A functional cysteine-less form of the hamster reduced folate carrier protein was generated by alanine replacement of the 14 cysteine residues. The predicted 12-transmembrane topology was examined by replacing selected amino acids, predicted to be exposed to the extracellular or cytosolic environments, with cysteines. The location of these cysteines was defined by their accessibility to biotin maleimide in the presence or absence of specific blocking agents. Amino acids predicted to be exposed to the extracellular environment (S46C, S179C, L300C, Y355C, and K430C) could be labeled with biotin maleimide; this modification could be blocked by prior treatment with nonpermeable reagents. Amino acids predicted to be within the cytosol (S152C, Cys198, and L475C) could be labeled only after streptolysin O permeabilization. In addition, the cysteine-less reduced folate carrier was exploited to evaluate a potential substrate-binding domain as suggested by previous studies. Nineteen cysteine replacements were generated between residues 39 and 75, a region located between the first and second transmembrane segments. From the biotinylation of these sites and the ability of various reagents to block this labeling, it appears that L41C, E45C, S46C, T49C, I66C, and L70C are exposed to the extracellular environment, whereas Q54C, Q61C, and T63C are slightly less accessible. Cysteines 39, 42, 44, 47, 51, and 73 were inefficiently biotinylated, suggesting that these sites are located in the membrane or within a tightly folded domain of the protein. Furthermore, biotinylation of cysteines 41, 46, 49, 70, and 71 could be prevented by prior treatment with either methotrexate or folinic acid, indicating that these sites form part of a substrate-binding pocket.

The reduced folate carrier (RFC), the major transporter for folates in mammalian cells (1), is a low capacity carrier with a high affinity for substrate and a preference for reduced folates (2). It is expressed as a membrane protein in a wide variety of tissues and cell types (1, 3, 4); and although highly conserved at the amino acid level, it varies in predicted size from 58 to 85 kDa depending upon the species (5–7). Hydrophobicity analyses have indicated that the RFC protein has 12 transmembrane-spanning segments with the N and C termini located intracellularly and a large intracellular loop between transmembrane domain (TM) 6 and TM7 (8). Epitope insertions into the predicted major loops (8, 9) and N-glycosylation scanning mutagenesis (9) support this model, although the latter study suggests that the C-terminal portion of the protein may demonstrate an alternative topology.

Various regions of the protein have been implicated in different aspects of function and biogenesis. For example, maintenance of both termini is important for ensuring the appropriate cellular localization of the RFC (10, 11), whereas the large intracellular loop between TM6 and TM7 is required for both protein stability and efficient substrate translocation (10, 12). Furthermore, several amino acids in the predicted TM1 (13–15) and elsewhere (16–18) have been implicated as important determinants of substrate selectivity and recognition.

Cysteine scanning mutagenesis has been useful in establishing various aspects of protein structure and function (19–23). This approach is based on the cysteine replacement of specific residues within a functional cysteine-less form of the protein. The modification of these replacement cysteines with a variety of sulfhydryl-reacting reagents enables a given site to be designated as extracellular or cytosolic facing. However, if a site is within the membrane or a tightly folded domain of the protein, it may not be accessible (21–23). Furthermore, modification of these Cys residues by substrates allows the identification of binding pockets or domains (23).

In this study, each of the 14 cysteine residues of the hamster RFC was replaced with alanine. Although three of these cysteines are conserved among the human, hamster (amino acids 33, 248, and 396), rat, and mouse proteins, the cysteine-less form of the protein (Cys(→) RFC) was functional. Cysteine residues were introduced into the putative extracellular or cytosolic facing regions of the RFC, and the altered proteins were subsequently modified with sulfhydryl-reacting reagents. Biotinylation of sites facing the extracellular environment could be blocked by nonpermeable sulfhydryl-reacting reagents, whereas intracellular sites required permeabilization of the membrane; these results are consistent with the 12-transmembrane model for the carrier. Furthermore, biotinylation of several positions between TM1 and TM2 could be specifically blocked by substrate, suggesting that this region forms part of the substrate-binding pocket.
**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases, ECL chemiluminescent reagent, and streptavidin-biotinylated horseradish peroxidase were from Amersham Biosciences. Biotin maleimide (BM; N’-3-maleimidylpropionyl)biocytin; 523 Da), lucifer yellow iodoacetamide (LYI) (659 Da), and rabbit anti-green fluorescent protein antibody were from Molecular Probes, Inc. Sulfosuccinimidyl 2-(biotinamido)ethyl-1,3,5-iminodi(propi- onate (EZ-Link Sulfo-NHS-SS-Biotin, biotin succinimide) was from Pierce, and (2-(trimethylammonium) ethyl)methanethiosulfonate bromide (MTSET; 278 Da) was from Toronto Research Chemicals Inc. PEG and (2-(trimethylammonium) ethyl)methanethiosulfonate bromide (MTSET; 278 Da) was from Toronto Research Chemicals Inc. Mouse anti-FLAG antibody, Polybrene, horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody, streptolysin O, and streptavidin-biotinylated horseradish peroxidase were from Amersham Biosciences. Biotin maleimide (BM; N’-3-maleimidylpropionyl)biocytin; 523 Da), lucifer yellow iodoacetamide (LYI) (659 Da), and rabbit anti-green fluorescent protein antibody were from Molecular Probes, Inc. Sulfosuccinimidyl 2-(biotinamido)ethyl-1,3,5-iminodi(propi- onate (EZ-Link Sulfo-NHS-SS-Biotin, biotin succinimide) was from Pierce, and (2-(trimethylammonium) ethyl)methanethiosulfonate bromide (MTSET; 278 Da) was from Toronto Research Chemicals Inc. PEG and (2-(trimethylammonium) ethyl)methanethiosulfonate bromide (MTSET; 278 Da) was from Toronto Research Chemicals Inc. Mouse anti-FLAG antibody, Polybrene, horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody, streptolysin O, and N-ethylmaleimide (NEM) were from Sigma. [3H]Methotrexate (Mtx; 35 Ci/mmol) was purchased from Moravek Biochemicals, Inc and purified by thin-layer chromatography before used as described previously (24).

**Construction of C-terminally Tagged RFC Proteins**—The construction of the wild-type RFC with enhanced green fluorescent protein (EGFP) fused to its C-terminal end was constructed in the following manner. Modified primers were used to PCR-amplify the 3’-end of the hamster RFC coding region with a non-stop codon followed by a BglII site. This fragment was cloned into the pGEM-T vector (Promega) and subcloned into the SacI and NcoI sites of pBluescript KS. A single FLAG cassette (coding for the epitope DYKDDDDK) flanked by BglII (5’') and XbaI (3’’) sites was generated using two hybridized oligonucleotides (Primer 1, GATCTGACTACAAAAGGACGACCCAGGAAAG; and Primer 2, CTAGATCACTTGTCGTCGTCGTCCTTGTAGTCCTTGTA) and inserted after the non-stop codon. This construct was designated RFC-FLAG. A second cassette with two FLAG epitopes flanked by BglII (5’’) and BamHI (3’’) sites (Primer 3, GATCTGACTACAAAAGGACGACCCAGGAAAG; and Primer 4, GATCCTTGTCGTGCTCTTGTAGTCTGTGTCGTGTCGCCTTGTA) was then inserted into the BglII site of the RFC-FLAG construct and screened for orientation. The EcoRI fragment from this construct was subcloned into the wild-type RFC in the pcDNA3 vector and sequenced for verification. Subsequently, the wild-type RFC sequence coding for the fusion protein in both of the tagged vectors (EGFP and FLAG) was replaced with the Cys(-) RFC by standard molecular biological procedures.

**Site-directed Mutagenesis**—In some cases, the construction of the Cys(-) RFC and the subsequent replacement of individual residues with Cys were done in an N-glycosylation-minus hamster RFC background (N56Q) to simplify subsequent analyses. The lack of glycosylation does not affect RFC transport function (25). For the majority of the Cys replacements, site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene). Plasmid DNA was PCR-amplified using complementary primers (Invitrogen) containing the mutagenic sequences and Ffu Turbo polymerase according to the supplier. Cycling conditions were 95 °C for 20 s, 55 °C for 60 s, and 72 °C for 200 s per kb of DNA. Non-mutagenized template DNA was digested with DpnI (New England Biolabs Inc.) prior to transformation. In some cases, mutagenesis was carried out using a two-step PCR with a proofreading polymerase (Expand, Roche Applied Science). Complementary mutagenic primers were used with appropriate upstream and downstream primers in two separate PCR reactions to generate fragments overlapping at the mutated site. Fragments were purified on acrylamide gels, and the DNA was eluted from excised plugs in 50 μl of 10 mm Tris-HCl (pH 7.5). These fragments were mixed and used as template with the flanking primers in the second PCR. The resultant fragment was cloned into pGEM-T, digested with restriction endonucleases, and cloned into the appropriate RFC backbone: wild type (Cys(+)) rfc-EGFP), Cys(-) rfc-EGFP, or Cys(-) rfc-FLAG. In this manner, the site with the

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**Fig. 1. Predicted topology of RFC.** The topology is based on hydrophathy plots and epitope insertion analysis (8, 9). Circled residues indicate those conserved among hamster, human, rat, and mouse species. Cys(33), Cys(248), and Cys(396) are conserved. The black circles indicate the locations of the Cys residues that were replaced with Ala. The arrows indicate where the Cys replacements were made to assist in topology determinations, and the black dots indicate those residues that were mutated to Cys to examine for biotinylation and substrate effects. The asterisk indicates Asn(56), which was changed to Gln in several constructs.
TABLE I

| Cell type*       | Colony formation/10 μg DNA/10^6 cells | Mtx (D_{50}°) | Folic acid (D_{50}°) | K_e^a | V_{max}/f |
|------------------|---------------------------------------|--------------|---------------------|--------|----------|
| Pro-3 (Wild-type)| n/a                                   | n/a          | n/a                 | 1.9 ± 0.2 | 1.0 ± 0.5 |
| MtxRII-3         | 12                                    | 12           | 1.3                 | 2.1 ± 0.6 | 0.8 ± 0.1 |
| Asn^{66} Cys(+) RFC-EGFP | 1280                          | 12         | 1.3                 | ND     | ND       |
| N56Q Cys(+) RFC-EGFP  | 1380                          | 14         | ND                 | 1.2 ± 0.4 | 0.5 ± 0.2 |
| N56Q Cys(-) RFC-EGFP  | 730                             | 40          | 3.0                 | ND     | ND       |

* The cell lines are designated as described in “Experimental Procedures.” Pro-3 is the parental wild-type line; MtxRII-3, the Mtx null line; Asn^{66} Cys(+) RFC-EGFP, the null line expressing the wild-type RFC-EGFP fusion construct with a N-glycosylation site at Asn^{66}; N56Q Cys(+) RFC-EGFP, the null line expressing the wild-type RFC-EGFP fusion construct with the N-glycosylation site removed (N56Q); and N56Q Cys(-) RFC-EGFP, the null line expressing the Cys(-) RFC-EGFP fusion protein with the N-glycosylation site removed (N56Q).

TABLE II

| Replacement | Location (between) | Cys(-) RFC-FLAG colony formation/10 μg DNA/10^6 cells |
|-------------|--------------------|-----------------------------------------------------|
| S46C        | TM1 and TM2        | 372                                                 |
| S152C       | TM4 and TM5        | 7 (656)*                                             |
| S179C       | TM5 and TM6        | 329                                                 |
| Cys^{324}   | TM6 and TM7        | ND (2144)                                            |
| L309C       | TM7 and TM8        | 1129                                                |
| Y355C       | TM9 and TM10       | 228                                                 |
| Q386C       | TM10 and TM11      | 17 (704)                                             |
| L475C       | C terminus         | 500                                                 |

* The numbers in parentheses are the colony formation in 10 nm folic acid as the selective growth medium.

Fig. 2. RNA, protein, and surface expression of RFC. A, RNA expression. Poly(A)^+ RNA from cell lines was analyzed by Northern blot analysis as described under “Experimental Procedures.” Lane 1, wild-type Pro-3 cells; lane 2, MtxRII-3 cells; lane 3, cells expressing Asn^{66} Cys(+) RFC-EGFP; lane 4, cells expressing N56Q Cys(-) RFC-EGFP. Upper panel, blot probed with rfc cDNA; lower panel, blot stripped and reprobed with actin cDNA. B, protein expression. Extracts were prepared and separated by SDS-PAGE, and RFC-EGFP fusion proteins were detected by Western blot analysis with anti-green fluorescent protein antibody as described under “Experimental Procedures.” Lane 1, extract from SW480; lane 2, extract from Asn^{66} Cys(+) RFC-EGFP; lane 3, extract from N56Q Cys(-) RFC-EGFP. C, surface expression. Equal numbers of cells expressing the RFC-FLAG fusion protein were labeled with biotin sulcinimidyl horseradish peroxidase complex; lower panel, blot stripped and probed for fusion proteins using anti-FLAG antibody as described under “Experimental Procedures.”

Cys replacement replaced a similar region of rfc in the fusion expression vectors. All final constructs were sequenced using an ABI 377 sequencer (Robarts Research Institute, London, Ontario, Canada) to confirm their authenticity.

DNA Transfections—Transfection of the mutant rfc plasmid clones into the recipient MtxRII-3 cells was performed using 10 μg of purified DNA in Polybrene/1.5 x 10^6 cells as described previously (26). This cell line is resistant to Mtx because it does not transport the drug and has no detectable rfc message (26). After transfection with DNA, the cells were selected for growth in low levels of folic acid (2 nm) or at 10 nm if low complementation capabilities were demonstrated. Single colonies were isolated and in most cases cloned by limiting dilution from individual transfection experiments.

Phenotype Testing—The transfectants were tested for Mtx resistance and folic acid growth requirement as described previously (27). For Mtx resistance, the D_{50} value is the drug concentration that reduced survival to 10%, whereas for folic acid growth, the D_{50} is the nutrient concentration that permitted 50% growth compared with the wild-type.

Kinetic Analysis—The kinetic analyses for the determination of V_{max} and K_e are described previously (26). V_{max} is expressed as pmol of substrate/min/mg of protein and K_e as μM substrate.

Northern Blotting—Poly(A)^+ RNA (5 μg) was separated on a 1.2% agarose gel in formaldehyde buffer as outlined by Sambrook et al. (29) and transferred to Hybond XL membrane by the capillary method. Membranes were probed with 32P-labeled rfc cDNA, washed, and exposed to x-ray film as described previously (26). Blots were stripped and rehybridized with 32P-labeled actin cDNA.

Western Analysis—Total cellular extracts were prepared from various numbers of cells by lysis on ice in lysis buffer (5% concentrated protease inhibitor mixture tablets (Roche Applied Science), 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 μM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μM orthovanadate).

After centrifugation at 12,000 x g for 2 min at 4 °C, the soluble lysate was separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Detection of the EGFP fusion proteins was as described previously (7). Detection of the FLAG-tagged proteins was carried out in a similar manner using mouse anti-FLAG antibody followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG. Images were scanned, and bands were quantitated using the Bio-Rad Multi-Analyt program. Only images in the linear range of the film were used for quantitation.
Biotin Labeling and Detection—Approximately $1 \times 10^6$ cells growing in 6-well tissue culture dishes and expressing either the Cys(-) RFC-FLAG fusion proteins or single Cys residues at the designated sites were washed three times with phosphate-buffered saline (PBS) containing 0.1 mM CaCl$_2$ and 1 mM MgCl$_2$ (PBS/CM) and overlaid with 1 ml of 0.15 M HEPES (pH 7.4) and 1 mM MgCl$_2$. Cells were treated at room temperature for 30 min in the absence or presence of 0.68 mM LYI or the indicated concentration of either Mtx or folinic acid followed by 1.5-h incubation, blots were washed with Tris-buffered saline/Tween containing 0.5% bovine serum albumin. After a dilution of streptavidin-biotinylated horseradish peroxidase followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG. In some cases, cells were pretreated with either 0.68 mM LYI or 3 mM NEM prior to permeabilization with streptolysin O (SLO) as described above. At least two biotin detections were performed on at least two independent cell lines for each Cys replacement and for each pretreatment procedure. For cell-surface biotinylation, equal numbers of cells ($7 \times 10^5$) were washed twice with PBS/CM containing 100 mM glycine and once with ice-cold PBS before lysis. Equal amounts of total protein from each cell line were separated by SDS-PAGE and processed as described above. Biotin was detected as described above. Images were scanned and quantitated as described above.
RESULTS

Construction of the Cys(-) RFC—The hamster RFC contains 14 Cys residues, with over half of these located in predicted transmembrane segments (Fig. 1). Of these residues, three are conserved (amino acids 33 (TM1), 248 (TM6–TM7 loop), and 396 (TM11) for hamster, mouse, human, and rat species. To determine whether any of the 14 cysteines are critical for function, site-directed mutagenesis was used to replace each with alanine. Because previous work had indicated that the lack of glycosylation did not affect the function of the RFC (25), constructs were initially generated in a N-glycosylation-minus background (N56Q). The resulting cysteine-less protein was expressed with either EGFP or FLAG fused to the C terminus to assess various functional features. We have previously shown that the C-terminal EGFP fusion does not affect RFC function, but serves as a useful marker for monitoring carrier expression and cellular localization (7, 8).

Properties of the Cys(-) RFC—To determine whether the N56Q Cys(-) RFC was functional, the plasmid DNA was transfected into mutant hamster cells lacking the RFC (MtxRII5-3 cells) and selected for growth in low levels of folinic acid. As shown in Table I, the N56Q Cys(-) RFC DNA was able to complement the folate uptake-defective phenotype of the mutant cells and allowed cell survival, although at a reduced frequency compared with the wild-type (Asn 56 Cys (+)) RFC DNA.

Two independently selected transfected clones expressing N56Q Cys(-) RFC-EGFP were assessed for their sensitivity to Mtx, for the growth requirement for folinic acid, and for the kinetic parameters associated with drug uptake. In general, cells expressing this fusion protein had a slightly increased sensitivity to Mtx and growth requirement for folinic acid compared with those expressing the wild-type protein (Table I). This is reflected in a marginal increase in the affinity for uptake as well as a reduced efficiency of transport.

Expression of rfc Message and RFC Protein—Northern blot analysis was used to examine the nature of the rfc mRNA in the transfected cells. As shown in Fig. 2A, the level of rfc mRNA in cells expressing N56Q Cys(-) RFC-EGFP was similar to that in cells expressing the wild-type form of the carrier (Asn 56 Cys (+) RFC-EGFP). The level of expression in the transfected cell lines was 4–10-fold higher than that in the non-transfected wild-type Pro 3 cells. The size of the message expressed for N56Q Cys(-) RFC-FLAG was smaller than the endogenous wild-type message or the message expressed for Asn 56 Cys (+) RFC-EGFP; this is a result of replacing the 3’-untranslated region with tags of different sizes.

Western blot analysis of lysates from equal numbers of cells indicated similar levels of fusion protein expression in the transfected cell lines (Fig. 2B). The cells expressing Asn 56 Cys (+) RFC-EGFP produced a protein that migrated as a broad band centering at ~97 kDa, similar to that previously reported (7, 10, 28). The lower band in lane 1 of Fig. 2B represents core and unglycosylated material (7). Cells expressing N56Q Cys(-) RFC-EGFP or N56Q Cys(-) RFC-FLAG produced a protein of ~65 kDa in size, reflecting the lack of the N-glycosylation site. This size is still less than the expected size of 85 kDa (RFC plus EGFP), and however, is likely due to incomplete denaturation of the protein since cellular extracts cannot be boiled prior to SDS-PAGE because of protein aggregation.

To examine the proportion of protein that was expressed at the cell surface, equal numbers of cells expressing N56Q Cys(-) RFC-FLAG or N56Q Cys(-) RFC-FLAG were treated with nonpermeable biotin succinimide (Fig. 2C). This fusion protein is ~50 kDa in size since it is not glycosylated. When biotinylated cell-surface protein levels were normalized in the linear range to total fusion protein expressed, the ratios were similar for both cell lines. Thus, the absence of cysteines and the lack of N-glycosylation do not appear to interfere with the membrane localization of the RFC protein.

Insertions of Cys Residues into the Cys(-) RFC Mutant—The availability of a cysteine-less mutant provided the opportunity to confirm the topology of the RFC using Cys scanning mutagenesis, a less disruptive strategy than either epitope inser-

| Replacement | Cys(-) RFC-FLAG colony formation/10 μg DNA/10̇̊ cells |
|-------------|----------------------------------------------------------|
| F37C        | <3 (<3)                                                  |
| A39C        | 1955                                                    |
| L41C        | 34                                                      |
| R42C        | <3 (<3)                                                  |
| P43C        | 295                                                     |
| E45C        | <3 (<1876)                                               |
| S46C        | 174                                                     |
| F47C        | <3 (<938)                                                |
| T49C        | 107                                                     |
| Y51C        | 898                                                     |
| Q54C        | 392                                                     |
| G61C        | 235                                                     |
| T63C        | <3 (<450)                                                |
| H66C        | 175                                                     |
| L70C        | 1340                                                    |
| P71C        | <3 (<450)                                                |
| S73C        | 1528                                                    |
| L75C        | <3 (<450)                                                |

* The numbers in parentheses are the colony formation in 10 nM folinic acid as the selective growth medium.
tion or N-glycosylation scanning. At selected sites within the RFC protein, single residues were replaced with cysteines (Fig. 1). Initially, the substitutions were made in the N56Q Cys(H11002) RFC-EGFP backbone and transfected into the transport-defective indicator cells (MtxRII5-3 cells) to assess functionality. The C-terminal EGFP tag was replaced with FLAG to enable the specific detection of the inserted Cys residues with sulfhydryl reagents. This was necessary because the EGFP tag contains two Cys residues essential for fluorescence (31).

In most cases, the fusion proteins with the assorted cysteine substitutions were able to complement the RFC-deficient cell line (Table II), indicating that RFC functionality was retained. One of the cysteine substitutions (S152C) yielded a protein that had a significantly decreased ability to complement the mutant cell phenotype under the standard folinic acid growth conditions. However, when the folinic acid levels were raised to 10 nM, the number of colonies was drastically increased, indicating that this mutant protein may transport substrate less efficiently.

**Biotinylation of Single Cys Residues in Predicted Extracellular Regions**—The accessibility of individual replacement Cys residues predicted to be facing the extracellular environment was assessed for functional proteins. Stable cell lines expressing these various constructs were treated with BM in the presence or absence of the membrane-impermeable sulfhydryl-reacting reagents LYG and MTSET. As expected, there was no detectable biotinylation of the N56Q Cys(-) RFC-FLAG fusion protein. For most of the cysteine replacements, biotinylated fusion protein was detected after treatment with 20 or 50 μM BM, although two mutants (Y355C and K430C) required higher levels (500 μM) (Fig. 3A). As well, the level of biotinylation for many of the replacements was significantly decreased by pretreatment with LYG, a nonpermeable sulfhydryl-reacting reagent. When normalized to the amount of fusion protein expressed, biotinylation was essentially eliminated for Cys replacements at positions 46, 179, and 430. Although biotinylation was decreased by only 50% for the residue 300 replacement and was minimally affected by the residue 355 replacement, pretreatment with the smaller sized cell-impermeable sulfhydryl reagent MTSET completely prevented biotinylation at either position (Fig. 3B). Thus, these results confirm the extracellular location of the regions containing residues 46, 179, 300, and 430 as described previously by other methods (8, 9) and also indicate that residue 355 is exposed to the extracellular environment, an observation contrary to previous work involving N-glycosylation scanning mutagenesis (9). However, residue 355 may be in a cleft since it is not very reactive with biotin maleimide and is not that accessible to LYG, but is accessible to the smaller sized reagent MTSET.

**Biotinylation of Cys Residues in Predicted Cytosolic Facing Regions**—Cell lines expressing fusion proteins with Cys replacements in the predicted cytosolic regions (S152C and
L475C) and the endogenous site (Cys224) were also examined in a similar manner as described above. Unlike the replacements in the extracellular loops, none of the Cys residues were biotinylated (Fig. 3C), indicating that, under the conditions used in this study, BM did not cross the cell membrane. However, permeabilization of the cells with streptolysin O prior to BM treatment enabled labeling of the Cys residues at these positions. This labeling could be partially prevented by pretreatment with the membrane-permeable reagent NEM, but not with the membrane-impermeable reagent LYI. This confirms the cytosolic location of regions containing residues 224 and 475 as previously determined (8, 9) and also positions residue 152 in a similar location as predicted by the 12-transmembrane model.

Effect of Substrate on Biotinylation of Extracellularly Located Residues—Sites exposed to the extracellular environment could be potentially involved in substrate interactions. If these regions are involved in substrate binding, then the biotinylation of Cys residues at these sites might be prevented by prior treatment with substrate. Alternatively, substrate binding could induce a conformational change in the protein that affects the reactivity of certain residues. To examine this, cells expressing the fusion proteins with extracellular Cys residues were treated with either Mtx or folinic acid prior to biotinylation. As shown in Fig. 4, biotinylation of S46C was essentially eliminated by prior treatment with Mtx and was reduced by 70% with folinic acid. In contrast, the other sites tested were minimally affected.

Analysis of Cys Replacements in the Region between TM1 and TM2—To explore the extent of this substrate protection around residue 46, a total of 19 Cys mutants were generated within the region from residues 37 to 75. Because initial work indicated that some of these constructs were unstable in the N56Q background, all constructs were made with the N-glycosylation site (Asn56) intact. As shown in Table III, a vast majority of these replacements were able to complement the RFC-minus phenotype of the indicator cells. Two constructs (F37C and P43C) were unable to complement the mutant phenotype even when selected under increased concentrations of folinic acid and were not studied further. As well, six constructs (R42C, E45C, F47C, T63C, P71C, and L75C) were able to complement only when selected under 10 nM folinic acid conditions, indicating that these mutant proteins may transport the substrate less efficiently.

Western blot analysis indicated that the fusion protein expression varied from 2- to 5-fold among the cell lines (data not shown). However, one line expressing L75C had an ~10-fold lower level than the others. The expressed fusion protein migrated as three components (Fig. 5): a high molecular mass broad band centering at ~75 kDa that represents the fully N-glycosylated form (7, 28), the core glycosylated form (~55 kDa) (7, 28), and the unglycosylated form (~48 kDa) (7, 28). The relative proportion of these lower molecular mass species varied among the cell lines; in the L41C-, S46C-, and P71C-expressing lines, there appears to be more of the unglycosylated form than the core glycosylated form, whereas the core glycosylated form appears more abundant in the E45C-expressing line. These differences may reflect maturation and processing consequences of the Cys replacements at these sites, although these were not further explored.

To assess the accessibility of the individual replacement Cys residues in these functional proteins, stable lines expressing the constructs were treated with BM in the presence or absence of the impermeable sulfhydryl reagents LYI and MTSET. As
indicated in Fig. 5, several of the Cys residues were readily biotinylated (L41C, E45C, S46C, T49C, Q54C, Q61C, T63C, I66C, L70C, and P71C). Furthermore, this biotinylation could be prevented by pretreatment with either LYI or MTSET for all replacements except P71C, indicating that these sites are exposed to the extracellular environment. It is of interest to note that, in some cases (L41C, S46C, and T63C), the unglycosylated form of the fusion protein was biotinylated and, furthermore, that this labeling could be blocked. This suggests that the unglycosylated form is expressed at the surface of the cell. In contrast, several of the Cys residues were labeled at a low level (A39C, R42C, and G44C) or not at all (F47C, Y51C, S73C, and L75C) even at high concentrations of BM. These sites may be buried in the membrane or have some structural feature that sterically hinders access of BM.

Effect of Substrate on Biotinylation of Sites in the Region between TM1 and TM2—The demonstration that several of the sites in the region between TM1 and TM2 were exposed to the extracellular environment provided the opportunity to assess whether they played a role in substrate recognition. In an analogous manner as shown previously for S46C (see above), cells expressing these replacements were treated with various concentrations of Mtx (Fig. 6) or folinic acid (Fig. 7) prior to exposure to BM. Based on the ability of the substrate to prevent biotinylation, the sites could be separated into three types depending on the ability of the substrate to prevent the biotinylation: 1) those sites in which biotinylation protection could be detected at 1 μM substrate (L41C, S46C, T49C, and L70C), 2) one site in which detectable blocking occurred at 10 μM substrate (P71C), and 3) those sites in which biotinylation was not prevented by substrate (Q54C, Q61C, T63C, and I66C). Modification of the residues in category 1 was blocked in a dose-dependent manner such that <20% biotinylation occurred at the highest concentration of substrate. For the Mtx and folinic acid substrates, a decrease of 50% in the biotinylation levels was achieved at ~0.7 and ~0.1 μM, respectively, for T49C (Fig. 8). For S46C, L41C, and P71C, the profiles were similar for both substrates, with 50% blockages at ~0.5, ~2–5, and 10 μM, respectively. For L70C, the profiles were most divergent; at ~2 μM folinic acid, 50% of the biotinylation was inhibited, whereas at least 10 μM Mtx was required to achieve the same effect. These results indicate that L41C, S46C, T49C, L70C, and P71C are in close contact with substrate, whereas Q54C, Q61C, T63C, and I66C are not.

DISCUSSION

In this study, a cysteine-less form of the hamster RFC was generated by alanine replacement of the 14 cysteine residues. The Cys(−) RFC protein was able to localize to the cell membrane and to transport reduced folates with slightly altered kinetics compared with the wild-type protein, indicating the nonessential role for this amino acid in RFC function. Recently, a functional cysteine-less form of the human RFC has also been generated (32). The availability of a functional Cys(−) RFC allowed the topology of the protein to be confirmed by cysteine scanning mutagenesis, a less disruptive and less restricted approach than epitope insertion or glycosylation scanning. The predicted extracellular location of specific regions was confirmed by the ability of the nonpermeable reagents to block biotinylation of Cys residues at positions 46, 179, 355, and 430. All of these residues were not equally accessible, as some required either high levels of biotin maleimide for labeling (Y355C and K430C) or a smaller molecule for blocking (L300C and Y355C). Although these sites are exposed to the extracellular environment, it appears that these regions may possess a structural feature such as a cleft or pocket that sterically hinders reagent accessibility. This is evident with residue 355, which is not that
reactive with biotin maleimide and requires a smaller sized reagent for complete blocking.

The observation that residue 430 between TM11 and TM12 is exposed to the extracellular environment is of interest, as we had initially mapped this region using an inserted epitope (8). Unfortunately, this insertion led to a transport-defective protein and raised doubts regarding the accuracy of the assigned location of this region. However, in the present study, the Cys replacement resulting in a functional protein confirms that the original assignment was accurate.

A previous study of the human RFC protein (9) suggested that the C-terminal half of the protein may demonstrate alternative topologies from the predicted 12-transmembrane model, where the region between TM9 and TM10 is exposed to the intracellular environment. This model is based on the absence of N-glycosylation at a site (residue 358) engineered into this region; however, N-glycosylation is spatially restricted to sites farther than 12 residues from the membrane (33). Thus, it is likely that this predicted small loop may be too close to the membrane to be N-glycosylated. In the present study, we used a less disruptive technique to demonstrate that the corresponding area between TM9 and TM10 (Y355C) in the hamster RFC protein is exposed to the extracellular environment. The only other support for this alternative topology is the glycosylation of a region (residue 387) between TM10 and TM11 in a nonfunctional isolate (9). Although it is possible that there are differences in topology between the human and hamster RFC proteins, this seems unlikely given the high level of amino acid similarity and transport properties of these two carriers.

Under the conditions used in this study, biotin maleimide did not cross the membrane. This is demonstrated by the lack of detectable biotinylation of regions exposed to the cytosol and in

The values from Figs. 6 and 7 were plotted to allow determination of the substrate concentration required for 50% protection of biotinylation. A, Mtx; B, folinic acid. The dotted line indicates 50% protection.

**FIG. 8. Summary of substrate protection of biotinylation of Cys replacements in the region between TM1 and TM2.**

**TABLE IV**

Summary of features of Cys replacements in the region between TM1 and TM2

| Replacement | BM | Extracellular facing | Substrate block |
|-------------|----|----------------------|-----------------|
| A39C        | (+) | +                    | +               |
| L41C        | +  | +                    | +               |
| R42C        | (+) | +                    | +               |
| G44C        | (+) | +                    | +               |
| E45C        | (+) | +                    | +               |
| S46C        | +  | +                    | +               |
| F47C        | -  | +                    | +               |
| T49C        | +  | +                    | +               |
| Y51C        | -  | +                    | +               |
| Q54C        | +  | ( + )                | -               |
| Q61C        | +  | (+)                  | -               |
| T63C        | (+) | +                    | -               |
| I66C        | +  | +                    | -               |
| L70C        | +  | +                    | +               |
| P71C        | +  | +                    | +               |
| S73C        | -  | +                    | -               |

(+ ) indicates no or low biotinylation or partial exposure to extracellular environment; blanks indicate situations where analyses could not be done due to low or no biotinylation; * indicates biotinylation, extracellular facing, or substrate block of biotinylation; | indicates no biotinylation or no substrate block of biotinylation.
the core glycosylated form of the carrier. This observation is similar to studies in other systems (22, 30), but also is in contrast to others (34). The reasons for this are not understood, but may be cell type-specific. Permeabilization of the cell membrane allowed the biotinylation of sites predicted to be in cytosolic exposed regions. Because biotinylation of Cys residues in transmembrane regions has been reported to be inefficient, presumably due to the inaccessibility of amino acids in this hydrophobic environment to biotin maleimide (22), these studies confirm the earlier work that sites 224 and 475 are in regions exposed to the cytosol and now also position site 152 in a similar region. The results presented in this work combined with the previously reported works (8, 9) strongly support the 12-transmembrane model for the reduced folate carrier.

Previous work had indicated that a region in the predicted TM1, encompassing at least residues 44–46, may be involved in substrate binding and recognition (13–15). In this report, we used Cys scanning mutagenesis to show that sites within a region extending from residues 39 to 75 form a substrate recognition pocket or domain (Table IV). Cys residues at several sites (L41C, S46C, T49C, and I66C) were readily biotinylated with 50 μM biotin maleimide, and this modification could be prevented by prior treatment with the nonpermeable reagent LYI. This indicates that these sites are easily accessible and exposed to the extracellular environment, in contrast to the predication of various models that placed this region in TM1 (13–15, 35). Cysteines at other sites (Q54C, Q61C, T63C, and P71C) required a higher level of biotin maleimide (200 μM) for efficient detection. Furthermore, three of these sites (Q54C, Q61C, and T63C) required a smaller sized nonpermeable reagent (MTSET) to block biotinylation, suggesting that these regions may face the cell exterior and may not possess a structural feature such as a cleft or pocket that sterically hinders reagent accessibility. The E45C and L70C sites required a high concentration of biotin maleimide (500 μM) for efficient labeling, although they could be blocked by LYI. This indicates that these sites are easily accessible and exposed to the extracellular environment, but its biotinylation is blocked by substrate. Perhaps the binding of substrate induces a conformational change in the protein such that the site becomes exposed, allowing biotinylation to be prevented. The results described here provide concrete support to the concept that this region has a prominent role in substrate recognition (13–15, 32).

The availability of a functional Cys(–) RFC protein will now assist in further defining the extent and nature of this binding pocket and the size of the loops between the transmembrane segments. Furthermore, this system can be utilized in combination with cross-linking approaches to define the proximity of various residues within this domain.

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REFERENCES

1. Siretnik, F. M., and Tolner, B. (1999) Annu. Rev. Nutr. 19, 91–122
2. Siretnik, F. M. (1985) Cancer Res. 45, 3992–4000
3. Wang, Y., Zhao, R., Russell, B. G., and Goldman, I. D. (2001) Biochim. Biophys. Acta 1513, 49–54
4. Wethsine, J. R., Flatley, R. M., and Matherly, L. H. (2002) Biochem. J. 367, 629–640
5. Matherly, L. H. (2001) Prog. Nucleic Acids Res. Mol. Biol. 67, 131–162
6. Zhao, R., Gao, F., Liu, L., and Goldman, I. D. (2000) Biochim. Biophys. Acta 1466, 7–10
7. Sadlish, H., Murray, R. C., Williams, F. M. R., and Flintoff, W. F. (2000) Biochem. J. 346, 509–518
8. Ferguson, P. L., and Flintoff, W. F. (1998) J. Biol. Chem. 273, 16289–16278
9. Liu, X. Y., and Matherly, L. H. (2002) Biochim. Biophys. Acta 1564, 333–342
10. Sadlish, H., Williams, F. M. R., and Flintoff, W. F. (2002) Biochem. J. 364, 777–786
11. Marchant, J. S., Subramanian, V. S., Parker, I., and Said, H. M. (2002) J. Biol. Chem. 277, 33325–33333
12. Sharina, I. G., Zhao, R., Wang, Y., Babani, S., and Goldman, I. D. (2002) Biochim. Biophys. Acta 1578, 1717–1724
13. Jansen, G., Mauritz, R., Drori, S., Sprecher, H., Kathmann, I., Bunni, M., Priest, D. G., Nordhuis, P., Schormagel, J. H., Pinedo, H. M., Peters, G. J., and Assaraf, Y. G. (1998) J. Biol. Chem. 273, 31818–31818
14. Zhao, R., Assaraf, Y. G., and Goldman, I. D. (1998) J. Biol. Chem. 273, 7873–7879
15. Rothem, L., Heragan, I., Kaufman, Y., Priest, D. G., Jansen, G., and Assaraf, Y. G. (2002) Biochem. J. 376, 741–750
16. Roy, K., Tolner, B., Chiao, J. H., and Sirotnak, F. M. (1998) J. Biol. Chem. 273, 2526–2531
17. Wilt, T. L., and Matherly, L. H. (2002) Biochim. Biophys. Acta 1567, 56–62
18. Zhao, R., Gao, F., and Goldman, I. D. (1999) Biochim. Biophys. Acta 58, 1615–1624
19. Casey, J. R., Ding, Y., and Kopita, R. R. (1995) J. Biol. Chem. 270, 8521–8527
20. Frillingos, S. U., Upwal, M. L., Sun, J., and Kabaek, H. R. (1997) Protein Sci. 6, 431–437
21. Cheung, M., and Akabas, M. H. (1997) J. Gen. Physiol. 109, 289–299
22. Tang, X.-B., Fujinaga, J., Kopita, R., and Casey, J. R. (1998) J. Biol. Chem. 273, 22545–22553
23. Loo, T. W., and Clarke, D. M. (1999) Biochim. Biophys. Acta 1461, 315–325
24. Flintoff, W. F., and Nagunis, C. R. (1983) Biochim. Biophys. Acta 715, 433–440
25. Wilt, T. L., and Matherly, L. H. (1998) Biochim. Biophys. Acta 1375, 6–12
26. Williams, F. M. R., Murray, R. C., and Flintoff, W. F. (1994) J. Biol. Chem. 269, 5810–5816
27. Underhill, T. M., and Flintoff, W. F. (1989) Somatic Cell Mol. Genet. 15, 49–59
28. Sadlish, H., Williams, F. M. R., and Flintoff, W. F. (2002) J. Biol. Chem. 277, 42105–42112
29. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 9.18–9.46, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Grunwald, M., Bendahan, A., and Kanner, B. I. (1998) Neuron 21, 623–632
31. Tenen, R. Y. (1998) Annu. Rev. Biochem. 67, 509–544
32. Cao, W., and Matherly, L. H. (2003) Biochem. J. 374, (Pt. 1) 27–36
33. Popov, M., Tam, L. Y., Li, J., and Reithmeier, R. A. (1997) J. Biol. Chem. 272, 18325–18331
34. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 843–848
35. Zhao, R., Sharina, I. G., and Goldman, I. D. (1999) Mol. Pharmacol. 56, 68–76