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Functional Effect of Point Mutations in the α-Folate Receptor Gene of CABA I Ovarian Carcinoma Cells

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Abstract The α-folate receptor (αFR) is overexpressed in 90% of nonmucinous ovarian carcinomas. In addition to the known role of αFR binding and mediating the internalization of folates, functional interaction of αFR with signaling molecules was recently shown. To identify a model to study the role of αFR in ovarian carcinoma, we characterized the αFR gene in the ovarian carcinoma cell line CABA I in comparison to a reference line, IGROV1. In CABA I cells, Northern blot analysis revealed an αFR transcript of the expected length and FACS analysis indicated receptor expression on the cell membrane; however, RNase protection assay revealed no specific signals. Southern blot and genomic PCR analysis suggested the presence of a rearrangement(s) involving the 5′ region of the gene in CABA I cells as compared to IGROV1 cells. Cloning and sequencing of CABA I αFR cDNA revealed several point mutations. The partitioning of αFR in membrane microdomains from CABA I cells and its association with regulatory molecules was comparable to that of IGROV1 cells. By contrast, the αFR expressed on the CABA I cell membrane bound folic acid with lower affinity, and ectopic expression of the corresponding cDNA in CHO cells confirmed impaired folic acid binding. Thus, CABA I cells may provide a tool to delineate functional domains of the αFR. J. Cell. Biochem. 81:488–498, 2001.

Key words: α-folate receptor; ovarian carcinoma; genetic rearrangement; folic acid binding; Triton-insoluble domain

The α-folate receptor (αFR) is a 38-kDa glycosylphosphatydilinositol (GPI)-anchored membrane protein that binds folic acid (FA) with high affinity and appears to be directly involved in the uptake of the physiological form of 5-methyltetrahydrofolate. Folate uptake is initiated by binding to the external αFR, which cycles to an internal, but membrane-bound, compartment. At 37°C, these two pools, which are acid-labile and acid-resistant, respectively, are constantly interchanging [Antony, 1996].

The αFR protein has been characterized as a marker of ovarian carcinoma [Miotti et al., 1987], since it is overexpressed in 90% of nonmucinous ovarian carcinomas and in some other malignant tissues while it is present at low levels in a few normal tissues. Furthermore, αFR expression, which is absent in normal ovary epithelial cells but which appears very early in the transformation process, appears to be very stable in this oncotype and even increases in association with tumor progression [Toffoli et al., 1997].

Antony proposed [1996] that αFR might affect cell proliferation by a mechanism that is independent of its role in folate internalization. Indeed, we found that αFR expression was only partially responsible for folate internalization in a panel of ovarian carcinoma cell lines [Miotti et al., 1997]. Our recent studies have provided evidence that αFR expression in ovarian cancer cells might affect cell proliferation by generating regulatory signals. In the ovarian carcinoma cell line IGROV1, we observed that αFR is partitioned in cellular-membrane microdomains, where it is physically and functionally associated with the src-family member p53–56 lyn and the Gαi-3 subunit of heterotrimeric G-proteins [Miotti et al., 2000]. More recently, we found that αFR expression in ovarian cancer cells is inversely related to that of the tumor suppressor gene caveolin-1 through a mechanism

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exerted at the transcriptional level [Bagnoli et al., 2000].

Molecular analysis has revealed the complexity of structure and organization of the human αFR gene family in a tandem array on chromosome 11q13 [Ragoussis et al., 1992]. The αFR gene, composed of seven exons, is transcribed from at least two different promoters, one located upstream and within exon 1 (named P1) and the second upstream of exon 4 (named P4) [Saikawa et al., 1995; Elwood et al., 1997]. The open reading frame (ORF) spans exons 4 through 7. Many distinct αFR mRNA species are detectable in ovarian carcinoma cell lines, originating from different promoter usage and alternative splicing. These transcripts differ in their 5′-untranslated region (UTR) sequences, but share a common ORF [Coney et al., 1991]. Our recent analysis of gene transcriptional regulation indicates that αFR overexpression is not related to reorganization of the gene but to a specific activation of P1 and P4 promoters only in αFR-expressing cell lines [manuscript in preparation].

Here, we have identified a model of ovarian carcinoma, CABA I cells, which provides a tool to analyze the relationship between the sequence, the subcellular distribution, and the different functions of αFR. We find evidence of genetic rearrangement of the αFR gene and of many point mutations in the protein sequence. We demonstrate that the CABA I mutated receptor still distributes in low-density membrane microdomains and associates with molecules regulating cell proliferation, similar to αFR of other ovarian carcinoma cell lines, but shows impaired binding of the high-affinity ligand FA.

METHODS

Materials, Cell Lines and Antibodies

EDTA, formamide, RNase A, protease K, PIPES, HEPES buffer, and FA were from Sigma (St. Louis, MO); octyl-β-glucoside and the random priming kit from Boehringer-Mannheim (Germany); Taq polymerase from FinnZyme (Finnzymes OY, Finland); reverse transcriptase and Ampli Taq polymerase from Perkin Elmer (Roche, Branchburg, NJ); restriction enzymes and β-actin riboprobe from New England Biolabs (Beverly, MA); lipofectin from Gibco (BRL, Paisley, UK); 5′[α-^{32}P]dCTP, [α-^{32}P]CTP, [^{3}H]FA (specific activity, 32 Ci/mmol), x-ray film, and Hybond N® nitrocellulose membranes from Amersham (Little Chalfont, UK).

Two human ovarian carcinoma cell lines of serous origin were used: CABA I, derived from the ascite of an ovarian cancer patient prior to drug treatment [Dolo et al., 1997], and the reference cell IGROV1 (kindly provided by Dr. J. Benard, Institut G. Roussy, Villejuif, France). For stable transfection, CHO-K1 cells American Type Culture Collection (ATCC) were used. Cells were maintained in RPMI 1640 (Gibco BRL) supplemented with 5% FCS and 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO_2 in air.

Murine monoclonal antibody (MAb) MOv19, which specifically detects the αFR protein, was produced in our laboratory [Miotti et al., 1987] and purified as described [Miotti et al., 1995]. Rabbit anti-Gαi-3 and anti-lyn polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Flow Cytometry Analysis

To evaluate αFR cell surface expression, cells were incubated at 4°C with MAb MOv19 (10 μg/ml) followed by fluorescein-conjugated goat anti-mouse IgG (1:80) in PBS with 0.03% BSA and analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Northern Blot and RNase Protection Assay (RPA)

Northern blot assay was conducted on 10 μg of total RNA, purified using the Total RNA Purification kit (Qiagen, Gmbh, Hilden, Germany), electrophoresed on a 1% agarose-formaldehyde denaturing gel, and transferred to a nylon membrane (Hybond N®). Hybridization was carried out with 2×10^6 cpm/ml of αFR ORF probe [Coney et al., 1991] labeled with 5′[α-^{32}P]dCTP by random priming. The filter was washed at 65°C with 0.1 × SSC, 0.1% SDS and exposed in a Phosphorimager screen (Molecular Dynamics Inc., Sunnyvale, CA) for 16–20 h. Band intensity was quantified using IMAGEQUANT software upon normalization of the total RNA loading with 28 nd 18 S rRNA bands.

For RPA, riboprobes IG1, KB1 and IG4 were prepared as described [Coney et al., 1991; Elwood et al., 1997] and radiolabeled with 20 μCi of [α-^{32}P]CTP. IG1 and KB1 are representative of P1 usage and contain the 5′-UTR of #4/6 cDNA cloned from IGROV1 cells and the KB1 cDNA cloned from KB nasopharyngeal

carcinoma cells, respectively. IG4 is derived from P4 promoter sequences and contains 54 bp at the 3′ end of the 5′-UTR of #51 cDNA cloned from IGROV1 cells. The three riboprobes share the initial 144 bp of the ORF until the *AvaI* site and 10 bp between the common splicing site and the beginning of the ORF. RPA was performed essentially as described [Page et al., 1993]. A β-actin riboprobe was hybridized with the same RNAs to calibrate RNA loading.

Southern Blot Analysis

Genomic DNA (10 μg) was digested overnight with PstI restriction enzyme, electrophoresed on 0.8% agarose gel, transferred to a nylon membrane (Hybond N®), and hybridized with the following ^{32}P-labeled probes: αFR ORF [Coney et al., 1991], P1, and P4 (spanning residues −40 to +256 and residues +2195 to +2760 relative to the first nucleotide of exon 1, respectively). The filter was washed at 65°C with 0.1 × SSC, 0.1% SDS and exposed in a Phosphorimager screen (Molecular Dynamics). After each hybridization, the filter was stripped of the previous probe by boiling for 10 min in 1% SDS.

Genomic PCR

Genomic DNA (750 ng) was amplified using 1.5 U of Taq polymerase and ≥10 pmoles of each primer matching various sequences inside the two promoters (see Results and Fig. 2). PCR was performed for 30 cycles with the following steps: denaturation at 95°C for 2 min, annealing at 58°C for 2 min, and polymerization at 72°C for 3 min.

cDNA Cloning

Total RNA (2 μg) was reverse-transcribed using 50 U of reverse transcriptase and a poly(dT) primer. The resulting cDNA (2 μl) was amplified using 2 U of Ampli Taq polymerase and a 30-cycle PCR (95°C, 1 min, 58°C, 1 min, 72°C, 2 min) followed by an elongation step of 72°C for 10 min. The forward and reverse primers matched the 5′ (5′-ATGGCTCAGCGGATGACAACA, forward) and 3′ (5′-TCAGCTGAGC-AGCCACAGCAG, reverse) regions of the ORF, respectively. The PCR product was cloned in the pCR3.1 vector using the TA Cloning System® as suggested by the manufacturer (InVitrogen, San Diego, CA).

Sequencing

Automated sequencing was performed with the ABI PRISM 377 DNA Sequencer (Perkin Elmer). cDNA was sequenced using the following primer pair: 5′-TAATACGACTCACTATAGGG-3′ (sense) and 5′-TAGAAGGCACAGTCGAG-3′ (antisense), matching the two ends of the vector cloning site. The genomic regions at the 5′ and 3′ ends of the ORF were PCR-amplified and sequenced respectively with the following primer pairs: 5′-ACTGTTTCACCCCAGAATAT-3′ (sense, matching inside intron 3) and 5′-TTCCTTGTGGTGCTTGGCGTTCATGCAGACATT-3′ (antisense, matching inside exon 4, 100 bp downstream from the first ATG); 5′-TGCATCCAGATGTGGTTCGA-3′ (sense, matching inside exon 7), and 5′-CCAAATAATTCTCAAGACAC-3′ (antisense, matching the end of exon 7, after the stop codon).

Folate Binding Assay

Cells were cultured for 9 days in folate-deficient medium [Miotti et al., 1997] and plated at 3×10^5/well in 24-well plates. After 24 h, cells were incubated at 0°C and 37°C with 10 nM [^{3}H]FA for 2 h and then treated with saline solution containing 40 mM acetic acid, pH 3. After two washes with cold 0.9% NaCl, acetic acid-treated and- untreated cells were lysed with 400 μl of buffer containing 1.1% octyl-β-glucoside. In another experiment, [^{3}H]FA was titrated from 0.01 to 1 μM in PBS, with or without 1 μM cold FA as competitor. Protein concentration and radioactivity of each sample was measured using aliquots of 50 and 300 μl, respectively. Bound pmol/mg of protein, internalization efficiency, and percent binding competition of cold FA with [^{3}H]FA were determined.

cDNA Stable Transfection in CHO Cells

CHO cells were transfected using the Lipofectin method as described [Tomassetti et al., 1999] with the pCR3.1 plasmid containing the CABA I or IGROV1 αFR cDNA. On Day 3, G418 (800 μg/ml) was added to the medium to select transfected cells. Starting from Day 10, single colonies were collected and αFR-positive clones were detected by immunofluorescence assay with MOv19 MAb.

Characterization of CABA I αFR Membrane Microdomains

αFR expressed on the membrane of CABA I cells was partitioned on a 5–30% linear sucrose gradient starting from a 1% TX-100 cell lysate as described [Miotti et al., 2000]. Twelve 1-ml-

fractions were collected and 100 μl of each were analyzed by 10% SDS-PAGE. Nitrocellulose-transferred proteins were analyzed by Western blot for FR distribution by using MAb MOv19 and a secondary rabbit antimouse HRP-conjugated antibody. The reaction was developed by ECL (Amersham). Low- (4–6) and high-density (8–9) fractions were pooled and immunoprecipitated with anti-FR as described [Miotti et al., 2000]. The anti-HLA class I MAb W6/32 was used as a control for specific immunoprecipitation. Briefly, goat antimouse magnetic beads (Dynal AS, Oslo, Norway) were incubated for 2 h at room temperature with MAbs MOv19 and anti-HLA class I diluted in PBS containing 0.1% BSA (1 μg antibody/25 μl bead suspension). After washing, the beads were incubated with an aliquot of pooled fractions from the sucrose gradient, diluted 1:2 with 50 mM Hepes buffer, pH 7.4 containing 0.75% TX-100, protease inhibitors, and 10 mM iodoacetamide (IAA). BSA (1%, 40 μl) was added to each sample during overnight incubation at 4°C with rotation. To separate bound from unbound material, the beads were washed once with the above buffer and four times with PBS containing 1% BSA, 10 mM IAA, and protease inhibitors (10 min/wash). Precipitated molecules were extracted using SDS-PAGE sample buffer and boiling for 5 min at 95°C and analyzed by Western blot with rabbit anti-Gαi-3 and anti-lyn polyclonal antibodies as described above.

RESULTS

CABA I Cell αFR Expression

FACScan analysis indicated cell surface expression of αFR on CABA I cells (Fig. 1A), consistent with the histological origin of this line (serous ovarian carcinoma). Expression levels were about 1/3 of that on IGROV1 cells.

Northern blot analysis using an αFR ORF probe revealed the αFR transcript in CABA I RNA, migrating at the same position as that from IGROV1 cell RNA. Densitometric analysis indicated that the band obtained with CABA I RNA was about four-fold less intense than that obtained with IGROV1 RNA (Fig. 1B).

To determine the promoter usage in CABA I cells, RPA was performed using IG1, KB1, and IG4 riboprobes (Fig. 1C), which contain the sequence spanning nucleotides −10 to +144 (relative to the first αFR ORF base) common to all αFR transcripts, but differ in their 5′ sequences corresponding to three different 5′-UTRs of αFR mRNAs [Coney et al., 1991; Elwood et al., 1997]. Surprisingly, no protection was observed with CABA I RNA using either probe, in the range between 250 and 300 bp in length, although longer exposure showed slight specific bands between 80 and 100 bp (data not shown) that could be generated by partial hybridization with either the 5′- or the 3′-end of the riboprobes. IGROV1 mRNA protected the expected fragments (Fig. 1C). Thus, αFR transcripts in CABA I cells differ from the previously described cDNA [Elwood, 1989] in the 5′-UTR and/or the initial 144 bp of the ORF.

CABA I αFR Gene Structure

Southern blot analysis was performed on CABA I and IGROV1 genomic DNA, digested with PstI and hybridized with ORF, P1, and P4 probes. *Pst*I sites are present in the introns, so that digestion with this enzyme allows determination of the length of αFR gene exons. Indeed, the PstI pattern obtained after hybridization with the three probes differed in CABA I vs. IGROV1 DNA (Fig. 2A); the only common band around 500 Kb corresponds to the region of exons 6 and 7. This result suggests genetic rearrangement/deletion at least in the ORF 5′-end of the CABA I αFR gene and might explain the absence of a signal in RPA, which can be affected even by a single-point mutation.

To analyze the region spanning from exon 1 through 5 of the CABA I cell αFR gene, genomic DNA from the cells was amplified by PCR using primers designed on the basis of the published sequence (Fig. 2B). The expected fragments of 1130 and 550 bp were obtained only using primer pair *a* and *b*, indicating that the region spanning exon 1 through 3 and the region containing exon 4 are maintained in both IGROV1 and CABA I DNA. However, we cannot exclude the presence of mutation in exon 1–3 region, which would explain the lack of hybridization with riboprobes IG1 and KB1 in the RPA experiment (see Fig. 1). Discrepancies were obtained in CABA I DNA with the other primer pairs, consistent with the presence of rearrangement/deletions in introns 3 and 4 and/or exon 5 as suggested by Southern blot analysis. Indeed, by Southern blot analysis of PCR products with P4 probe (see panel A), none of the major bands stained with ethidium bromide in lanes c–e hybridized, while hybridization with P4 probes was detected with the 550 bp frag-

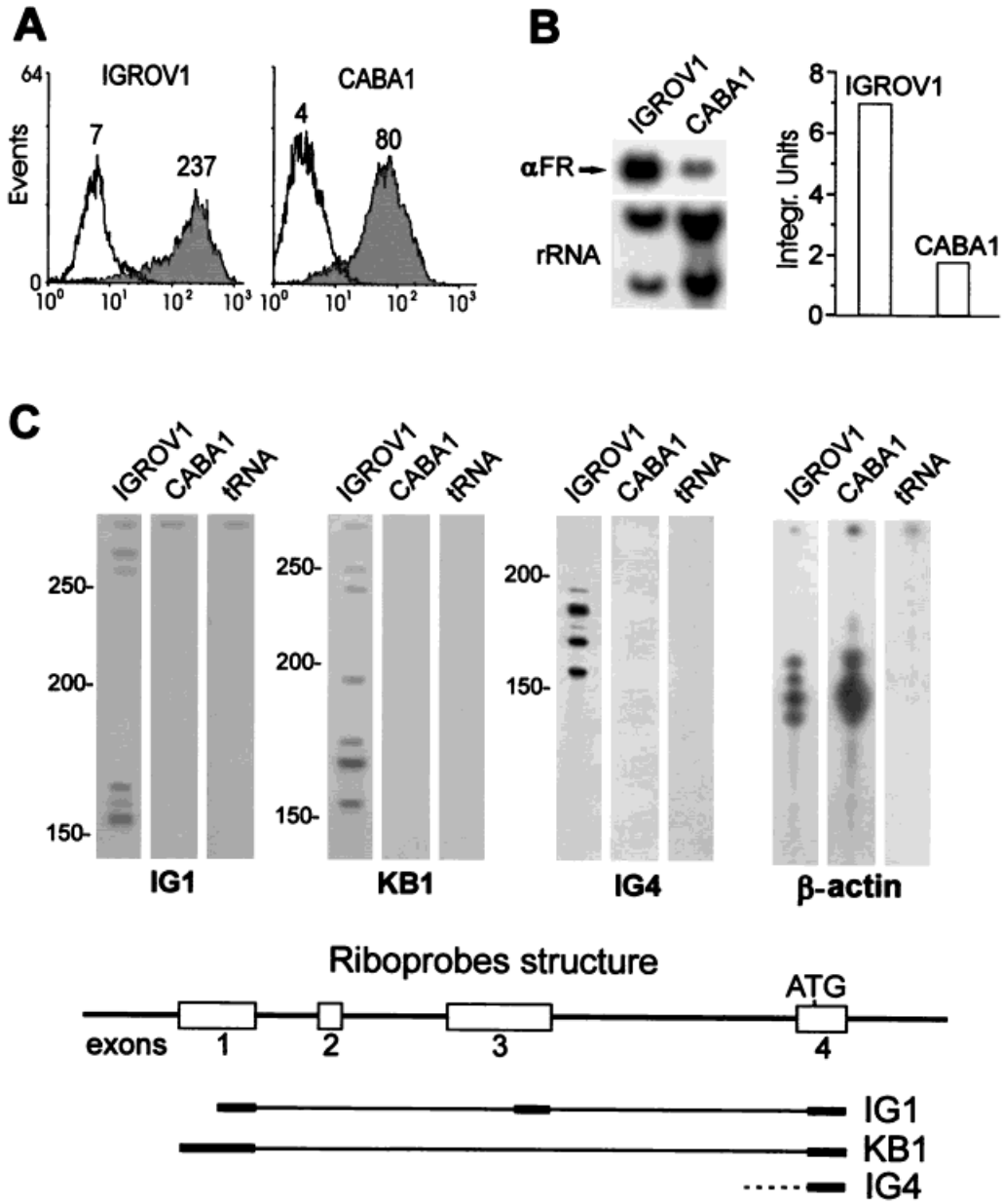

Fig. 1. Characterization of αFR expressed on CABA I cells. **A**. FACScan analysis of αFR cell surface expression on CABA I and IGROV1 cells incubated with MAb MOv19. Open curve: no primary antibody. Numbers at curve peaks are mean fluorescence intensity. **B**. Northern blot analysis of mRNA from IGROV1 and CABA I. Graph on the right shows quantitation of αFR mRNA in relative integration units. **C**. Determination of transcriptional start sites and promoter usage of IGROV1 and CABA I cells by RPA. Size marker was a sequencing reaction (not shown). A schematic diagram of the riboprobes is shown at the bottom of the figure.

ment of lane b (data not shown). With IGROV1 DNA, all the amplified fragments showed the expected length (Fig. 2C), and their specificity was confirmed by Southern blot analysis with P4 probe (data not shown).

Sequence of the αFR ORF in CABA I Cells

To determine whether deletions/mutations were present in the CABA I αFR ORF, CABA I and IGROV1 αFR cDNAs were synthesized by RT-PCR using primers corresponding to the 5′- and 3′-ends of the αFR ORF published sequence and cloned in the pCR3.1 vector. Sequencing analysis of the cloned CABA I αFR ORF, as well as that of the PCR-amplified 5′ and 3′ genomic regions of the ORF, indicated 21 point mutations as compared with the published sequence (accession number HSU20391 [Elwood, 1989])

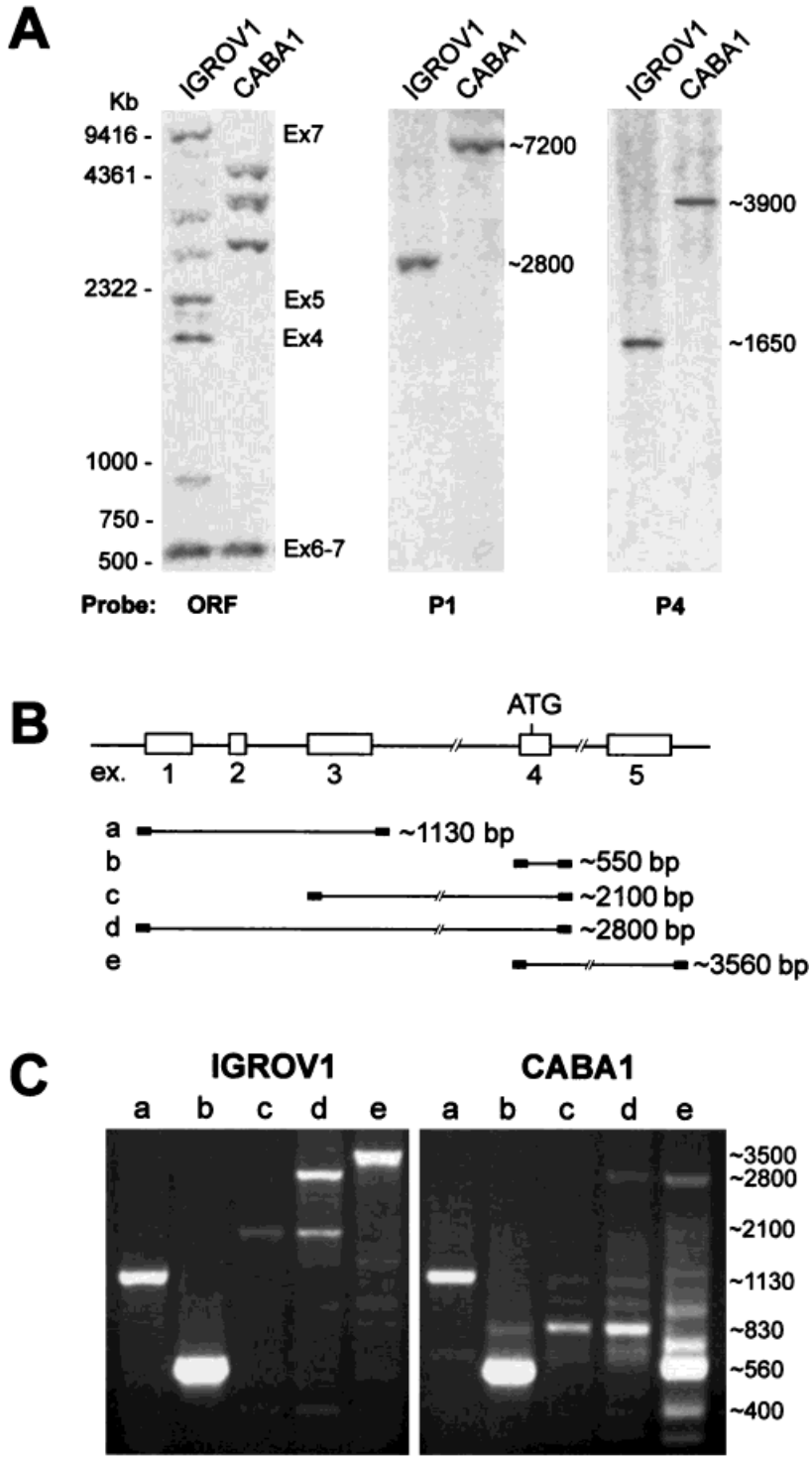

Fig. 2. Molecular analysis of the αFR gene in IGROV1 and CABA I cells. **A**. Southern blot analysis of the αFR gene; the three panels correspond to the indicated probes. *Hin*d III digested λ-phage DNA was used as a size marker. **B**. Five primer pairs used for PCR with region of alignment and expected size of amplified products. **C**. PCR amplification of genomic DNA from IGROV1 and CABA I cells using primers in B.

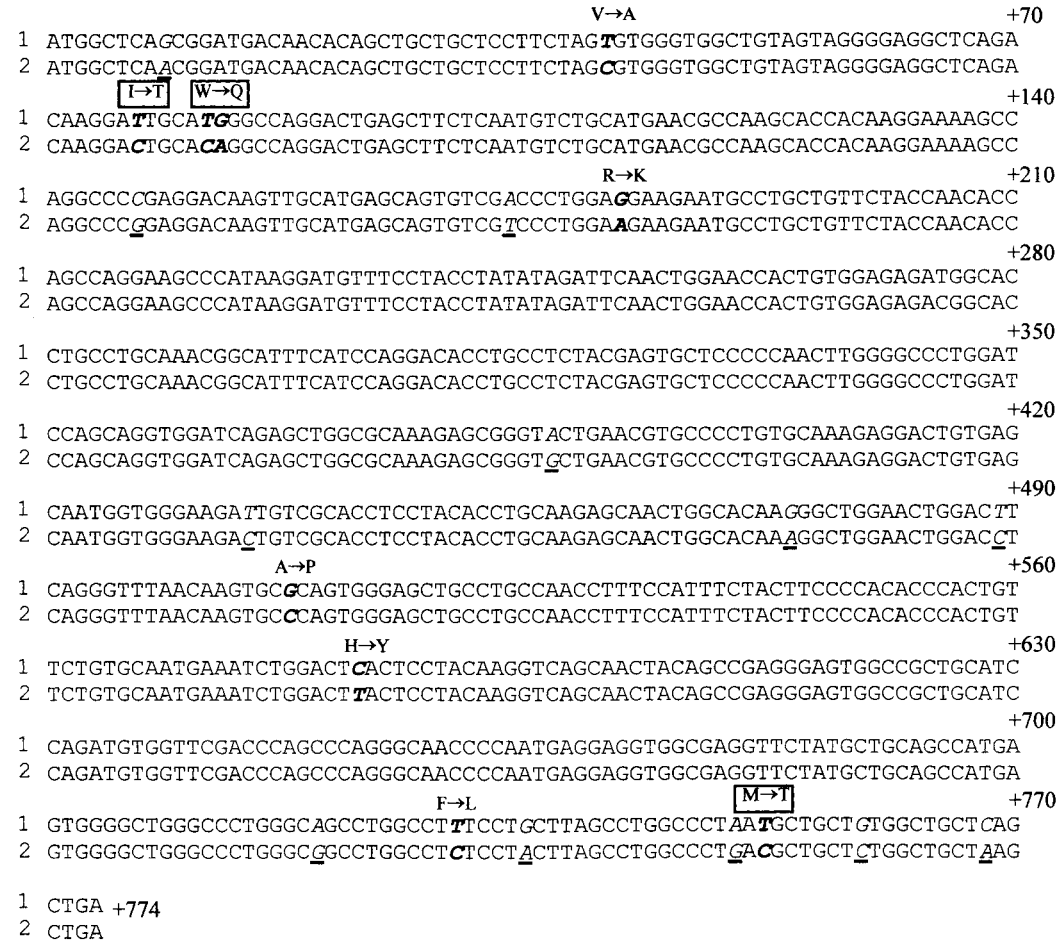

Fig. 3. Sequence of the CABA I αFR. The nucleotide sequence of the αFR ORF cloned from CABA I cDNA (2) is aligned with the wild-type αFR sequence (Gene Bank, accession number U20391) (1). Silent mutations are underlined; improper mutations are in bold, and amino acid substitutions are indicated with the single letter code, boxed in the case of class change.

(Fig. 3). Consistent with the results of Southern analysis, the *Pst*I site inside exon 7 was not mutated; however, within exon 7 beyond the stop codon, numerous mutations were detected (data not shown). Parallel sequencing of the cloned IGROV1 αFR cDNA confirmed the published sequence of the ORF (not shown).

Nine of the 21 CABA I αFR point mutations were missense, leading to eight amino acid substitutions (the 3rd and 4th mutations were in the same codon), and none caused a frameshift. The following eight amino acid substitutions were found: Val14→Ala, Ile26→Thr, Trp28→Gln (exon 4 mutations), Arg61→Lys (exon 5 mutation), Ala171→Pro, His195→Tyr, Phe244→Leu, and Met251→Thr (exon 7 mutations). In positions 26, 28, and 251, a hydrophobic amino acid was substituted with a polar species.

Folate Binding on CABA I Cells

While cell surface expression levels of αFR on CABA I cells were about one third of those on IGROV1 cells, [^{3}H]FA binding at 0°C on CABA I cells was only about 10% of the corresponding binding on IGROV1 cells (Fig. 4). Moreover, internalization of [^{3}H]FA, measured as the ratio between the acid-resistant and the total bound ligand, was greatly impaired in CABA I cells. Indeed, at 0 and 37°C CABA I cells internalized the same small amount of FA (<10%), while internalization of [^{3}H]FA by IGROV1 cells at 37°C was about sevenfold greater than that at 0°C (ca. 32 vs. 5%). Furthermore, competition of cold FA with [^{3}H]FA did not reflect the ratio between the cold and radiolabeled species in CABA I cells, unlike the competition observed with IGROV1 cells (data not shown). Together,

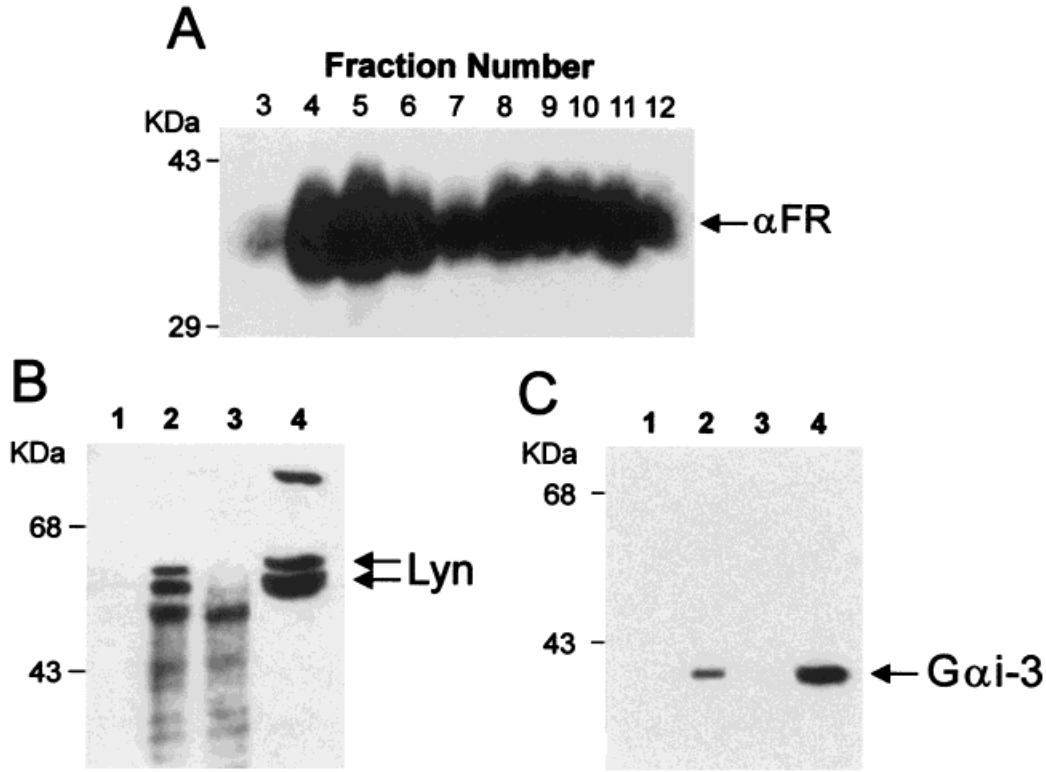

Fig. 4. Partitioning of αFR expressed on the CABA I cell membranes. **A**. TX-100 lysate was separated on a 5–30% linear sucrose gradient and 100 μl of each 1-ml fraction collected was analyzed by 10% SDS-PAGE. Nitrocellulose-transferred proteins were analyzed by Western blot for distribution of αFR with MOv19 MAb (low-density fractions, 3–7; high-density fractions, 8–12). **B** and **C**. Low-(**lane 2**) and high-(**lane 3**)density fractions from the sucrose gradient were pooled and incubated with anti-αFR MAb MOv19 bound to antimouse IgG-conjugated magnetic beads. Separated proteins were analyzed by 10% SDS-PAGE and Western blot for the presence of lyn and $G\alpha_{i-3}$. Total material before immunoprecipitation (**lanes 1** and **4**) represents of that used for immunoseparation.

these results suggest that the αFR protein produced by CABA I cells has a reduced affinity for folates.

Folate Binding on CABA I- and IGROV1- αFR Stable CHO Transfectants

To directly compare the ability of the IGROV1 and CABA I αFR to bind folates, CHO cells were stably transfected with the pCR3.1 vector containing the CABA I or IGROV1 αFR cDNA, or with an empty vector. αFR membrane expression on αFR_{IGROV1}-transfected CHO cells was comparable to that of $\alpha FR_{CABA\ I}$ transfectants (Fig. 4). Folate binding assay, in which cells were incubated with 10 nM [^{3}H]FA either at 0 or 37°C, revealed no detectable binding of [^{3}H]FA in mock-transfected cells (not shown), and only low-level binding by $\alpha FR_{CABA\ I}$ transfectants at 37°C, whereas αFR_{IGROV1} transfectants bound 40-fold more FA at either 0 or 37°C (Fig. 4).

Partitioning of CABA I αFR Into Lipid Rafts

We previously reported that 40–60% of the αFR from IGROV1 cell partition in low-density membrane microdomains, forming macromolecular complexes together with p53–56 lyn and the Gαi-3 subunit of heterotrimeric G-proteins [Miotti et al., 2000]. Separation of Triton-insoluble CABA I lipid rafts by sucrose gradient, to determine whether the presence of point mutations in the CABA I αFR might affect αFR membrane distribution and molecular associations, demonstrated that this mutated αFR maintains the reported distribution both in low- and high-density fractions (Fig. 5A). Also, as previously found in IGROV1 cells [Miotti et al., 2000], immunoprecipitation with anti-αFR MAb MOv19 showed enrichment of p53–56 lyn and the Gαi-3 subunit (Fig. 5B, C) in low-density immunoprecipitates, confirming the association with signaling molecules. No copre-

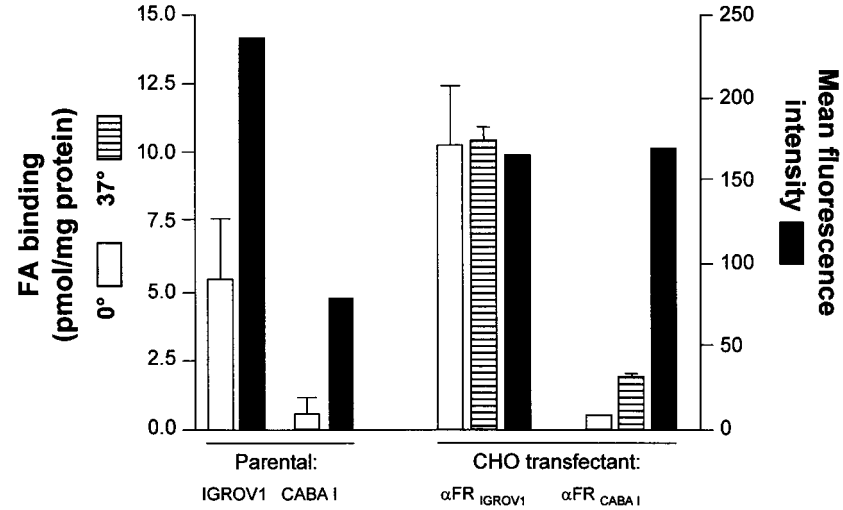

Fig. 5. FA binding and αFR expression on parental cells (IGROV1 and CABA I) and transfected (αFR_{IGROV1}- and $\alpha FR_{CABA\ I}$) CHO cells. Left *y*-axis: [^{3}H]FA binding measured at 0°C (open bars) and 37°C (stripped bars). Data are given as mean (SE) of four separate experiments carried out in triplicate in the case of parental cells, and as mean (SD) of a single experiment carried out in triplicate, with a second independent experiment giving similar results in the case of transfected cells. Right *y*-axis (solid bars): surface αFR expression, measured as immunofluorescence with MOv19 MAb using the FACScan.

cipitation was observed in high-density samples. No αFR immunoprecipitation was observed with the control anti-HLA antibody (not shown).

DISCUSSION

In this study, we provide evidence of genetic rearrangements/mutations and point mutations in the ORF of the αFR gene in the ovarian carcinoma cell line CABA I. The alteration in the αFR protein sequence in CABA I cells strongly impaired binding and internalization of FA but did not affect the membrane distribution or association with cytoplasmic signaling molecules.

Molecular analysis showed that the regions likely to be involved in the rearrangements/mutations are between and downstream of the two transcriptional regulatory sequences. Indeed, P1- and P4-reporter gene constructs are efficiently transcribed in CABA I cells (our unpublished observations), consistent with our recent finding that αFR transcription is regulated in a tissue- and ovarian carcinoma-specific manner [manuscript in preparation]. The presence of genetic rearrangements in the αFR gene would be consistent with its location in 11q13, the site of many disease-associated genes such as MEN-1, FGF 4, and INT 2, including tumor suppressor genes. Moreover a correlation has been reported between chromosome instability and various carcinomas [Yang et al., 1999; Bockmuhl et al., 2000] and other cancers [Campo et al., 1999]. Further, genomic PCR analysis of shorter fragments in the region spanning exons 1–5 and obtained sequences amplified would lead to a better definition of the nature of the observed alterations of the αFR gene.

The αFR protein has been characterized as a marker of ovarian carcinoma, and its role in folate internalization and signal pathway transduction has been investigated [Miotti et al., 2000], together with its gene transcriptional regulation. The contribution of αFR to folate internalization gives cells a growth advantage in a low-folate environment [Bottero et al., 1993], and the role of the receptor in proliferation control is suggested by its physical association with mitogenic signal transducers such as lyn and G proteins. Sequence mutations apparently affect neither the membrane distribution of CABA I αFR nor its association with signal transduction molecules, raising the possibility that the role of αFR in transducing stimuli might be relevant for the transformed epithelial cells of the ovary.

Ligand-binding analysis of CABA I αFR functionality suggested a reduced affinity for FA as compared to that of the IGROV1 αFR. Indeed,

even considering that expression levels of the protein were about one third of those on the reference cell line, both total binding and competition were drastically reduced.

Ectopic transfection of the αFR cDNAs cloned from CABA I and IGROV1 cells into an αFR-nonexpressing cell line enabled direct comparison of the binding ability of the two αFR forms in the same cellular context. Again, the folate-binding capability of CABA I αFR was found to be impaired. In the transfected cells, the difference between the binding capabilities of the two αFRs was further increased: at 0°C, the IGROV1 αFR exhibited a higher FA binding suggesting that in the parental cells, this capability was regulated by the membrane/cellular environment. In contrast the affinity of the CABA I receptor for folates in the transfected cells was similar to, or even lower than in parental cells. These results further support the notion that the impaired folate-binding capability of the CABA I αFR is due to the mutations in the ORF.

Few reports have addressed the identification of functional domains in the αFR protein. Orr and Kamen [1995] described a nonfunctional mutant with a Ser→Pro substitution at position 67, which was completely unable to bind FA. We recently reported that the region spanning amino acids 233–237 is required for GPI processing of the receptor and anchoring to the membrane [Tomassetti et al., 1999]. The eight missense mutations detected in the CABA I αFR ORF in the present study appear to be distributed along the sequence with a slight preferential localization in the N- and the C- termini of the protein. Two mutations (Phe244→Leu and Met251→Thr) are near the stretch required for GPI anchorage, however, they do not affect GPI processing since the protein is released by treatment with phosphatydilinositol phospholipase C (data not shown). One of the mutations in the N-terminal the third of the chain (Arg61→Lys) is near the site reported by Orr and Kamen [1995], and in two others, at positions 26 (Ile→Thr) and 28 (Trp→Gln), a hydrophobic amino acid was replaced with an uncharged hydrophilic species. Such changes in the amino acid class might lead to an altered tertiary structure and, in turn, impaired ligand binding. Based on our data and those of others [Orr and Kamen, 1995], we speculate that the N-terminal region might be responsible for αFR ligand binding.

In light of the more recent publications, αFR expression might be considered related to tumor transformation. Indeed, we have previously found that folate internalization in ovarian cancer cells is mediated by cooperation between the αFR and the reduced folate carrier [Miotti et al., 1997]. On the other hand, the association of αFR with G proteins and the Src-family member lyn indicated that αFR could generate signals in ovarian cancer cells [Miotti et al., 2000]. More recently, we demonstrated the inverse correlation between αFR and caveolin expression. In fact, loss of caveolin expression has been associated with tumor transformation since it can recruit signaling molecules in an inactive form [Bagnoli et al., 2000]. The present findings on CABA I cells further support the hypothesis that αFR expression is not induced in ovarian cancer cells in connection with folate internalization. For this reason, investigations are underway in order to evaluate in ovarian cancer specimens, changes in the αFR gene and proteins found in CABA I cells. Furthermore, the CABA I cell line, with its mutated αFR, might provide an excellent tool to further explore the relationship between amino acid sequence and functional domains of this protein.

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