Expression levels of functional folate receptors α and β are related to the number of N-glycosylated sites

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In a previous study with inhibitors of N-glycosylation, it was proposed that core glycosylation of the folate receptor (FR) is required for the proper folding of the protein [Luhrs (1991) Blood **77**, 1171–1180]. The human FR isoforms type α and type β have three and two candidate sites for N-glycosylation respectively, only one of which is conserved. The significance of N-glycosylation at each of these loci in the expression and function of FR was examined by eliminating the sites both individually and in combination by introducing Asn → Gln substitutions. Translation experiments *in vitro* showed that the mutations did not alter the synthetic rates of the polypeptides. The recombinant proteins were expressed in human 293 fibroblasts. Treatment with N-glycanase and analysis by Western blotting of the wild-type and mutant proteins revealed that all of the candidate sites in both FR-α and FR-β are glycosylated. When all of the N-glycosylation sites were abolished, 2 % and 8 % of FR-α and FR-β respectively were expressed on the cell surface compared with the corresponding wild-type proteins; the residual FR polypeptides in the cell lysates were unable to bind [^{3}H]folic acid. In both the proteins, the inclusion of each additional N-glycosylation site partly contributed to restoration of cell surface [^{3}H]folic acid binding and receptor-mediated folate transport. Further, in FR-β the introduction of an additional unnatural site of N-glycosylation resulted in the enhancement of the expression of the cell surface receptor compared with the wild-type protein. The results indicate that the total mass of N-glycosylation, not a specific locus of the modification, is critical for the efficient folding and optimal expression of functional FR-α and FR-β.

INTRODUCTION

The mammalian folate receptor (FR) is a single polypeptide that binds folic acid with a relatively high affinity ($K_d < 1$ nM) and with a stoichiometry of 1 : 1 [1]. The receptor also binds the major circulating folate coenzyme, (6*S*)-5-methyltetrahydrofolate and various anti-folate drugs [1]. Multiple isoforms of FR have been identified from human (hFR-α, hFR-β and hFR-γ) and murine (mFR-α and mFR-β) sources, and they share amino acid sequence identity of 68–79 % including 16 cysteine residues that are all conserved [2–6]. The FR isoforms are also functionally distinct. Thus FR-α and FR-β have opposite stereospecificities for reduced folate coenzymes and different affinities for folate compounds and anti-folate drugs [7,8]. Further, FR-α and FR-β are attached to the cell surface by a glycosyl phosphatidylinositol (GPI) membrane anchor and are released into the medium by treatment with the bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) [3,9]. FR-γ, in contrast, is a constitutively secreted protein [10]. Ligands and folate-conjugated macromolecules bound to the GPI-anchored FR are internalized by an endocytic mechanism [11–14]. The FR isoforms are also differentially tissue-specific and their levels are differentially elevated in several malignant tissues [15]. FR has been utilized as a tumour target in a variety of experimental cancer therapies [16–23].

The FR isoforms have two (hFR-β, mFR-β) or three (hFR-α, hFR-γ, mFR-α) candidate sites for N-linked glycosylation; only one of these sites is conserved between the human and murine proteins (Figure 1). It has been demonstrated that deglycosylation of FR-α from KB cells with endoglycosidase H or of a mixture of FR-α and FR-β purified from placenta with N-glycanase does not affect the ability of the proteins to bind folate [24,25]. In contrast, in a systematic study with inhibitors of glycosylation, it was demonstrated that core glycosylation is essential for the acquisition of the ligand-binding property of FR-α in KB cells [24].

The present study was undertaken to identify the candidate sites of N-glycosylation in hFR-α and hFR-β at which the modification can occur *in vivo* and to test the role of specific sites of N-glycosylation in the expression of functional FR. Our working hypothesis was that the single candidate site of N-glycosylation that is conserved among the five human and murine FR polypeptides is either the most significant or the only contributor to the formation of a functional receptor. To address these issues, we used a mutagenesis approach to either eliminate potential glycosylation sites in hFR-α and hFR-β systematically or introduce an unnatural glycosylation site in hFR-β. We studied the effects of these changes on the glycosylation of the FR polypeptides, their ability to bind [^{3}H]folic acid and the extent of their expression on the cell surface as functional folate-binding and transport proteins.

MATERIALS AND METHODS

Mutagenesis and recombinant plasmids

All of the mutants were constructed by using PCR. The reactions were performed with Vent DNA polymerase (New England Biolabs) and the cDNA for either FR-α or FR-β as template. Two complementary oligonucleotides (Table 1), containing the desired mutations, were used in combination with upstream and downstream primers containing appropriate restriction sites. The resulting PCR products were digested at both ends with the restriction enzymes and substituted for the corresponding fragments in the cDNA species for FR-α and FR-β in the plasmid

Abbreviations used: FR, folate receptor; GPI, glycosyl phosphatidylinositol; h, human; HBSS, Hanks balanced salt solution; m, mouse; PI-PLC, phosphatidylinositol-specific phospholipase C.

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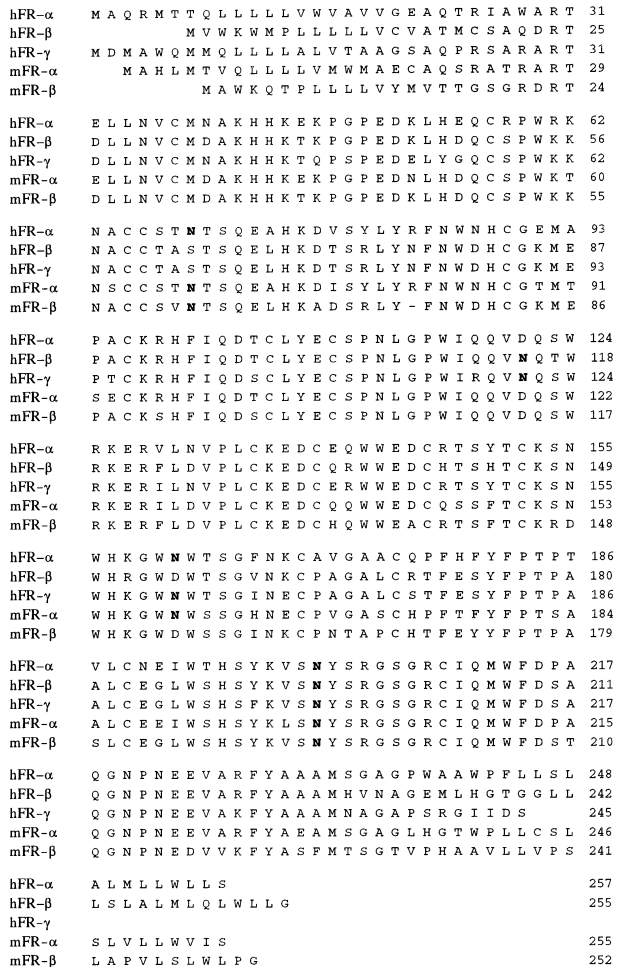

Figure 1 Aligned amino acid sequences of hFR-α, hFR-β, hFR-γ, mFR-α and mFR-β

Highlighted residues are potential sites for N-linked glycosylation.

pCDNAIneo (Invitrogen). The recombinant plasmids were amplified in *Escherichia coli* MC1061/p3 and the entire cDNA sequence was verified by dideoxy sequencing with Sequenase Version 2.0 (USB).

Cell culture and transfection

Human 293 fibroblasts were grown in Eagle's minimal essential medium (Irvine Scientific, Santa Anna, CA, U.S.A.) supplemented with fetal bovine serum (10%, v/v), penicillin (100 i.u./ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). Transfections were performed with lipofectamine (Gibco BRL, Grand Island, NY, U.S.A.) in accordance with the manufacturer's protocol. For the transport assay, cells were grown in folate-free RPMI medium (Irvine Scientific) supplemented with fetal bovine serum (10%, v/v), penicillin (100 i.u./ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) for at least one generation before transfection. Transfection was performed in folate-free RPMI supplemented with transferrin (4 g/ml; Sigma), insulin (20 μg/ml; Sigma) and epidermal growth factor (10 ng/ml; Sigma). Transfection efficiencies were normalized to a co-transfected β-galactosidase standard. The transfection efficiencies were generally uniform.

Table 1 Oligonucleotides (5′ → 3′) used to generate the glycosylation mutants of FR-α and FR-β

Underlined bases in the oligonucleotide sequences indicate the mutated sites. For hFR-α, N1, N2 and N3 indicate the retention of Asn residues at positions 69, 161 and 201 respectively, and Q1, Q2 and Q3 refer to Asn → Gln substitutions at N1, N2 and N3 respectively. For hFR-β, N1 and N2 indicate the retention of Asn residues at positions 115 and 195 respectively, and Q1 and Q2 indicate Asn → Gln substitutions at N1 and N2 respectively; (N3) denotes an unnatural candidate site of N-glycosylation.

Mutant construct	Sequences of mutagenic oligonucleotides
hFR-$\alpha_{Q1,N2,N3}$	TGTTCTACCCAGACCAGCCAG
	CTGGCTGGTCTGGGTAGAACA
hFR-$\alpha_{N1,Q2,N3}$	AAGGGCTGGCAGTGGACTTCA
	TGAATCCACTGCCAGCCCTT
hFR-$\alpha_{N1,N2,Q3}$	AAGGTCAGCCAGTACAGCCGA
	TCGGCTGTACTGGCTGACCTT
hFR-$\beta_{Q1,N2}$	CAGCAGGTGCAGCAGACGTGG
	CCACGTCTGCTGCACCTGCTG
hFR-$\beta_{N1,Q2}$	AAGGTCAGCCAGTACAGCCGA
	TCGGCTGTACTGGCTGACCTT
hFR-$\beta_{N1,N2,(N3)}$	TGCACAGCTAACACCAGCCAG
	CTGGCTGGTGTTAGCTGTGCA

[^{3}H]Folic acid binding assay

The radioactive binding assay for cell surface FR was performed as described previously [10]. Cells (3×10^6) at 48 h after transfection were washed in a 35 mm tissue culture dish at 4 °C once with 1 ml of Hanks balanced salt solution (HBSS), twice with 1 ml of acid saline [10 mM sodium acetate (pH 3.5)/150 mM NaCl], and once again with 1 ml of HBSS. The cells were then incubated with 2 pmol of [^{3}H]folic acid (Moravek) in 1 ml of HBSS at 4 °C for 1 h. After the cells had been washed once with 1 ml of HBSS, [^{3}H]folic acid bound to the cell surface was recovered in 0.5 ml of acid saline and subjected to liquid-scintillation counting. The specific [^{3}H]folic acid binding was determined by subtracting the value for the negative control cells, which had been transfected with the vector alone. The specificity of [^{3}H]folic acid binding was also established by competition with unlabelled folic acid (1 μM).

Intracellular folic acid binding protein was estimated as described [10]. Cells (3×10^6) were dissolved in 10 mM sodium phosphate (pH 7.5)/150 mM NaCl/1% (v/v) Triton X-100 and insoluble material was removed by centrifugation at 10000 ***g*** for 2 min. The pH of the supernatant was lowered to 3.5 by the addition of 1/10 vol. of 100 mM sodium acetate, pH 2.5, at 4 °C. Simultaneously, Triton X-100 was added to a final concentration of 1%. Then an equal volume of ice-cold Norit A charcoal (80 mg/ml) was added to remove endogenous folic acid. The pH of the solution was adjusted back to 7.5 with a pretitred amount of NaOH. Aliquots of the treated cell lysates were taken for the assay in a final volume of 0.5 ml. The samples were incubated with 2 pmol of [^{3}H]folic acid at 37 °C for 2 h; [^{3}H]folic acid-binding protein was assayed by the charcoal binding assay described previously [7]. Specific [^{3}H]folic acid binding was determined by subtracting the value for the negative control cells, which had been transfected with the vector alone. The specificity of [^{3}H]folic acid binding was also established by competition with unlabelled folic acid (1 μM).

Measurement of [^{3}H]folic acid uptake

Cells (6×10^6) in duplicate 35 mm tissue culture dishes were grown and transfected in folate-free RPMI as described above.

At 48 h after transfection the cells were washed at 4 °C once with 1 ml of HBSS, twice with 1 ml of acid saline and once again with 1 ml of HBSS. The cells were then incubated with 1 ml of HBSS containing 12.5 pmol of [^{3}H]folic acid with or without 1 μM folic acid at 37 °C for 2 h. After the cells had been washed once with 1 ml of HBSS, [^{3}H]folic acid bound to the cell surface was recovered in 0.5 ml of acid saline and subjected to liquid-scintillation counting. After one wash with 1 ml of cold HBSS, the cells were scraped off the dishes with a rubber 'policeman', resuspended in 0.5 ml of HBSS and subjected to scintillation counting. The specific uptake of [^{3}H]folic acid was determined by subtracting the value for the negative control cells, which had been transfected with the vector alone. The specificity of [^{3}H]folic acid uptake was also established by competition with unlabelled folic acid (1 μM).

Treatment with PI-PLC

Cleavage of the GPI membrane anchor of hFRs-α and hFRs-β and their quantitative release from the cell surface were accomplished by treating the cells with PI-PLC as described previously [10].

Deglycosylation and Western blot analysis

Cells (6×10^6) were dissolved in 100 μl of 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM NaCl, 0.5 % Triton X-100, 10 mM EDTA and 50 mM 2-mercaptoethanol. Insoluble material was removed by centrifugation at 10000 ***g*** for 2 min. A portion of the sample (10 μl) was used for protein estimation by the Bio-Rad protein assay (Bio-Rad Laboratories). The remaining sample was divided into two equal portions; 3 μl of N-glycanase (Genzyme) was added to one of them. The samples were incubated at 37 °C for 20 h followed by SDS/PAGE [12.5 % (w/v) gel] and Western blot analysis as described [7] with affinity-purified rabbit antibodies to FR from human placenta [4] as the probe.

Translation *in vitro*

Translation *in vitro* was performed with the TNT® Coupled Reticulocyte Lysate System (Promega) in the presence of Canine Pancreatic Microsomal Membranes (Promega) in accordance with the manufacturer's protocol. In a 25 μl reaction, 12.5 μl of TNT lysate, 0.5 μl of TNT reaction buffer, 0.5 μg of plasmid DNA, 2.5 μl of canine microsomal membranes and 0.5 μl of RNA polymerase were added. The amino acid mixture (lacking methionine), RNasin ribonuclease inhibitor and [^{35}S]methionine were added to final concentrations of 20 μM, 0.8 unit/μl and 0.8 mCi/ml, respectively. The reaction was incubated at 30 °C for 90 min and a 100-fold excess of unlabelled methionine was added to stop the reaction. Products translated *in vitro* (4 μl) were mixed with 20 μl of 1 × SDS sample buffer and half of each sample was subjected to electrophoresis on an SDS/12.5 % (w/v) polyacrylamide gel. The gel was fixed in 10 % (w/v) trichloroacetic acid, dried and exposed to X-ray film.

RESULTS

Identification of N-glycosylated sites in hFR-α and hFR-β

To identify the sites of N-linked glycosylation in hFR-α and hFR-β, the putative modification sites (Figure 1) were disrupted both individually and in all of the possible combinations. The sites were altered by substitution of the Asn residues with the structurally similar Gln, which cannot be modified. The total cell lysates from human 293 fibroblasts expressing the wild-type and mutant proteins were subjected to Western blot analysis (Figures 2A to 2C). Wild-type hFR-α, containing three candidate N-glycosylation sites, and the mutant forms of the receptor, containing any one or two of these sites, showed multiple or diffuse bands, as expected, from microheterogeneity in N-glycosylation [4] and the presence of incompletely modified intracellular protein in the cell lysates [9]. The relative mobilities of the bands increased progressively with a decrease in the number of the putative glycosylation sites (Figure 2A). In the absence of N-glycosylation sites, the protein gave a single relatively sharp band whose mobility was greater than that of the mutants containing at least one site of glycosylation (Figure 2A). Treatment with N-glycanase of the mutant forms of FR-α containing any one of the three available sites for N-glycosylation resulted in at least partial deglycosylation, yielding the appearance of a sharp band corresponding to the aglycosylated hFR-α (Figure 2B). The above results demonstrate that N-glycosylation occurs *in vivo* at all of the three candidate sites in hFR-α.

A similar analysis of the wild-type and mutant constructs of hFR-β revealed multiple bands for wild-type hFR-β, the lowest of which corresponded to the aglycosylated form of the poly-

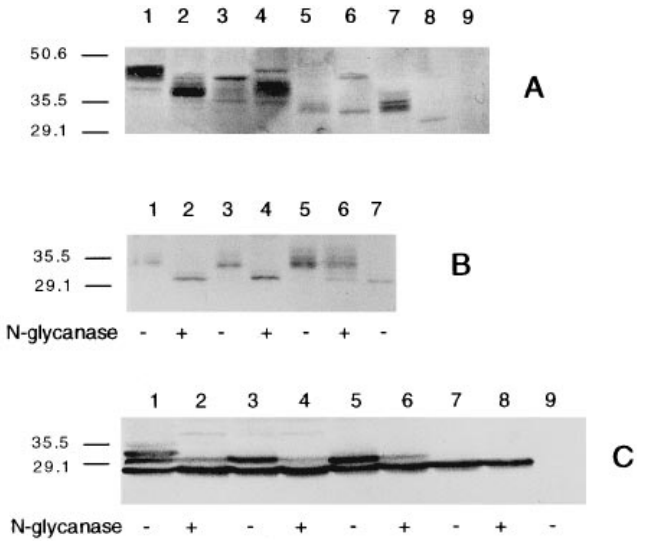

Figure 2 Western blot analysis of total cell lysates from human 293 fibroblasts transfected with hFR-α, hFR-β and mutated FRs

In this legend N1, N2 and N3 of hFR-α indicate the retention of the Asn residue at positions 69, 161 and 201 respectively; Q1, Q2 and Q3 of hFR-α indicate Asn → Gln substitutions at N1, N2 and N3 respectively; N1 and N2 of hFR-β indicate the retention of Asn at positions 115 and 195 respectively; and Q1 and Q2 of hFR-β indicate Asn → Gln substitutions at these positions respectively. Numbers at the left of each panel are molecular masses in kDa. (**A**) Wild-type hFR-α and its mutant forms containing two, one or none of the three candidate N-glycosylation sites. The blot was probed with affinity-purified rabbit polyclonal antibodies to hFR-α as described in the Materials and methods section. Lane 1, hFR-$\alpha_{(N1,N2,N3)}$; lane 2, hFR-$\alpha_{(Q1,N2,N3)}$; lane 3, hFR-$\alpha_{(N1,Q2,N3)}$; lane 4, hFR-$\alpha_{(N1,N2,Q3)}$; lane 5, hFR-$\alpha_{(Q1,Q2,N3)}$; lane 6, hFR-$\alpha_{(N1,Q2,Q3)}$; lane 7, hFR-$\alpha_{(Q1,N2,Q3)}$; lane 8, hFR-$\alpha_{(Q1,Q2,Q3)}$; lane 9, negative control (cells transfected with vector alone). (**B**) Deglycosylation of the mutant forms of hFR-α containing a single N-glycosylation site. The blot was probed with affinity-purified rabbit polyclonal antibodies to hFR-α. Lanes 1 and 2, hFR-$\alpha_{(Q1,Q2,N3)}$; lanes 3 and 4, hFR-$\alpha_{(N1,Q2,Q3)}$; lanes 5 and 6, hFR-$\alpha_{(Q1,N2,Q3)}$; lane 7, hFR-$\alpha_{(Q1,Q2,Q3)}$. (**C**) Deglycosylation of wild-type hFR-β and its mutant forms containing either one or neither of the two candidate N-glycosylation sites. The blot was probed with affinity-purified rabbit polyclonal antibodies to hFR-β. Lanes 1 and 2, FR-$\beta_{(N1,N2)}$; lanes 3 and 4, FR-$\beta_{(Q1,N2)}$; lanes 5 and 6, FR-$\beta_{(N1,Q2)}$; lanes 7 and 8, FR-$\beta_{(Q1,Q2)}$; lane 9, negative control (cells transfected with vector alone).

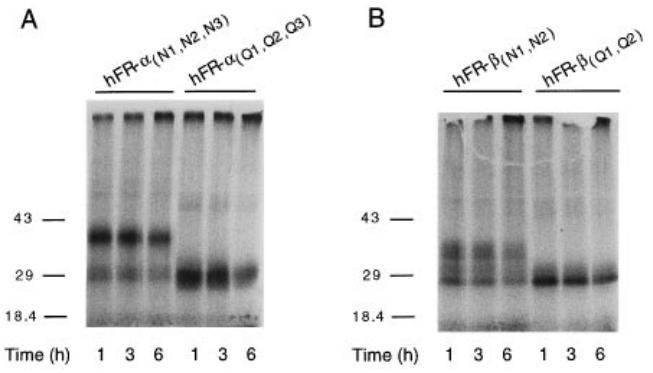

Figure 3 Translation *in vitro* of wild-type FRs and mutant FRs lacking N-glycosylation sites

FR-α, FR-β and the aglycosylated forms of FR-α and FR-β were translated *in vitro* in the presence of [^{35}S]methionine in dog pancreas microsomes as described in the Materials and methods section. After 1.5 h of translation, a 100-fold excess of unlabelled methionine was added; after different intervals at 37 °C aliquots were frozen and subsequently analysed by SDS/PAGE and autoradiography. N1, N2 and N3 of hFR-α indicate the retention of Asn at positions 69, 161 and 201 respectively; Q1, Q2 and Q3 of hFR-α indicate Asn → Gln substitutions at N1, N2 and N3 respectively; N1 and N2 of hFR-β indicate the retention of Asn at positions 115 and 195 respectively; and Q1 and Q2 of hFR-β indicate Asn → Gln substitutions at these positions respectively. Numbers at the left of each panel are molecular masses in kDa.

peptide (Figure 2C). Treatment of hFR-β with N-glycanase resulted primarily in a band corresponding to the aglycosylated form of hFR-β (Figure 2C). Mutants of hFR-β containing a single N-glycosylation site showed a band corresponding to the aglycosylated form of the protein and an additional band with a lower mobility, which on treatment with N-glycanase co-migrated with the band corresponding to the aglycosylated protein (Figure 2C). It is thus concluded that the two candidate sites of N-glycosylation in hFR-β are both modified *in vivo*.

Translation of FR-α, FR-β and their aglycosylated forms *in vitro*

Before the effects of the mutations in FR-α and FR-β on their cell surface expression and function were tested, it was desirable to ensure that the mutations did not affect the efficiency of mRNA translation. Therefore FR-α, FR-β and their aglycosylated forms were synthesized by a translation system *in vitro*, analysed on denaturing electrophoresis gels and detected by autoradiography (Figure 3). Both the fully glycosylated FRs and the aglycosylated forms showed bands of similar intensities (Figure 3), indicating similar levels of translation for the two forms of the proteins.

Effects of glycosylation at specific sites on the expression of functional hFR-α and hFR-β

The expression of functional FR on the surface of cells expressing the wild-type and mutant constructs of hFR-α and hFR-β was measured from the binding and transport of [^{3}H]folic acid. In general, the hFR-α constructs with three, two, one or no N-glycosylation sites showed progressively lower levels of [^{3}H]folic acid binding at the cell surface (Table 2). Removal of a single N-glycosylation site from hFR-α resulted in levels of cell surface FR that were 30–80 % of the level of the wild-type protein; removal of two glycosylation sites further decreased the values to 12–40 %. In the absence of any N-glycosylation site, there was only approx. 2 % of FR expression on the cell surface relative to wild-type FR-α. Similarly, disruption of one of the two N-glycosylation sites in hFR-β resulted in cell surface FR that was 60–66 % of the level of the wild-type protein (Table 3). In the absence of both the glycosylation sites in hFR-β, approx. 8 % of FR was expressed on the cell surface compared with wild-type FR-β.

Table 2 Relative expression levels of [^{3}H]folic acid-binding protein on the surface of, and relative uptake of [^{3}H]folic acid by, cells expressing the wild-type and mutant constructs of hFR-α

The assays were repeated at least four times as described in the Materials and methods section and concordant results were obtained. N1, N2, N3, Q1, Q2 and Q3 have the same meanings as in Table 1.

	[^{3}H]Folic acid (% of the value for wild-type FR-α)	
hFR construct	Cell surface	Intracellular
hFR-$\alpha_{(N1,N2,N3)}$	100.00 ± 8.22	100.00 ± 0.73
hFR-$\alpha_{(Q1,N2,N3)}$	54.41 ± 2.46	61.08 ± 3.29
hFR-$\alpha_{(N1,Q2,N3)}$	28.11 ± 0.36	22.58 ± 0.55
hFR-$\alpha_{(N1,N2,Q3)}$	79.70 ± 2.68	76.60 ± 7.60
hFR-$\alpha_{(Q1,Q2,N3)}$	18.84 ± 0.43	16.90 ± 0.41
hFR-$\alpha_{(N1,Q2,Q3)}$	12.76 ± 0.10	16.30 ± 1.60
hFR-$\alpha_{(Q1,N2,Q3)}$	42.86 ± 2.12	34.15 ± 3.24
hFR-$\alpha_{(Q1,Q2,Q3)}$	1.74 ± 0.16	3.30 ± 0.06

The relative expression levels of [^{3}H]folic acid-binding protein on the surface of cells expressing the wild-type and mutant constructs of hFR-α and hFR-β also reflected the relative uptakes of [^{3}H]folic acid by these cells (Tables 2 and 3). This result suggests that the transport of folate by FR is not dependent on the presence of N-glycosylation but is instead dependent on the expression level of [^{3}H]folic acid-binding protein on the cell surface.

From Tables 2 and 3 it is clear that the single conserved glycosylation site in hFR-α and hFR-β (Figure 1) is not the only site or even the most critical site at which N-glycosylation is required for the expression of functional FR. Although it seems that in the absence of glycosylation at position 161 in hFR-α, there is a greater decrease in the expression of FR, it might be concluded that, in general, every glycosylation site in both hFR-α and hFR-β contributes to the optimal expression of functional FR.

To test further the relationship between the number of N-glycosylation sites and the cell surface expression of FR, a third unnatural N-glycosylation site was introduced in hFR-β by

Table 3 Relative expression levels of [^{3}H]folic acid-binding protein on the surface of, and relative uptake of [^{3}H]folic acid by, cells expressing the wild-type and mutant constructs of hFR-β

The assays were repeated at least four times as described in the Materials and methods section and concordant results were obtained. N1, N2, Q1 and Q2 have the same meanings as in Table 1.

	[^{3}H]Folic acid (% of the value for wild-type FR-β)	
hFR construct	Cell surface	Intracellular
hFR-$\beta_{(N1,N2)}$	100.00 ± 2.91	100.00 ± 0.50
hFR-$\beta_{(Q1,N2)}$	60.79 ± 3.42	56.27 ± 4.78
hFR-$\beta_{(N1,Q2)}$	66.08 ± 0.88	57.16 ± 2.38
hFR-$\beta_{(Q1,Q2)}$	8.80 ± 0.50	7.69 ± 0.48

Table 4 Relative expression levels of [^{3}H]folic acid-binding protein on the surface of cells expressing the wild-type hFR-β and its mutant containing an additional N-glycosylation site

The assays were repeated at least four times as described in the Materials and methods section and concordant results were obtained. N1, N2, Q1 and Q2 have the same meanings as in Table 1; N3 indicates an unnatural potential N-glycosylation site that was introduced by mutagenesis.

hFR construct	Cell surface [^{3}H]folic acid bound (% of binding to wild-type FR-β)
hFR-$\beta_{(N1,N2)}$	100.00 ± 2.91
hFR-$\beta_{(N1,N2,N3)}$	154.56 ± 8.98

converting Ser-63 into Asn. When this construct was expressed in human 293 cells, the level of cell surface [^{3}H]folic acid-binding protein was approx. 1.5 times that of the wild-type hFR-β (Table 4). The results in Tables 2–4 thus indicate that it is the total mass of N-glycosylation rather than a specific site of glycosylation that is critical for the expression of functional FR.

Binding of [^{3}H]folic acid by FR polypeptides in intracellular compartments

To test whether the aglycosylated FR polypeptides might be retained in intracellular compartments as [^{3}H]folic acid-binding proteins, cells expressing hFR-α or hFR-β as well as cells expressing the aglycosylated mutants of hFR-α and hFR-β were first treated with PI-PLC to remove cell surface [^{3}H]folic acid-binding protein in a quantitative manner (Table 5). The cells were then solubilized with Triton X-100 and the lysates were assayed for the binding of [^{3}H]folic acid. Whereas a significant amount of intracellular [^{3}H]folic acid-binding protein was detected in the cells expressing the wild-type FRs, there was no detectable binding of the radioligand by the intracellular proteins in cells expressing the aglycosylated proteins (Table 5) despite the fact that immunoreactive protein was found in all of these cells by using anti-FR antibodies (Figures 2A and 2C). These results indicate the absence of intracellularly trapped [^{3}H]folic acid-binding protein from cells expressing either hFR-α or hFR-β in which all of the N-glycosylation sites were disrupted.

DISCUSSION

The application of the mutagenesis approach to examine the significance of N-glycosylation in hFR-α and hFR-β offers the advantage that the pleiotropic changes due to interference with the glycosylation machinery encountered in experiments with glycosylation inhibitors can be avoided. Further, the effects of glycosylation at specific sites in the proteins can be studied. The Asn → Gln mutations generated in this study to disrupt each candidate site of N-glycosylation are conservative and are therefore unlikely to directly cause significant alterations in the tertiary structures of the proteins. The results of this study have clearly demonstrated that each of the candidate sites of N-glycosylation in both hFR-α and hFR-β is actually modified *in vivo*. Further, the ability of both the FR isoforms to transport folate is dependent on the expression level of [^{3}H]folic acid-binding protein on the cell surface but not on the glycosylation status of the proteins.

In the absence of N-glycosylation, both hFR-α and hFR-β were expressed in a functional form on the cell surface at very low levels. However, Western blot analysis under reducing conditions after deglycosylation with N-glycanase showed the occurrence of the intact polypeptide from the total lysates of cells expressing the aglycosylated form of FR compared with the wild-type protein. After the removal of the cell surface FR by treatment with PI-PLC, there was no detectable [^{3}H]folic acid binding in the cell lysates containing the aglycosylated hFR-α and hFR-β polypeptides, in contrast with the lysates from cells expressing the wild-type proteins. These results are consistent with those of Luhrs [24], who demonstrated that, in KB cells, inhibition of core glycosylation by tunicamycin resulted in the expression of an aglycosylated form of hFR-α that was unable to bind to folate. It has also been demonstrated that FR purified from human placenta, which contains a mixture of hFR-α and hFR-β, can be deglycosylated with N-glycanase without alteration of its affinity for [^{3}H]folic acid [25]. Similarly, hFR-α from KB cells, when deglycosylated with endoglycosidase H, retained its ability to bind folate. It therefore seems, as previously suggested [24], that N-glycosylation is required to aid in the formation of the folded state of the FR polypeptide defined by the acquisition of its property of binding folic acid.

Given the uniformity in cDNA transfection, any observed differences in the total amount of FR present in the cells expressing native compared with aglycosylated forms of FR (Figure 2) are not likely to be due to differences in their synthetic rates because the proteins could be translated *in vitro* with comparable efficiencies. In contrast, the lack of N-glycosylation might enhance the rate of intracellular degradation of the FR polypeptide. The correct folding of proteins, their sorting and the rates of their intracellular degradation are interrelated phenomena because it has been demonstrated that misfolded proteins

Table 5 Binding of [^{3}H]folic acid by FR polypeptides in intracellular compartments

The assays were repeated at least four times as described in the Materials and methods section and concordant results were obtained. hFR-$\alpha_{(N1,N2,N3)}$ and hFR-$\beta_{(N1,N2)}$ denote wild-type hFR-α and hFR-β respectively; hFR-$\alpha_{(Q1,Q2,Q3)}$ and hFR-$\beta_{(Q1,Q2)}$ denote aglycosylated forms of hFR-α and hFR-β respectively. −PI-PLC and +PI-PLC indicate cells that were untreated and treated with PI-PLC respectively, as described in the Materials and methods section. Errors were less than 10%.

	[^{3}H]Folic acid bound (pmol/10^7 cells)			
	Cell surface		Intracellular	
Transfected cDNA	−PI-PLC	+PI-PLC	−PI-PLC	+PI-PLC
hFR-$\alpha_{(N1,N2,N3)}$	1.08	0.00	0.33	0.32
hFR-$\alpha_{(Q1,Q2,Q3)}$	0.018	0.00	0.00	0.00
hFR-$\beta_{(N1,N2)}$	1.10	0.07	0.29	0.30
hFR-$\beta_{(Q1,Q2)}$	0.08	0.0036	0.00	0.00

are retained in the endoplasmic reticulum and eventually degraded [26]. Although glycosylation is required for targeting lysosomal proteins, a general role for core glycosylation in protein sorting has been ruled out because there are a number of examples of glycoproteins that are efficiently targeted to the plasma membrane or normally secreted in the absence of glycosylation [27]. However, a possible direct role for core glycosylation in protein sorting in specific instances cannot be ruled out as demonstrated by the requirement for N-glycosylation in constitutive apical secretion but not in regulated secretion [28–30]. It should be noted that, for many proteins that in the absence of N-glycosylation are retained inside the cell, there are no practical means of monitoring folding in intracellular compartments. For FR, when the N-glycosylation sites were abolished, there was no detectable [^{3}H]folic acid binding by the intracellular protein. Instead, the small amounts of the aglycosylated proteins that were capable of binding [^{3}H]folic acid seemed to be targeted efficiently to the cell surface. Other examples of proteins that are dependent on core glycosylation for the initial acquisition of ligand binding include the epidermal growth factor receptor [31,32] and corticosteroid-binding globulin [33].

The proper folding of FR might be critically dependent on the formation of the correct disulphide bonding pattern involving its 16 cysteine residues. This is likely, because the number and positions of the cysteine residues are conserved in the five known homologous FR polypeptides from human and murine sources (Figure 1). Further, the fully folded FR, with the correct disulphide bonds, is a highly stable protein and the mature FR can be denatured by treatment at extremely acidic conditions (pH 2.5) but will immediately regain its ligand-binding properties when returned to near neutral pH. It might therefore be reasonable to speculate that in the nascent FR polypeptide the formation of the appropriate set of disulphide bonds is extremely inefficient in the absence of core glycosylation.

The results from this study clearly demonstrate that N-glycosylation at only the single conserved site in FR will not result in optimal expression of [^{3}H]folic acid-binding protein. In fact, glycosylation at this site is not even the major contributor to the expression of functional FR. The expression level of wild-type FR results from the combined effect of glycosylation at all of the candidate sites, each of which by itself results in partial expression of functional FR. These observations, together with the finding that the expression of hFR-β can be further enhanced by the introduction of an additional unnatural site of N-glycosylation, provide strong evidence that it is the total mass of N-glycosylation rather than any specific site of the modification that is critical for the proper folding of FR. An obvious implication of this finding is that core glycosylation is not likely to be involved in a specific step in the folding pathway of the polypeptide backbone of FR but, rather, glycosylation might facilitate the folding of FR in a general manner, either by a direct effect on the physical properties of the polypeptide or by indirect means. A relationship between N-glycosylation and the energetics of protein folding is evident from studies of model proteins such as ribonuclease B and IgM-tailpiece glycopeptide [34]. N-glycosylation stabilizes the partly folded states of the proteins and also enhances their free energy of unfolding. In contrast, a possible indirect role for glycosylation in protein folding *in vivo* involving interactions with enzymes and chaperones in the endoplasmic reticulum is conceivable and needs to be investigated.

We thank Mr. William Kohler for technical assistance and Mrs. Jenny Zak for typing the manuscript. This work was supported by NCI grant no. R29CAS-7598 and a Junior Faculty Research Award from the American Cancer Society to M. R.

REFERENCES

1 Antony, A. C. (1992) Blood **79**, 2807–2820
2 Elwood, P. C. (1989) J. Biol. Chem. **264**, 14893–14901
3 Lacey, S. W., Sanders, J. M., Rotherberg, K. G., Anderson, R. G. W. and Kamen, B. A. (1989) J. Clin. Invest. **84**, 715–720
4 Ratnam, M., Marquardt, H., Duhring, J. L. and Freisheim, J. H. (1989) Biochemistry **28**, 8249–8254
5 Shen, F., Ross, J. F., Wang, X. and Ratnam, M. (1994) Biochemistry **33**, 1209–1215
6 Brigle, K. E., Westin, E. H., Houghton, M. T. and Goldman, I. D. (1991) J. Biol. Chem. **266**, 17243–17249
7 Wang, X., Shen, F., Freisheim, J. H., Gentry, K. E. and Ratnam, M. (1992) Biochem. Pharmacol. **44**, 1898–1901
8 Brigle, K. E., Spinella, M. J., Westin, E. H. and Goldman, I. D. (1994) Biochem. Pharmacol. **47**, 337–345
9 Yan, W. and Ratnam, M. (1995) Biochemistry **34**, 14594–14600
10 Shen, F., Wu, M., Ross, J. F., Miller, D. and Ratnam, M. (1995) Biochemistry **34**, 5660–5665
11 Rothberg, K. G., Ying, Y., Kolhouse, J. F., Kamen, B. A. and Anderson, R. G. W. (1990) J. Cell Biol. **110**, 637–649
12 Leamon, C. P. and Low, P. A. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 5572–5576
13 Birn, H., Selhub, J. and Christensen, E. I. (1993) Am. J. Physiol. **264**, 302–310
14 Turek, J. J., Leamon, C. P. and Low, P. S. (1993) J. Cell Sci. **106**, 423–430
15 Ross, J. F., Chaudhuri, P. K. and Ratnam, M. (1994) Cancer **73**, 2432–2443
16 Habeck, L. L., Leitner, T. A., Shakelford, K. A., Gossett, L. S., Schultz, R. M., Andis, S. L., Shih, C., Gridey, G. B. and Mendelsohn, L. L. (1994) Cancer Res. **54**, 1021–1026
17 Leamon, C. P. and Low, P. S. (1992) J. Biol. Chem. **267**, 24966–24971
18 Leamon, C. P. and Low, P. S. (1994) J. Drug Targeting **2**, 101–112
19 Leamon, C. P., Pastan, I. and Low, P. S. (1993) J. Biol. Chem. **268**, 24847–24854
20 Lee, R. J. and Low, P. S. (1994) J. Biol. Chem. **269**, 3198–3204
21 Bolhuis, R. L., Lamers, C. H., Goey, S. H., Eggermont, A. M., Trimbos, J. B., Stoter, G., Lanzavecchia, A., di Re, E., Miotti, S. and Raspagliesi, F. (1992) Int. J. Cancer **7**, 78–81
22 Canevari, S., Mezzanzanica, D., Menard, S., Ferrini, S., Moretta, L. and Colnaghi, M. I. (1992) Int. J. Cancer **7**, 42–44
23 Ferrini, S., Cambiaggi, A., Storzini, S., Canevari, S., Mezzanzenica, D., Colnaghi, M. I. and Moretta, K. (1993) Cancer Detect. Prevent. **17**, 295–300
24 Luhrs, C. A. (1991) Blood **77**, 1171–1180
25 Ratnam, M., McAlinden, T. P. and Freisheim, J. H. (1988) Proc. Am. Assoc. Cancer Res. **29**, 355a (Abstr.)
26 Gething, M. J. and Sambrook, J. (1992) Nature (London) **355**, 33–45
27 Varki, A. (1993) Glycobiology **3**, 97–103
28 Urban, J., Parczyk, K., Leutz, A., Kayne, M. and Kondor-Koch, C. (1987) J. Cell Biol. **105**, 2735–2743
29 Scheiffele, P., Peranen, J. and Simons, K. (1995) Nature (London) **378**, 96–98
30 Appel, D., Pilarsky, C., Gnaichen, R. and Koch-Brandt, C. (1996) Eur. J. Cell Biol. **70**, 142–149
31 Olson, T. S. (1989) FASEB J. **3**, 1618–1624
32 Slieker, L. J. (1986) J. Biol. Chem. **261**, 15233
33 Ghose–Dastidar, J. (1991) Proc. Natl. Acad. Sci. **88**, 6408–6412
34 Edge, J. C., Joas, H. C., Woods, R. J. and Wormald, M. R. (1993) Biochem. Soc. Trans. **21**, 452–455

Received 2 April 1997/23 June 1997; accepted 27 June 1997