown to mediate receptor internalization (42); however, no such sequence is found in RET. The motif, Tyr-Xaa-Xaa-hydrophobic, where Xaa stands for any amino acid, also appears to mediate receptor internalization (43). The sequence Tyr^{1015}-Leu-Asp-Leu in the RET cytoplasmic domain fulfills the criteria for an internalization signal. Whether this sequence serves this function remains to be determined.

Although the EGFR/RET chimera is internalized more rapidly than the EGF receptor, it is down-regulated less extensively. Scatchard analyses indicated that 90% of cell surface EGF receptors were lost after a 24-h treatment with EGF, whereas only 50% of cell surface EGFR/RET chimeras were lost. The observation that EGF induces the down-regulation of 50% of the cell surface EGFR/RET receptors suggests that ligand binding promotes internalization of the chimera. However, pulse-chase studies as well as Western blotting demonstrated that EGF did not enhance the rate of degradation of the EGFR/RET chimera. This contrasts markedly with the findings observed for the EGF receptor for which ligand promoted both receptor internalization and receptor degradation. These find-

ings suggest that Ret may lack an effective lysosomal targeting signal and demonstrate a marked difference in the intracellular trafficking of the EGFR/RET chimera and the wild type EGF receptor.

Perhaps as a result of reduced lysosomal targeting, the EGFR/RET chimera appeared to be recycled to the plasma membrane more extensively than the EGF receptor. Approximately two-thirds of the internalized ^{125}I-EGF was released intact from cells expressing EGFR/RET chimeras, whereas cells expressing wild type EGF receptors released only one-third of their internalized ^{125}I-EGF intact. Assuming that the release of intact ^{125}I-EGF is due to recycling of the receptor to the plasma membrane, this suggests that EGFR/RET is preferentially recycled to the plasma membrane, whereas the EGF receptor is preferentially degraded. The combination of reduced receptor degradation and enhanced receptor recycling apparently gives rise to the limited down-regulation observed in cells expressing the EGFR/RET chimera.

Interestingly, the internalized EGFR/RET chimeras retained their levels of tyrosine phosphorylation throughout a 24-h incubation with EGF. Since approximately half of the chimeras are present in an intracellular compartment at the 24-h time point, this suggests that the internalized receptors remain active and capable of signaling. Furthermore, the 50% of the receptors remaining on the cell surface appeared to be functional, at least in terms of ligand internalization. These observations suggest that the maintenance of receptor function under conditions that typically lead to a loss of receptor activity is an intrinsic feature of the Ret tyrosine kinase.

Protein kinase C has been shown to phosphorylate the EGF receptor leading to decreases in EGF binding and receptor tyrosine kinase activity. Although treatment of cells expressing the wild type EGF receptor with PMA to activate protein kinase C clearly led to a marked decrease in ^{125}I-EGF binding and receptor autophosphorylation, cells expressing the EGFR/RET chimera showed little response to PMA. Thus, the Ret tyrosine kinase does not appear to be regulated by a protein kinase C-dependent mechanism. The observation that the activity and function of the EGFR/RET chimera are not diminished markedly by either down-regulation with EGF or activation of protein kinase C indicates that Ret is unresponsive to two major forms of regulation designed to suppress receptor-mediated signaling. This suggests that physiologically it may be important for cells to maintain a constant sensitivity to Ret ligand and to prolong the lifetime of the activated form of the Ret tyrosine kinase.

Recently the Ret ligand has been identified as GDNF (13–17). GDNF has been shown to promote the survival of dopaminergic and motor neurons following axotomy or treatment with dopaminergic neurotoxins (44–48). GDNF undergoes receptor-mediated retrograde axonal transport (49), suggesting that it is a target-derived neurotrophic factor. In such systems the growth factor-receptor complex is internalized at the nerve terminal and subsequently transported to the cell body, a process that can take a significant amount of time. If the goal of this process is to deliver a signaling-competent, ligand-receptor complex to the cell body, then the ability of Ret to internalize ligand rapidly, maintain its activated state, and avoid degradation in the lysosomes is consistent with a role for this receptor in mediating the effects of a target-derived neurotrophic factor.

These studies have identified unique functional properties of the EGFR/RET chimeric receptor. The absence of ligand-stimulated receptor degradation as well as the persistent autophosphorylation of the chimera indicate that Ret is not subject to traditional mechanisms used to regulate receptor function. These characteristics, which differ from those of the EGF receptor, may be relevant to the physiological role of the Ret tyrosine kinase in growth and development.

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