Cytoplasmic domains of the reduced folate carrier are essential for trafficking, but not function

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The reduced folate carrier (RFC) protein has a secondary structure consistent with the predicted 12 transmembrane (TM) domains, intracellular N- and C-termini and a large cytoplasmic loop between TM6 and TM7. In the present study, the role of the cytoplasmic domains in substrate transport and protein biogenesis were examined using an array of hamster RFC deletion mutants fused to enhanced green fluorescent protein and expressed in Chinese hamster ovary cells. The N- and C-terminal tails were removed both individually and together, or the large cytoplasmic loop was modified such that the domain size and role of conserved sequences could be examined. The loss of the N- or C-terminal tails did not appear to significantly disrupt protein function, although both termini appeared to have a role

in the efficiency with which molecules exited the endoplasmic reticulum to localize at the plasma membrane. There appeared to be both size and sequence requirements for the intracellular loop, which are able to drastically affect protein stability and function unless met. Furthermore, there might be an indirect role for the loop in substrate translocation, since even moderate changes significantly reduced the $V_{\max}$ for methotrexate transport. Although these cytoplasmic domains do not appear to be absolutely essential for substrate transport, each one is important for biogenesis and localization.

Key words: biogenesis, intracellular domain, membrane protein, polytopic, topogenesis.

INTRODUCTION

The uptake and intracellular accumulation of folates in mammalian cells is mediated by the reduced folate carrier (RFC) protein, which has been examined in the hamster [1,2], mouse [3,4], rat [5] and human [6–10] systems. This low-capacity carrier transports reduced folates such as folinic acid, as well as the anti-folate methotrexate (Mtx), with relatively high affinity. Reduced folate transport is essential for normal cell growth and differentiation, and is an important determinant of anti-folate resistance encountered in chemotherapeutic applications [11–13]. Furthermore, folate deficiencies have been implicated in a wide variety of health conditions including heart disease and neural tube defects, underlining the importance of characterizing the transporter [14].

The RFC protein is localized to the plasma membrane, and has a secondary structure consistent with the predicted 12 transmembrane (TM) domains [15]. Both N- and C-termini are located in the cytoplasm, as is a large loop between TM6 and TM7. Although a number of studies have examined the effect of single point mutations on RFC protein stability and function [16–22], little is known about the mechanism of folate translocation into the cell. Furthermore, although there has been preliminary analysis of the RFC biogenic pathway [20,23], many questions remain regarding the regulation of protein levels at the plasma membrane.

With recent developments in proteomics, it is becoming apparent that interacting molecules can affect localization, function, specificity or stability of a protein [24]. Accordingly, there may be cellular components that define and modify the post-

translational regulation of the RFC protein, possibly by interacting with either of the termini or the large cytoplasmic loop between TM6 and TM7. Although there is little amino acid similarity among species in these RFC domains, the cytoplasmic localization and general structure are both conserved and markedly similar to many members of the major facilitator superfamily of proteins [25]. Thus it is possible that these domains have universally important functional and/or structural roles.

In order to determine the role of the large cytoplasmic domains within the RFC protein, they were removed either individually or in combination, and the resulting gene products were characterized both functionally and biochemically. Within the Chinese hamster ovary system, neither terminus was essential for function, although they both appeared to have a role in protein maturation. Alterations to the central loop indicate there is a requisite length for protein stability, and that a sequence and/or structural motif in this domain might be important in the early stages of protein biogenesis.

EXPERIMENTAL

Reagents

Polybrene was purchased from Sigma Chemical Co (St Louis, MO, U.S.A.). Geneticin (G418) was purchased from Life Technologies (Gaithersburg, MD, U.S.A.) [³H]-Mtx (35 Ci/mmol) was purchased from Moravsek Biochemicals Inc. (Brea, CA, U.S.A.) and purified by TLC before use, as described previously [26]. The brefeldin A-BODIPY® (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) 558/568 conjugate was obtained from

Abbreviations used: BODIPY®, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; (E)GFP, (enhanced) green fluorescent protein; CA-EGFP, C-terminal-deletion construct; NA-EGFP, N-terminal-deletion construct; NΔCA-EGFP, N- and C-termini-deletion construct; LoopΔ-1 → 5-EGFP, loop-deletion constructs 1–5; ER, endoplasmic reticulum; HRP, horseradish peroxidase; Mtx, methotrexate; poly(A)⁺, polyadenylated; RFC, reduced folate carrier; TM, transmembrane; WT, wild-type.

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Table 1 Primers used in designing the terminal deletion constructs

The restriction sites for *KpnI* and *BglII* are shown underlined in primers 1 and 4 respectively.

Primer	bp	Sequence (5' → 3')
1	1	GAGGTACCATGGTGGCCACTGGCCAGAAGTCCTGGAGATGCCTAGTATTC
2	500	ACAGAGCTGGTGAAGACTCC
3	1050	GTACATGGTCCACTACGTGA
4	1383	TCAGATCTCTCCAGCCGGCAGTACCTCAC

Table 2 Sequence of the loopΔ constructs

Construct	Sequence	Total number of amino acids
	204	270
WT-Loop	KRPKHS�FFNRSALVHKALPCELDQMHPGPGRPEPGKLERVLGSCRNSFLVCMSELVGNLRRPQLR	67
LoopΔ-1	KRPKHS�FFNRS LR	14
LoopΔ-2	KR VGNLRQPQLR	12
LoopΔ-3	KRPKHS�FFNRS VGNLRQPQLR	22
LoopΔ-4	KR NSFLVCMSELVGNLRRPQLR	23
LoopΔ-5	KRPKHS�FFNRS NSFLVCMSELVGNLRRPQLR	33

Molecular Probes Inc. (Eugene, OR, U.S.A.), as were both the polyclonal and monoclonal anti-[green fluorescent protein (GFP)] antibodies, whereas the secondary mouse anti-rabbit horseradish-peroxidase (HRP) antibody was from Jackson Laboratories (West Grove, PA, U.S.A.). The biotinylation compound, sulphosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate ('EZ-Link sulfo-NHS-SS-biotin'), was obtained from Pierce (Rockford, IL, U.S.A.), whereas the biotinylated streptavidin-HRP complex was from Sigma Chemical Co.

Cell lines

Clonal cell lines of Chinese hamster ovary wild-type (WT) Pro⁻³ cells and mutant Mtx-resistant Pro⁻³ MtxR^{II} 5-3 (MtxR^{II} 5-3) cells, and their maintenance, have been described previously [27,28]. The Mtx-resistant line does not transport Mtx, and contains no detectable RFC message on Northern blot analysis [2].

Construct cloning

N-terminal-deletion [NΔ-enhanced GFP (EGFP)] construct

Primers 1 and 2 (Table 1) were used to amplify a fragment from the hamster RFC cDNA [2], where amino acids 7–22 were removed. The PCR product was TA-cloned into pGEM-T (Promega, Madison, WI, U.S.A.), and the *KpnI*–*NcoI* fragment was used to replace the 5'-coding sequence of the RFC-EGFP construct in a pcDNA₃ vector backbone [20].

C-terminal-deletion (CΔ-EGFP) construct

Primers 3 and 4 (Table 1) were used to amplify a 'non-stop' truncated fragment of the hamster RFC cDNA, which was lacking the final 57 amino acids of the coding region. After this PCR fragment was TA-cloned into pGEM-T, the *SacII*–*BglII* fragment was subcloned into the pEGFP-N1 vector (Clontech, Palo Alto, CA, U.S.A.). The *SacII*–*NotI* fragment of this construct was used to replace the corresponding fragment in the RFC-EGFP construct [20].

N- and C-termini-deletion (NΔCΔ-EGFP) construct

The *HindIII*–*SacII* fragment of NΔ-EGFP was used to replace the corresponding fragment of the CΔ-EGFP construct.

Loop-deletion (LoopΔ-1 → 5-EGFP) constructs

Blunt-cutting restriction endonuclease sites (*StuI* at Arg²⁰⁵, *Aor51HI* at Ser²¹⁵, *HpaI* at Arg²⁴⁹ and *Eco721* at Leu²⁶⁰) were introduced into the region coding for the predicted large cytoplasmic loop of RFC using site-directed mutagenesis (Stratagene Quik-Change kit; La Jolla, CA, U.S.A.). These sites did not affect the coding sequence of the final constructs and, with a native *PvuII* site at Q268, were used to modify the RFC-EGFP construct [20] to generate the variants summarized in Table 2. All constructs were sequenced to confirm alterations.

DNA transfections

Transfection of the construct plasmids into the recipient MtxR^{II} 5-3 cells was performed using 10 µg of purified DNA in polybrene/2 × 10⁵ cells, as described previously [2]. After transfection with DNA, the cells were selected for growth in either low levels of folinic acid (2 nM) or normal media containing 1.2 mg/ml G418, as described previously [15]. Colonies were picked from individual transfection experiments, and cloned by limiting dilution. At least two independently generated isolates from separate transfection experiments with each construct were used for analysis. Cell lines expressing the LoopΔ-3- and LoopΔ-5-EGFP constructs were isolated with higher levels of folinic acid (10 nM), whereas the LoopΔ-4-EGFP line was isolated under G418 selection. All other lines were isolated and maintained in the low folate (2 nM) media. As both isolates for each construct showed similar characteristics, representative data from only one of each type is shown.

Dose-response experiments

Clonal cell lines stably expressing the appropriate RFC molecule were incubated in various concentrations of Mtx or folinic acid for 7 days, after which cells were stained with Methylene Blue

and colonies were counted. Dose-response curves were generated, and D_{10} (concentration allowing 10% survival under Mtx selection) or D_{50} (concentration allowing 50% growth under folinic acid restrictions) values were determined.

Folate uptake

Kinetic analyses for the determination of $V_{\max}$ and K_t (the K_m for the transporter) for [3H]Mtx were performed as described previously [29]. The $V_{\max}$ values were normalized relative to surface protein, and corrected for cellular expression levels (see the Results section).

Northern blot analysis

Polyadenylated [poly(A)⁺] RNA (approx. 5 μ g) was separated on a 1.2% (w/v) agarose gel in formaldehyde buffer, and transferred to Hybond XL membrane by the capillary method, as outlined by Sambrook et al. [30]. Membranes were probed, washed and exposed to X-ray film, as described previously [2].

Western blot analysis

Total cellular extracts were prepared, separated by SDS/PAGE [7.5% (w/v) gels], and transferred electrophoretically to nitrocellulose. Proteins were detected using polyclonal rabbit anti-GFP antibodies, as described previously [20], and visualized using the ECLTM reagent from Amersham Biosciences (Piscataway, NJ, U.S.A.).

Cell-surface biotinylation

Equal numbers of cells (approx. 7×10^5) expressing the various RFC deletion constructs were washed twice with PBSCM (137 mM NaCl/2.7 mM KCl/8 mM Na₂HPO₄/1.5 mM KH₂PO₄/1 mM MgCl₂/0.1 mM CaCl₂), and incubated with 1.5 mg/ml biotin succinimide for 1 h at room temperature. Cells were washed twice in 100 mM glycine/PBSCM, and once in ice-cold PBS before lysis on ice [1% (v/v) Nonidet P40/0.1% (w/v) SDS/0.5% (w/v) sodium deoxycholate/50 mM Tris/HCl (pH 8)/150 mM NaCl/0.2 mM orthovanadate/0.5 mM PMSF/5 $\times$ protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany)]. EGFP-fusion proteins were immunoprecipitated using monoclonal anti-GFP antibody and Protein G-Sepharose beads (Amersham Biosciences). Beads were washed twice in buffer [0.1% Nonidet P40/50 mM Tris/HCl (pH 8)/50 mM NaCl/1 $\times$ protease inhibitor cocktail], resuspended in loading buffer, heated at 55 °C for 20 min, and separated by SDS/PAGE before electrophoretic transfer to nitrocellulose. Membranes were blocked in 5% BSA/TBS-T [20 mM Tris base/137 mM NaCl/0.2% (v/v) Tween 20 (pH 7.6)], before detection with streptavidin-HRP (diluted 1:2000 in 0.5% BSA/TBS-T), and were subsequently visualized using ECLTM reagent. Blots were then stripped, probed for EGFP levels as described previously [20], and the band intensities were subsequently quantified using the BioRad Multi-Analyst program. The amount of signal for each EGFP-fusion protein, relative to WT-EGFP, was used to normalize the level of biotinylated protein.

Fluorescence detection of GFP

For confocal microscopy, cells were grown on glass coverslips and stained with the tracking reagent (brefeldin A-BODIPY[®] 558/568 conjugate), as described previously [20]. Detection was performed on an LSM410 inverted Zeiss laser scanning microscope, with LSM410 software, using a krypton/argon laser and a 63 $\times$ oil immersion lens under standard conditions.

Analysis of glycosylation

Cell lysates were acetone-precipitated, re-suspended in lysis buffer [50 mM Tris/HCl (pH 8.0)/0.15 M NaCl/0.1% SDS/1% Nonidet P40/0.5% sodium deoxycholate/0.2 mM orthovanadate/5 $\times$ concentrated protease inhibitor cocktail tablets (Boehringer Mannheim)] and N-glycosidase F (Boehringer Mannheim) was added to a final concentration of 50 units/ml, before incubation at 37 °C for 15 h. Samples were separated by SDS/PAGE and analysed by Western blot analysis, as described above.

Turnover analysis

For examination of protein turnover, 0.2 mg/ml cycloheximide was added to cells, which were then incubated at 37 °C. Samples (approx. 2×10^5 cells) were lysed at intervals over the appropriate time period, separated by SDS/PAGE and analysed by Western blot analysis, as described above. Images were scanned, and bands were quantified using the BioRad Mutli-Analyst program.

RESULTS

Construct design

On the basis of RFC topology, as predicted by hydropathy plots and defined by epitope mapping [15], various modified hamster proteins were generated in which the different cytoplasmic domains were removed (Figure 1 and Table 2). A few amino acids were left near the hypothetical membrane boundaries in the design of these constructs, which was an attempt to minimize the disruption of potential TM domains. Additionally, although a total of 16 residues (amino acids 7–22) were eliminated in the NA-EGFP construct, the first six amino acids were maintained, since they might be critical in the early stages of targeting and insertion into the endoplasmic reticulum (ER) membrane. In the CA-EGFP construct, the final 57 residues (amino acids 461–518) were removed, whereas both the N- and C-terminal modifications were combined to generate the NACA-EGFP construct. In order to examine the cytoplasmic loop domain between TM6 and TM7, five separate variations were examined (Table 2). In Loop Δ -1-EGFP and Loop Δ -2-EGFP, the majority of the residues were eliminated with the N-terminal conserved patch (Figure 1; A $\rightarrow$ B), or the corresponding C-terminal non-conserved region (D $\rightarrow$ E) maintained. Loop Δ -3-EGFP contains both these segments, with only the most central part of the loop being deleted (B $\rightarrow$ D). In order to address the role of the conserved patch at the N-terminal end of the loop, Loop Δ -4-EGFP (C $\rightarrow$ E) was designed with a similar number of residues as for Loop Δ -3-EGFP, but without these conserved amino acids. Loop Δ -5-EGFP contains the largest number of residues, and includes the conserved patch at the N-terminal end of the loop. All of the modified RFC constructs included EGFP as a C-terminal fusion, previously shown not to affect protein function or localization [15,20].

Complementation ability of modified RFC constructs

The constructs were transfected into MtxR115-3 cells and examined for their ability to complement the deficiency of RFC under low folate growth conditions (Table 3). The NA-, CA- and NACA-EGFP constructs were able to complement the transport-deficient cell line at a frequency similar to that of WT-EGFP, whereas each of the Loop Δ deletions were variable in this ability. Loop Δ -1-, -2- and -4-EGFP-transfected cells were unable to survive under selective conditions and, while both the Loop Δ -3- and -5-EGFP variants were able to complement folate transport,

Figure 1 Topology of the reduced folate carrier

The topology is on the basis of hydropathy plots and epitope insertion analysis [15]. Circled residues indicate those conserved among hamster, human, rat and mouse species. Residues that were deleted in construct design are indicated with bold lines. For the modifications to the central loop, dotted lines indicate residues removed in only some of the constructs, whereas the solid line indicates that region was consistently deleted. The 'conserved patch' (see the Results section) is as indicated. Restriction endonuclease sites used for generating the modified loop constructs (see the Experimental and Results sections) are as follows: **A**, *Stu*I; **B**, *Aor*51HI; **C**, *Hpa*I; **D**, *Eco*721; and **E**, *Pvu*II.

Table 3 Summary of functional and kinetic data for cells transfected with RFC constructs

ND, not determined.

Cell type	Colony formation/10 μ g protein per 10 ⁶ cells	Mtx (D_{10} ; nM)*	Folinic acid (D_{50} nM)*	K_t (μ M)	V_{max} (pmol/min per mg)	Normalized V_{max} †
WT	—	12	1.3	1.9 $\pm$ 0.2	1.0 $\pm$ 0.8	—
Mutant	—	250	400	—	—	—
WT-EGFP	1368	12	1.3	2.1 $\pm$ 0.6	7.6 $\pm$ 1.2	7.6
NΔ-EGFP	1305	12	1.3	0.9 $\pm$ 0.6	0.8 $\pm$ 0.6	4.1 $\pm$ 1.2
CΔ-EGFP	1400	20	1.3	0.8 $\pm$ 0.5	2.3 $\pm$ 0.8	1.0 $\pm$ 0.06
NΔCΔ-EGFP	1540	40	1.3	0.9 $\pm$ 0.5	1.3 $\pm$ 0.8	2.8 $\pm$ 0.1
LoopΔ-1-EGFP	< 7	ND	ND	ND	ND	ND
LoopΔ-2-EGFP	< 7	ND	ND	ND	ND	ND
LoopΔ-3-EGFP	54 (1850)‡	30	3.0	1.4§	0.7§	0.2 $\pm$ 0.06§
LoopΔ-4-EGFP	< 7	ND	ND	ND	ND	ND
LoopΔ-5-EGFP	710 (2010)‡	12	3.4	1.6§	0.5§	0.4 $\pm$ 0.2§

* D_{10} is the Mtx concentration reducing growth to 10% of the control value; D_{50} is the folinic acid concentration allowing 50% survival.

† Normalized to surface protein and expression levels using the value from Table 4; column C (see the Results section).

‡ Number in parentheses indicates growth in 10 nM folinic acid selective medium.

§ Analyses were performed on a single clone, with duplicate values within 10% of each other.

Figure 2 RNA expression levels of transfected cell lines

Poly(A)⁺ RNA from representative cell lines stably expressing the fusion constructs was analysed by Northern blot analysis as described in the Experimental section. Lanes were loaded as follows: lane 1, WT Pro3 cells; lane 2, RFC-deficient MtxRII 5-3 cells; lane 3, WT-EGFP; lane 4, NΔ-EGFP; lane 5, CΔ-EGFP; lane 6, NΔCΔ-EGFP; lane 7, LoopΔ-3-EGFP; lane 8, LoopΔ-4-EGFP; lane 9, LoopΔ-5-EGFP. Upper panel, blot probed with RFC cDNA; the asterisks indicate longer exposure times required for some samples; lower panel, the same blot was stripped and probed with actin cDNA. The numbers to the left indicate the size of markers, in kb.

it was at a lower frequency than the WT. Interestingly, when an increased level of folinic acid in the selective medium (10 nM) was used for the latter two constructs, the transfection frequency was greater than for cells transfected with the WT-EGFP construct.

RNA expression of transfected cells

Expression of the RFC transcript in all clonally isolated cell lines was evaluated by Northern blot analysis (Figure 2). Cells expressing LoopΔ-1- and -2-EGFP were not characterized further, since stable isolates could not be obtained. The levels of RFC message were similar in all the transfected lines, at approx. 15–20-fold greater than for WT cells (Table 4). The exception was the line expressing LoopΔ-4-EGFP, which was about 4-fold higher than that for WT cells. As expected, the message produced by the transfectants was similar in size to the endogenous RFC (approx. 2.8 kb); a result of the addition of the EGFP tag and the corresponding loss of the 3'-untranslated region during cloning.

Figure 3 Cell-surface biotinylation of transfected cell lines

Biotin-succinimide was used to label cells stably expressing the functional deletion mutants, and the EGFP-fusion proteins were subsequently immunoprecipitated and examined by Western analysis, as described in the Experimental section. Lanes were loaded as follows: lane 1, WT-EGFP; lane 2, NΔ-EGFP; lane 3, CΔ-EGFP; lane 4, NΔCΔ-EGFP; lane 5, LoopΔ-3-EGFP; lane 6, LoopΔ-5-EGFP; and lane 7, LoopΔ-5-EGFP selected under low folate conditions. Upper panel, blot was probed using streptavidin-biotinylated-HRP complex; lower panel, the blot was stripped and probed for EGFP-fusion proteins, as described in the Experimental section. The numbers to the left indicate the size of protein markers, in kDa.

Functional analysis of modified RFC proteins

In order to determine whether the cytoplasmic domains are involved in compound recognition, the sensitivity to and dependence of the stable transfectants on Mtx and folinic acid respectively was assayed (Table 3). In general, it appears that sensitivity to both Mtx and folinic acid is not affected significantly in any of the deletion mutants capable of complementing the RFC-deficient cells.

The functional capabilities of the deletion mutants were characterized further by evaluating the kinetics of Mtx uptake. As described previously [15,20], fusion of the EGFP moiety to the C-terminus of the RFC protein does not alter K_t (the K_m for the

Table 4 Summary of relative RNA and protein expression levels for cells transfected with RFC constructs

ND, not determined.

Cell type	RNA expression level*	EGFP-fusion protein expression†‡ (A)	Surface protein normalized to WT-EGFP§ (B)	Relative surface protein corrected for expression level (A × B = C)
WT-EGFP	1.0	1.0	1.0	1.0
NΔ-EGFP	0.9	0.8	0.3	0.2
CΔ-EGFP	0.9	0.6	3.9	2.3
NΔCΔ-EGFP	1.0	0.2	2.3	0.5
LoopΔ-3-EGFP	1.0	1.7	1.9	3.2
LoopΔ-4-EGFP	0.2	0.1	ND	ND
LoopΔ-5-EGFP	0.6	1.1	1.5	1.7
LoopΔ-5-EGFP	ND	1.2	1.8	2.2

* Values relative to WT-EGFP mRNA expression (see Figure 2).

† Values relative to WT-EGFP when equal amounts of total protein are loaded (see Figure 5A).

‡ Values are the average of at least two independent analyses.

§ Values relative to surface WT-EGFP after correcting for the amount of EGFP-fusion protein loaded (see Figure 3).

|| Clone selected in 2 nM folinic acid medium.

Figure 4 Confocal microscopy of transfected cell lines

The left-panel images for **A–G** shows GFP fluorescence, whereas the centre panels show specific staining of ER and Golgi complex with brefeldin A–BODIPY®. The right panels within each lettered Figure part represent an overlay of the two. (**A**) WT–EGFP; (**B**) NA–EGFP; (**C**) CA–EGFP; (**D**) NACA–EGFP; (**E**) soluble EGFP control protein; (**F**) LoopΔ-3–EGFP; (**G**) LoopΔ-4–EGFP; (**H**) LoopΔ-5–EGFP.

transporter) for Mtx. The value for WT–EGFP is comparable with that of WT cells (approx. $1.9 \mu\text{M}$), whereas the increased V_{max} probably reflects a greater number of WT–EGFP molecules at the plasma membrane, as it corresponds with the increased transcript expression (see above). For all the deletion mutants capable of complementing the RFC-deficient cells, the K_i for Mtx was within 2-fold of $2 \mu\text{M}$ (Table 3), although there were significant differences in the V_{max} as compared with the WT–EGFP-transfected cells.

Variations in protein expression were accommodated by normalizing V_{max} values against both the levels of EGFP-fusion protein for each transfected cell line relative to WT–EGFP (Table 4; also see Figure 5) and the amount of total RFC protein at the cell surface as determined using a membrane-impermeant biotinylation reagent and Western blot analysis (Figure 3 and Table 4). For example, when the amount of the biotinylated CA–EGFP fusion protein is normalized to account for EGFP protein loading (Figure 3), there is a 3.9-fold higher level of CA–EGFP protein than WT–EGFP at the cell surface. However, as the CA–EGFP cell line expresses only 60 % of the WT–EGFP-fusion protein levels (see Figure 5A), the relative difference in surface protein is only 2.3-fold. For each of the cell lines, the corrected level of protein at the cell surface was subsequently used to normalize the V_{max} data (Table 3). In spite of the fact that the normalized V_{max} for NA–EGFP is within 2-fold of that of the WT–EGFP protein, both CA- and NACA–EGFP V_{max} values are

even lower. Furthermore, it appears that modifications to the loop have significant structural effects that disrupt substrate translocation, since the V_{max} value is 20–40-fold lower than WT–EGFP for both LoopΔ-3- and -5–EGFP.

Cellular localization of modified RFC proteins

The localization of the altered RFC–EGFP molecules was evaluated relative to the brefeldin A–BODIPY® reagent that accumulates in the ER and Golgi complex (Figure 4). With WT–EGFP (Figure 4A), it is apparent that the majority of the RFC protein is at the cell surface, and a very small amount is localized to the ER and Golgi complex: this is probably newly translated material in the early secretory pathway, prior to localization at the plasma membrane. The majority of the modified RFC proteins, with the exception of LoopΔ-4–EGFP (Figure 4G), have similar localization patterns as for WT–EGFP, with visible EGFP-tagged material at the plasma membrane. The localization of the NA–EGFP molecule is slightly punctate, and is less abundant at the cell surface (Figure 4B). This, as well as the slightly increased level of intracellular protein relative to WT–EGFP cells, suggests that the removal of the N-termini may be detrimental to processing of the molecule.

The removal of the C-termini does not prevent the trafficking of the RFC protein, since there is an obvious plasma membrane localization of EGFP signal (Figure 4C). However, there also

Figure 5 Western blot analysis of EGFP-tagged proteins

(A) Cells stably expressing the modified RFC-EGFP proteins were examined for protein expression. Of the cell lysate, 2.5 μ g aliquots were examined in each case, except for Loop Δ -4-EGFP, where 25 μ g of protein was used. Lane 1, WT-EGFP; lane 2, N Δ -EGFP; lane 3, C Δ -EGFP; lane 4, N Δ C Δ -EGFP; lane 5, Loop Δ -3-EGFP; lane 6, Loop Δ -4-EGFP; lane 7, Loop Δ -5-EGFP; lane 8, Loop Δ -5-EGFP selected under low folate conditions. (B) Protein extracts were treated with N-glycosidase F (lanes 2, 4, 6, 8, 10, 12 and 14) or not (lanes 1, 3, 5, 7, 9, 11 and 13), and analysed as described in the Experimental section. Lanes 1 and 2, WT-EGFP; lanes 3 and 4, N Δ -EGFP; lanes 5 and 6, C Δ -EGFP; lanes 7 and 8, N Δ C Δ -EGFP; lanes 9 and 10, Loop Δ -3-EGFP; lanes 11 and 12, Loop Δ -4-EGFP; lanes 13 and 14, Loop Δ -5-EGFP. The numbers to the left indicate the size of protein markers in kDa.

seems to be an accumulation of C Δ -EGFP protein within the Golgi body as compared with the WT-EGFP, suggesting that the later portion of the secretory pathway is affected by this modification. When both N- and C-termini are removed, the majority of the protein appears to be located intracellularly, with hardly any visible at the cell surface (Figure 4D), although it should be noted that the percentage of total molecules labelled by the membrane-impermeant biotinylation agent is greater than for WT-EGFP (Table 4).

Only the Loop Δ -3-, -4- and -5-EGFP-expressing cells were examined by confocal microscopy, since the instability of expression of Loop Δ -1- and -2-EGFP proteins interfered with the isolation of clonal lines. Both the Loop Δ -3- and Loop Δ -5-EGFP gene products are localized predominantly to the plasma membrane, with very little accumulation in the ER or Golgi body (Figures 4F and 4H). The distribution of the Loop Δ -4-EGFP protein (Figure 4G) is very similar to that of the soluble EGFP control (Figure 4E) indicating that, although this mutant is completely translated, it is dispersed throughout the cell, rather than localizing to the secretory pathway.

Protein and glycosylation analysis of the modified RFC proteins

When the gene products of the deletion constructs are visualized by SDS/PAGE (Figure 5), the migration patterns correspond

with the expected sizes, confirming that all proteins are properly translated and, with the exception of Loop Δ -4-EGFP, are fully glycosylated. The variations in signal for the EGFP-fusion proteins indicate that the individual deletions have variable effects on cellular RFC levels. Over the course of multiple Western analyses, the expression of the Loop Δ -3- and -5-EGFP gene products was approximately equivalent to that of WT-EGFP, whereas that of both the N Δ - and C Δ -EGFP gene products was slightly less. Most striking were the low levels of the N Δ C Δ -EGFP and Loop Δ -4-EGFP proteins, which were approx. 20 % and 10 % of the WT respectively (Table 4).

The high-molecular-mass 'smear' in the WT-EGFP sample (Figure 5A, lane 1) is protein that has obtained the complex glycosylation modification while in the Golgi, as demonstrated previously [20]. When the protein sample is deglycosylated with N-glycosidase F, there is a complete conversion of the high-molecular-mass smear to the lower band (Figure 5B, lanes 1 and 2). This lower band is core- and un-glycosylated material within the ER that has not yet matured through the secretory pathway [20]. Quantification of these two bands from several sample preparations indicated that approx. 90 % of the protein is fully glycosylated, whereas the remainder is core- and un-glycosylated.

With the N Δ -EGFP sample (Figure 5A, lane 2), the amount of incompletely glycosylated material (approx. 30 % of the total) is approx. 3-fold greater than in the WT-EGFP sample, although the slight shift after N-glycosidase F treatment (Figure 5B, lanes 3 and 4) indicates this protein is core-glycosylated. This abundance of core- and un-glycosylated material, also approx. 40 % of the total for the N Δ C Δ -EGFP sample, implies that the removal of the N-terminus affects exit from the ER. In contrast is the pattern observed for the C Δ -EGFP gene product (Figure 5A, lane 3), where the amount of core-glycosylated material (< 5 % of total) is approx. half of that seen in the WT-EGFP sample.

In contrast with the terminal-deletion gene products, the loop modifications either do not affect the glycosylation profile, as for both the Loop Δ -3- and -5-EGFP proteins (Figure 5A, lanes 5 and 7), or there is no visible glycosylation (Loop Δ -4-EGFP, Figure 5B, lanes 11 and 12). The absence of a shift in Loop Δ -4-EGFP protein migration after treatment with N-glycosidase F indicates it is not core-glycosylated, suggesting this molecule does not pass out of the ER. Furthermore, the low level of detectable Loop Δ -4-EGFP protein (Figure 5A, lane 6), in combination with the intracellular localization (see above), suggests that these molecules are degraded very soon after translation.

Stability of the modified RFC proteins

The stability of the EGFP-tagged gene products was evaluated over time by inhibiting protein synthesis with cycloheximide (Figure 6). The half-life ($t_{1/2}$) of WT-EGFP was approx. 27 h, and, although the removal of the N-termini did not affect the rate of protein turnover significantly, the modifications to the C-terminus and in the Loop Δ -3-EGFP construct resulted in a slight decrease (2-fold) in $t_{1/2}$. The removal of both termini (N Δ C Δ -EGFP), or the conserved patch in the cytoplasmic loop (Loop Δ -4-EGFP) significantly decreased stability, leading to a 5–10-fold increase in the rate of turnover. The $t_{1/2}$ for the Loop Δ -5-EGFP gene product was similar to that of WT-EGFP and, interestingly, this was equally stable in cells selected under low levels of folinic acid.

For the WT-EGFP protein, the disappearance of the lower band after approx. 2–4 h represents molecules in the ER that have moved into the Golgi prior to the complex glycosylation

Figure 6 Turnover analysis of EGFP-tagged RFC molecules

Clonal cell lines expressing the various modified 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 of each lane, and subjected to Western analysis as described in the Experimental section. The asterisks denote protein molecules with a shorter half-life, which were harvested at 0, 0.5, 1, 2, 4, 6 and 8 h. '†' denotes the cell line isolated under low folate conditions. The numbers to the right of the gels indicate the half-life ($t_{1/2}$) of each respective protein, in h.

process. However, the lower band in the NA-EGFP and NΔCA-EGFP samples persists, and has a $t_{1/2}$ similar to that of the fully glycosylated material, supporting further the hypothesis that removal of the N-terminus results in a slower release of protein from the ER to the secretory pathway. In contrast, the only unglycosylated material evident for the CA-EGFP sample is when the protein is overloaded ($t = 0$), indicating that this deletion might enhance protein exit from the ER.

DISCUSSION

Examination of the secondary structure of the RFC protein suggests it is consistent with 12 TM-spanning domains, where both termini and a large loop between TM6 and TM7 are located in the cytoplasm. Little is known about the role of these domains, although size and location make each a likely candidate for intermolecular interactions, or for defining the structural and/or functional characteristics of the RFC protein. The effects of their removal were evaluated using a series of functional assays, in combination with characterization of the gene products. It appears each of the three domains has important roles in conferring both protein stability and mobility via the secretory pathway, but are not absolutely essential for function.

Removal of the N-terminus is not significantly disruptive for the RFC, as indicated by the similarity to WT-EGFP for both the number of functional transfectants and the K_t and V_{max} parameters for Mtx transport (Table 3). However, a number of observations indicate that the N-terminus might be important in the early stages of protein biogenesis, namely: (i) a greater percentage of core- and un-glycosylated protein; (ii) a lower level of NA-EGFP protein at the cell surface; and (iii) the punctate plasma membrane distribution. This correlates with studies that suggest that the N-terminal domain of polytopic proteins such as lac permease [31] and the plasma membrane calcium pump [32] assist in efficient protein folding. Interference with the sequence causes proteins to be less efficiently inserted and/or processed, although function is not affected if the molecule is able to get to the plasma membrane.

On the basis of complementation efficiency and phenotype assays, the CA-EGFP molecule also appears to retain much of its transport capability, although the V_{max} for Mtx transport is somewhat lower than normal (Table 3), and rate of turnover is approx. 2-fold greater than for WT-EGFP. The absence of an essential functional role for this domain reflects studies on the lac permease protein, which requires a few amino acids distal to TM12 for protein stability [33], but is otherwise unaffected by the absence of the tail [34]. Furthermore, while the cystic fibrosis transconductance regulator protein molecules are significantly destabilized by deletions to their C-termini [35], these molecules are also fully functional. However, in spite of the fact that the RFC C-terminus does not have a critical role in transporter activity, this domain appears to be important for protein trafficking. This conclusion is supported by the following observations: (i) the near absence of core- and un-glycosylated molecules; (ii) the visible intracellular accumulation of protein; and (iii) the (4-fold)-increased level of total protein at the cell surface.

The C-terminal domains of polytopic membrane proteins have been shown to hold complex and competing signals that direct protein movement through the secretory pathway [36]. Interestingly, within the RFC C-terminus there appears to be both an RXR (Arg-Xaa-Arg) motif (at amino acid position 460) and a di-leucine sequence (at amino acid positions 498 and 499) conserved within the rat, hamster and mouse proteins. The RXR sequence serves as an ER-retention signal in numerous polytopic proteins [36–38], such that its removal increases protein expression at the plasma membrane. Moreover, a number of studies have described di-leucine-containing motifs that direct protein localization or internalization [39–41]. Interestingly, the RFC protein has been reported to have varying polar localizations in several mouse tissues [42,43], suggesting the cellular organization of the RFC may be dictated by tissue-specific proteins. Furthermore, the human RFC demonstrates a polar localization when expressed in *Xenopus* oocytes [23], which is differentially affected by a partial or complete removal of the C-terminal. Taken together, it seems plausible that unidentified intracellular components interact with various aspects of the C-termini to direct the RFC protein through the secretory pathway, as well as to a final destination.

Given that the removal of both N- and C-termini significantly destabilizes the protein, the complementation efficiency indicates that sufficient levels can localize to the cell surface and transport substrate. In fact, when the protein at the surface is normalized to total cellular levels, it is 2.5-fold higher than WT-EGFP (Table 4), perhaps as a result of the C-terminal deletion (see above). However, the high percentage of unglycosylated material and the significantly increased rate of turnover demonstrate this protein has difficulty in moving through the secretory pathway.

These characteristics indicate further that, even though neither terminus is essential for functionality of the RFC protein, they have an important role in processing and trafficking.

The effects of the central loop deletions on the RFC protein suggest that both the domain size and the sequence comprising it are important. The inability of the Loop Δ -1- and -2-EGFP variants to complement the RFC-deficient cells, in combination with the difficulty in generating clonal cell lines, suggests these molecules are highly unstable. This is probably due to a size constraint for this domain; a conclusion supported by the increased number of functional transfectants when a greater number of amino acids are maintained, as in Loop Δ -3-EGFP (22 amino acids) and Loop Δ -5-EGFP (33 amino acids) constructs (Table 3).

The enhanced complementation efficiency for the larger loop constructs after a modest increase in folate levels does not appear to be a result of enhanced protein stability. This is a phenomenon reported for both cytoplasmic and membrane proteins [44,45], where the addition of substrate steadies structurally unstable proteins and facilitates passage of these molecules through the maturation pathway. In contrast with those reports, the half-life of the Loop Δ -5-EGFP molecule in the present study appeared to be independent of the folate levels, as did the percentage of total mutant protein at the cell surface (Figures 3 and 6). The only discernible difference was a slightly increased level of total protein for those cells selected under low folate conditions (Figure 5). Thus it is likely that the two selective conditions are on either side of a functional threshold: only highly expressing cells can transport sufficient substrate to survive under low folate conditions, whereas a wider range of expression is adequate when folate levels are elevated. The effect of the loop modifications on Mtx transport is corroborated further by the considerably lower $V_{\max}$ values obtained for both Loop Δ -3-EGFP- and Loop Δ -5-EGFP-expressing cell lines. However, the affinity (K_m) for Mtx is not significantly affected, and the confocal images and glycosylation analysis suggest that the molecules are processed properly under conditions of higher levels of folinic acid. Taken together, these results imply that a shortening of the loop may lead to a conformational stress that affects substrate translocation.

The destabilizing effect of the cytoplasmic loop modifications correlates with studies on the lac permease [46]. This is also a 12 TM protein with a large intracellular loop (33 amino acids), of which one-third could be removed without significantly affecting function. Interestingly, function could still be maintained with a larger region removed, but only when a hydrophilic sequence was inserted or the protein was expressed as two halves. It was suggested that, during lac permease translation, the translocon can accommodate up to six TMs, which are then inserted into the membrane as a unit. The long loop appears to be necessary to accommodate the stretch between the N-terminal membrane-localized portion of the protein and the C-terminal half that is being synthesized. The results presented here suggest that RFC protein translation through the translocon may operate under similar principles, and suggest further this domain has a role in structural conformation during substrate translocation. These speculations provide an explanation for, and are supported by, the common appearance of a long intracellular loop in large polytopic proteins.

In addition to a size requirement, it appears there is a sequence and/or structural requirement for the large central loop. While the Loop Δ -3- and -4-EGFP variants are similar in size (22 and 23 amino acids respectively) and have a comparable number of charged residues, they differ in the presence or absence of the conserved patch of amino acids. This difference is significant,

since the Loop Δ -3-EGFP protein localizes to the membrane and is able to transport substrate, whereas the Loop Δ -4-EGFP gene product is non-functional and appears to be rapidly degraded soon after translation. Although hydropathy plots predict that these nine amino acids are in the cytoplasm, the instability of the Loop Δ -4-EGFP gene product would be expected if they formed part of TM6 [47]. Although three of the nine amino acids are highly charged, it is still possible they are found in the membrane as predicted, but with their presence minimized by charge-pair interactions. An alternative explanation for the differences between the Loop Δ -3- and -4-EGFP gene products is that the conserved patch, either due to sequence or structure, might provide a signal necessary for biogenesis; for example, prompting the release of the first six TMs from the translocon into the membrane. This motif may be something as simple as the unique structure of proline appearing early in the loop domain, as observed in many 12 TM protein families [48–50], or may be more complex. Although this study does not directly address the role of the conserved patch in the central intracellular loop, these results suggest that this region is important in the initial stages of membrane protein biogenesis.

Because there is little conservation in the RFC cytoplasmic domains at the amino acid level among species, it is not surprising that they do not seem to have a direct role in transporter function. However, it cannot be ruled out that these domains might be functionally more important under more complex cellular conditions. For example, a unique characteristic of the RFC protein is that there are reported differences in substrate specificity and optimum transport conditions among molecules located in the intestine relative to other tissues [51]. The cytoplasmic domains may have a role under these conditions, via interactions with other cellular components.

This work supports the growing body of evidence that suggests the large cytoplasmic structures of polytopic proteins have a necessary role in biogenesis, and have a lesser part in function.

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