UMSCC38 Cells Amplified at 11q13 for the Folate Receptor Synthesize a Mutant Nonfunctional Folate Receptor

Rebecca B. Orr and Barton A. Kamen

Department of Pediatrics and Pharmacology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235–9063

ABSTRACT

Some cells accumulate folate via a receptor-coupled process termed potocytosis. The folate receptor, a glycosyl phosphatidylinositol anchored Mf, 38,000–39,000 glycoprotein, is coded for by at least two genes (FRα and FRβ) at 11q13. The karyotype of UMSCC38, a human squamous cell carcinoma cell line, suggests that it may contain multiple copies of the folate receptor gene(s). Southern blot analysis confirms the presence of four to six copies. Using polymerase chain reaction methodology, Northern blot analysis, immunoblotting, and immunofluorescence, we find relatively limited expression of FRα and no FRβ in UMSCC38 cells when compared to nonamplified lines. Antigen is observed on the cell surface in a punctate pattern, and the protein is anchored via a glycosyl phosphatidylinositol anchor. Transport of 5-[methyl-3H]tetrahydrofolic acid is blocked by 5-methyltetrahydrofolic acid and probenecid, which suggests anion transport. Monensin, an inhibitor of potocytosis, and folic acid, a high-affinity ligand for the receptor, do not effectively block 5-methyltetrahydrofolic acid transport. Taken together, the results suggest that UMSCC38 cells, although gene amplified, synthesize surprisingly small amounts of receptor and that receptor is nonfunctional. In order to establish further the nature of the receptor, 16 clones were obtained, and the complementary DNA was sequenced. Three mutations were found.

INTRODUCTION

The FR,^ an Mr, 38,000–39,000 glycoprotein, is thought to play a key role in folate uptake in some cells (1). It is the membrane form of the soluble folate-binding protein found in plasma (2, 3) and milk (4, 5) of various animal species (reviewed in Refs. 6–8). It is a cell surface receptor anchored in the membrane via a GPI linkage (9, 10). This protein is characterized by its high affinity for FA as well as a number of reduced FA derivatives (11, 12). Several laboratories simultaneously obtained cDNA from CaCo2 (9), KB (13), and human placenta cells (13, 14). The open reading frames and 3′-untranslated regions are identical, but the 5′-untranslated regions are heterogeneous in length and sequence. In addition, gp38, an overproduced cell surface glycoprotein (IGROV-1 cells) recognized by two monoclonal antibodies, was identified as the folate receptor (16, 17). This protein had been termed MFP2 and more recently FRα. A second sequence isolated from placenta had been termed MFP1 and is now FRβ. It bears a 68% sequence homology with FRα (14). A recent study suggested that FRβ may have a lower affinity for some folates than FRα (12). Murine homologues of both species have also been described (18). Data suggest a third member of this family were recently presented by Page et al. (19). This cDNA is homologous to the FRβ but contains a unique 5′ terminus and sequence differences in the open reading frame. Folate receptors, therefore, seem to be coded for by a family of genes whose physiological role(s) have not yet been completely identified.

We are interested in the regulation of the synthesis and function of the folate receptor. Our studies of MA104 cells, an established kidney epithelial cell line expressing moderate amounts of receptor compared to some malignant cells in vitro, have shown that the folate receptor mediates the internalization of 5CH3FH4, via a process termed “potocytosis” (20–22). This process takes place in four steps: (a) high-affinity binding of folate to the receptor; (b) translocation of the ligand-receptor complex into an internal, membrane-bound compartment; (c) dissociation of the folate from the receptor in response to a proton gradient and subsequent movement across the membrane by an anion carrier; and (d) polyglutamation of folate in the cytoplasm to retain the folate within the cell. When cells expressing the folate receptor are growing in physiological concentrations of the vitamin, potocytosis will increase efficiency of folate uptake 30–50 fold. In this regard receptor-negative cells transfected with receptor cDNA have a growth advantage compared to control cells when grown in limiting concentrations of folate (23–25).

The folate receptor genes have been mapped to chromosome 11q13 (15, 26). Amplification of this region is observed in 20% of human esophageal tumors (27), 15–20% of primary breast cancers (28), and 30–50% of squamous cell carcinomas of the head and neck (29), making this region one of the most frequently amplified loci in human carcinomas (reviewed in Ref. 30). In addition, chromosomal rearrangements involving the 11q13 region are found in some B-lymphocyte malignancies and parathyroid tumors. DNA markers located in this region include int-2, hst-1, and bel-1 (30). It is assumed that amplification represents an important step in cancer evolution, but biologically relevant gene(s) within this region, overexpressed with an 11q13 amplification, have not yet been defined. Recent evidence does suggest that cyclin D (also known as prad1) may be a functionally important component of the amplicon (31). It has not been determined whether overexpression of cyclin D is the selective force of the amplification.

In an effort to determine what effect amplification of 11q13 might have on the regulation of function and synthesis of the folate receptor, we have studied UMS MCC38, a squamous cell carcinoma line that is hexaploid at 11q13. This is a result of four copies of chromosome 11, two of which contain an inverse duplication (11q24.3–11q13.4) (32). We present evidence that this cell line is a relatively “low producer” of folate receptor and the protein synthesized does not bind folic acid or 5-methyltetrahydrofolate. In addition, folate internalization does not seem to occur via potocytosis.

MATERIALS AND METHODS

Chemicals/Reagents. Radiolabeled folic acid and 6S-5-methyltetrahydrofolate were purchased from Moravek Biochemicals (City of Industry, CA). Bacillus thuringiensis PIPLC was obtained from ICN. MOV 19 (monoclonal antibody) was provided by Centocor. RNA from uterine sarcoma (used as control for FRα detection by RT-PCR) was kindly provided by Dr. Manohar Ratnam (University of Toledo). T-75 culture flasks and 35-mm dishes were from Costar. RPMI 1640 salts and amino acids were purchased from Sigma. RPMI vitamers (without folic acid) were added sepa-
rately using Sigma products. Medium 199 was made according to instructions from Sigma. Trypsin/EDTA was from Sigma. Fetal calf serum was purchased from Sigma (lot 31H-0234) and Gibco (lot 31P4124). Penicillin/streptomycin was from Sigma. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate was purchased from Zymed Laboratories (South San Francisco, CA). Nytran and nitrocellulose were from Schleicher and Schuell. Other chemicals were purchased from Sigma.

**Cell Culture.** UMSCC38 and UMSCC21A cells (human squamous cell carcinoma cell lines) were kindly provided by Dr. Thomas Carey (University of Michigan). IGROV-1 cells, a human ovarian carcinoma cell line (33), MA104 cells, a monkey kidney epithelial cell line, UMSCC38, and UMSCC21A were grown continuously as a monolayer in FA-free RPMI media. UMSCC38 cells were grown in medium M199 without sodium bicarbonate, pH 7.4. Cells were kept chilled throughout the experiment. Cells were washed with medium 199 for 20 min and then with medium 199 containing BSA (0.15% BSA-fraction V) for 30 min. The coverslips were incubated in 10 µg/ml primary antibody for 1 h (150 µl/cover slip) and washed quickly 2 times and then 3 times for 10 min each with medium containing BSA. Secondary antibody (20 µg/ml) was applied for 1 h (150 µl/cover slip), after which the cells were washed quickly 2 times in PBS and then 3 times for 10 min each. For visualization of the fluorescence, the cells were fixed in 3% paraformaldehyde in PBS for 30 min, washed, and mounted. These methods were kindly provided by Dr. Karen G. Rothberg.

**Phospholipase C Analysis.** PIPLC analysis was performed as described in Ref. 9.

**Cell Preparation for Folic Acid-binding Assay.** Cells were washed for 30 s with acid saline, pH 3.5. The acid saline was removed, and cells were washed with PBS, released with trypsin, and collected by centrifugation for 10 min at 500 × g. The cell pellet was frozen in solubilization buffer (50 mm Tris, pH 7.4-150 mm NaCl-25 mm octylglucoside-5 mm EDTA-0.02% sodium azide), thawed, and allowed to incubate for 30 min at 4°C. The pH was then lowered to 3.5 with glacial acetic acid, and dextran-coated charcoal was added. After incubation for 20 min on ice (inverting tubes every 2–3 min), the extract was centrifuged at 500 × g for 5 min. The supernatant was removed and centrifuged again at 500 × g for 5 min. The supernatant pH was adjusted to 7.0 with concentrated ammonium hydroxide and chilled, and any precipitate was removed by centrifugation at 500 × g for 5 min.

**Binding Assay.** Assay buffer (50 mm potassium phosphate, pH 7.0-1 mg/ml charcoal-treated BSA), sample, and [3H]folic acid were combined in a microtube. Equal amounts of solubilization buffer were in each tube (including blank) because the detergent tended to increase the background. After the tube was incubated on ice for 30 min, dextran-coated charcoal was added. After 1 min of incubation on ice, charcoal was removed by centrifugation (12,000 rpm, 1.5 min) in a microfuge. Radioactivity in the supernatant was quantified by liquid scintillation at 55% efficiency.

**Dot Blotting.** Dot blotting was done based on methods described previously (45). Briefly, 5 µg total RNA isolated from either UMSCC38 (parent) or IGROV-1 (normal) were dotted on to Nytran, UV cross-linked, and pre-hybridized with 2× SSC-10× Denhardt’s solution-150 µg/ml salmon sperm DNA for 3–5 h. Oligonucleotides were designed with and without each base pair mutation. Oligonucleotides were n5:5'-CGCGACGCAAAGTGTTGTGGTGG-3', 5'-CGCGACGCAAAGTGTTGTGGTGG-3', n13:5'-CGCGACGCAAAGTGTTGTGGTGG-3', n15:5'-C-GCGACGCAAAGTGTTGTGGTGG-3', and 15:5'-C-GCGACGCAAAGTGTTGTGGTGG-3'. Each oligonucleotide was labeled with [γ-32P]ATP using T4 kinase, added to the prehybridization mix, and incubated for 72 h. Each filter was then washed with 2× SSC at the T, T, + 3, T, + 6, and T, + 9. Filters were exposed on film 12–24 h.

**Other Methods.** Protein determinations were made using the Bio-Rad protein assay dye reagent. Cells were counted using a standard hemocytometer.

**RESULTS**

**UMSCC38 Have Increased Copies of the FR Gene.** Amplification of the folate receptor gene was assessed by Southern blot analysis. UMSCC38 cells were compared to another squamous cell carcinoma line, UMSCC21A, which is diploid at chromosome 11. Fig. 1 shows the amplified signal detected in UMSCC38 cells. Quantitation of the signal revealed a 5.3-fold increase.

**Folate Receptor RNA and Protein Expression Is Limited to FRα and Is Decreased in UMSCC38 Cells Compared to Diploid Cells.** Expression of folate receptor RNA in UMSCC38 cells was characterized using RT/PCR. Primers specific for either FRβ or FRα (described in “Materials and Methods”) were used to show that FRα is expressed exclusively in UMSCC38 cells. Expression was also assessed by Northern blot analysis using full-length cDNA (isolated from cancerres.aacjrnl.org on April 13, 2017. © 1994 American Association for Cancer Research.
from CaCo₂ cells) as a probe. UMSCC38 cells express approximately 4-fold less receptor mRNA than MA104 or UMSCC21A cells (Fig. 2A). Western blot analysis, using monoclonal antibody MOV 19, shows only a small amount of antigen in UMSCC38 cells compared to IGROV-1, MA104, and UMSCC21A cells (Fig. 2B), thus correlating well with measurement of the RNA.

When it was confirmed that UMSCC38 cells expressed the folate receptor protein, although in less than quantities expected based on gene copy, it was important to determine whether the protein was found on the cell surface and whether it was functional. Indirect surface immunofluorescence, using the monoclonal antibody MOV 19, shows that UMSCC38 cells express folate receptor on the cell surface in a punctate pattern similar to MA104 cells, albeit at much lower amounts (Fig. 3).

Folate Receptor Is Released from the Membrane by PIPLC. To determine whether the folate receptor expressed on the surface of UMSCC38 cells was anchored via a GPI anchor, PIPLC was used to anchor the cell surface. It was still necessary, however, to determine whether the folate receptor protein expressed by UMSCC38 cells participates in folate accumulation.

5-Methyltetrahydrofolate Accumulation by UMSCC38 Cells Does Not Occur According to the Paradigm for Potocytosis. To further define the mechanism of folate uptake in the UMSCC38 cells, we utilized several known inhibitors of either receptor-coupled or anion transport-mediated uptake. Cells were incubated with 5CH₃FH₄ in the presence of FA, monensin, or probenecid. FA is used to block receptor-mediated uptake because it binds to the receptor with much greater affinity (Kᵦ = 5–10 pm) than 5CH₃FH₄ (Kᵦ = 1 nM) (11, 41). Monensin will also block receptor-mediated uptake because it prevents the acidification of vesicles, a condition predicted for ligand release from the receptor (1, 37). Probenecid inhibits anion transport, and because anion transport is thought to mediate both receptor independent and receptor dependent uptake, probenecid should and has been shown to block both receptor-dependent and receptor-independent uptake in MA104 cells (1). Fig. 6 shows the effects of these inhibitors on both MA104 cells and UMSCC38 cells. FA (2 μM) inhibits >90% of 5CH₃FH₄ uptake in MA104 cells but inhibits <2% of uptake in UMSCC38 cells. Monensin (25 μM) inhibits >80% of uptake in MA104 cells and inhibits only 28% of uptake in UMSCC38 cells. Probenecid (10 mM) inhibits 75–80% of folate uptake in both MA104 and UMSCC38 cells. These results
are consistent with the hypothesis that 5CH₃FH₄ uptake in UMSCC38 cells is receptor independent and is mediated via an anion transporter.

**Folate Receptor Expressed by UMSCC 38 Cells Does Not Bind Folic Acid.** Interestingly, our uptake studies showed that the amount of 5CH₃FH₄ bound to the membrane is virtually undetectable (maximum, 0.04 pmol/10⁶ cells) (Fig. 5). Because the affinity of FA for the receptor is very high, additional binding studies were done using [³H]folic acid as a ligand. These experiments also showed that folate binding does not exceed 0.02 pmol FA/10⁶ cells (data not shown). This value is in contrast to MA104 cells (2 pmol FA/10⁶ cells) and IGROV cells (10–12 pmol/10⁶ cells). Given that UMSCC38 cells express approximately 4-fold less receptor than do MA104 cells (assessed by immunoblot), one would expect these
to bind approximately 25% (0.50 pmol/10^6 cells). The fact that binding is considerably less than this implies a nonfunctional protein.

To analyze more completely the binding characteristics of the folate receptor expressed by UMCC38, Scatchard analysis was done using solubilized whole cell extracts from both MA104 and UMCC38 cells. To ensure equal sensitivity, the amount of folate receptor was equalized using equal amounts of antigen (i.e., 4-fold more UMCC38-derived protein). An MA104 cell preparation binds FA with a K_d of 0.054 nM. There is no detectable binding in the UMCC38 cells over the range (0.19–24 nM) of ligand concentration used (data not shown). To determine whether the amount of protein used to assay the UMCC38 cells interferes with binding, Scatchard analysis was done using 50 μg UMCC38 protein and 200 μg MA104 protein. Again, binding is undetectable in UMCC38 cells. These results suggest that the UMCC38 cells express a folate receptor that does not bind folate with high affinity. To address the possibility that this lack of function could be attributed to a processing error or aberrant protein synthesis, UMCC38 cells were transfected with FRA cDNA but produce a nonfunctional receptor points to the existence of a mutation(s) rendering the expressed protein nonfunctional. Sixteen full-length folate receptor cDNA clones were isolated from UMCC38 cells and are not an RT/PCR artifact.

**DISCUSSION**

Receptor-coupled transport (potocytosis) is an efficient, endocytotic pathway for internalization of folate and perhaps other small
molecules (20). Folate accumulation via receptor-coupled transport has been most extensively studied in kidney epithelial cells (MA104). These cells express a moderate amount of easily detectable receptor (1–2 pmol/10^6 cells) compared to some malignant cells having significantly more binding (35). The latter binding greatly exceeds intracellular folate requirements. Recently, three laboratories simultaneously showed that transfection of the folate receptor cDNA into receptor-negative cells conferred a growth advantage when cells are grown in small amounts of folate (nm versus μM) (23–25). This suggests a functional significance for the receptor with regard to cell growth. Tissue distribution of FRα (assessed by Northern blot, immunoblotting, and immunocytochemistry) indicates that expression is restricted primarily to epithelial type cells (42, 17).

Regulation of folate receptor synthesis has been only partially characterized in a malignant cell line (43). Therefore a number of important questions remain. Why does a cell express the folate receptor when it can depend completely on receptor-independent folate uptake to survive? Why does a cell express far more receptor than it needs for receptor-dependent uptake of folate? These are the dilemmas that are stimulating questions as to the role of the receptor in the regulation of folate uptake as well as what other functions or capacities this GPI-anchored protein may have.

The amplification of the folate receptor gene in UMSCC38 cells seemed to make these cells an ideal line for addressing some questions regarding the regulation of the folate receptor and its involvement with folate homeostasis. Finding that expression did not correlate with this amplification at either the RNA or protein levels was initially surprising and paradoxical. It is possible that the translocation plays a role in expression. The translocation is mapped at 11q13.4, and the receptor has been mapped at 11q13.3–11q13.5 (15, 26, 32). Perhaps the translocation results in the active repression of transcription of the folate receptor, or removal of an enhancer, resulting in decreased expression. It is also possible that the receptor may exist in a hyper-methylated state in this cell line, a condition recently demonstrated to result in down-regulation of folate receptor expression in methotrexate-resistant cells.4

In addition to the observation that these cells expressed decreased quantities of receptor (both at RNA and protein levels), we also noted that the antigen does not bind folic acid. There is no precedent for this observation. The operational definition of a folate receptor is a protein with a high affinity for FA and no enzyme activity. Even the recent characterization of folate receptors (FRα, FRβ, FRγ) demands that additional efforts are made to determine the effects of FR(s) on the sensitivity or resistance to classic antifolos, such as methotrexate (for which there is a relatively poor affinity compared to 5-methyltetrahydrofoleric acid), and the newer folate analogues, such as lometrexol and tomudex (which have a much higher affinity).

REFERENCES


4 B. J. Delnick and C. T. Hsueh, personal communication.

Downloaded from cancercres.aacrjournals.org on April 13, 2017. © 1994 American Association for Cancer Research.
UMSCC38 CELLS SYNTHESIZE NONFUNCTIONAL FOLATE RECEPTOR


UMSCC38 Cells Amplified at 11q13 for the Folate Receptor Synthesize a Mutant Nonfunctional Folate Receptor

Rebecca B. Orr and Barton A. Kamen


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/14/3905