Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1690 (2004) 110–117

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Functional analysis of altered reduced folate carrier sequence changes identified in osteosarcomas

Wayne F. Flintoff[a,*], Heather Sadlish[a,1], Richard Gorlick[b], Rui Yang[b], Frederick M.R. Williams[a]

[a] *Department of Microbiology and Immunology, University of Western Ontario, Dental Sciences Bldg., Dock 15, London, ON, Canada N6A 5C1*
[b] *Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA*

Received 5 April 2004; received in revised form 26 May 2004; accepted 28 May 2004
Available online 23 June 2004

Abstract

Osteosarcomas are common primary malignant bone tumors that do not respond to conventional low-dose treatments of methotrexate (Mtx), suggesting an intrinsic resistance to this drug. Previous work has shown that cDNAs generated from osteosarcoma mRNA from a fraction of patients contain sequence changes in the reduced folate carrier (RFC), the membrane protein transporter for Mtx. In this study, the functionality of the altered RFC proteins was assessed by fusing the green fluorescent protein (GFP) to the C-terminal, and examining the ability of the transfected constructs to complement a hamster cell line null for the carrier. Confocal microscopy and cell surface biotinylation indicated that all altered proteins were properly localized at the cell membrane. Only one of those examined, Leu291Pro, was unable to complement the null carrier line, but did bind Mtx at the cell surface. Thus, this alteration confers drug resistance since the carrier is unable to translocate the substrate across the cell membrane. Three alterations, Ser46Asn, Ser4Pro and Gly259Trp, while able to complement the carrier null line, conferred some degree of resistance to Mtx via a decreased rate of transport (V_{max}). Another set of alterations, Glu21Lys, Ala7Val, and the combined changes Thr222Ile, Met254Thr, complemented the carrier null line and did not confer resistance to Mtx. Thus, some, but not all of these identified alterations in the RFC may contribute to the lack of responsiveness of osteosarcomas to Mtx treatment.

Keywords: Osteosarcomas; Reduced folate carrier; Fusion protein; Methotrexate; Biotinylation

1. Introduction

Osteosarcomas are common primary malignant bone tumors occurring in children and adolescents, and are generally treated with high doses of the antifolate methotrexate (Mtx). [1–5]. The conventional low-dose treatments are ineffective, suggesting that such tumors may be intrinsically resistant to Mtx [6,7]. Previous studies [8–10] have shown that approximately half of the osteosarcoma samples examined at diagnosis have decreased expression of the mRNA coding for the reduced folate carrier (RFC), the membrane protein responsible for the transport of Mtx into the cell [11]. As well, sequence changes in the coding regions of *rfc* cDNAs generated from mRNAs in this clinical material have also been identified in a small percentage of patients [12]. Interestingly, the resulting amino acid changes in the RFC protein primarily occur in regions predicted to face the cytosol or the extracellular environment, but not in transmembrane (TM) segments. It is unclear whether these changes alter the function of the RFC protein and contribute to the observed clinical resistance to Mtx.

In this report, the function of these altered RFC proteins was examined by expressing each as a fusion protein with enhanced green fluorescent protein (EGFP) in a hamster cell line lacking RFC. One change confers a high level of resistance to the drug while some others, a low level suggesting that these alterations may be contributing factors to in vivo resistance.

Abbreviations: Biotin succinimide, sulfo-NHS-SS biotin; sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate; (E) GFP, (enhanced) green fluorescent protein; HRP, horseradish peroxidase; Mtx, methotrexate; PMSF, phenylmethylsulfonyl fluoride; RFC, reduced folate carrier; TM, transmembrane segment

* Corresponding author. Tel.: +1-519-661-3438; fax: +1-519-661-3499.
E-mail address: flintoff@uwo.ca (W.F. Flintoff).
[1] Present address: Division of Molecular Medicine, Oregon Health Sciences University, Portland, OR, USA.

0925-4439/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2004.05.008

2. Materials and methods

2.1. Patient material

The source of the osteosarcoma tumor material in which the sequence changes in *rfc* were identified was previously described [12].

2.2. Reagents

Restriction endonucleases, ECL chemiluminescent reagent, and streptavidin-biotinylated horseradish peroxidase (HRP) were from Amersham Pharmacia Biotech. Rabbit polyclonal and monoclonal anti-GFP antibodies, and brefeldin A BODIPY 558/568 conjugate were from Molecular Probes. Goat anti-rabbit IgG conjugated to HRP was from Jackson Laboratories. Sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (EZ-Link sulfo-NHS-SS-biotin) (biotin succinimide) was from Pierce Chemical Co. Polybrene was from Sigma-Aldrich Chemical Company and Geneticin (G418) from Gibco. [3′,5′,7′-^{3}H] Mtx (23 Ci/mmol) was purchased from Moravek Biochemicals Inc. and purified by thin-layer chromatography before use as described previously [13].

2.3. Constructs

The construction and characterization of human RFC tagged at the C-terminus with the EGFP has been previously described [14]. This modification has minimal effects on the function and cellular localization of the protein [14,15]. An *Eco*R1–*Xho*1 fragment containing the 3' end of the *rfc* cDNA fused to EGFP was subcloned into the pCDNA$_3$ expression vector to allow the insertion of the altered osteosarcoma *rfc* cDNAs.

Full-length human RFC cDNAs were obtained by reverse transcription-polymerase chain reaction (RT-PCR) using RFC-specific primers on RNA isolated from osteosarcoma samples. The full-length cDNAs with the indicated sequence changes [12] were cloned into the BamH1 site of pCDNA$_3$. The BamH1–*Eco*R1 fragments from each of these cDNAs were then subcloned into the BamH1–*Eco*R1 site of the 3′ *rfc*-EGFP pCDNA$_3$ vector (see above), sequenced to confirm the presence of only the indicated alterations and used for transfection studies.

2.4. DNA transfections

Transfection of the altered *rfc* plasmid clones into the recipient MtxRII 5-3 cells was performed using 10 μg of purified DNA in polybrene/1.5×10^5 cells as previously described [16]. This cell line is resistant to Mtx because it does not transport the drug and has no detectable *rfc* message. After transfection with DNA, the cells were selected for growth in low levels of folinic acid (2 nM) or in 1.2 mg/ml G418 for the line expressing Leu291Pro (alteration J). Single colonies were isolated and cloned by limiting dilution from individual transfection experiments. Two independent clones for each alteration were analyzed.

2.5. Phenotype testing

The transfectants were tested for Mtx resistance and folinic acid growth requirement as previously described [17]. Briefly, concentration response curves were generated with various concentrations of the compounds with varying numbers of cells and colony formation scored at 8 days of growth. In both cases, the media lacked nucleosides. For Mtx resistance, the D_{10} value is the drug concentration that reduced survival to 10% while the D_{50} for folinic acid growth is the nutrient concentration permitting 50% growth as compared to wild-type. For each of these values, two analyses were carried out for each cell line with less than 15% difference between each sample.

2.6. Kinetic analysis

The kinetic analyses for the determination of V_{max} and K_t for were carried out as previously described [13,17,18] with at least two determinations for each cell line.

2.7. Western analysis

Total cellular extracts were prepared from various numbers of cells by lysis on ice in lysis buffer (5 × concentrated protease-inhibitor-cocktail tablets [Roche], 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], and 2 μM orthovanadate). After centrifugation at $12,000 \times g$ for 2 min at 4 °C, the soluble lysates were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes. Detection of the fusion proteins was as previously described [18]. Images were scanned, and bands quantitiated using the BioRad Multi-Analyst program. Only images in the linear range of the film were used for quantitation.

2.8. Biotin labeling and detection

For cell surface biotinylation, equal numbers of cells (~7×10^5) were washed twice with PBSCM (phosphate-buffered saline buffer containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$), overlayed with 1 ml of PBSCM and biotinylated with 1.5 mg/ml sulfo-NHS-SS biotin for 1 h at room temperature. Cells were washed twice in 100 mM glycine/PBSCM and once in ice-cold PBS. The cells were lysed as described above and equal amounts of total cellular protein were treated with mouse monoclonal anti-GFP antibody. The lysates were rotated at 4° for 1 h. Protein G-Sepharose was added and the incubation continued for 2 h. The resin–lysate mixture was centrifuged for 2 min at $12,000 \times g$, and washed three times with a 10-fold excess of wash buffer

(0.5 × protease inhibitor cocktail tablets, 0.1% NP-40, 50 mM Tris–HCl, pH 7.4, 50 mM NaCl). Samples were eluted from the resin by incubation for 20 min at 56 °C with SDS-PAGE buffer. The solubilized immunoprecipitates were separated by SDS-PAGE (8% acrylamide) and electroblotted onto nitrocellulose membranes.

The blots were blocked in 5% bovine serum albumin in TBST (20 mM Tris–HCl, pH 7.6, 0.137 M NaCl, 0.2% Tween 20) and biotinylated proteins detected with 1:2000 dilution of streptavidin-biotinylated HRP in TBST buffer containing 0.5% BSA. After 1.5 h incubation, blots were washed with TBST and visualized using ECL reagent and Amersham Hyperfilm ECL film. Blots were stripped and reprobed with polyclonal rabbit anti-GFP antibody followed by goat anti-rabbit IgG conjugated to HRP. Images were scanned and transfected protein levels were quantitated using EGFP signal intensities and expressed relative to wild-type (Table 2, column 2). The amount of surface protein was normalized to an equivalent amount of wild-type EGFP fusion protein (Table 2, column 3, ratio of biotinylated protein to biotinylated wild-type). The relative amount of surface protein expression was then corrected for cellular expression levels by multiplying the relative fusion protein levels by the relative surface labeled protein (Table 2, column 4).

2.9. Confocal microscopy

For confocal microscope analysis, cells were grown on glass cover slips, treated with brefeldin A-BODIPY, and fixed with 4% paraformaldehyde. Fluorescence was detected using a LSM410 inverted Zeiss laser scanning microscope with LSM410 software using a krypton/argon laser as previously described [15]. Due to variations in the intensities of the signals, different integration values were used for each cell line to give the clearest representation of the fusion protein distribution.

3. Results

Previous studies have indicated that approximately 14% of samples from patients with osteosarcoma contained nucleotide sequence changes in RFC [12]. The RFC proteins expressed from these mRNAs could potentially have altered function that might provide an explanation for the intrinsic resistance of these tumors to Mtx treatment. To evaluate the consequences of these changes (Fig. 1), RFC-deficient hamster cells were transfected with constructs containing these human altered RFCs fused to EGFP. This is a useful system for assessing RFC characteristics since

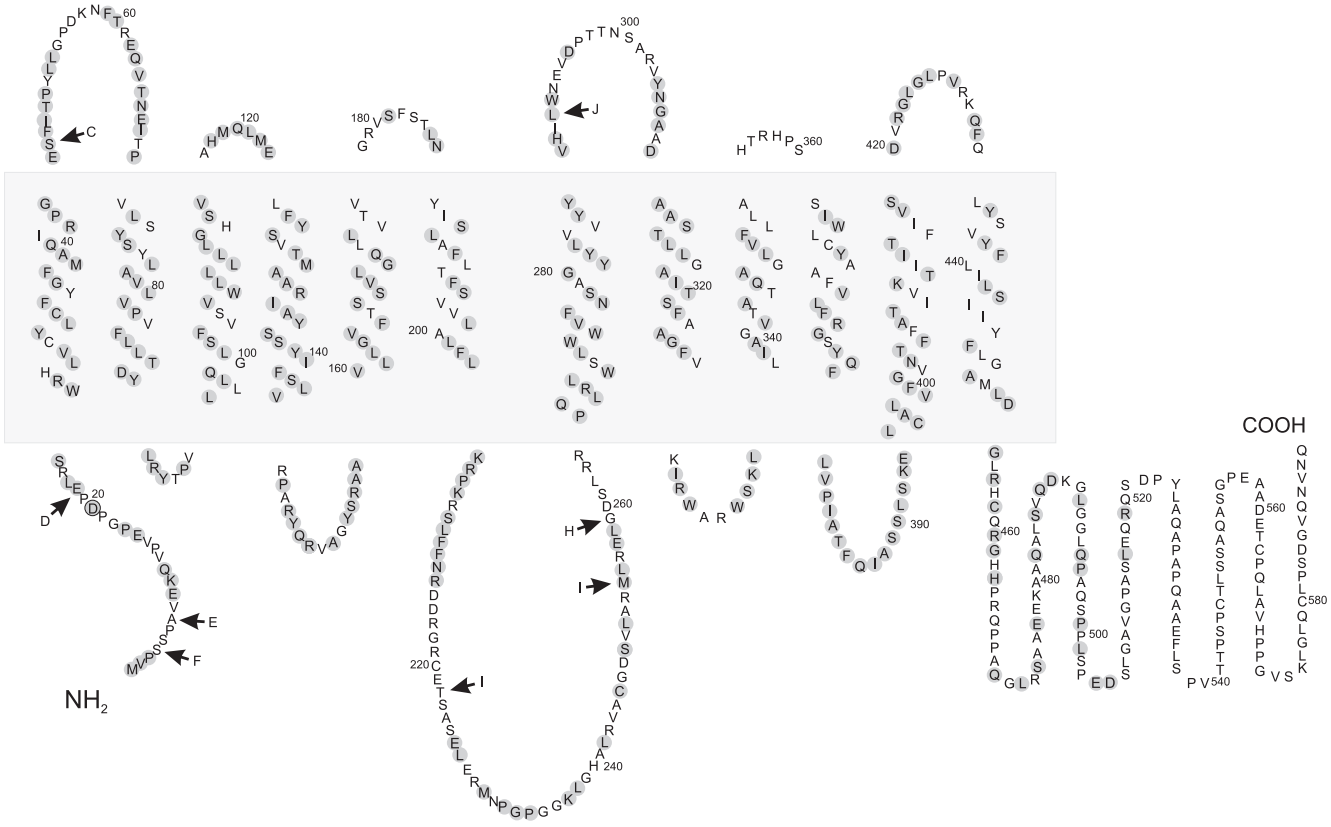

Fig. 1. Topology of RFC. The topology is on the basis of hydropathy plots, epitope insertion analysis [12,28], N-glycosylation [28], and cysteine scanning mutagenesis [18]. Closed circled residues indicate those conserved among hamster, human, rat and mouse species. Arrows indicate the location of the alterations.

Table 1
Functional characteristics of cell lines expressing EGFP altered fusion proteins

Cell line[a]	Location	Colonies/ μg/10^6 cells[b]	Folinic acid D_{50} (nM)	Mtx D_{10} (nM)	Mtx K_t (μM)[c]	Mtx V_{max} (pmol/min/mg)[c]	Normalized V_{max} (pmol/min/mg)[d]	V_{max}/K_t
Mtx RII 5-3	–	–	200	250	–	–	–	–
Wild-type	–	2077	2	10	0.95 ± 0.3	2.0 ± 0.2	2.0	2.1
C (S46N)	Between TM1–TM2	1823	1.5	30	0.95 ± 0.2	0.4 ± 0.08	0.05	0.05
D (E21K)	N-terminal	1018	2	8	0.7 ± 0.2	1.2 ± 0.16	1.8	2.5
E (A7V)	N-terminal	1447	2	3	0.58 ± 0.2	2.0 ± 0.3	0.5	0.9
F (S4P)	N-terminal	1608	1.8	40	0.6 ± 0.1	0.18 ± 0.05	0.25	0.4
H (G259W)	Between TM6–TM7	1960	1.5	30	0.92 ± 0.12	3.25 ± 1	0.9	1.0
I (T222I; M254T)	Between TM6–TM7	1420	1.5	12	0.7 ± 0.1	1.7 ± 0.6	1.4	2.0
J (L291P)	Between TM7–TM8	<7	100[e]	180[e]	–	–	–	–

[a] Mtx RII 5-3 does not contain any RFC-EGFP constructs.
[b] Frequency of colony formation of constructs transfected into Mtx RII 5-3 recipient cell line.
[c] Values are averages of at least two independent analyses ± standard error of the mean.
[d] V_{max} was normalized for surface protein and corrected for cellular expression levels (Fig. 2, Table 2).
[e] Clones selected in G418.

cells expressing a functional RFC can grow in nanomolar concentrations of folinic acid [14]. As shown in Table 1, all but one of these fusion proteins was able to form colonies in 2 nM folinic acid. Colony formation varied between 50% and 95% of wild-type, indicative of a functional RFC protein. Cells expressing alteration J (Leu291Pro) were unable to grow under these selective conditions, and cell lines expressing this fusion protein were obtained using G418 drug selection.

To further characterize these altered RFCs, the growth requirements for folinic acid and sensitivity to Mtx were assessed for cells expressing the proteins. As shown in Table 1, nearly all the cell lines had growth requirements for folinic acid that were similar to the wild-type. In contrast, cells expressing the J alteration (Leu291Pro) required a similar level to that for the RFC-minus line (Mtx RII 5-3) to sustain growth. The cellular sensitivity to Mtx enabled a separation of the alterations into three general classes: (1) little change in the Mtx sensitivity of the RFC-minus cell line (alteration J); (2) similar or 3-fold lower sensitivities compared to wild-type (alterations D, E, and I); and (3) sequence changes that led to 3- to 4-fold increased resistance compared to wild-type (alterations C, F, and H).

The transport properties for Mtx were further examined by kinetic analysis. Cell lines expressing either alterations C or H had similar affinities for Mtx as the wild-type expressing line, while those expressing changes D, E, F, and I had slightly lower K_t values (Table 1). Sequence alterations E and F were the most divergent, with ~1.6-fold higher affinity for Mtx. Before comparing the V_{max} values, variations in protein expression levels or the proportion of RFC at the cell surface were accommodated for using a previously described method [18]. Cells stably expressing each altered protein were labeled with a membrane-impermeant amide-reactive biotinylation reagent, and the fusion proteins were immunoprecipitated from equal amounts of total cellular protein using an excess of monoclonal antibody. Subsequent Western analysis allowed a relative quantification of the amounts of both biotinylated (cell surface) and fusion protein (Fig. 2, Table 2). After correcting for differences in total fusion protein levels, there was a 0.6- to 8.5-fold range in the relative surface protein expressed in the mutant lines as compared to the wild-type expressing line. When these expression variations were taken into account to normalize the V_{max} values for transport, the alterations could be placed into one of two groups. One group, containing alterations D and I, were able to transport Mtx with a similar rate as wild-type. The second group containing changes C, E, F and H expressing cell lines were less effective in transporting the drug. Alteration C was least efficient with a

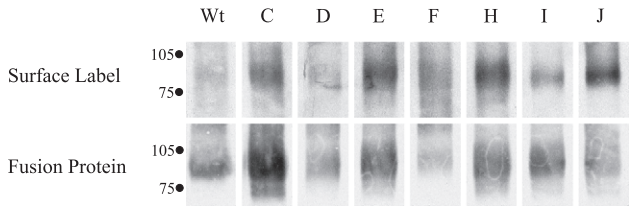

Fig. 2. Relative levels of EGFP fusion protein at the cell surface. Equal numbers of cells expressing the various EGFP-fusion proteins were labeled with biotin succinimide. EGFP-fusion proteins were immunoprecipitated from equal amounts of total cellular protein, separated by SDS-PAGE and examined by Western analysis as described in "Materials and methods". Upper panel: the blot was probed using streptavidin-biotinylated HRP complex. Lower panel: the blot was stripped and probed for EGFP fusion proteins as described in "Materials and methods". The numbers on the left indicate the size of protein markers in kilodaltons. The fusion protein migrates as a broad band between 90 and 105 kDa since RFC is N-glycosylated at N58 [29]. WT, wild-type; C (Ser46Asn); D (Glu21Lys); E (Ala7Val); F (Ser4Pro); H (Gly259Trp); I (Thr222Ile, Met254Thr), J (Leu291Pro).

Table 2
Quantitation of EGFP fusion proteins at the cell surface

Cell line	EGFP fusion protein level[a]	Surface protein normalized to protein wild-type[b]	Relative surface corrected for expression level[c]
Wild-type	1.0	1.0	1.0
C (S46N)	3.2	2.7	8.5
D (E21K)	0.6	1.0	0.6
E (A7V)	1.2	3.3	4.1
F (S4P)	0.4	1.8	0.7
H (G259W)	1.1	3.3	3.6
I (T22I; M254T)	1.0	1.3	1.3
J (L291P)	0.7	3.2	2.2

[a] Numbers were generated from image 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 wild-type.

[b] Values indicate the normalized amount of surface protein when cellular EGFP fusion expression levels are equivalent to wild type (ratio of biotinylated protein to biotinylated wild type/column 1).

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

rate 40-fold less than that of the wild-type, whereas E, F and H changes had rates that were 4-, 8- and 2-fold less, respectively. This is also reflected in the overall efficiency of transport (V_{max}/K_t, Table 1) where the C alteration is 40-fold less efficient than wild-type, whereas the others vary between 0.2 and 1.25 of wild-type.

Cells expressing alteration J were unable to transport the drug; however, the biotinylation studies indicated that RFC-fusion protein was being expressed on the cell surface (Fig. 2, Table 2). Binding studies indicated that such cells bound Mtx with a K_d of 0.9 μM, similar to that of the wild-type cells (0.8 μM), and with a normalized B_{max} of 0.6 pmol/mg, which was also similar to the wild-type value of 0.5 pmol/mg. Thus, it appears that the defect conferred by this alteration leads to an inability of the RFC protein to translocate the drug across the cell membrane.

The biotinylation and transport studies indicate that in all cases, the fusion protein was present at the cell surface. Confocal microscopy was utilized to evaluate the distribution of the various fusion proteins throughout the entire cell (Fig. 3). For all of the sequence changes, a vast majority of the fusion protein was localized to the cell membrane with a minor amount co-localizing with an endoplasmic reticulum–Golgi-specific stain (brefeldin A BODIPY). In general, the fusion protein was discretely localized to the plasma membrane similar to wild-type, but the fluorescence appeared somewhat more diffuse in cells expressing alteration D and especially alteration J. There also appeared to be a slightly higher level of intracellular material for C and F expressing cell lines. It should be noted that the EGFP signal intensities do not reflect the relative expression levels among the cell lines (see Materials and methods).

4. Discussion

Osteosarcoma tumors typically have a poor response to conventional dose protocols with Mtx. Various sequence changes within the RFC gene have been identified in osteosarcoma samples, raising the possibility that alterations

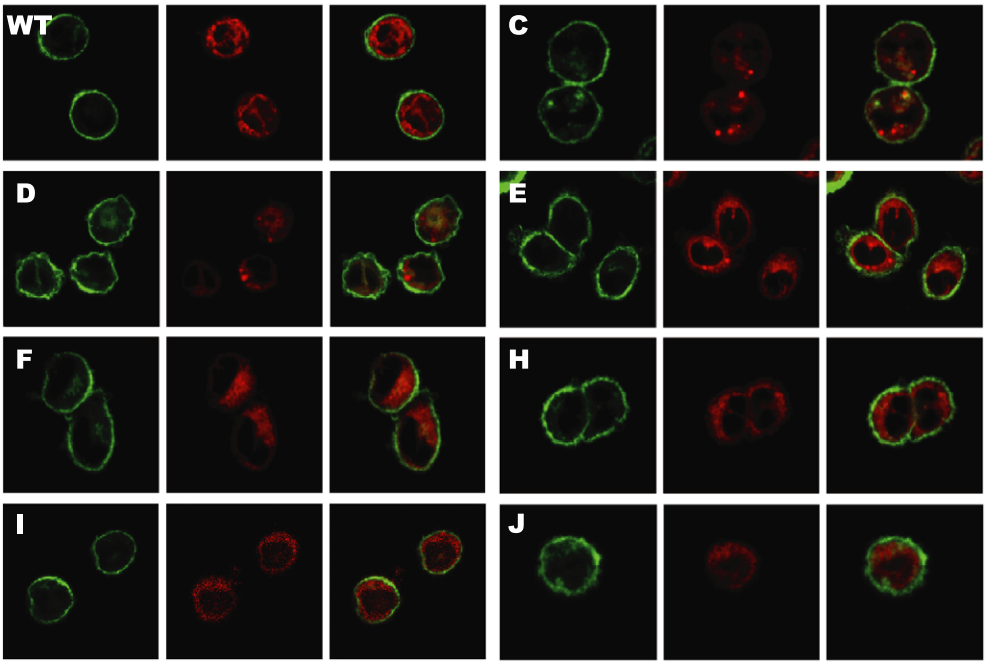

Fig. 3. Confocal microscopy of transfected cell lines. The left image in each panel is EGFP fluorescence, and the center image is specific staining of the endoplasmic and Golgi complex with brefeldin A BODIPY as described in ‘‘Materials and methods’’. The right panel is an overlay of the two preceding images. WT, wild-type; C (Ser46Asn); D (Glu21Lys); E (Ala7Val); F (Ser4Pro); H (Gly259Trp); I (Thr222Ile, Met254Thr), J (Leu291Pro).

to the carrier may account for the inherent resistance to the Mtx treatments. It is of interest to note that sequence changes in RFC in childhood acute lymphoblastic leukemia do not play a major role in resistance to Mtx [19]. In this report, we have examined the consequences of several clinically identified sequence changes on protein function, by expressing the altered human RFCs as EGFP fusion proteins in an RFC-deficient hamster cell line. The clinically identified sequence changes in the RFC gene chosen for analysis have previously been reported by Yang et al. [12] and were identified from a panel of osteosarcoma patients.

Expression of the altered human RFC-EGFP fusion proteins in the RFC-deficient hamster cell line indicated that none of the alterations affected the ability of the protein to localize to the cell membrane. The pattern of fluorescence of the mutant fusion proteins as observed with confocal microscopy is similar to that of the wild-type fusion protein, and implies that the altered proteins are trafficking properly and efficiently. Furthermore, most of the altered proteins appeared functional, as assessed by the ability of the transfected protein to rescue the RFC-deficient cell line under low folate conditions. Phenotypic studies have indicated that some of the alterations can confer resistance to Mtx, albeit at low levels, while others have wild-type or increased sensitivities to the drug.

The alteration Leu291Pro (J) was the only one unable to complement the RFC deficiency of the recipient cell line. Furthermore, cells expressing this RFC alteration retained resistance to Mtx and increased growth requirements for folinic acid. This is not a result of an inability of the protein to traffic to the cell surface, as demonstrated by both confocal microscopy and binding assays. As this alteration is predicted to be located in the extracellular loop between TM7 and 8, it could play a role in substrate binding. However, the drug affinity of this mutant is the same as for wild-type protein indicating little involvement in a binding domain. Thus, it appears likely that the change to proline at this site alters the conformation of the RFC protein such that it interferes with translocation of the substrate across the cell membrane.

Alteration of serine at position 46 to asparagine (C) results in a 3-fold resistance to Mtx as compared to wild-type. This phenotype is likely due to a dramatic decease in the efficiency to translocate the substrate, as the V_{max} of Mtx transport is about 40-fold lower than for the wild-type fusion protein. Interestingly, the same amino acid substitution (Ser46Asn) in mouse RFC has been identified in the L1210 cell line, and leads to a 10-fold increase in resistance to Mtx and a $\sim$40-fold reduction in V_{max} compared to the wild-type line [20]. These similar findings for RFC proteins from different species indicate that this residue plays a key role in the transport of the substrate. Furthermore, this site is in a region predicted to be part of the substrate binding domain [21,22]; numerous mutations in the loop between TM1 and TM2 of RFC from several systems have been shown to affect the binding affinity for substrates [23–25]. While residue 46 does not appear to be directly involved in the binding site, as the affinity for transport is unaffected by the change (Table 1), it is nonetheless critical for efficient substrate translocation. Although this alteration affects the efficiency of transport, the folinic acid growth requirement and the sensitivity to Mtx are not that different from those alterations with 40-fold higher efficiency. This may reflect the nature of the 8–10 day assays employed to measure growth which allows sufficient accumulation of the either the nutrient or drug to permit or limit growth, respectively.

Two other alterations (F and H) had an increased resistance (3–4-fold) to Mtx as compared to the wild-type expressing cells. Although alteration F (Ser4Pro) demonstrates a slightly increased affinity for the drug, this effect is likely offset by the significantly decreased V_{max} of transport, which is 8-fold lower than wild-type. Furthermore, this change may affect the efficiency of trafficking to the cell surface, as indicated by the surface protein biotinylation data and confocal images. In contrast, it is unclear how the change of glycine at 259 to tryptophan (H) leads to an increased resistance to Mtx. The distribution of fusion protein throughout the cell closely resembles the wild-type protein, and while the V_{max} of transport is 2-fold less than wild-type, the affinity for Mtx is unaffected.

Three alterations, D (Glu21Lys), E (Ala7Val), and I (Thr222Ile; Met254Thr) do not appear to have a role in conferring an intrinsic Mtx resistance to osteosarcomas. While change E demonstrates a 2-fold decrease in substrate translocation efficiency as compared to wild-type, there is also an increase in affinity, resulting in a 3-fold increase in sensitivity to Mtx. The phenotypic parameters for alterations D and I closely resemble those of the wild-type expressing cells, with a slight decrease in V_{max} offset by a slightly higher affinity for the substrate. The increased affinity for these altered proteins are likely secondary consequences of conformational changes rather than an involvement in the initial substrate binding, as they are located in regions that face the cytosol.

Thus, in most cases, these changes confer a low level of resistance to Mtx but not at the levels that are observed in vivo where doses of the drug often reach the millimolar range. Thus, the intrinsic resistance in vivo may be a combination of several mechanisms and these sequence level changes could be contributing factors. The potential of several mechanisms of resistance including both quantitative and qualitative changes in RFC may contribute to the rationale for individualized chemotherapeutic strategies [26].

It remains possible, however, that the full effect of these alterations on RFC function has not been observed, as this functional analysis was carried out in a heterologous system where the human RFC was expressed in a hamster background. Although a wild-type human RFC can complement the RFC deficiency in the hamster cell [27], previous work has indicated that other factors may be required for the full spectrum of transport activities associated with the human

RFC [28]. Also, there is evidence suggesting that cell type may influence the properties of RFC; mouse RFC expressed in rat intestinal IEC-cells yields two kinetically different transport activities differing in pH optima, whereas expression in murine leukemia cells yields a single transport activity [29]. Thus, it may be informative to express these fusion proteins in an osteosarcoma cell line to ascertain whether there is an increased resistance to Mtx.

It is important to point out that the sequence changes identified in the initial material were heterozygous, being present with the wild-type allele [12]. Previous work has indicated that alterations conferring resistance to Mtx as a result of defective folate transport are genetically recessive [30] and thus for the resistant phenotype to be detected, the wild-type allele must be inactivated. Lack of RNA from these tumor samples precluded an investigation of the wild-type and sequence-altered RFC in these tumor samples. Silencing of RFC expression by promoter methylation has been reported in cell lines [31]. It is possible that the wild-type allele is not expressed in these tumor cells and, if this is the case, then some of these altered forms of RFC could contribute to low levels of intrinsic resistance to Mtx. Thus, studies similar to those described here on additional samples with altered RFCs in conjunction with analyses of RNA expression will need to be performed to assess the role sequence changes and allele inactivation have in relation to intrinsic drug resistance in osteosarcomas.

Acknowledgements

This work was supported by an operating grant (to WFF) and a studentship (to HS) from the Canadian Institute of Health Research.

References

[1] P.A. Meyers, R. Gorlick, Osteosarcomas, Pediatr. Clin. N. Am. 44 (1997) 973–990.
[2] P.A. Meyers, G. Heller, J. Healey, A. Huvos, J. Lane, R. Marcove, A. Applewhite, V. Vlamis, G. Rosen, Chemotherapy for nonmetastatic osteogenic sarcoma: the Memorial Sloan-Kettering experience, J. Clin. Oncol. 10 (1992) 5–15.
[3] P.A. Meyers, R. Gorlick, G. Heller, E. Casper, J. Lane, A.G. Huvos, J.H. Healy, Intensification of preoperative chemotherapy for osteogenic sarcoma: the results of the Memorial Sloan-Kettering (T12) protocol, J. Clin. Oncol. 16 (1998) 2452–2458.
[4] N. Jaffe, E. Frei, H. Watts, D. Traggis, High-dose methotrexate in osteogenic sarcoma: a 5-year experience, Cancer Treat. Rep. 62 (1978) 259–264.
[5] G. Seater, T.A. Alvegard, I. Elomaa, A.E. Stenwig, T. Holmstrom, O.P. Solheim, Treatment of osteosarcoma of the extremities with the T-10 protocol, with emphasis on the effects of preoperative chemotherapy with single-agent high-dose methotrexate: a Scandinavian Sarcoma group study, J. Clin. Oncol. 9 (1991) 1766–1775.
[6] G. Bacci, S. Ferrari, N. Delepine, F. Bertoni, P. Picci, M. Mercuri, P. Bacchini, A. Brach del Prever, A. Tienghi, A. Comandone, M. Campanacci, Predictive factors of histologic response to primary chemotherapy in osteosarcoma of the extremity: study of 272 patients preoperatively treated with high-dose methotrexate, doxorubicin, and cisplatin, J. Clin. Oncol. 16 (1998) 658–663.
[7] N. Delepine, G. Delepine, H. Cornille, F. Brion, P. Arnaud, J.C. Desbois, Dose escalation with pharmacokinetics monitoring in methotrexate chemotherapy of osteosarcoma, Anticancer Res. 15 (1995) 489–494.
[8] W. Guo, J.H. Healey, P.A. Meyers, M. Ladanyi, A.G. Huvos, J.R. Bertino, R. Gorlick, Mechanisms of methorexate resistance in osteosarcoma, Clin. Cancer Res. 5 (1999) 621–627.
[9] I. Ifergan, I. Meller, I. Issakov, Y.G. Assaraf, Reduced folate carrier protein expression in osteosarcoma, Cancer 98 (2003) 1958–1966.
[10] M. Serra, G. Reverter-Branchat, D. Maurici, S. Benini, J.-N. Shen, T. Chano, C.-M. Hattinger, M.-C. Manara, M. Pasello, K. Scotlandi, P. Picci, Analysis of dihydrofolate reductase and reduced folate carrier gene status in relation to methotrexate resistance in osteosarcoma cells, Ann. Oncol. 15 (2004) 151–160.
[11] F.M. Sirotnak, B. Tolner, Carrier-mediated membrane transport of folates in mammalian cells, Annu. Rev. Nutr. 19 (1999) 91–122.
[12] R. Yang, R. Sowers, B. Mazza, J.H. Healey, A. Huvos, H. Grier, M. Berstein, G.P. Beardsley, M.D. Krailo, M. Devidas, J.R. Bertino, P.A. Meyers, R. Gorlick, Sequence alterations in the reduced folate carrier are observed in osteosarcoma tumor samples, Clin. Cancer Res. 9 (2003) 837–844.
[13] W.F. Flintoff, C.R. Nagainis, Transport of methotrexate in Chinese hamster ovary cells: a mutant defective in methotrexate uptake and binding, Arch. Biochem. Biophys. 233 (1983) 433–440.
[14] P.L. Ferguson, W.F. Flintoff, Topological and functional analysis of the human reduced folate carrier by hemagglutinin epitope insertion, J. Biol. Chem. 274 (1999) 16269–16278.
[15] H. Sadlish, R.C. Murray, F.M.R. Williams, W.F. Flintoff, Mutations in the reduced folate carrier affect protein localization and stability, Biochem. J. 346 (2000) 509–516.
[16] F.M.R. Williams, R.C. Murray, T.M. Underhill, W.F. Flintoff, Isolation of a hamster cDNA clone coding for a function involved in methotrexate uptake, J. Biol. Chem. 269 (1994) 5810–5816.
[17] T.M. Underhill, W.F. Flintoff, Mutant Chinese hamster ovary cells with defective methotrexate uptake are distinguishable by reversion analysis, Somat. Cell Mol. Genet. 15 (1989) 49–59.
[18] H. Sadlish, F.M.R. Williams, W.F. Flintoff, Functional role of Arginine 373 in substrate translocation by the reduced folate carrier, J. Biol. Chem. 277 (2002) 42105–42112.
[19] Y. Kaufman, S. Drori, P.D. Cole, B.A. Kamen, J. Sirota, I. Ifergan, M.W. Ben Arush, D. Sahar, G.J.L. Kaspers, G. Jansen, L.H. Matherly, G. Rechave, A. Toren, Y.G. Assaraf, Reduced folate carrier mutations are not the mechanism underlying methotrexate resistance in childhood acute lymphoblastic leukemia, Cancer 100 (2004) 773–782.
[20] R. Zhao, Y.G. Assaraf, I.D. Goldman, A reduced folate carrier mutation produces substrate-dependent alterations in carrier mobility in murine leukemia cells and methotrexate resistance with conservation of growth in 5-formyltetrahydrofolate, J. Biol. Chem. 273 (1998) 7873–7879.
[21] W.F. Flintoff, F.M.R. Williams, H. Sadlish, The region between transmembrane domains 1 and 2 of the reduced folate carrier forms part of the substrate binding pocket, J. Biol. Chem. 278 (2003) 40861–40876.
[22] W. Cao, L.H. Matherly, Characterization of a cysteine-less human reduced folate carrier: localization of a substrate binding domain by cysteine-scanning mutagenesis and cysteine accessibility methods, Biochem. J. 374 (2003) 27–36.
[23] R. Zhao, F. Gao, P.J. Wang, I.D. Goldman, Role of the amino acid 45 residue in reduced folate carrier function and ion-transport as characterized by site-directed mutagenesis, Mol. Pharmacol. 57 (2000) 317–323.
[24] S. Drori, G. Jansen, R. Mauritz, G.J. Peters, Y.G. Assaraf, Clustering of mutations in the first transmembrane domain of the human reduced folate carrier in GW1843U89-resistant leukemia cells with impaired

antifolate transport and augmented folate uptake, J. Biol. Chem. 275 (2000) 30855–30863.
[25] A.J. Gifford, M. Haber, T.L. Witt, J.R. Whetsine, J.W. Taub, L.H. Matherly, M.D. Norris, Role of the E45K-reduced folate carrier gene mutation in methotrexate resistance in human leukemia cells, Leukemia 16 (2002) 2379–2387.
[26] G. Rosen, B. Caparros, A.G. Huvos, C. Kosloff, A. Nirenberg, A. Cacavio, R.C. Marcove, J.M. Lane, B. Mehta, C. Urban, Preoperative chemotherapy for osteogenic sarcoma: selection of postoperative adjuvant chemotherapy based on the response of the primary tumor to preoperative chemotherapy, Cancer 49 (1982) 1221–1230.
[27] F.M.R. Williams, W.F. Flintoff, Isolation of a human cDNA that complements a mutant hamster cell defective in methotrexate uptake, J. Biol. Chem. 270 (1995) 2987–2992.
[28] S.C. Wong, S.A. Proefke, A. Bhushan, L.H. Matherly, Isolation of human cDNAs that restore methotrexate sensitivity and reduced folate carrier activity in methotrexate transport-defective Chinese hamster ovary cells, J. Biol. Chem. 270 (1995) 17468–17475.
[29] A. Rajgopal, E.E. Sierra, R. Zhao, I.D. Goldman, Expression of the reduced folate carrier SLC19A1 in IEC-6 cells results in two distinct transport activities, Am. J. Physiol., Cell Physiol. 281 (2001) C1579–C1586.
[30] W.F. Flintoff, S.M. Spindler, L. Siminovitch, Genetic characteristics of methotrexate resistant Chinese hamster ovary cells, In Vitro 12 (1976) 749–757.
[31] J. Worm, A.F. Kirkin, K.N. Dzhandhugazyan, P. Guldberg, Methylation-dependent silencing of the reduced folate carrier gene in inherently methotrexate-resistant human breast cancer cells, J. Biol. Chem. 276 (2001) 39990–40000.