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SHORT COMMUNICATION

Gene Amplification and Increased Expression of the Reduced Folate Carrier in Transport Elevated K562 Cells

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ABSTRACT. The molecular bases for the 6-fold elevated methotrexate transport capacity of K562.4CF cells (Matherly *et al.*, *Cancer Res.* **51:** 3420–3426, 1991) were studied with reduced folate carrier (RFC) cDNA, genomic, and antibody probes. Southern analysis showed that *RFC* gene copies were increased (≈4- to 5-fold) in K562.4CF over wild-type K562 cells. Fluorescence *in situ* hybridization using a genomic *RFC* probe confirmed the localization of the RFC gene to the q-arm of chromosome 21. In K562.4CF cells, the frequent loss of a normal copy of chromosome 21 (61% of metaphases) was accompanied by *RFC* gene amplification and translocations of amplified *RFC* gene fragments to several (2 to 6) different chromosomal loci not seen in wild-type cells. Particularly intense *RFC* signals were mapped to homogeneously staining regions in chromosomes 2 and 15. Increased *RFC* gene copies were accompanied by a similar increase in the major 3.1 kb RFC transcript by northern blotting and an ≈7-fold elevated level of the broadly migrating (80–95 kDa) RFC protein on a western blot probed with an RFC C-terminal peptide antibody. These results demonstrate that selection of cells with a growth-limiting concentration of reduced folates (0.4 nM of leucovorin) is sufficient to promote chromosomal aberrations, including gene amplification and translocations that result in increased RFC expression and folate transport. BIOCHEM PHARMACOL **55**;7:1135–1138, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. methotrexate; transport; gene amplification; folates; homogeneously staining region

In mammalian cells, active transport of reduced folates, essential cofactors for *de novo* purine and pyrimidine biosynthesis, mainly involves the RFC.¶ Kinetic and physical properties of this transport system, including its role in transporting 4-amino antifolates such as Mtx, have been described thoroughly [1–3]. Membrane transport of Mtx and related antifolates by RFC is generally considered as limiting to antitumor activity [1]. Further, in both clinical and experimental tumor cells, resistance is frequently associated with impaired transport activity [4–6].

The ability to elevate RFC activity by culturing cells in low physiologic or sub-physiologic concentrations of reduced folates, described by this laboratory as well as others [7–9], strongly implies that membrane transport is responsive to changes in endogenous cofactors and/or folate-dependent biosynthetic products. Although evidence for an acute regulation of Mtx transport in response to folates and purines has been described previously in some of these lines [7, 8], the molecular alterations that characterize the transport-elevated phenotype have not been well described.

In this communication, we demonstrate that selection of K562 cells that grow in limiting reduced folates is accompanied by amplification and translocations of the *RFC* gene. Increased *RFC* gene copies result in elevated levels of RFC transcripts, membrane protein, and folate transport activity.

MATERIALS AND METHODS

Chemicals

[3′,5′,7-^{3}H]Mtx (20 Ci/mmol) was purchased from Moravek Biochemicals. Unlabeled Mtx and (6*R*,*S*)-5-formyl-tetrahydrofolate (leucovorin) were obtained from the Drug Development Branch of the National Cancer Institute. Both labeled and unlabeled Mtx were purified by reversed-phase HPLC prior to use [10]. Dialyzed fetal bovine serum and folate-free RPMI 1640 were purchased from GIBCO/BRL.

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¶ *Abbreviations:* CBS, cystathione β-synthase; DAPI, 4,6-diamino-2-phenylindole; FISH, fluorescence *in situ* hybridization; GTG banding, Giemsa-trypsin G banding; HSR, homogeneously staining region; Mtx, methotrexate; and RFC, reduced folate carrier.

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Cell Culture

The characteristics and maintenance of wild-type K562, transport up-regulated K562.4CF, and transport-defective K500E cells were described previously [7, 11].

Southern and Northern Analyses

Genomic DNAs from K562 and K562.4CF cells were isolated using the Puregene® system (Gentra System, Inc.), and aliquots (10 μg) were digested with restriction enzymes. Following electrophoresis on a 0.6% agarose gel, resolved DNAs were transferred onto a nylon membrane (Genescreen plus, DuPont) and hybridized with [α-^{32}P]dCTP-labeled full-length human RFC cDNA (KS43, [12]). The blot was autoradiographed, and the membrane was stripped and rehybridized with a ^{32}P-labeled human CBS cDNA probe [13].

Total RNAs were isolated by the method of Chomczynski and Sacchi [14]. Agarose gel fractionation and northern blotting were performed exactly as previously described [12]. Blots were probed with ^{32}P-labeled RFC cDNA, and loading was normalized with β-actin. Densitometry on the autoradiograms was performed using a Molecular Dynamics computing densitometer and analyzed using Image Quant software.

Analysis of RFC Gene by FISH

A genomic fragment of the *RFC* gene (insert size ≈17 kb), RFC-g1, was isolated from a leukocyte genomic library constructed in EMBL3 SP6/T7 (Clontech). Detailed characterization of the *RFC* genomic clone will be described elsewhere. *RFC*-g1 DNA was purified from phage lysate using a phage DNA purification kit (Qiagen). Metaphase spreads from K562 and K562.4CF lines were prepared following standard protocols [15]. For FISH studies, RFC-g1 phage DNA was directly labeled with spectrum orange-labeled dUTP (Vysis) using a nick translation kit (Oncor) according to the vendor's specifications. The probe (10 ng/μL) was hybridized to the slides for 48 hr using reagents and protocols described by the manufacturer (Vysis). The slides were counter-stained with DAPI, which gives a banding pattern similar to GTG banding for chromosome identification. The hybridization signals were analyzed with a Zeiss Axioskop fluorescence microscope equipped with a DAPI filter and a triple band pass filter for FITC/DAPI/Texas-Red. In addition, GTG banding karyotype analyses for both K562 and K562.4CF cells were also performed.

Preparation of RFC-Specific Peptide Antiserum and Western Analysis of RFC Protein

Polyclonal antibodies, designated anti-RFC/ps, were prepared in New Zealand white rabbits by Research Genetics using a synthetic peptide conjugated to keyhole limpet hemocyanin as the immunogen. The peptide sequence (PEDSLGAVGPASLEQRQS) was based on the predicted RFC amino acid sequence [12] from residues 504–521. Pre-immune and immune sera were purified on columns of protein-A agarose [16].

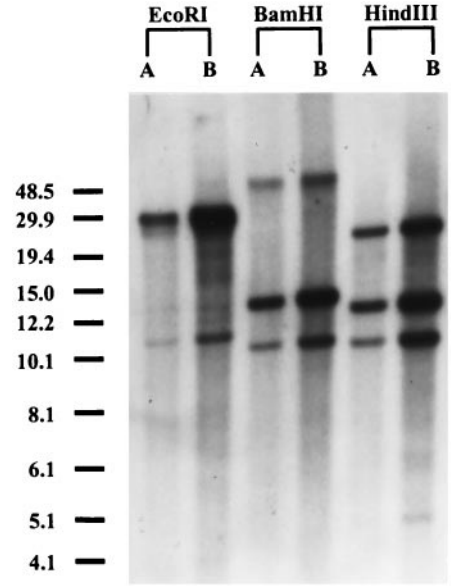

FIG. 1. *RFC* gene amplification in K562.4CF cells. *Hin*dIII, *Bam*HI, and *Eco*RI digested genomic DNA (10 μg) from K562 (A) and K562.4CF (B) cells were analyzed by Southern blotting. The membrane was hybridized with a ^{32}P-labeled full-length human RFC cDNA. DNA standards in kb are indicated.

Sucrose gradient-purified membrane proteins were prepared as described previously [7]. Protein samples were electrophoresed on 4–10% gradient gels in the presence of SDS [17] and transferred to polyvinylidene difluoride membranes (DuPont/NEN; [11]). Immunoblot analysis was performed using an enhanced chemiluminescence kit (Boehringer Mannheim). Light emission was recorded on x-ray film, and the intensities of the signals were quantitated by densitometry. Multiple exposure times were used in order to ensure that the linear range of the film was not exceeded.

RESULTS AND DISCUSSION

Experiments were performed to establish the molecular changes in the RFC accompanying selection of K562 cells to grow in 0.4 nM of leucovorin as the sole source of folates [7]. The ability of the resulting K562.4CF subline to proliferate under these restrictive conditions results from a 6-fold increased RFC activity over that of wild-type cells [7].

Genomic DNAs from wild-type K562 and K562.4CF cells were digested with restriction enzymes (*Hin*dIII, *Bam*HI and *Eco*RI) and analyzed by Southern blotting. *RFC* gene copies were increased 4- to 5-fold in the K562.4CF subline (Fig. 1), when compared with wild-type K562 cells. There were no differences in the patterns of restriction fragments hybridizing with the RFC cDNA probe between the cell lines, suggesting that *RFC* gene amplification was

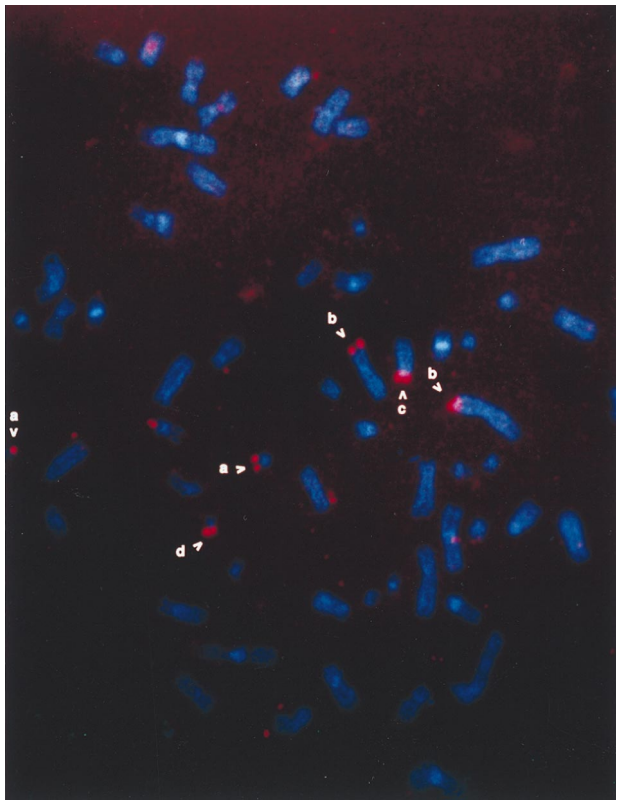

FIG. 2. FISH analysis of the *RFC* gene in K562.4CF cells. Right panel: A 17 kb RFC genomic fragment in EMBL SP6/T7 was labeled by nick translation with spectrum orange-labeled dUTP and hybridized to metaphase spreads from K562.4CF cells. The slides were counter-stained with DAPI for chromosome identification. A representative metaphase is shown, illustrating RFC gene amplification and translocations of amplified *RFC* gene fragments to chromosome 2 (b), chromosome 15 (c), and a small unidentifiable metacentric chromosome (d). Normal copies of chromosome 21 are also seen (a). Left panel: GTG banded chromosomes 2 and 15. HSRs were identified at the distal end of the chromosome 2 and at the proximal end of chromosome 15 (indicated by arrows) from K562.4CF cells. Corresponding normal chromosomes are shown to the left of each abnormal chromosome for comparison.

not accompanied by major alterations of *RFC* gene structure.

The same Southern blot was stripped and rehybridized with a cDNA to human CBS (data not shown), a gene that maps to the same region of chromosome 21 (21q.22.2–3; [13]) as the RFC [18, 19]. In spite of its close proximity to the *RFC* locus, *CBS* gene copy numbers were identical between wild-type and K562.4CF cells.

FISH analysis of *RFC* gene copies and chromosomal localization based on the DAPI banding patterns revealed that *RFC* gene amplification in K562.4CF cells was associated with translocations of the *RFC* gene fragment to various chromosomes (Fig. 2). Of 31 randomly selected metaphases from K562.4CF cells, the number of *RFC* signals observed ranged from 2–8, with the majority showing 4–5 signals. Overall, the large numbers of *RFC* signals by FISH were consistent with amplification of the *RFC* gene seen by Southern analysis. Only a single copy of a normal chromosome 21 RFC signal was observed in the majority (61%) of the metaphases examined. Likewise, the loss of one copy of chromosome 21 was observed consistently in the karyotype analysis (data not shown). Amplified *RFC* gene copies were translocated at high frequencies to chromosomes 2 (90%) and 13 (52%); and at lower frequencies to chromosomes 15 (32%) and 22 (16%). An unidentifiable small metacentric chromosome labeled with the *RFC* probe also was observed frequently (45%). The intensities of signal varied between chromosomes, which may reflect differences in *RFC* gene copy number. Most notably, the translocated RFC signals in chromosomes 2 and 15 were markedly more intense than the other loci, and each co-localized with an HSR identified by GTG banding (Fig. 2). In a small number ($< 3\%$) of metaphases, a large interstitial HSR region was associated with an exceedingly intense *RFC* signal in another unidentifiable chromosome (not shown in Fig. 2). *RFC* signals were also localized to double minutes and DNA fragments (or minutes). HSRs and double minutes (or minutes) are classic chromosomal aberrations associated with gene amplification [20].

By contrast, in the wild-type K562 cells (data not shown), two *RFC* signals were consistently observed, corresponding to normal copies of chromosome 21. However, a third *RFC* signal, localized to chromosome 1, was also observed in some metaphases. The HSRs identified in the K562.4CF subline were not detected in wild-type K562 cells.

RFC gene amplification in the K562.4CF cells was associated with a similar increase (3.98 ± 0.33; mean $\pm$ SEM, $N = 5$) in the major 3.1 kb RFC transcript on northern blots over wild-type cells (Fig. 3, right panel). Low levels of larger RFC transcripts were also detected; this may result from differences in splicing or polyadenylation from the major transcript form, or alterations resulting from the *RFC* gene translocations. A somewhat greater increase in the broadly migrating (80–95 kDa) RFC protein (7.2 ± 1.9; mean $\pm$ SEM, $N = 3$) was measured on western blots probed with human RFC specific antiserum (Fig. 3, left panel). The broadly migrating RFC protein at times appeared as multiple bands (particularly obvious for K562.4CF cells), reflecting its heterogeneous glycosylation [7]. For the wild-type K562, transport-deficient K500E and transport-elevated K562.4CF lines, immunoreactive RFC levels approximate relative transport capacities [11].

In conclusion, our results demonstrate that the selective pressure of growth-limiting concentrations of reduced folate cofactors, in the absence of cytotoxic drugs, is sufficient to promote chromosomal aberrations, including gene amplification and translocations. These alterations result in increased RFC expression and folate transport, therefore ensuring a selective growth advantage under these restrictive conditions.

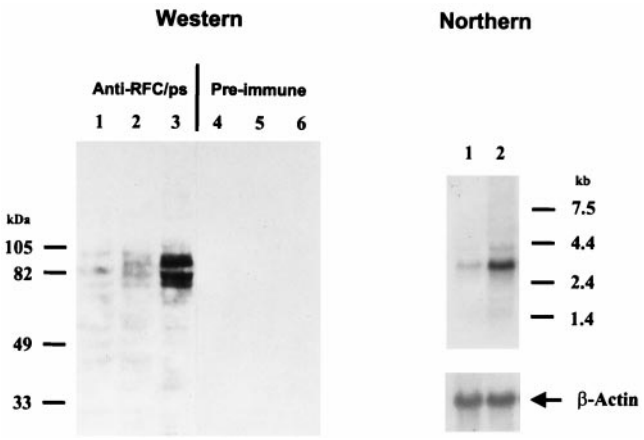

FIG. 3. RFC expression in wild-type K562 and K562.4CF cells. Right panel: RNAs (20 μg) from wild-type K562 (lane 1) and K562.4CF cells (lane 2) were electrophoresed, and then transferred to a nylon membrane; the blot was hybridized with a ^{32}P-labeled full-length RFC cDNA and autoradiographed (upper panel). In the lower panel, the same membrane was stripped and reprobed with a ^{32}P-labeled β-actin probe to demonstrate equal loading. RNA standards in kb are indicated. Left panel: Sucrose gradient-purified membrane proteins (150 μg/lane) were fractionated on 4–10% SDS-polyacrylamide gels for immunoblot analysis with immune serum (lanes 1–3) and preimmune serum (lanes 4–6). Data are shown for K500E (lanes 1 and 4), wild-type K562 (lanes 2 and 5), and K562.4CF cells (lanes 3 and 6). The molecular masses (in kDa) of standard proteins are indicated.

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