The RET proto-oncogene encodes a member of the receptor tyrosine kinase family. Multiple endocrine neoplasia type 2B (MEN 2B) is caused by the mutation of a conserved methionine to a threonine in the catalytic domain of the RET kinase. When the MEN 2B point mutation was introduced into the epidermal growth factor (EGF) receptor (M857T EGFR), the intrinsic tyrosine kinase activity of the mutant receptor was similar to that of wild-type EGF receptor and remained ligand-dependent. However, the mutant receptor showed an enhanced transforming capacity compared to the wild-type receptor as judged by its ability to mediate the growth of NIH 3T3 cells in soft agar. Using the oriented peptide library approach to examine substrate specificity, the M857T mutation was found to be associated with a decrease in the selectivity for Phe and an increase in the selectivity for acidic residues at the P + 1 position as compared to wild-type EGF receptor. Short-term responses to EGF were similar in cells expressing wild-type and M857T EGF receptors. However, significant differences in receptor down-regulation were observed between the two receptors. These data demonstrate that the MEN 2B point mutation alters the substrate specificity of receptor tyrosine kinases and suggest that the enhanced oncogenesis associated with the MEN 2B mutation may be due in part to alterations in receptor regulation.

RET was first identified as a transforming gene by transfection experiments using DNA isolated from a human T-cell leukemia (1). Subsequently, physical and genetic mapping data have implicated the RET gene in several dominantly inherited human neoplasias, including multiple endocrine neoplasia type 2A (MEN 2A),1 multiple endocrine neoplasia type 2B (MEN 2B), and familial medullary thyroid carcinoma (FMTC) (2–6). MEN 2A and FMTC are both characterized by the development of medullary thyroid carcinoma between the ages of 20 and 50. Approximately one-third to one-half of MEN 2A patients also develop pheochromocytomas and parathyroid hyperplasia. MEN 2B is a more severe form of the disease with onset of symptoms during the first decade of life. Patients exhibit ganglioneuromas of the intestinal tract, mucosal neuromas, and ophthalmologic and skeletal abnormalities, in addition to a particularly aggressive form of medullary thyroid carcinoma.

The RET proto-oncogene encodes a protein that is a member of the family of transmembrane tyrosine kinase growth factor receptors. The RET protein is comprised of a 635-amino acid extracellular domain, a single transmembrane spanning region, and an intracellular tyrosine kinase domain that contains a short, 26-amino acid kinase insert domain. Residues 515 to 634 of the extracellular domain constitute a region of high cysteine content similar to those found in the EGF receptor and the insulin receptor (7, 8). I immediately amino-terminal to this high cysteine region is a domain of ~110 amino acids that is 20–30% homologous to cadherins (9) The presence of this cadherin-like domain is unique among receptor tyrosine kinases and its function within the RET protein is not known. A ligand for the RET receptor tyrosine kinase has not been identified.

Mutations in several conserved cysteine residues in the high cysteine region of the RET extracellular domain account for the majority of cases of MEN 2A and FMTC (2, 3). However, mutations in the tyrosine kinase domain of RET have also been associated with FMTC (10). Biochemical characterization of MEN 2A RET proteins in which Cys-634 is altered to tyrosine, arginine, or tryptophan suggests that the mutation causes constitutive dimerization and activation of the RET tyrosine kinase (11, 12). The MEN 2B phenotype is associated with a single missense mutation in the tyrosine kinase domain of RET that converts Met-918 to threonine (4, 5). To date, this is the only mutation that has been detected in patients with MEN 2B. Interestingly, Met-918 is conserved as a methionine among the receptor tyrosine kinases but is present as a conserved threonine residue in the cytosolic, non-receptor tyrosine kinases such as src (13).

Comparison of the sequence of the RET tyrosine kinase domain with the structure of the cyclic AMP-dependent protein kinase with bound inhibitor peptide suggests that Met-918 forms part of the binding site for the substrate residue immediately COOH-terminal to the site of phosphorylation (14, 15). Based on this information, Carlson et al. (4) predicted that the MEN 2B mutation would alter the substrate specificity of the RET tyrosine kinase rather than constitutively activate the enzyme. The properties of the MEN 2B RET protein appear to be consistent with this hypothesis. Only a modest increase in basal autophosphorylation has been demonstrated but there are differences between the two-dimensional gel electrophoresis patterns of tyrosine phosphorylated proteins in cells ex-

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1 The abbreviations used are: MEN 2A, multiple endocrine neoplasia type 2A; MEN 2B, multiple endocrine neoplasia type 2B; Bom, benzyl oxymethyl; Br-z, 2-bromobenzyloxycarbonyl; Bzl, benzyl; Cl-Z, chlorobenzyloxycarbonyl; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; OBzl, benzyl ester; OHex, cyclohexyl ester; Tos, p-toluensulfonyl; MES, 4-morpholineethanesulfonic acid; MAP, microtubule-associated protein; PI, phosphatidylinositol; FMTC, familial medullary thyroid carcinoma; FAK, focal adhesion kinase.

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1 The abbreviations used are: MEN 2A, multiple endocrine neoplasia type 2A; MEN 2B, multiple endocrine neoplasia type 2B; Bom, benzyl oxymethyl; Br-z, 2-bromobenzyloxycarbonyl; Bzl, benzyl; Cl-Z, chlorobenzyloxycarbonyl; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; OBzl, benzyl ester; OHex, cyclohexyl ester; Tos, p-toluensulfonyl; MES, 4-morpholineethanesulfonic acid; MAP, microtubule-associated protein; PI, phosphatidylinositol; FMTC, familial medullary thyroid carcinoma; FAK, focal adhesion kinase.

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pressing MEN 2A RET and MEN 2B RET (11, 12). More recently, Songyang et al. (16) have shown that wild-type RET and MEN 2B RET exhibit different activity toward three synthetic tyrosine-containing peptides.

Because the ligand for RET has not yet been identified, previous studies of the MEN 2B RET protein have been unable to determine the effect of this point mutation on either the ligand dependence of RET tyrosine kinase activity, the activation of downstream signaling pathways, or receptor regulation. To address these issues, we introduced the MEN 2B point mutation into the kinase domain of the EGF receptor and stably expressed this construct in NIH 3T3 cells. Met-857 of the EGF receptor is equivalent to Met-918 in RET. Thus, we refer to this mutant as the M857T EGFR. Substrate specificity studies using the oriented peptide library approach demonstrated that the wild-type and M857T EGFR receptors preferentially phosphorylated different tyrosine-containing peptides confirming the hypothesis that the MEN 2B mutation changes the substrate specificity of RET. The mutation was found to enhance the transforming capacity of the EGF receptor but had little effect on the ability of EGF to stimulate downstream signaling pathways. However, M857T EGFR receptors exhibited differences in down-regulation, resensitization, and trafficking compared to wild-type receptors. The data suggest that the MEN 2B mutation is generally transforming for growth factor receptors and that the enhanced oncogenesis may result from a change in receptor kinase specificity that leads to alterations in long-term receptor regulation. These data provide insight into the mechanism by which the MEN 2B mutation induces malignant activation of RET.

**Experimental Procedures**

Materials

Antibodies to phosphoryl-tyrosine (PY-20) and PY20 conjugated with horseradish peroxidase were purchased from Transduction Laboratories. Antibodies to MAP kinase, FAK, Ras-GAP and PI 3-kinase were horseradish peroxidase were purchased from Transduction Laboratories. Antibodies to MAP kinase, FAK, Ras-GAP and PI 3-kinase were horseradish peroxidase were purchased from Transduction Laboratories. Antibodies to MAP kinase, FAK, Ras-GAP and PI 3-kinase were horseradish peroxidase were purchased from Transduction Laboratories.

**Antibodies to MAP kinase, FAK, Ras-GAP and PI 3-kinase**

Antibodies to MAP kinase, FAK, Ras-GAP and PI 3-kinase were horseradish peroxidase were purchased from Transduction Laboratories. Antibodies to MAP kinase, FAK, Ras-GAP and PI 3-kinase were horseradish peroxidase were purchased from Transduction Laboratories. Antibodies to MAP kinase, FAK, Ras-GAP and PI 3-kinase were horseradish peroxidase were purchased from Transduction Laboratories.

**Preparation of pSAC1**

pSAC1 contains an EcoRI fragment from 2317 to 3085 of the EGF receptor cDNA subcloned into the BSKSII+ vector. Primer extension and all other reactions were carried out as described in the protocol provided with the kit. This procedure generated pSAC4M with the T → C mutation at nucleotide position 2828 which converts the encoded sequence from Met to Thr at residue 857 of the EGF receptor (M857T EGFR). The mutation was confirmed by DNA sequencing (Sequenase, U. S. Biochemicals).

**Construction of pSAC1**

pSAC1 contains an XbaI-HindIII (HindIII site, nucleotide 4207) full-length fragment of EGF receptor cDNA subcloned into BSKSII+ vector. pSAC4 contains an EcoRI fragment from 2317 to 3085 of the EGF receptor cDNA subcloned into BSKSII+ vector. Primer extension and all other reactions were carried out as described in the protocol provided with the kit. This procedure generated pSAC4M with the T → C mutation at nucleotide position 2828 which converts the encoded sequence from Met to Thr at residue 857 of the EGF receptor (M857T EGFR). The mutation was confirmed by DNA sequencing (Sequenase, U. S. Biochemicals).

**Cell Culture and Transfection**

NIH 3T3 cells were grown in DMEM containing 10% calf serum. The cells were transfected with plasmids pSP14 and pSP15 using a calcium phosphate precipitation method (21). After 2 days, cells were trypsinized and replated in media containing 400 μg/ml G418 on 150-mm dishes. Individual colonies were selected after 2–3 weeks of growth in G418-containing medium.

**125I-EGF Binding—Cells were incubated for 2 h at 4°C in DMEM containing 40 μCi 125I-EGF, pH 7.4, 0.1% bovine serum albumin and the indicated concentration of 125I-EGF.** At the end of the incubation, cells were washed three times in Hanks’ balanced buffer solution. Monolayers were dissolved by the addition of 1 ml of NaOH and cell associated 125I-EGF determined by γ counting (17). Nonspecific binding was determined in duplicate wells containing 500 nCi unlabeled EGF. All assays were performed in triplicate. Results of Scatchard analyses were analyzed using the LIGAND computer program (23).

**Arg-Arg-Src Peptide Phosphorylation—Preparation of membranes**

was carried out at 4°C. A 100-mm plate of cells was washed once with
phosphate-buffered saline and then scraped into 10 ml of buffer containing 10 mM Tris-HCl, pH 7.2, 12,000 g for 30 min at 4°C, and then centrifuged for 5 min. The supernatant was removed and the monolayers washed twice with the same Triton X-100-containing buffer and twice with phosphate-buffered saline. The Triton-insoluble material was scraped into 1 ml of RIPA buffer and homogenized by passage through a 25-gauge needle. The homogenate was then clarified by centrifugation at 12,000 g for 10 min. The protein present in lysates and cytoskeletal preparations was determined by BCA assay and aliquots containing 100 μg of protein were analyzed by SDS-polyacrylamide gel electrophoresis.

Immunoprecipitation and Western Blot Analyses

For immunoprecipitation, primary antibodies were added to aliquots of lysates containing 300 μg of protein and incubated for 2 h at 4°C. Immune complexes were precipitated using Pansorbin. Immunoprecipitates were washed in RIPA buffer and resuspended in 50 μl of RIPA buffer plus 50 μl of Laemmli sample buffer (25). After boiling and centrifugation to pellet the Pansorbin, samples were subjected to SDS-polyacrylamide gel electrophoresis. For Western blotting, proteins were electroblotically transferred from the gels to nitrocellulose, which was then blocked using 10% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20. Nitrocellulose membranes were incubated with primary antibody for 1–2 h at room temperature, washed, and bands visualized using enhanced chemiluminescence according to the manufacturers instructions.

Phosphatidylinositol 3-Kinase Assay

Phosphatidylinositol 3-kinase was assayed in anti-phosphotyrosine immunoprecipitates as described previously (17, 26).

RESULTS

Characterization of Cells Expressing Wild-type and M857T EGF Receptors—Conducts encoding the wild-type and M857T human EGF receptor were transfected into NIH 3T3 cells and stable, G418-resistant clones were selected as described under “Experimental Procedures.” Scatchard analysis of 125I-EGF binding was carried out on several clones expressing wild-type or M857T EGF receptors. All lines examined exhibited a single class of 125I-EGF binding sites with an affinity of 0.6 ± 0.1 nM (data not shown).

Two clones expressing wild-type EGF receptor and two clones expressing M857T EGF receptor were assayed for tyrosine kinase activity. Kinase activity was assessed by quantitating the ability of membranes derived from the different cell lines to catalyze the phosphorylation of the Arg-Arg-Src synthetic peptide. Parental NIH 3T3 cells exhibited no 125I-EGF binding sites with an affinity of 0.6 ± 0.1 nM (data not shown).

Preparation of Cell Lysates and Cytoskeletons

In 60-mm dishes were treated with or without EGF in DMEM containing 0.1% bovine serum albumin. After washing once with phosphate-buffered saline, monolayers were lysed by scraping into 300 μl of RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 500 μM sodium orthovanadate, and 10 μM p-nitrophenol phosphate. In some experiments, the RIPA buffer was replaced by Nonidet P-40 buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40). Lysates were incubated for 10 min on ice with periodic vortexing and then clarified by centrifugation at 12,000 × g for 10 min.

Cytoskeletons were prepared by incubating cells in 100-mm dishes for 30 min at 4°C with constant agitation in 10 ml of buffer containing 40 mM HEPES, pH 7.2, 2 mM MgCl2, 2 mM MnCl2, 1 mM CaCl2, 250 mM NaCl, 0.5% Triton X-100 plus protease and phosphatase inhibitors as described above. The buffer was removed and the monolayers washed twice with the same Triton X-100-containing buffer and twice with phosphate-buffered saline. The Triton-insoluble material was scraped into 1 ml of RIPA buffer and homogenized by passage through a 25-gauge needle. The homogenate was then clarified by centrifugation at 12,000 × g for 10 min. The protein present in lysates or cytoskeletal preparations was determined by BCA assay and aliquots containing 100 μg of protein were analyzed by SDS-polyacrylamide gel electrophoresis.
human EGF receptor is expressed at approximately 50-fold higher levels in WT9 and Mut6 cells than is the endogenous mouse EGF receptor.

Membranes were prepared from cells expressing the wild-type EGF receptor (WT) or the M857T EGF receptor (Mut) and assayed for the ability of increasing concentrations of EGF to stimulate the phosphorylation of Arg-Arg-Src as described under “Experimental Procedures.” Data shown represent the average of duplicate determinations. Panel B, Lineweaver-Burk analysis of Arg-Arg-Src phosphorylation by WT9 and Mut6 cells. Membranes were prepared from WT9 or Mut6 cells and equal amounts of membrane protein were assayed for their ability to catalyze the phosphorylation of increasing concentrations of Arg-Arg-Src in the presence of 10^{-7} M EGF. Data shown represent the average of triplicate determinations.

Although the binding and kinase activities of the wild-type and M857T EGF receptors were similar, Mut6 cells exhibited a markedly enhanced transforming potential in soft agar assays. When WT9 cells were plated in soft agar, EGF induced a dose-dependent increase in the ability of the cells to form colonies (Fig. 2A). Likewise, EGF induced an increase in the growth of Mut6 cells in soft agar. However, at optimal concentrations of EGF, approximately 4-fold more colonies were formed by the Mut6 cells than by the WT9 cells. Furthermore, the colonies formed by the Mut6 cells were significantly larger than those formed by WT9 cells and exhibited a less compact morphology (Fig. 2B). Parental NIH 3T3 cells did not produce colonies in soft agar at any concentration of EGF (not shown).

Substrate Specificity of Wild-type and M857T EGF Receptors—Comparison of the sequence of RET and the EGF receptor with that of the cAMP-dependent protein kinase has suggested that the Met→Thr MEN2B mutation is likely to lie in the substrate binding pocket that recognizes the residue immediately COOH-terminal to the phosphorylated tyrosine (the P+1 residue) (14, 15). To test the hypothesis that the M857T mutation alters the substrate specificity of the EGF receptor, we utilized the oriented peptide library approach (16, 24). A degenerate peptide library with the sequence EVPEYXXS-PLL was phosphorylated using membranes derived from cells expressing wild-type or M857T EGF receptors. The phosphorylated peptides were isolated and sequenced to identify amino acids preferred by each kinase at the degenerate positions (Table I).

The wild-type EGF receptor selected primarily hydrophobic residues at the P+1 position, including those with both aliphatic and aromatic side chains. A slight preference for glutamic acid was also observed. The M857T EGF receptor exhibited a different set of preferences. While amino acids with aliphatic side chains, such as Val, Ile, and Leu, were selected at this position, the M857T receptor showed no selectivity for the
The MEN 2B Mutation in the EGF Receptor

Substrate preference of wild-type and M857T EGF receptors

| Position (P = phosphorylated Tyr) | Wild-type receptor | M857T EGF receptor |
|----------------------------------|-------------------|--------------------|
| P + 1 V (2.0)                   | V (2.2)           |
| I (1.9)                         | E (2.1)           |
| L (1.8)                         | I (1.9)           |
| F (1.7)                         | L (1.7)           |
| E (1.5)                         | D (1.5)           |
| P + 2 E (3.1)                   | E (4.2)           |
| D (2.1)                         | P (2.6)           |
| F (1.7)                         | D (2.2)           |
| P (1.7)                         | V (1.6)           |
| P + 3 F (3.5)                   | F (4.1)           |
| I (2.4)                         | L (2.2)           |
| L (2.0)                         | I (2.0)           |

Aromatic side chain at the P1 position increases the phosphorylation of substrates with acidic residues at this position. The M857TEGF receptor selected against Asp at this site appeared. These data suggest that the M857T mutation decreases the phosphorylation of substrates with aromatic side chains at the P + 1 position and increases the phosphorylation of substrates with acidic residues at this position.

Subtle changes in substrate specificity were also observed at the P + 2 position. Both the wild-type and M857T EGF receptors showed strong selectivity for acidic residues at this position but also showed some preference for amino acids with hydrophobic side chains. However, the wild-type receptor selected the large aromatic Phe residue whereas the M857T EGF receptor selected the much smaller Val residue. Preferences at the P + 3 position were similar for the two receptors.

One noteworthy finding not reported in Table I was that both wild-type and M857T EGF receptors strongly selected against Arg in either of the first two degenerate positions. The ratio of Arg present at the P + 1 and P + 2 positions in the phosphorylated peptides to Arg present in these positions in the total peptide library was 0.3 to 0.4 for both receptors. In addition, both receptors selected against Asp at the P + 1 position (ratio = 0.5). Wild-type EGF receptors also selected against Arg at the P + 3 position (ratio = 0.5) whereas the M857T EGF receptor did not (ratio = 0.8).

To determine whether the observed changes in selectivity in vitro lead to changes in the phosphorylation of substrates in vivo, the EGF-stimulated phosphorylation of endogenous proteins in the two cell lines was compared (Fig. 3). In total cell lysates the phosphorylation of 60- and 90-kDa proteins was stimulated more strongly by EGF in WT9 cells than in Mut6 cells. Conversely, in cytoskeletal preparations, the phosphorylation of several proteins of ~95 kDa was stimulated by EGF more strongly in Mut6 cells than in WT9 cells. These data suggest that the differences in substrate specificity between wild-type and M857T EGF receptors observed in vitro translate into differences in substrate phosphorylation in vivo.

Autophosphorylation of Wild-type and M857T EGF Receptors—To further compare the kinase activities of the wild-type and M857T EGF receptors, the autophosphorylation of the two receptors was examined (Fig. 4). Western blotting with anti-EGF receptor antibodies detected similar levels of EGF receptors in WT9 and Mut6 cells. Treatment with 50 nM EGF increased the apparent mass and diffuseness of the receptor in both cell lines. This is characteristic of phosphorylated EGF receptors. Western blotting with anti-phosphotyrosine antibody demonstrated that receptor phosphorylation was apparent by 2 min and declined over the following 30 min. The extent of receptor autophosphorylation was greater in WT9 cells than in Mut6 cells. Since WT9 and Mut6 cells exhibit similar numbers of receptors, these observations suggest that the M857T mutation is associated with diminished EGF receptor autophosphorylation.

Activation of Downstream Signaling Pathways—MAP kinase is activated by phosphorylation on Tyr and Thr residues. Phosphorylation decreases the mobility of the p42 and p44 MAP kinase bands on SDS gels and this shift can be used to monitor MAP kinase activation. As shown in Fig. 5A, the time course of EGF-stimulated MAP kinase activation was similar in WT9 cells and Mut6 cells. MAP kinase activation was apparent by 2 min, peaked at 5 min, and declined thereafter. Activation of the PI 3-kinase by EGF was also similar in WT9 and Mut6 cells. The PI 3-kinase activity present in anti-phosphotyrosine immunoprecipitates was essentially identical in both cell lines over a wide range of EGF concentrations (Fig. 5B).

The tyrosine phosphorylation of several downstream signaling molecules was also examined (Fig. 6). EGF stimulated the phosphorylation of the focal adhesion kinase, FAK, to the same extent in WT9 and Mut6 cells. In addition, the EGF-stimulated phosphorylation of Ras-GAP and the associated p190 and p62 proteins was similar in WT9 and Mut6 cells. EGF also induced the phosphorylation of the p46, p52, and p66 forms of SHC in both cell lines. Therefore, the failure to observe significant differences in EGF-stimulated MAP kinase activity and PI 3-kinase activity in WT9 and Mut6 cells is not surprising.
WT9 and Mut6 cells. A tyrosine phosphorylated protein at 
~170 kDa, which most likely represents the EGF receptor, was also present in anti-SHC immunoprecipitates from both cell lines indicating that in both cell types phosphorylated SHC was also associated with the EGF receptor. The slightly lower level of phosphorylated EGF receptor present in immunoprecipitates from Mut6 cell probably reflects the lower level of receptor autophosphorylation observed in these cells (Fig. 4). The band at approximately 145 kDa that was present in anti-SHC immunoprecipitates from both cell lines may be the p145 protein observed by Kavanaugh et al. (27) in anti-SHC immunoprecipitates.

Down-regulation of Wild-type and M857T EGF Receptors—Prolonged treatment of cells with EGF causes the loss of cell surface EGF receptors by internalization and degradation. This process is referred to as down-regulation. To determine whether wild-type and M857T EGF receptors exhibited differences in down-regulation, WT9 and Mut6 cells were incubated with 2 nM EGF for 0 to 60 min and the remaining cell surface EGF receptors were quantitated. As shown in Fig. 7A, the rate and extent of EGF receptor down-regulation was substantially greater in Mut6 cells than in WT9 cells. The rate of 125I-EGF internalization was similar for both cell types (Fig. 7B) indicating that the increased down-regulation by Mut6 cells is not explained by accelerated endocytosis.

EGF receptor down-regulation leads to a decrease in the responsiveness of cells to EGF. Because a major difference in the biological response of WT9 cells and Mut6 cells to EGF occurred in an assay (soft agar) in which the cells were incubated with EGF for many days, we wondered whether the observed differences in down-regulation led to differences in the long-term responsiveness of these cells to EGF. To address this question, duplicate cultures of WT9 and Mut6 cells were incubated with increasing concentrations of EGF for 24 h to down-regulate the EGF receptors. One monolayer from each set was then re-challenged with 50 nM EGF for 2 min. Receptor levels and autophosphorylation were then assessed by Western blotting to evaluate the ability of the cells to respond to stimulation by EGF. As shown in Fig. 8, control WT9 cells showed a strong increase in receptor autophosphorylation in response to acute stimulation with EGF. Pretreatment of WT9 cells with 0.5 nM EGF for 24 h did not significantly alter the ability of these cells to respond to a subsequent challenge with 50 nM EGF. However, down-regulation for 24 h with concentrations of EGF of 2 nM or greater completely blunted the ability of the receptors to autophosphorylate in response to an acute challenge with EGF. Anti-EGF receptor blots (Fig. 8) demonstrated a similar dose response to EGF for the decline in total cellular EGF receptor levels. Mut6 cells appeared to be significantly less sensitive to down-regulation by EGF than WT9 cells. Even following down-regulation with 2 nM EGF for 24 h, Mut6 cells showed little loss of total cellular EGF receptors and exhibited a nearly normal autophosphorylation response to EGF. Only at doses of EGF of 10 nM or greater were the M857T EGF receptors significantly down-regulated with respect to either receptor number or receptor autophosphorylation.

These differences in receptor down-regulation were also apparent in the ability of the wild-type and M857T EGF receptors to mediate activation of MAP kinase. As shown in Fig. 8, activation of MAP kinase following treatment of WT9 cells with 50 nM EGF for 2 min was only apparent in control cells and cells down-regulated for 24 h with 0.5 nM EGF. As was true for
receptor autophosphorylation, the ability of the wild-type receptor to mediate activation of MAP kinase was completely ablated following down-regulation with 2 nm EGF for 24 h. By contrast, a significant decrease in the ability of M857T EGF receptors to stimulate MAP kinase occurred only after long-term down-regulation was carried out with doses of 10 nm or greater EGF.

Further analysis of the long-term down-regulation of the EGF receptor in WT9 and Mut6 cells was carried out. WT9 cells and Mut6 cells were incubated with 2 nm EGF for periods of time ranging from 1 to 24 h. At the end of the incubation, cell surface 125I-EGF binding was assessed as well as total cellular receptor levels and receptor autophosphorylation. The data in Fig. 9A demonstrate that when cells were incubated with 2 nm EGF for up to 24 h, cell surface 125I-EGF declined in both cell types. However, after 24 h, Mut6 cells exhibited approximately twice as much 125I-EGF binding. Total cellular levels of EGF receptors also declined rapidly in WT9 cells and remained low throughout the incubation (Fig. 9B). By contrast, treatment of Mut6 cells with EGF led to a modest decrease in total cellular receptor levels over the first 6 h but the levels of EGF receptor increased thereafter. Receptor autophosphorylation was markedly different over this time course in the two cell lines (Fig. 9B). Whereas the wild-type EGF receptor was phosphorylated to some extent throughout the entire 24-h time course, the M857T EGF receptor appeared to be completely dephosphorylated within 1 h after treatment with EGF and remained unphosphorylated throughout the incubation.

**DISCUSSION**

Multiple endocrine neoplasia type 2B is the result of a Met → Thr mutation in the catalytic domain of the RET tyrosine kinase. Because the RET protein has not been extensively characterized and no ligand has been identified for this receptor, we investigated the effects of this Met → Thr mutation within the context of the well studied EGF receptor. Our findings demonstrate that this mutation is capable of enhancing the transforming capacity of the EGF receptor as judged by the ability of cells to grow in soft agar. This suggests that transformation elicited by the MEN 2B mutation is not specific for the RET tyrosine kinase and implies that signaling or regulatory pathways utilized by all receptor tyrosine kinases are likely to be the basis of MEN 2B-induced oncogenesis.

The MEN 2B mutation lies at position 857 in the cytoplasmic domain of the EGF receptor. Consistent with the intracellular location of this mutation, we found no evidence for a change in the ability of EGF to interact with its receptor. Lineweaver-Burk analysis of the phosphorylation of the synthetic Arg-Arg-Src peptide by cells that expressed similar numbers of wild-type or M857T EGF receptors yielded similar K_m and V_max values for the phosphorylation of this peptide by both receptors. This demonstrates that the intrinsic tyrosine kinase activity of the wild-type and mutant EGF receptors is the same.

It is noteworthy that the M857T EGF receptor was not constitutively activated. EGF was still required to induce a biological response through this mutant receptor. These findings suggest that the oncogenicity of MEN 2B RET is not due to...
the constitutive activation of the kinase. This conclusion contrasts with the findings from studies of the activity of the MEN 2A RET protein. MEN 2A mutations are found primarily in the high cysteine region of the extracellular domain (2, 3). Characterization of MEN 2A RET suggests that it is constitutively dimerized and active (11, 12). The fact that the MEN 2A and MEN 2B phenotypes are clinically distinguishable is consistent with the apparent differences between a constitutively activated MEN 2A RET protein and a ligand-dependent but structurally altered MEN 2B RET.

The Met → Thr mutation in MEN 2B RET was hypothesized to change the substrate specificity of the RET receptor kinase, in particular at the P + 1 position (4). Using the oriented peptide library approach (24), we found that the corresponding Met → Thr mutation in the EGF receptor decreased its selectivity for aromatic residues and increases its selectivity for acidic residues at the P + 1 position. Minor changes relating to the size of the preferred hydrophobic side chains also occurred at the P + 2 position but no significant differences were observed at the P + 3 position. These findings directly demonstrate that the MEN 2B mutation alters the substrate specificity of a receptor tyrosine kinase. The preference of the M857T EGF receptor for Glu and Asp residues at the P + 1 position is similar to that observed for the src family kinases (16). Since the src family kinases all contain a threonine residue at the position equivalent to that of Met-857 in the EGF receptor, a hydrophilic residue at this position in the kinase sequence appears to be a strong determinant for the selection of acidic residues at the P + 1 position of the kinase substrate.

Our experiments with the wild-type and M857T EGF receptor utilized a degenerate peptide library that differed from that used by Songyang et al. (16). In addition, our studies were performed with cell membranes as the source of tyrosine kinase activity rather than with purified recombinant proteins. Nonetheless, our findings with respect to the selectivity of the wild-type EGF receptor are essentially identical to the results previously reported by Songyang et al. (16). Thus, the oriented peptide library method appears to be a consistent and reliable approach to characterize differences in protein kinase specificity. However, primary sequence selectivity probably represents only one component of substrate specificity, as none of the known sites of autophosphorylation in the EGF receptor (28–30) conform to the amino acid preferences identified in the oriented peptide library experiment. Hence, substrate localization due to intramolecular interactions or SH2-dependent protein binding probably plays a major role in substrate selection by receptor tyrosine kinases.

The observation that there are differences in the phosphorylation of endogenous proteins in response to EGF in cells expressing wild-type or M857T EGF receptors suggests that the changes in substrate specificity identified using the in vitro oriented peptide library approach reflect significant changes in substrate specificity in vivo. Together with the demonstration that the wild-type and M857T EGF receptors possess similar intrinsic kinase activities, these findings support the hypothesis that MEN 2B-induced oncogenesis is the result of a change in kinase substrate specificity rather than an increase in total enzyme activity.

There are several mechanisms through which alterations in substrate specificity could enhance the transforming potential of the EGF receptor in vivo. First, changes in substrate specificity could lead to the phosphorylation of novel downstream targets resulting in the activation of new signaling pathways. Alternatively, changes in substrate specificity could affect the normal pattern of EGF receptor regulation leading to an alteration in receptor down-regulation or desensitization. Our findings with respect to the M857T EGF receptor support the latter hypothesis.

Although the M857T EGF receptor was more transforming than the wild-type EGF receptor and exhibited differences in substrate specificity, many of the downstream signaling pathways activated in response to EGF were unchanged in cells expressing the M857T EGF receptor. This included activation of MAP kinase and PI 3-kinase as well as EGF-stimulated phosphorylation of SHC, focal adhesion kinase, Ras-GAP and its associated p62 and p190 proteins. Thus the enhanced transforming capacity of the M857T EGF receptor apparently is not due to changes in the acute activation of these common signaling pathways.

By contrast, regulation of receptor function appeared to be significantly altered by the Met to Thr mutation. Treatment with EGF for up to 60 min led to the loss of cell surface 125I-EGF binding activity in cells expressing both forms of the receptors. However, the loss of cell surface receptors was more rapid and more extensive in cells expressing the M857T EGF receptor than in cells expressing the wild-type EGF receptor. The enhanced clearance of M857T receptors from the cell surface was not due to an increased rate of receptor internalization (Fig. 7). As down-regulation of cell surface receptors at short times represents a balance between the rate of receptor internalization and the rate of receptor recycling back to the surface, these findings suggest that there is less recycling of the M857T
EGF receptor than of the wild-type receptor. Thus, receptor trafficking appears to have been affected by the MEN 2B mutation. EGF receptor kinase activity has been shown to be important in receptor trafficking. Kinase-negative EGF receptors undergo ligand-induced internalization but do not become down-regulated because the receptors are recycled back to the cell surface rather than targeted to the lysosomes for degradation (31, 32). It is possible that the change in substrate specificity in the M857T EGF receptor leads to alterations in the phosphorylation of a protein involved in sorting such that more M857T EGF receptors are targeted to the lysosomes and fewer are shuttled into the recycling pathway.

In addition to differences in short-term down-regulation, WT9 and Mut6 cells also exhibited alterations in their long-term responsiveness to EGF. When incubated with EGF for 24 h to down-regulate the receptors, Mut6 cells required a 5-fold higher concentration of growth factor to ablate their responsiveness to EGF than did WT9 cells. That this difference in regulation can lead to changes in the growth properties of cells is apparent from the soft agar growth curves shown in Fig. 2. In that experiment, the response of WT9 cells peaked at 0.7 nM EGF, a concentration at which Mut6 cells showed approxi-

that the MEN 2B mutation into the EGF receptor. Our data indicate that there was little change in a variety of short-term responses to EGF. However, in assays examining the response of cells to long-term treatment with EGF, we observed marked differences in the behavior of the wild-type and M857T EGF receptors. These results suggest that the enhanced oncogenicity associated with the MEN 2B mutation correlates with changes in substrate specificity and may be due to alterations in the long-term response to growth factor rather than changes in the acute response to EGF. We have identified significant alterations in the down-regulation of the M857T EGF receptor. These changes in the long-term regulation of EGF receptor function may be responsible, at least in part, for the enhanced transforming potential of the M857T EGF receptor.

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