Point Mutations Alter the Cellular Distribution of the Human Folate Receptor in Cultured Chinese Hamster Ovary Cells1,2

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ABSTRACT Diminished cellular need for folate results in decreased function of the human folate receptor (FR) but increased expression of this protein in cells grown at different rates. Much of this FR is intracellular and not available for vitamin transport, raising the following question: what is the function of this excess receptor? In this study, we characterized the effects of three point mutations on FR regulation in Chinese hamster ovary cells stably transfected to express either wild-type receptor or FR containing mutations at positions 67S → P, 144E → D, and/or 201N → D. The 201N → D FR responded functionally like the wild-type receptor but was localized predominantly at the cell surface (90% vs. <40% for wild-type). This mutation disrupted a N-linked glycosylation site and generated a partially deglycosylated receptor. The 67S → P mutation also shifted the cellular distribution such that more FR was surface accessible (80%) but did not affect glycosylation. Because previous results showed that these mutations influence the conformation of FR, our findings suggest that structural changes in the receptor facilitate its trafficking to the cell surface. FR containing the 67S → P mutation with either a 144E → D or 201N → D change was not processed from the high-mannose to complex glycoform but was still transported to the cell surface and able to transport folates. Thus, conformational changes introduced by specific point mutations can influence FR processing and/or trafficking to the cell surface. Furthermore, the fact that mutated FR can be trafficked to the cell surface more efficiently suggests that the native receptor may be retained intracellularly to perform some function there. J. Nutr. 134: 308–316, 2004.

KEY WORDS: • folate receptor • folate uptake • regulation • point mutation

There are two well-characterized systems for folate uptake into the cell. The first utilizes a high-capacity, low-affinity transmembrane transporter known as the reduced folate carrier (RFC4) (1, 2). The second employs the folate receptor, which binds physiologic folates with high affinity [Kd = 0.4 nmol/L for folic acid and 3 nmol/L for 5-methyltetrahydrofolate (5-MTHF)] and transports them into the cytosol via fluid-phase endocytosis (3–6). There are three isoforms of the folate receptor (α, β, and γ) of which folate receptor-α (FR) is the most biologically relevant (7–10). Although the RFC is found in most, if not all tissues, FR expression is limited to normal differentiated epithelial cells including those of the choroid plexus, placenta, kidney, retina pigment epithelium, and lung (11, 12). The FR has also been found to be overexpressed in many malignant tissues of epithelial origin, including the uterus, ovary, breast, and colon (13).

The affinity of FR for 5-MTHF and folic acid is roughly 2 and >5 orders of magnitude, respectively, greater than the RFC (14). Therefore, FR is expected to be much more effective at taking up these folates from serum, where they are found in nanomolar concentrations (15). That this is the case is supported by the findings of experiments in which cultured cells that do not normally express FR acquired the ability to grow in nanomolar concentrations of folate only after transfection with this receptor (16, 17). However, in different cells and under different conditions, such as when higher concentrations of folate are available, the RFC appears to be the dominant transporter (18, 19). How the FR is regulated, either alone or in relationship to the RFC, is poorly understood. The levels of both FR mRNA and protein (20–22) increase in cells grown in low folate, suggesting that this receptor can be upregulated when it is needed to effectively transport folates. However, whether increased FR levels actually reflect increased activity of this receptor under these circumstances remains unstated. In a previous study, we found that folate uptake decreased as cell growth slowed, despite an increase in cellular levels of endogenously expressed receptor (23). This suggested that total FR levels and the activity of this receptor are not coordinately regulated. Conversely, we found that FR...
Western blotting of cells that did and did not express FRα. The mouse monoclonal anti-β-tubulin antibody was purchased from Sigma. Endoglycosidase H (endo H) was purchased from Roche Diagnostics. Bacillus thuringiensis phosphatidylinositol-specific phospholipase C (PI-PLC) was prepared and purified as described previously (28).

**Cells and cell culture.** Chinese hamster ovary (CHO) cells, purchased from the American Type Culture Collection, were routinely grown in monolayer cultures in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 105 U/L penicillin, and 100 mg/mL streptomycin in an atmosphere of 5% CO2 at 37°C. Cells were routinely passaged with 0.5 mmol/mL EDTA in PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na2HPO4, and 1.4 mmol/L KH2PO4). Cell numbers were determined by counting with a hemocytometer.

For all experiments, cells were plated at 2.5 × 105 cells/25 cm2 flasks in folate-free RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 105 U/L penicillin, and 100 mg/mL streptomycin and grown to the indicated confluence. The medium was changed every 2 d (to prevent depletion of nutrients other than folate). Cultures were judged to be subconfluent (SC) or confluent (C) when the cells covered ~75 or 100% of the flask’s growth surface, respectively. Cells were defined as postconfluent (PC) when they had grown for 2 d after reaching confluence.

**Recombinant plasmids and site-directed mutagenesis.** The recombinant plasmid pZeos/FR, containing the pZeosSV2(+) mammalian expression vector (Invitrogen) and cDNA encoding the human FRα protein, was constructed using standard cloning methods and subsequently used as a template for mutagenesis. Mutations at sites 67S to 67D, 144E to 144D, 201N to 201D, and 144S to 144D were constructed by the site-directed mutagenesis method using the QuickChange kit from Stratagene. Each was confirmed by nucleotide sequence analysis. The recombinant plasmid pEGFP-GaIT, containing the pEGFP-N3 green fluorescent protein (GFP) expression vector (Clontech) and cDNA encoding β1,4-galactosyltransferase (GaIT) was a generous gift from Dr. Victor Faundez (Emory University).

**Stable transfection by electroporation.** Stable transfectants were generated by electroporation (2.5 kV) of CHO cells with a BTX Electro Cell Manipulator and selection with 175 mg/L G418 (Clontech) for the pEGFP-GaIT vector as previously described (23).

**[H]Folic acid purification.**[3,5,7,9-3H]Folic acid was purified as previously described (23). The purified [3H]folic acid was either used immediately or stored at ~80°C and used within 7 d.

**[H]Folic acid binding.** Folic acid binding was used to estimate the amount of receptor with and without access to the cell surface, as well as the total amount of FR in the cell. To determine the total amount of FR in the cell, cellular membranes were solubilized with 1% (v/v) Triton X-100 before the binding assay to allow the [3H]folic acid access to all pools of receptor in the cell. To delineate FR with access to the cell surface (which includes receptors on the plasma membrane and in the endocytic pathway) from intracellular receptors, viable cells maintained in culture conditions at 37°C were first treated with PI-PLC to remove surface-accessible receptors. The binding assay was then performed on the supernatant (removed cell-surface receptors) and solubilized cell pellet (intracellular receptors) fractions. We defined these two pools of receptors as the "surface-accessible" (SA) and "intracellular" (IC) FR pools.

Ligand binding to FR was measured as previously described with a few minor changes (23). To separate the SA from the IC receptors, living cells were first treated with 1 mL serum-free growth medium containing 1 mL/L PI-PLC. After incubation of the cells at 37°C for 30 min, the cells were placed on ice for 5 min, centrifuged at 4°C (300 × g for 5 min), and the supernatant transferred to a new tube and placed on ice. The cell pellet was rinsed with 1 mL of ice-cold PBS, centrifuged as above, the supernatant removed, and the [3H]folic acid binding in the pellet (corresponding to the IC FR) and reserved supernatant (corresponding to the SA FR) quantified as previously described (23).

**Materials.** Fetal bovine serum was purchased from Atlanta Biologicals. RPMI 1640 and folate-free RPMI 1640 media were purchased from Life Technologies. [3,5,7,9-3H]Folic acid (25–30 Ci/mmol, 99% pure) was obtained from Moravek Biochemicals. Eco-Lite fluid was purchased from ICN. Folic acid, Triton X-100, leupeptin, aprotinin, charcoal, and all other chemicals were purchased from Sigma. The reagents for the bicinchoninic acid protein assay were purchased from Pierce.

**Enzymes and antibodies.** The mouse monoclonal antibody MoV19, a gift from Centocor, was used for immunofluorescence microscopy and a rabbit polyclonal anti-FRα antibody (anti-FRα) was used for Western blotting. Anti-FRα was produced by rabbits immunized with a recombinant FRα-glutathione-S-transferase fusion protein. The specificity of the resulting antiserum was determined by Western blotting of cells that did and did not express FRα. The mouse monoclonal anti-β-tubulin antibody was purchased from Sigma. Endoglycosidase H (endo H) was purchased from Roche Diagnostics. Bacillus thuringiensis phosphatidylinositol-specific phospholipase C (PI-PLC) was prepared and purified as described previously (28).

**Stable transfection by electroporation.** Stable transfectants were generated by electroporation (2.5 kV) of CHO cells with a BTX Electro Cell Manipulator and selection with 175 mg/L G418 (Invitrogen) for pZeos/FR vectors or 500 mg/L G418 (Clontech) for the pEGFP-GaIT vector as previously described (23).

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**Folic acid uptake.** The internalization of folic acid into the cytosol was quantified as previously described (23). Nonspecific uptake was determined in each experiment by measuring [3H]folic acid uptake in the presence of 750-fold excess unlabeled folic acid. Specific uptake values were determined by subtracting the nonspecific value from the respective total radioactivity.

**Western blot analysis.** For whole-cell extract preparation, cells were washed twice with PBS, scraped, centrifuged (300 \( \times g \), 5 min), the supernatant removed, and the pellet resuspended in denaturation buffer [10 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L EDTA, pH 7.4, and 0.2% (wt:v) SDS]. Samples were denatured at 100°C for 5 min and centrifuged (10,000 \( \times g \), 1 min) to remove insoluble material. The total protein concentration was determined and proteins were resolved on a 15% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Osmonics). Nonspecific binding was blocked by incubating the nitrocellulose in Tris-buffered saline (TBS)-TWEEN [20 mmol/L Tris, 137 mmol/L NaCl, 3.8 mmol/L HCl, and 0.1% Tween 20 (v:v), pH 7.5] containing 5% nonfat milk (wt:v) and 0.02% NaN\(_3\) (wt:v), at room temperature (RT) for 1 h, probed with a primary anti-human FR rabbit polyclonal antibody (1:2000 dilution) in blocking solution for 3 h at RT, washed (3 \( \times 10 \) min) with TBS-Tween, and incubated for 1 h at RT with a secondary anti-rabbit horse radish peroxidase (HRP)-conjugated antibody (1:3000 dilution) in TBS-Tween containing 5% nonfat milk (wt:v). Membranes were washed as above and proteins detected by chemiluminescence with ECL Western blotting detection reagents (Amer sham Biosciences). For simultaneous detection of \( \beta \)-tubulin, an anti- \( \beta \)-tubulin monoclonal primary antibody (1:20,000 dilution) and antiperoxidase HRP-conjugated secondary antibody (1:3000 dilution) were added to the primary and secondary incubation solutions, respectively.

**Glycosidase treatment.** Cell fractions containing 5–20 \( \mu \)g protein were diluted to a final volume of 35 \( \mu \)L to which 10 \( \mu \)L of 250 mmol/L Na\(_2\)HPO\(_4\), pH 5.5, and 2.5 \( \mu \)L of denaturation buffer containing 2.0% SDS (wt:v) and 1 mol/L \( \beta \)-mercaptoethanol were added. The samples were denatured at 100°C for 5 min, cooled to room temperature, 0.5 mL endo H added, and incubated for 16 h at 37°C. After treatment, samples were acetone-precipitated with ice-cold acetone and washed with CHCl\(_3\):MeOH (1:2, v:v). The samples were then dried and prepared for electrophoresis by resolubilization with SDS reducing buffer [62.5 mmol/L Tris-HCl, pH 6.8, 10% glycerol (v:v), 2% SDS (wt:v), 1 mol/L \( \beta \)-mercaptoethanol, and 0.05% (wt:v) bromophenol blue] and denatured at 100°C for 5 min.

**Immunofluorescence microscopy.** For indirect immunofluorescence, cells were grown for 2–3 d plated on 12-mm glass cover slips (Fisher Scientific). To better visualize the IC FR pool, the SA receptor was removed by treating cells with 1 mL/L PIP-PLC, which can gain access to endocytic compartments, was used to distinguish SA receptor (consisting of cell surface receptor and that cycling through the endocytic pathway) from IC FR. To verify that the PIP-PLC treatment removed all of the SA receptor, [3H]folic acid binding was also measured in cells immediately after PIP-PLC treatment. There was no detectable specific binding for any of the cell lines, indicating that PIP-PLC had effectively removed the SA receptor (data not shown). In addition, the combined SA and IC FR value was within 10% of the measured total cellular FR for all cell lines, indicating that these pools accurately reflect the majority of the receptor in the cell. For purposes of clarity, only the SA and IC data are shown in subsequent figures.

**RESULTS**

**Expression of FR constructs containing various point mutations**

To test the influence of the 144E\( \rightarrow \)D, 201N\( \rightarrow \)D, and 67S\( \rightarrow \)P changes on FR regulation, these point mutations were introduced into the FR cDNA singly and in pairwise combinations. The relative expression and molecular weight of each of the FR constructs were evaluated by Western blotting. Because growth rate can influence the expression of FR (23), confluent cultures of each cell line were used. \( \beta \)-Tubulin expression was measured simultaneously to confirm equal protein loading for each cell line. The amount of FR expressed by the various transfectants varied greatly (Fig. 2). All three FR mutants containing the 201N\( \rightarrow \)D point mutation (lanes 4, 6 and 7) exhibited an \( \sim 3 \) kDa shift in mobility, which is consistent with the loss of one of the N-linked oligosaccharides normally attached at this site (26). Two bands were seen in all of the cell lines except FR67,144 and FR67,201 (lanes 5 and 6). Previous characterization of the FR expressed by cultured cells (24–26) showed that the two bands represent different glycoforms of the receptor with either high-mannose or complex oligosaccharides. Thus, this Western analysis indicated that the 201 mutation blocks N-linked glycosylation and the processing of the oligosaccharides in the 67,201 and 67,144 mutants was different from that of the other FR forms.

**Effect of the 144E\( \rightarrow \)D and 201N\( \rightarrow \)D mutations on FR regulation.** Sensitivity to cleavage by the membrane-impermeant enzyme PI-PLC, which can gain access to endocytic compartments, was used to distinguish SA receptor (consisting of cell surface receptor and that cycling through the endocytic pathway) from IC FR. To verify that the PI-PLC treatment removed all of the SA receptor, [3H]folic acid binding was also measured in cells immediately after PI-PLC treatment. There was no detectable specific binding for any of the cell lines, indicating that PI-PLC had effectively removed the SA receptor (data not shown). In addition, the combined SA and IC FR value was within 10% of the measured total cellular FR for all cell lines, indicating that these pools accurately reflect the majority of the receptor in the cell. For purposes of clarity, only the SA and IC data are shown in subsequent figures.

FR regulation was investigated by measuring changes in the SA and IC pools of the receptor as cellular requirements for folate changed in response to different rates of cell growth.
using both specific [3H]folic acid binding and Western blot analysis. The levels of the wild-type FR pools stayed fairly constant as cells reached confluency, and then increased (0.8 to 3.2 for SA and 1.4 to 4.9 pmol/mg protein for IC) through the slower postconfluent growth phase (Fig. 3A). This trend was also observed in the Western blot analysis (Fig. 3A, inset). The FRWT receptor was distributed so that more FR was in the IC pool (~60%) than in the SA pool, suggesting that the receptor is not processed and/or trafficked efficiently to the cell surface in these cells.

The changes in the levels of the FR containing the 144E→D and 201N→D mutations are shown in Figure 3B. The amount of SA receptor in the FR144,201 cells, which was very high relative to the FRWT cells, increased (85.3 to 109.0 pmol/mg protein) as cell growth slowed. However, the IC pool decreased (10.1 to 3.4 pmol/mg protein) as the rate of growth slowed. These results, which were confirmed by the Western analysis (Fig. 3B, inset), were somewhat different than we had reported previously (23). Although the decrease in IC FR was consistent, we showed previously that the total amount of receptor in the FR144,201 cells (previously referred to as FRGPI-16 cells) also decreased. Careful investigation of the assay conditions previously used to measure total FR with [3H]folic acid binding revealed that increasing percentages of receptor were not reliably measured when the total amount of this protein was very high, as in the FR144,201 cells. Results from measurements of total FR after modifying the assay to deal with extremely high levels of FR, confirm that the SA receptor with mutations at positions 144 and 201 did increase as cell growth slows (data not shown). This response is similar to that seen for the wild-type FR (Fig. 3A). The decrease in the IC receptor in the FR144,201 cells was different from the response in the FRWT cells. Although the IC receptor represents only a small fraction (at most ~10%) of the total FR in the FR144,201 cells, this difference could indicate that one or both of the mutations influence the regulation of this pool of receptor. A more dramatic difference between the wild-type and doubly mutant FRs is the proportion of receptor in the SA pool; >90% of the total FR in the FR144,201 cells was accessible from the cell surface, whereas <40% of the wild-type receptor was in the SA pool. This result suggests that the sequence alterations facilitate the processing and/or trafficking of the mutant FR to the cell surface.

**Effect of single mutations at position 144 and 201 on FR regulation.** The differences between the wild-type and doubly mutant FR led us to investigate whether they were caused by one of the two mutations or required both in combination. To address this question, the response of FR levels to changes in growth rates was measured in cells expressing FR containing either the 144N→D or 201N→D point mutation. The level of FR in the SA and IC pools increased in both the FR144 (SA, 0.8 to 2.0; and IC, 1.0 to 8.2 pmol/mg protein) and FR201 (SA, 3.1 to 26.8 and IC, 2.0 to 3.3 pmol/mg protein) cells as their growth rate decreased (Figs. 4A and B). Western analysis of these cells confirmed these trends (Fig. 4A and 4B, insets).

The response of both of the single mutation receptors was similar to the wild-type FR in that the amount in both pools increased as cell growth slowed. However, only the FR144 receptor behaved like the unaltered FR in its cellular distribution. The majority of the FRWT and FR144 receptor was in the IC pool, whereas more of the FR201 was in the SA pool than the IC pool. This distribution is similar to that of the FR144,201 cells, suggesting that the 201N→D point mutation was responsible for the altered trafficking and/or processing of FR in the doubly mutated receptor. Furthermore, because the 201N→D mutation eliminated one of the receptor’s three N-linked glycosylation sites, these results suggest that glycosylation at this site plays an important role in the cell surface expression of this receptor.

**Effect of the 144E→D and 201N→D point mutations on FR function.** To determine the effect of the various point mutations on the ability of the FR to transport folates, [3H]folic acid uptake was measured at SC, C, and PC growth in cells expressing the various FR forms. The rate of cellular folate uptake remained fairly constant for the FRWT and FR144 cells and decreased for FR201 and FR144,201 cells as growth slowed (Fig. 5). These results are consistent with those found above for the changes in FR levels in which the 144E→D mutant behaved more like the wild-type FR and the 201N→D mutant was more similar to the doubly mutated FR. Thus, the 201N→D change in sequence that leads to loss of the N-linked
oligosaccharide at this site influences the regulation of both FR function and surface expression.

Effect of a 67S→P mutation on the regulation of FR levels. Orr and Kamen (27) previously reported that a mutation at position 67 in FR that changed a serine to a proline conferred a "dominant-negative" phenotype on this receptor. To compare the effect of the 67S→P point mutation with that of the 144E→D and 201N→D mutations on receptor regulation, cell lines were generated that stably expressed FR constructs containing the 67S→P mutation alone, in combination with the 144E→D or the 201N→D mutation. The changes in levels of the various FRs were then measured as cell growth slowed.

The effect of the 67S→P mutation alone on the response to different growth rates (Fig. 6A) was similar to the wild-type FR. The SA and IC pools in FR67 cells both increased (1.8 to 20.9 and 1.7 to 5.0 pmol/mg protein, respectively) as cell growth slowed. This trend was confirmed by Western blot analysis (Fig. 6A, inset). This mutated FR differed from the wild-type receptor in its distribution within the cell. Like the FR144,201 and FR201 receptor, more of the 67S→P receptor was in the SA pool than the IC pool. Thus, this point mutation at position 67 influenced the processing and/or trafficking of FR in a similar manner as loss of the N-linked glycosylation at position 201. However, based on the relative mobility of the FR67 protein (Fig. 2), the 67S→P mutation did not appear to alter the core glycosylation of the FR.

Combining the 67S→P mutation with either the 201N→D or 144E→D point mutation resulted in responses to changes in growth rate that were markedly different from those of all of the other FR constructs we examined. For both FR67,201 and FR67,144 cells, SA and IC FR levels decreased as cell growth slowed (Fig. 6B and 6C). The decrease in total cellular FR levels was confirmed by Western blot analysis (Fig. 6B and 6C, inset). Comparison of the SA and IC pools showed that ~25–35% (FR67,201) and 30–40% (FR144,201) of the total cellular receptor had access to the cell surface. However, only one FR species was detected for both cell types. The apparent molecular weight (30 kDa, FR67,201; and 32 kDa, FR67,144) suggested that this is the high-mannose form of the receptor.

To determine what effect the 67S→P and 67S→P/201N→D mutations have on the ability of the FR to internalize folates, the rate of cellular [3H]folic acid uptake was measured in confluent FR67 and FR67,201 cells. The rate of folate uptake for the FR67 mutants was similar to that of the wild-type cells at confluence, whereas the FR67,201 cells internalized ~50% less [3H]folic acid (data not shown).

Characterization and localization of the SA and IC FR pools in FR67,201 cells. The FR67,201 cells expressed a single glycoform of FR which, based on its size, appeared to contain only two high-mannose oligosaccharides. To confirm the extent of processing of the glycoform, the sensitivity of the FR67,201 receptor to cleavage by endo H was evaluated. The digestion of a whole-cell lysate (collected at confluence when the FR in these cells is almost equally divided between the SA and IC pools) with endo H reduced the size of the FR to that of the high-mannose form of the receptor.

FIGURE 4 SA and IC FR levels in CHO cells stably transfected with either the FR144 (A) or FR201 (B) vector. SA and IC [3H]folic acid binding was measured at each of the indicated confluences. Data are expressed as pmol [3H]folic acid bound/mg protein. Values are means ± SEM, n = 3. Western blot analysis of FR expression prepared from whole-cell lysates was also measured at each of the confluences (inset). The relative molecular weight standard (in kDa) is indicated on the left. *Different from SC, P < 0.05.

FIGURE 5 FR function in CHO cells stably transfected with the FRWT, FR201, FR144,201 or FR144 vector. [3H]folic acid uptake was measured at each of the indicated confluences. Data are expressed as pmol [3H]folic acid internalized/mg cellular protein. Values are means ± SEM, n = 3.
seen for the totally deglycosylated receptor (lanes 2 and 5; the minor bands in lane 5 represent partial digestion products) (Fig. 7). Because endo H cleaves high-mannose but not complex oligosaccharides, this result indicates that the FR67,201 receptor contains only high-mannose N-glycans (29,30). This finding was confirmed by digestion of the SA and IC receptor separately. Endo H treatment of these fractions resulted in complete digestion and reduction in size of the FR (lanes 3, 4, 6, and 7). Thus, the FR with mutations at the 67 and 201 positions contains immature, high-mannose carbohydrates and likely represents the core-glycosylated protein that received N-linked glycosylations in the ER, but was not further modified to the complex species.

The difference in the forms of receptors expressed by the FRWT and FR67,201 cells suggests that the 67S3P/201N3D mutation somehow altered the post-translational processing of the receptor, an event that occurs in the Golgi apparatus. One explanation for the altered glycosylation could be that the 67S3P/201N3D mutant is retained in the ER. To investigate this possibility, we examined the distribution of the wild-type and 67S3P/201N3D mutant FR by immunofluorescence microscopy with specific markers for the ER and Golgi apparatus.

FRWT and FR67,201 cells were costained with the monoclonal FR antibody MOv19 and an antibody to BiP (Grp78), a molecular chaperone used as a marker for the ER (31). To better visualize the IC pool, cells were treated with PI-PLC before immunostaining. As shown in Figure 8A, both the mutant and wild-type FR (red, panels A and D, respectively) were detected in a perinuclear location (blue, Hoechst stain), a portion of which overlapped with the ER marker when the images were merged (yellow/orange, panels C and F). The fact that both the wild-type and 67S3P/201N3D mutant forms of FR were found in the ER is consistent with the immature high-mannose glycoform observed in the cells expressing each of these receptors (Figs. 2 and 7).

To determine whether the high-mannose 67S3P/201N3D mutant is also located in the Golgi, we assessed colocalization with GalT-GFP, a specific marker for the trans-Golgi (32). FR immunostaining (Fig. 8B) revealed a punctate perinuclear
changes in cellular growth rates similarly to the receptor previously characterized in MA-104 cells (23). Vitamin transport changed very little between the different confluencies, suggesting that the function and amount of the FRWT were not coordinately regulated. This result suggests that CHO cells are capable of regulating FR introduced by transfection in the same way as cells that normally express this protein. Furthermore, because the cDNA encoding the transfected protein does not contain the normal FR promoter or other components of the gene not included in the coding sequence, this implies that these elements are not used in the normal regulation of this receptor.

Comparison of the wild-type and $144_{E}^{-201_{N}}$ mutant receptor responses to slower growth rates demonstrated that these proteins differed significantly in their cellular distribution. The majority of the wild-type FR was intracellular (IC >60%), whereas most of the doubly mutant receptor was in the SA pool (>90%). Evaluation of the $144_{E}^{-D}$ or $201_{N}^{-D}$ mutations separately showed that the $201_{N}^{-D}$ change was responsible for this altered distribution. The finding that the total levels of both receptors respond similarly to changes in cellular growth rates despite the large difference in their cellular distributions suggests that regulation of FR levels is independent of the location of this receptor in the cell.

The $201_{N}^{-D}$ mutation eliminated one of the receptor’s three N-linked glycosylation sites and the expressed protein was only partially glycosylated. The altered cellular distribution of this receptor suggests that oligosaccharide modification at position 201 plays an important role in determining the cellular distribution of this receptor. This could be because partial deglycosylation facilitates the maturation and/or transport of the intracellular receptor to the cell surface. Alternatively, if receptor in the IC pool is unable to be further processed and transported to the cell surface, partial deglycosylation may prevent FR from reaching the IC pool, allowing more to reach the SA pool.

In a previous investigation of the importance of N-linked glycosylation for FR, Shen et al. (24) found that the expression of FR missing the carbohydrate at position 201 was decreased by ~20% on the cell surface and ~25% intracellularly relative to the wild-type receptor. Whether this represents findings similar to ours cannot be determined because the actual amounts of intracellular and surface FR were not presented. Roberts et al. (26) also studied a FR construct in which position 201 was mutated. They found that loss of the 201 oligosaccharide decreased the affinity of the receptor for folic acid by ~60% (26), suggesting that this modification plays an important role in the formation of the high-affinity FR binding site. The FR201 mutant receptor acquired endo H resistance with the same kinetics as the wild-type FR, indicating that the maturation of the remaining N-linked oligosaccharides was not altered and the partially deglycosylated receptor was not misfolded enough to target it for degradation in the ER. When combined with the results of Roberts et al. (26), our finding that FR lacking the N-linked oligosaccharide at position 201 appears to gain access to the cell surface more readily than the wild-type receptor suggests that loss of this modification influences the trafficking, rather than the processing, of FR to the cell surface.

The $67_{S}^{-p}$ mutation had effects on the FR very similar to those of the $201_{N}^{-D}$ mutation. The majority of the receptor with either of these changes was in the SA pool. The effect of introduction of a proline at position 67, which is just two amino acids away from the N-linked glycosylation site at position 69, on the folding capacity or conformation of the FR has not been studied. However, because proline will introduce

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**DISCUSSION**

Correction of the point mutations at positions 144 and 201 to the wild-type residues resulted in a FR that responded to pattern (red) in both the FR67,201 and FRWT cells (panels A and D, respectively). There was overlap of the some of wild-type FR perinuclear distribution with the green fluorescent marker for the trans-Golgi (panel H). (A) FR67,201 (panels A–C), and FRWT (D–F) cells were treated with PI-PLC before fixation and permeabilization. IC FR was visualized with the monoclonal antibody, MOv19 and anti-mouse Texas Red-conjugated IgG (red, panels A and D). BiP was immunostained with anti-BiP and anti-rabbit FITC-conjugated IgG (green, panels B and E). A yellow/orange color in panels C and F (Merge) indicates regions of overlap. For visualization of DNA (blue), Hoechst dye was included in the second-to-last wash. (B) FR67,201 (panels A–C) and FRWT (panels D–F) cells stably transfected to express GaIT-GFP, a specific marker for the trans-Golgi (B). (A) FR67,201 (panels A–C), and FRWT (D–F) cells treated with PI-PLC as described above. The cells were then fixed, permeabilized, and immunostained with the monoclonal FR antibody, MOv19, and IC FR was visualized with a Texas Red-conjugated secondary antibody (red, panels A and D). Co-localization of FR with the fluorescently tagged GaIT (green, panels B and E) was observed as a yellow/orange color (Merge, panels C and F).
new conformational constraints on the protein, it is highly likely that this substitution resulted in some perturbation in the tertiary structure of this receptor. The mobility of the 67S→P mutant receptor on SDS polyacrylamide gels indicated that it was normally glycosylated. Thus, the 67S→P and 201N→D mutated receptors both appear to have some subtle conformational changes and gain access to the cell surface more readily than wild-type FR. However, because only the FR201 transporter is partially deglycosylated, these findings suggest that the conformational change caused by the point mutations is responsible for the altered cellular distribution of these receptors.

FR with the 67S→P mutation in combination with either the 144E→D or the 201N→D mutation was very different from all of the other FR constructs. These double mutants were expressed at low levels, were primarily in the IC pool, and contained only the immature, high-mannose form of the receptor. A small amount of each mutant was found in the SA pool and was capable of transporting folate into the cell, indicating that maturation of the N-linked carbohydrates to the complex form was not required for either transport of FR to the cell surface or the function of the receptor. The FR67,201 receptor was partially deglycosylated, whereas the FR67,144 receptor appeared to be normally glycosylated. That both double mutants demonstrated similar behavior suggests that the effects of the two point mutations of the conformation of the protein rather than the loss one of the N-linked oligosaccharides was likely responsible for the altered processing and intracellular trafficking. The availability of these mutants should facilitate the elucidation of the signals that regulate the trafficking of FR.

The 67S→P mutation was reported by Orr and Kamen (27) to yield a “dominant-negative” phenotype because it blocked the ability of wild-type FR to bind and transport folates. These investigators also found that this mutation caused the receptor to be unable to bind folic acid and, as judged by immunofluorescence microscopy, to be trapped inside the cell. These results are very different from ours, which showed that the only difference between the 67S→P mutant and the wild-type receptor was in their cellular distribution. Although we have not investigated whether this mutation affects the affinity of the receptor for folic acid, comparison of the amounts of protein detected by Western blotting (Fig. 2) with the [3H]folic acid binding levels of the cells expressing the various FR constructs (Figs. 3, 4, and 6), suggests that it was not seriously perturbed.

The reason for the differences between the results of Orr and Kamen’s (27) and those in this study is not apparent. However, the behavior of the receptors with the 67S→P mutant in combination with either 201N→D or 144E→D mutations seems more similar to the mutant described by Orr and Kamen (27). Both our binding measurements and immunofluorescent microscopy results indicated that the majority of these mutants are intracellular, located in either the ER or Golgi. Furthermore, at confluence and postconfluence, the amount of [3H]folic acid binding in the FR67,201 and FR67,144 cells was very low. Therefore, one possible explanation for the discrepancy in our results is that the receptor studied by Orr and Kamen (27) actually had a second mutation that mimicked the effect of the 144 or 201 point mutations. We attempted to determine whether the 67,144 or 67,201 receptors act in a dominant-negative manner but have been unable to obtain stable transfectants of a cell line that expresses both the wild-type and either of these doubly mutant FRs despite numerous tries with several different cell lines. The reason for this is unclear and is currently being investigated.

Several studies have characterized the effect of naturally occurring mutations (27,33) or point mutations introduced by site-directed mutagenesis (24,34,35) on FR expression, ligand binding properties, and the ability to transport folates. One of the mutant FRs created by site-directed mutagenesis at position 142 (34) exhibited characteristics very similar to our 67S→P, 144E→D and 67S→P, 201N→D receptors. Replacing the tryptophan at position 142 with phenylalanine resulted in a protein that was expressed on the cell surface at relatively low levels and was less stable than the wild-type receptor. Like our double mutants containing the 67S→P change, the carbohydrates on the 142W→F receptor were not processed to the complex form. Despite this, this mutant FR was still able to bind folic acid. Thus, based on the behavior of our 67S→P, 144E→D and 67S→P, 201N→D receptors and the previously characterized 142W→F transporter, we can conclude that FR with immature, high-mannose oligosaccharides of the ICs can be transported to the cell surface, although with considerable inefficiency. Furthermore, maturation of the N-linked carbohydrates is not necessary for the receptor to be able to bind folic acid.

Our finding that a high percentage of the wild-type FR was located in the ER and Golgi suggests that the trafficking of this receptor to the cell surface is normally inefficient. We speculated previously that this might be because the high levels of FR in our transfectants overwhelmed the cellular machinery used for this (23). This can now be ruled out because the FR144, 201 cells expressed much higher levels of receptor and processed the majority of it to the SA pool. Altering the conformation of a protein usually results in its retention in the ER and early degradation. The conformational changes introduced by the 201N→D and 67S→P mutations appear to have the opposite effect. Why a FR with a non-native conformation is trafficked more readily to the cell surface is not known. This may be related to the overexpression of FR by some tumors, which is thought to facilitate the growth of the malignant cells (13). Mutated forms of the FR have been isolated from tumors (27,33); if they are more readily expressed on the cell surface, they would be expected to be more effective at transporting folate to these rapidly growing cells.

One possible explanation for the retention of FR in the IC pool is that this is a reservoir from which receptors could be mobilized when cellular needs for folate are increased. Alternatively, the receptor could have an important function intracellularly that is independent of its normal vitamin transport role. Either of these explanations could account for why FR levels increase as the amount of vitamin transported decreases. Further study into the fate of the IC receptor will be required to define the role of this pool of FR.

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