The reduced folate carrier (RFC) plays a critical role in the cellular uptake of folates. However, little is known regarding the mechanism used to transport substrates or the tertiary structure of the protein. Through the analysis of a Chinese hamster ovary cell line deficient in folate uptake, we have identified a single residue in TM10 (Arg-373) of RFC that appears to play a critical role in the translocation of substrate. Replacement of this position with various amino acids (KHQNA) diminished the rate of translocation by 16–50-fold, although substrate binding, protein stability, and localization were unaffected. Furthermore, the translocation capabilities of an R373C mutant in a cysteine-less form of the reduced folate carrier were enhanced 2.5-fold by the positively charged methanethiosulfonate reagent, confirming the essential role of positive charge at this position. When considering the membrane-impermeable nature of this reagent, the data further suggest that the Arg-373 residue is located within the substrate translocation pathway of the RFC protein. Moreover, cross-linking analysis of the Arg-373 residue demonstrates that it is within 6 Å of residue Glu-394 (TM11), providing the first definitive tertiary structural information for this protein.

Folates are essential compounds required by mammalian organisms for numerous biosynthetic pathways, including the synthesis of pyrimidines, purines and several essential amino acids (1). These nutrients are transported into the cell primarily by the reduced folate carrier (RFC)1 system. This transporter has been implicated in clinical resistance to the chemotherapeutic drug, methotrexate (Mtx) (2–4), further underlining the importance of characterizing the RFC protein and its mechanism of transport.

The RFC protein is consistent with a predicted 12-transmembrane (TM) topology and cytoplasmically located N and C termini, as determined by epitope mapping (5) and cysteine scanning.2 Recent work has also demonstrated that these cytoplasmic termini, as well as the loop between TM6 and TM7, do not appear to play a direct role in protein function although they are essential for protein stability and trafficking (6, 7). The characterization of various mutations in the RFC protein using human, mouse, and hamster systems has demonstrated that single amino acid changes can lead to drastic alterations in substrate affinity (8–15), substrate translocation (16, 17), protein stability, and trafficking (18, 19). Preliminary analysis has suggested an interaction between amino acid residues in TM2 and TM4 (20) providing some insight regarding the tertiary structure. Overall, however, there is limited information available on the folding of the RFC protein or the mechanism of transporting folates.

In this report, we examined the RFC protein encoded by a folate transport-deficient Chinese hamster ovary (CHO) line. The protein has a single point mutation in the predicted TM10, resulting in the substitution of arginine for histidine (R373H). Functional analysis of modified proteins with amino acid replacements for Arg-373 indicates that this residue plays a critical role in substrate translocation and may form part of the translocation pathway. Furthermore, the Arg-373 (TM10) residue and another (Glu-394; TM11) are shown to be within close proximity of each other, presenting the first definitive tertiary structural information for the RFC protein.

**Experimental Procedures**

**Reagents**—Polybrene was purchased from Sigma and geneticin (G418) from Invitrogen. [3,5',7',9-H]Mtx (35 Ci/mmol) was purchased from Moravek Biochemicals Inc. and purified by thin-layer chromatography before use as described previously (21). The brefeldin A BODIPY 558/568 conjugate was obtained from Molecular Probes Inc. as were both the polyclonal and monoclonal anti-GFP antibodies; secondary mouse anti-rabbit-HRP antibody was from Jackson Laboratories. The streptavidin-HRP complex and N,N'-o-phenylenediamine were from Sigma, and factor Xa was from Amersham Biosciences. The methanethiosulfonate (MTS) reagents, sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES) and 2-(trimethylammonium)ethyl methanethiosulfonate bromide (MTSET), were purchased from Toronto Research Chemicals Inc., and the sulfo succinimidyl-2-(biotinamid) ethyl-1,3, dithiopropionate (EAZY-link sulfo-NHS-SS-biotin; biotin succinimide) was from Pierce.

**Cell Lines**—The maintenance of clonal cell lines of CHO wild-type Pro 4, and mutant Mtx-resistant Pro 3 MtxRII 5-3 (MtxRII 5-3) have been described previously (22, 23). The Pro 4 MtxRII 4-5 (MtxRII 4-5) cell line was generated using single-step selection after ethyl methane sulfonate mutagenesis in a manner similar to that used for the MtxRII 5-3 line, except it was derived from a different parental line (Pro 4). Neither the MtxRII 5-3 nor the MtxRII 4-5 lines transport Mtx and, although the former contains no detectable rfc message by Northern analysis (24), the latter has levels similar to those in wild-type cells (25).

**Dot Blot Analysis**—Genomic DNA was isolated from cell lines as

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1 The abbreviations used are: RFC, reduced folate carrier; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; MTS, methanethiosulfonate; MTSES, sodium (2-sulfonatoethyl) methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate; Mtx, methotrexate; o-PDM, N,N'-o-phenylenediamine; CHO, Chinese hamster ovary; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; aa, amino acid; TM, transmembrane segment; dhfr, dihydrofolate reductase gene.

2 W. F. Flintoff, H. Sadlish, and F. M. R. Williams, manuscript in preparation.
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described previously (26) and digested overnight with BamHI restrictionendonuclease. The DNA was denatured with 3 M NaOH prior to loading onto nitrocellulose membrane in dot blot apparatus. Samples were washed with sodium acetate and baked onto membranes, which were subsequently hybridized using 32P-labeled rfc-specific probes as described previously (24). After autoradiography, membranes were stripped and hybridized with dithioflavate reductase (dhfr)-specific probes.

Constructs—Site-directed mutations were incorporated into the hamster rfc cDNA background using either the Stratagene QuikChange kit or a two-step PCR method. In the latter case, complementary primers encoding the desired sequence were designed and used in combination with an upstream forward primer or a downstream reverse primer, respectively, in two separate reactions. After the first round of amplification, PCR products were digested with SacI, mixed together, and reamplified using the same nonmutagenic upstream and downstream primers. These fragments were cloned into pGem-T (Promega) and, along with appropriate restriction endonucleases, were used to replace the homologous region of the hamster RFC-EGFP backbone in pCDNA3 (19). The C termini of all proteins were tagged with enhanced green fluorescent protein (EGFP), which had previously been shown not to affect the properties of RFC (6).

The cysteine-less RFC was generated as described elsewhere.2 Factor Xa sites were inserted into the double cysteine mutants using one of two methods that did not otherwise interfere with the coding region: 1) a blunt-cutting enzyme site was generated at the desired location by site-directed mutagenesis, and a primer cassette containing the desired recognition sites was inserted (ATC GAG GGA CGC ATC GAG GGT AGG); or 2) primers with 5' extensions of tandem factor Xa recognition sites were used as described in the Stratagene QuikChange kit. The factor Xa site was inserted into the D86C,R373C mutant in the large central loop (P231), although it was located within inner loop 5 (Ile-387) of the R373C,E394C mutant. All mutations were sequenced to kit. The factor Xa site was inserted into the D86C,R373C mutant in the recognition sites were used as described in the Stratagene QuikChange kit. The factor Xa site was inserted into the D86C,R373C mutant in the large central loop (P231), although it was located within inner loop 5 (Ile-387) of the R373C,E394C mutant. All mutations were sequenced to confirm the presence of alterations.

DNA Transfections—Transfection of the construct plasmids into the recipient MtxRII 5-3 cells was performed using 10 µg of purified DNA in polybrene/1 × 105 cells as described previously (24). After transfection, the cells were selected for growth in either low levels of folinic acid (4 nM) or in normal medium containing 1.2 mg/ml G418 as described previously (5). Colonies were picked from individual transfection experiments and cloned by limiting dilution; those under G418 selection were subsequently hybridized using 32P-labeled rfc-specific probes. In some cases, mutants were selected and cloned under higher levels of folinic acid (10 nM). At least two independently generated isolates from separate transfection experiments with each construct were used for analysis. As both isolates for each construct showed similar characteristics, representative data from only one of each type are shown.

Northern Analysis—Poly(A)+ RNA (5 µg) was separated on a 1.2% agarose gel in formaldehyde buffer and transferred to Hybond XL membrane by the capillary method as described (27). Membranes were subsequently hybridized using 32P-labeled rfc-specific probes, washed, and exposed to x-ray film as described previously (24). Membranes were then stripped and hybridized with dhfr- or actin-specific probes.

Dose Response—Clonal cell lines stably expressing the appropriate RFC molecule were incubated in varying concentrations of Mtx or folinic acid for 7 days. At this point, cells were stained with methylene blue, and colonies were counted. Dose-response curves were generated and D50 (concentration allowing 50% survival under Mtx selection) or D10 (concentration allowing 10% growth with folinic acid) values were determined.

Folate Binding and Uptake—Kinetic analyses for the determination of Vmax and Kf for [3H]Mtx were carried out as described previously (21, 28) with the exception that the cells were grown in flasks until ~90% confluent and then gently trypsinized. After two washes in phosphate-buffered saline (PBS) and a third in uptake buffer (0.15 m HEPES, pH 7.4, 125 m NaCl, 0.1 m MgCl2) cells were resuspended to ~1 × 106 or ~3 × 107 cells/ml for binding and uptake analyses, respectively. The Vmax values were normalized relative to surface protein and corrected for cellular expression levels (see “Results”). At least two independent sets of data were analyzed for significant differences using the two-tailed Student’s t test.

To assess the effects of Mtx reagents on [3H]Mtx uptake, cells expressing cysteine-less RFC-EGFP or cysteine-less RFC-EGFP with an R373C substitution were used. Adherent cells (~7 × 106/sample) were washed twice in uptake buffer, and the appropriate Mtx reagent was added to a final concentration of 10 µM. After gentle shaking for 20 min at 37 °C, the buffer was removed and [3H]Mtx added (0.2–0.4 µCi).
were identified. As there are normally two genomic DNA and, although sequencing of multiple products confirmed that the MtxRII 4-5 gene was subsequently PCR-amplified from MtxRII 5-3. Under folinic acid-restricted (4 nM) conditions, the presence of a single mutation indicated that the MtxRII 4-5 cell line (29), the presence of a single mutation that either both alleles are similarly altered or there is only a single copy of the gene. This latter possibility was addressed by dot blot analysis of the genomic DNA, where the intensity of specific signal was compared among the mutant cell line and two wild-type lines (Fig. 1A). Normalization of the signal to the dihydrofolate reductase gene confirmed that the MtxRII 4-5 cell line has a single copy of the rfc gene.

**Functional Characterization of the R373H Mutation**—The functional effects of the arginine to histidine substitution were examined by transfecting a construct encoding the R373H mutant with a C-terminal green fluorescent protein fusion (referred to as R373H-EGFP) into the RFC-deficient cell line (MtxRII 5-3). Under folinic acid-restricted (4 nM) conditions, fewer colonies were observed as compared with wild type (Arg-373-EGFP) (Table I). Although this finding indicates that the mutant protein has some ability to transport substrate, it is contrary to the phenotype of the parental MtxRII 4-5 cell line, which demonstrates approximately a 20-fold higher requirement for folinic acid than the Arg-373-EGFP line. Expression levels could account for this discrepancy, and thus Northern analysis was used to examine the rfc message. As described previously (24), the recipient cell line, MtxRII 5-3, has undetectable levels of specific message, whereas the MtxRII 4-5 is similar to that of wild-type cells (Fig. 1B). However, the rfc mRNA levels in two independent cloned cell lines stably expressing the R373H construct were much higher (20–50-fold) as compared with both wild type and the parental MtxRII 4-5 cells (Fig. 1C).

![Comparison of rfc and dhfr gene and mRNA levels](image)

**TABLE I**

| Colonies/10⁶ µg/10⁴ cells | Folinic acid D₅₀ | Mtx D₅₀ | Kᵢ | Vₘᵢₓ | Normalized Vₘᵢₓ |
|--------------------------|------------------|---------|-----|------|-----------------|
| Pro⁻ | 4 nm | 10 nm | 4 nm | 10 nm | μM | pmol/mg/min | |
| MtxRII 5-3 | 2030 | — | 1.8 | 17.5 | 3.9 ± 1.8 | 0.82 ± 0.08 | 1.0 |
| MtxRII 4-5 | 2190 | — | 1.0 | 5.4 | 0.9 ± 0.4 | 0.30 ± 0.07 | 0.06 |
| R373K | 300 | — | 2.0 | 26 | 3.0 ± 0.2 | 0.43 ± 0.04 | 0.05 |
| R373F | 70 | — | 1.3 | 22 | 2.9 ± 1.2 | 0.19 ± 0.09 | 0.02 |
| R373N | 10 | 350 | 40" | 250" | — | — | — |
| R373A | 30 | 1200 | 2.4" | 40.5" | 3.8" | 0.12" | 0.02 |
| R373E | <10 | <10 | <10 | <10 | — | — | — |

A & B

**Fig. 1. Comparison of rfc gene and mRNA levels.** A, genomic DNA was isolated from cell lines, and 2-fold dilutions from 10 to 0.63 µg were dotted onto nitrocellulose membrane (see “Experimental Procedures”). Blots were stripped and reprobed with radiolabeled dhfr cDNA, and the signal was quantitated. Pro⁻ and Pro⁻ are wild-type CHO cell lines, whereas the MtxRII 4-5 line is Mtx-resistant. The numbers on the right indicate the amount of rfc signal after normalization to dhfr. B and C, poly(A⁺) RNA from cell lines was analyzed by Northern analysis as described under “Experimental Procedures.” B, lane 1, MtxRII 5-3; lane 2, wild-type Pro 3; lane 3, wild-type Pro 3; lane 4, cells stably expressing R373H altered rfc cDNA; lane 5, cells expressing R373H-EGFP rfc cDNA. Upper panel, blot probed with rfc cDNA; lower panel, the same blot stripped and probed with dhfr cDNA as a loading control. The numbers on the left indicate the size of markers in kb. C, lane 1, MtxRII 5-3; lane 2, wild-type Pro 3; lane 3, MtxRII 4-5; lane 4, cells stably expressing R373H altered rfc cDNA; lane 5, cells expressing R373H-EGFP rfc cDNA.

**Site-directed Mutagenesis of Arg-373 and Functional Characterization**—To clarify the role of the Arg-373 residue in protein function, site-directed mutagenesis was used to replace the arginine with lysine, glycine, asparagine, glutamate, and are similar to those found in cells expressing Arg-373-EGFP (Table I).
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Alanine; this allowed an examination of the size, charge, and polarity requirements at this site. Each mutant was tagged at the C terminus with the EGFP protein and transfected into the RFC-deficient cell line (MtxRII 5-3). Table I summarizes the transfection frequencies under low folate growth conditions as well as the folinic acid requirements and Mtx sensitivity of stable transfectants. The alteration to lysine, similar in size and charge to arginine, resulted in a comparable ability to rescue the RFC-deficient cell line as Arg-373-EGFP. Additionally, the folinic acid requirement and Mtx sensitivity of cells stably expressing R373K-EGFP suggest that this altered protein is efficient at transporting both substrates. In fact, the sensitivity to Mtx was ~3-fold greater than in Arg-373-EGFP-expressing cells.

The transfection frequency of R373Q-EGFP is lower than that of R373H-EGFP, whereas the R373A-EGFP and R373N-EGFP constructs required a higher level of folinic acid (10 nM) to demonstrate a significant ability to rescue the RFC-deficient cell line. However, although the cells stably expressing the R373N-EGFP require a ~20-fold higher level of folinic acid than Arg-373-EGFP, the R373H-EGFP, R373Q-EGFP, and R373A-EGFP mutants are similar to Arg-373-EGFP (Table I). Although the R373H-EGFP and the R373Q-EGFP cells show an Mtx sensitivity similar to that of Arg-373-EGFP, the R373A-EGFP and the R373N-EGFP are more resistant with the latter demonstrating a phenotype similar to the RFC-deficient cell lines. The R373E-EGFP and R373D-EGFP mutant constructs were unable to rescue the RFC-deficient cell line (MtxRII 5-3) under either of the folate-selective conditions (4 or 10 nM), and both the folinic acid requirement and Mtx sensitivity of R373E-EGFP-expressing cells (selected in G418) resembled that of the recipient cells (Table I).

Kinetic Analysis of Arg-373 Substitution—The kinetic parameters of Mtx uptake were determined in order to elucidate the basis for the differences in transfection frequency of the replacement amino acids as compared with Arg-373-EGFP. The substitution to lysine seems to increase the affinity for Mtx by ~4-fold; however, this appears to be due to unique properties of this residue, as the $K_a$ for the R373H-EGFP, R373Q-EGFP, and R373A-EGFP mutants are similar to Arg-373-EGFP. Furthermore, although the R373E-EGFP mutant is transport-defective, it demonstrates a binding affinity for Mtx ($K_d = 3.1 \mu M$) that is similar to Arg-373-expressing cells ($K_d = 2.7 \mu M$). Thus, it appears that the 373 residue makes little contribution to the substrate-binding site of the RFC protein.

Interestingly, the $V_{\text{max}}$ values for Mtx uptake of all the 373-aa mutants are significantly reduced (2–7-fold) when compared with Arg-373-EGFP. To determine whether this was due to variations in cell line expression levels or the amount of protein at the cell surface, the values were normalized as follows. Cells stably expressing each of the mutant proteins were labeled with a membrane-impermeant amide-reactive biotinylation reagent, and the fusion proteins were immunoprecipitated from equal amounts of cellular lysate using an excess of monoclonal antibody. Subsequent Western analysis (see “Experimental Procedures”) allowed a relative quantification of the amounts of both biotinylated (cell surface) and fusion protein (Fig. 2, Table II). After normalizing for differences in fusion protein levels, it appears there is <2-fold variation in the proportion of protein at the cell surface for the mutant cell lines as compared with Arg-373-EGFP; the lowest values belong to those proteins most impaired in folate transport (Table II). However, there are significant differences in cellular EGFP-protein expression levels relative to Arg-373-EGFP that, when taken into account, decrease the $V_{\text{max}}$ values of the mutants further (Table I). Thus, it appears that the alterations to the 373 residue lead to a 16–50-fold reduction in the efficiency of substrate transport.

Role of Arg-373 in Protein Biogenesis—The effect of the substitutions on protein localization was evaluated using confocal microscopy to visualize cells expressing the various fusion proteins (Fig. 3). The majority of the Arg-373-EGFP protein appears to be at the plasma membrane, with a lesser amount co-localizing with an endoplasmic reticulum-Golgi-specific stain (brefeldin A BODIPY). The localization of each of the 373 mutant proteins is very similar, including the transport-defective R373E-EGFP mutant with a reversal of the wild-type charge. It should be noted that the EGFP signal intensities do not reflect relative expression levels between the cell lines (see “Experimental Procedures”).

The stability of the 373 mutant EGFP fusion proteins was evaluated over time and was determined not to be significantly different from Arg-373-EGFP (Fig. 4). These Western analyses further confirm that the majority of each of the mutant proteins are able to obtain a complex glycosylation pattern, with a small amount (~2–5%) that is core- or unglycosylated (19). Based on these analyses, it is apparent that the 373 residue does not play a fundamental role in RFC protein stability or localization.

Arg-373 Appears to be Part of the Translocation Pathway—Although the RFC topology predictions place Arg-373 within the 10th transmembrane domain, functional analyses of the various amino acid substitutions indicate that the 373 position requires a large, positively charged residue for optimum transport capabilities. The presence of a charged residue within a hydrophobic lipid bilayer is energetically unfavorable and can be tolerated only if neutralized by a polar interaction with another amino acid or by exposure to the hydrophilic environment, perhaps through a channel or pore. The latter possibility was examined by attempting to determine whether the 373 residue was accessible to MTS reagents. These small membrane impermeable compounds react specifically with cysteines, and can impart a positive (MTSET) or negative (MTSES) charge on the residue. A cysteine-less form of the RFC protein (Cys-RFC-EGFP) that retains function was generated recently. An R373C substitution was made in this backbone and subsequently transfected into the RFC-deficient cells. A single colony was ex-
panded from the small number obtained with increased levels of folinic acid (20 nM) for growth and evaluated for the ability to take up [3H]Mtx (Fig. 5). As compared with cells expressing the Cys-RFC-EGFP, the amount of drug transported by the R373C-EGFP protein was decreased by 90%. However, when these cells were treated with the MTSET reagent, there was a 3-fold increase in the accumulation of the Mtx over time. This effect was unique to the MTSET (+) compound, as the MTSES (−) treatment had little effect. These results further confirm that for substrate translocation a positive charge at position 373 is required.

Identification of Charged Residues Potentially Interacting with Arg-373

The possibility that another polar residue may be interacting with aa 373 was also evaluated. As the topology of the RFC protein is not defined precisely, it is difficult to

| EGFP fusion protein level | Surface protein normalized to Arg-373-EGFP | Relative surface protein corrected for expression levels |
|---------------------------|------------------------------------------|--------------------------------------------------|
| R372                      | 1                                        | 1.0                                              |
| R373K                     | 4.6                                      | 1.1                                              |
| R373H                     | 5.0                                      | 1.7                                              |
| R373Q                     | 5.7                                      | 2.1                                              |
| R373N                     | 0.7                                      | 0.7                                              |
| R373A                     | 4.6                                      | 1.6                                              |
| R373E                     | 3.9                                      | 0.7                                              |

* Numbers were generated from images in which the EGFP signal was within the linear range of the film. Transfected protein levels were quantitated using EGFP signal intensities and expressed relative to Arg-373-EGFP.

* Values indicate the normalized amount of surface protein when cellular EGFP fusion expression levels are equivalent to Arg-373-EGFP (ratio of biotinylated protein to biotinylated Arg-373-EGFP/column 1).

* Values indicate the relative amount of surface protein expression corrected for cellular expression levels (column 1 × column 2).

Fig. 3. Confocal microscopy of 373 mutant RFC cell lines. The left image in each panel is EGFP fluorescence, and the center image is specific staining of the endoplasmic reticulum and Golgi complex with brefeldin A BODIPY as described under “Experimental Procedures.” The right panel is an overlay of the two preceding images.

Fig. 4. Turn-over analysis of aa 373 mutant EGFP-tagged molecules. Clonal cell lines expressing the various aa 373 mutant RFC molecules were incubated with cycloheximide to inhibit protein synthesis. Samples were harvested over a 24-h period, as indicated by the numbers at the top, and subjected to Western analysis as described under “Experimental Procedures.” The numbers on the right indicate an approximate half-life (t_1/2). *, indicates core- or unglycosylated protein. Upon longer exposure of R373, these forms were also evident.
identify those residues on a similar plane within the lipid bilayer as the 373 position. Thus, three conserved and negatively charged residues predicted to be within the membrane were chosen for further analysis: Asp-86, Glu-394, and Asp-453 (Fig. 6).

Each of the candidate interacting positions was mutated to arginine, tagged with the EGFP protein, and examined for its ability to rescue the RFC-deficient cell line (MtxRII 5-3) (Table III). If the residue is involved in a critical interaction, then reversing the charge should affect functionality. Additionally, because the protein may regain transport capabilities if an interaction has been maintained within the molecule, each of the arginine substitutions was paired with the nonfunctional R373D mutation and examined. Each of the single alterations demonstrated some ability to rescue the RFC-deficient cell line (Table III), although the D86R mutation led to a significant reduction in transfection frequency. None of the paired constructs was able to rescue the nonfunctional R373D mutation.

**Functional Characterization of the Asp-86 and Glu-394 Residues**—As there was nearly a 90% decrease in the number of functional transfectants with the alteration to D86R, it appears this residue is important for substrate transport. This is supported by the significant reduction in transfection frequency when this position was changed to a structurally similar (D86E) or uncharged (D86A) amino acid (Table IV). Furthermore, although the reversal of both the 86- and 373-aa charges in the same molecule did not restore function, an interaction between these two residues cannot be ruled out; an opposite charge may have significant secondary effects in a different local environment. Thus, both the 86 and 373 residues were mutated to alanine in the same molecule, as removal of both charges may be less disruptive. For comparison, the E394 residue was also altered to alanine and paired with R373A, as the E394R substitution only depressed the transfection frequency by 55%. Surprisingly, both combinations showed a level of functionality greater than with R373A alone (Table IV). Kinetic analysis of Mtx uptake indicated that the D86A replacement in the same molecule as the R373A mutation (normalized \( V_{max} = 0.10 \) pmol/min/mg) restored some of the substrate translocation efficiency of the latter alteration alone (normalized \( V_{max} = 0.02 \) pmol/min/mg). In contrast, the combined alanine mutations in the R373A,E394A,EGFP construct had little effect (normalized \( V_{max} = 0.03 \) pmol/min/mg).

**Cross-linking Analysis of Asp-86 and Glu-394**—An alternative approach was used to evaluate the potential Arg-373 interactions between Asp-86 or Glu-394 utilizing the cysteineless form of the RFC protein. In the Cys-RFC-EGFP backbone, double cysteine mutants (D86C,R373C-EGFP and

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**Table III**

| Construct                  | Colonies/10 \( \mu g \)/10\(^6\) cells |
|----------------------------|----------------------------------------|
| Arg-373                    | 2030                                   |
| D86R                       | 260                                    |
| E394R                      | 950                                    |
| R373D                      | <10                                    |
| D453R                      | 1000                                   |
| D86R,R373D                 | <10                                    |
| R373D,E394R                | <10                                    |
| R373D,D453R                | <10                                    |

**Table IV**

| Construct                  | Colonies/10 \( \mu g \)/10\(^6\) cells | Folinic acid \( D_{50} \) nM | Mtx \( D_{10} \) nM |
|----------------------------|----------------------------------------|----------------------------|-------------------|
| Arg-373                    | 2030                                   | 2                          | 15                |
| R373A                      | 30                                     | 2.5                        | 40                |
| D86R                       | 260                                    | 4                          | 20                |
| D86E                       | 300                                    | 6                          | 18                |
| D86A                       | 110                                    | 0.8                        | 10                |
| D86A,R373A                 | 80                                     | 1.8                        | 25                |
| E394R                      | 950                                    | 18                         | 25                |
| E394A                      | 1390                                   |                            |                   |
| R373A,E394A                | 410                                    | 1.8                        | 25                |

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Alteration of Arg-373 Disrupts RFC Substrate Translocation
Alteration of Arg-373 Disrupts RFC Substrate Translocation

The Mtx-resistant phenotype of the MtxR II 4-5 cell line is the combined result of the loss of one rfc allele, possibly during the initial mutagenesis procedure, and the presence of a mutation in the remaining allele. This point mutation resulted in the substitution of histidine for arginine at position 373. As this amino acid is conserved within the RFC protein throughout eukaryotic organisms including the *Caenorhabditis elegans* homologue, it is likely to play a pivotal role in defining protein structure or function. Biochemical and functional analysis of an array of amino acids substituted into this position indicated that there are strict requirements to allow any degree of substrate transport. First, the amino acid at position 373 appears to require the ability to form hydrogen bonds, as the frequency of functional transfectants diminishes as the polar tendencies of the residues decline (Arg > Lys > His > Gln > Ala). Second, the amino acid needs to be of a certain size, as exemplified by the transfection frequency of the R373Q and R373N mutant proteins. Although the asparagine residue differs by only a single carbon in length, the transfection frequency is significantly reduced from that of R373Q. However, the alanine substitution was also slightly better tolerated than the asparagine, suggesting that the ability to form hydrogen bonds can be detrimental if the residue is too small due to destabilizing polar interactions within the local environment.

The transfection frequencies are drastically reduced for most of the alterations at aa 373 with the exception of lysine, a residue similar in size and charge to arginine. However, most of the other replacements (R373H, R373Q and R373A) yield cell lines similar to the wild-type in folinic acid requirements and Mtx sensitivity; this seems to be the result of increased EGFP expression (3–6-fold) over Arg-373-EGFP lines. The low folinic acid growth conditions select for cells with a high level of protein expression, which can compensate for the decreased rate of substrate transport. Thus, in some cases in which clones were not selected for functionality but merely for G418 resistance carried by the plasmid, the protein expression levels are lower (i.e. R373N-EGFP). The low level of R373N-EGFP expression as compared with the other 373 mutant cell lines is reflected in the folinic acid requirements and Mtx sensitivity.

Based on algorithms, epitope mapping (5), and cysteine scanning, the RFC protein is consistent with a 12-TM topology with aa 373 predicted to be in the 10th transmembrane domain. It appears that the presence of this charged amino acid in the hydrophobic membrane environment may be tolerated as a result of accessibility to the extracellular environment, perhaps as part of the translocation pathway. The kinetic parameters of Mtx uptake demonstrate that any alteration to the Arg-373 residue leads to a 16–50-fold reduction in the efficiency of substrate translocation ($V_{max}$), indicating that this residue has a vital role in the structure or function of the protein. As evaluated by protein stability, cellular distribution, and the portion of total molecules at the plasma membrane, the amino acid at position 373 does not significantly affect the
folding or localization of RFC. However, the addition of the positively charged MTSET reagent to cells expressing an R373C-EFGFP gene product led to a 2.5-fold increase in the amount of Mtx drug accumulation as compared with untreated cells, confirming the functional importance of the positive charge at Arg-373.

The Arg-373 residue is predicted to be located within a membrane-spanning segment of the RFC protein and thus should not be accessible to the extracellular environment. Preliminary experiments indicate that this is the case, as a cysteine at this position cannot be labeled by the membrane-impermeable biotin maleimide compound (data not shown). However, although the MTSET reagent is also membrane-impermeable, its small size enables the molecule to permeate protein pores. Taken together with the functional role of this amino acid in substrate transport, it appears the Arg-373 residue forms part of the translocation pathway.

It is difficult to determine conclusively whether the Arg-373 residue interacts with another amino acid based on functional analyses of an array of mutant proteins. It is only with the replacement of Asp-86 or Glu-394 with alamines in combination with R373A to limit potential secondary effects that there is a positive influence on protein function. When transfected into the RFC-deficient cell line, each combination demonstrates an increased transfection frequency as compared with R373A-EFGFP alone (3–13-fold), although it is still less than for Arg-373-EFGFP. Furthermore, the Mtx uptake kinetics of the cell lines expressing the D86A,R373A-EFGFP mutant indicates that this protein has a 5-fold better rate of translocation compared with R373A-EFGFP alone. This is suggestive of an interaction between these two residues, although secondary structural effects may also play a role. In contrast, it appears that combining the E394A alteration with R373A has little functional effect on the rate of substrate translocation.

The potential interactions between Arg-373 and the Asp-86 or Glu-394 residues were further examined using a technique previously utilized for the elucidation of membrane protein helix packing (30–32). Cysteines replaced the residues of interest in a cysteine-less RFC backbone, and a protease site inserted between them was used to evaluate the effectiveness of the cross-linking agents. Previous work has indicated that removal of the cysteines has little effect on functionality of the RFC and thus, should not interfere with helix packing. Based on these data, it is clear that E394 is within 6 Å of the Arg-373 residue in the tertiary structure of the RFC protein. However, there was no apparent cross-linking between the D86C and R373C residues after the addition of the ε-PDM cross-linker or under oxidative conditions.

The demonstration that Glu-394 (TM11) is within 6 Å of Arg-373 (TM10) is highly significant, as it provides the first evidence for a tertiary structure for this protein. Furthermore, it reflects studies on the bacterial lactose permease protein, where TM10 and TM11 are also located adjacent to each other (33). The two proteins are structurally similar in many respects, in that they are both polytopic 12-TM domain proteins with a large cytoplasmic loop between TM6 and TM7. As the helix packing of very few polytopic proteins has been documented, it is not clear whether there are common tertiary structural characteristics. A recent report of a potential charge-pair interaction in the human RFC protein (20) implicated the residues corresponding to Asp-86 and Arg-131 in the hamster protein. In an attempt to confirm these observations in the hamster system, the same alterations were made (D86V,R131L). However, neither this, nor a double alanine mutant (D86A,R131A) was able to complement the MtxR11 5-3 cell line under restricted folinic acid growth conditions (data not shown). Based on these two opposing results, it appears that there may be differences in tertiary folding of the RFC protein between the human and hamster species. This is somewhat surprising, as there is a high degree of amino acid similarity (~60%) between the two species as well as similar folate transport properties.

The 373 position has strict charge and size requirements such that none of the amino acids tested was able to provide comparable substrate translocation efficiency. The data presented here suggest that the Arg-373 residue is accessible to the extracellular space and may directly interact with the substrate, although it does not appear to be part of the substrate-binding site. Furthermore, the close proximity of this residue to TM11 and the slight stabilizing effect of the D86A alteration suggest that Arg-373 may also have a structural role in defining the translocation pathway. As the structural nature of the arginine residue allows multiple and simultaneous interactions, it is not improbable that the Arg-373 position in RFC may have many different roles.

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