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Interaction of folate receptor with signaling molecules lyn and $G\alpha_{i-3}$ in detergent-resistant complexes from the ovary carcinoma cell line IGROV1

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SUMMARY

Using as a model the ovary carcinoma cell line IGROV1, we analyzed the partitioning of the glycosyl-phosphatidylinositol-anchored folate receptor into lipid rafts based on its relative detergent insolubility, with a focus on physically and functionally associated signaling molecules. A variable amount (40-60%) of folate receptor was found in low-density Triton X-100 insoluble complexes together with subunits of heterotrimeric G-proteins and the src-family non-receptor tyrosine kinases p53-56 lyn. In the same fraction the structural component of caveolae, caveolin, was not detected at the protein level, although the corresponding mRNA was detected in trace amounts. Comodulation of folate receptor and signalling molecules was observed in the detergent-insoluble complexes during cell proliferation or induced by phosphatidylinositol-specific phospholipase C treatment or by interaction with anti-folate receptor monoclonal antibodies. Moreover, complexes of folate receptor, lyn and the $G\alpha_{i-3}$ subunit were immunoprecipitated using either anti-folate receptor or anti-lyn antibodies. In vitro kinase assay of the immunoprecipitates revealed stimulation of phosphorylation of common and specific proteins. In particular, the p53 form of lyn appeared to be enriched and phosphorylated in the anti-folate receptor MOv19 monoclonal antibody immunoprecipitate, whereas a 40 kDa band common to anti-folate receptor and anti-lyn immunoprecipitates was the phosphorylated form of the $G\alpha_{i-3}$ subunit. These findings point to the functional interaction between folate receptor and associated signaling molecules.

Key words: Triton-insoluble domain, GPI-linked protein, p53-56 lyn, Folate receptor, Heterotrimeric G protein

INTRODUCTION

A new dynamic model of the plasma membrane was recently proposed, in which clusters of sphingolipids, cholesterol and specific proteins move as rafts within the fluid bilayer (Simons and Ikonen, 1997). The use of various approaches (Song et al., 1996; Schnitzer et al., 1995; Smart et al., 1995; Chang et al., 1994; Brown and Rose, 1992), based on the biochemical and biophysical properties of these membrane domains, has led to the identification of specialized membrane regions that correspond to classically defined plasmalemma caveolae, characterized by the presence of the structural marker protein caveolin (Rothberg et al., 1992). However, membrane fractions with the same properties (caveolae-like) but devoid of caveolin have also been identified (Liu et al., 1997; Wu et al., 1997; Gorodinsky and Harris, 1995; Schnitzer et al., 1995; Fra et al., 1994). Lipid modified-proteins, including glycosylphosphatidylinositol (GPI)-anchored species, are generally enriched in these membrane regions (Anderson, 1998; Sargiacomo et al., 1993; Ying et al., 1992), suggesting that compartmentalization serves in regulating their activity. Increasing evidence points to the involvement of GPI-proteins in complex patterns of interactions in which they can exert different roles, including a costimulatory effect on T cell receptor activation (Robinson, 1991), a receptor-like behavior on cells of the nervous system (Buj-Bello et al., 1997; Klein et al., 1997; Peles et al., 1997) and a ligand effect, when in released form, on transmembrane receptors (Park and Sanchez, 1997). A key determinant of functional activity of GPI-proteins (Hanada et al., 1995; Rothberg et al., 1990) appears to rest in the cholesterol enrichment characteristic of lipids rafts (Schroeder et al., 1998), and, as shown in T lymphocytes, cholesterol depletion can negatively influence GPI-protein-mediated signal transduction (Stulnig et al., 1997). Indeed lipid rafts contain the machinery for cell signaling, including heterotrimeric G proteins, members of the src-family of non-receptor tyrosine kinases (NRTK), prenylated proteins and signal transducers (for review see Anderson, 1998; Okamoto et al., 1998), but specific activation pathways of signaling molecules downstream GPI-proteins have not been identified, except for urokinase plasminogen activator receptor (uPAR; Dumler et al., 1998; Koshelnick et al., 1997).

Rothberg et al. (1992), provided the first biochemical demonstration that the GPI-protein folate receptor (FR) is associated with caveolae in MA104 cells where functional coupling of FR with a reduced folate carrier (RFC) was thought

to provide a high-affinity route of folate internalization by a mechanism called potocytosis (Anderson et al., 1992; Kamen et al., 1991). The FR is overexpressed in human ovarian carcinoma cells (Coney et al., 1991; Alberti et al., 1990; Miotti et al., 1987) but is only partially responsible for folate internalization since another mechanism with the characteristics of RFC is present in these cells (Miotti et al., 1997).

No data are available on the interaction of FR with signaling molecules, but it has been suggested that FR affects cell proliferation not only by mediating folate uptake, but also by generating other regulatory signals (Antony, 1996). We have shown that FR expression provides a growth advantage to FR-transfected cells in vitro and in vivo (Bottero et al., 1993). Since ovarian tumor cells constitutively display high levels of the receptor (Miotti et al., 1987) and in tumor specimens FR overexpression has been associated with tumor progression (Toffoli et al., 1997), ovarian carcinoma offers an attractive model to study the possible functions of FR other than folate internalization. Using the well-characterized IGROV1 carcinoma cell line (Miotti et al., 1995, 1997; Coney et al., 1991), we analyzed the partitioning of FR into lipid rafts based on its relative detergent insolubility and examined molecules that might physically and functionally associate with FR in order to elucidate the involvement of the receptor in signal transduction mechanisms.

MATERIALS AND METHODS

Materials, antibodies and cell lines

Phenylmethylsulfonyl fluoride (PMSF), aprotinin, iodoacetamide (IAA), Triton X-100, 2-[*N*-morpholino]ethane-sulfonic acid (MES), HEPES buffer, Na_3VO_4 and cold folic acid (FA) were from Sigma (St Louis, MO); leupeptin and octyl-β-glucoside from Boehringer-Mannheim (Germany).

Murine monoclonal antibodies (mAb) MOv18 and MOv19, produced in our laboratory (Miotti et al., 1987) and recognizing two non-competing epitopes of FR, and anti-HLA class I mAb W6/32 from the American Tissue Culture Collection (ATCC, Rockville, MD), were purified as described (Miotti et al., 1995). The anti-FR mAb LK26 was a gift from Dr K. O. Lloyd (Memorial Sloan-Kettering Cancer Center, New York, NY). Chimeric MOv19 (CHI-19; Coney et al., 1994) was kindly supplied in purified form by L. Coney (Apollon, Malvern, PA). Rabbit polyclonal anti-uPAR was kindly provided by Dr F. Blasi (DIBIT, S. Raffaele, Milan, Italy). Commercially obtained antibodies were as follows: rabbit anti-$G\alpha_{i\text{-}3}$, α_{q11}, $\alpha_{i,o,z,t}$, α_s, β-subunit, lyn, fyn, c-yes (Santa Cruz Biotechnology, Santa Cruz, CA); anti-c-src mAb (Oncogene Sciences, Manhasset, NY); rabbit anti-lck, lyn, fyn, and anti-phosphotyrosine mAb (Upstate Biotechnology Inc., Lake Placid, NY); anti-caveolin mAb (clone C060 and 2234; Transduction Laboratories, Lexington, KY); and rabbit anti-β actin (Sigma).

IGROV1 cells (Dr J. Benard, Institut G. Roussy, Villejuif, France) were maintained in folate-deficient RPMI 1640 medium (L-RPMI) containing 20 nM FA supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine and 100 U/ml gentalyn (Miotti et al., 1995). A431 human vulval carcinoma cells (obtained from ATCC) were mantained in standard RPMI 1640 (Sigma) supplemented as above described.

Detergent solubilization

Cells grown to confluence in a T25 flask were rinsed twice with phosphate-buffered saline (PBS) and treated with lysis buffer (50 mM Tris-HCl, pH 7.4, or 25 mM MES, pH 6.5, plus 0.15 M NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM PMSF, 0.24 U/ml aprotinin, 5 μg/ml leupeptin, 1 mM Na_3VO_4) containing 1% Triton X-100 for 1 hour at 0°C. Detergent lysates were clarified by centrifugation at 4°C (15,000 ***g*** for 15 minutes) and the supernatant (TX-100 S) recovered. For separation of the TX-insoluble (TX-100 I) fraction, pellets were solubilized for 30 minutes at 0°C in lysis buffer as above containing 1.1% octyl-β-glucoside (OG) and the supernatant recovered by centrifugation. Alternatively, TX-100 lysates were ultracentrifuged (100,000 ***g*** for 1 hour) at 4°C and the pellet solubilized by 1.1% OG as above described. Protein concentration of TX-100 S fraction was determined by BCA protein assay (Pierce, Rockford, IL).

Sucrose gradient

Sucrose gradients were prepared according to reported protocols (Sargiacomo et al., 1993). Cells were grown to confluence in T75 flasks and 1 to 3 flasks were used for each gradient, depending from the experiment. After three washes with ice-cold PBS, 1 ml of ice-cold lysis buffer (25 mM MES, pH 6.5, 0.15 M NaCl, 1% Triton X-100 plus 0.75 mM PMSF) was added. Cells were collected with a scraper and the flask washed with an additional 1 ml of lysis buffer. The pool (1 ml + 1 ml) was brought to 40% sucrose by addition of 80% sucrose prepared in MES-saline and placed at the bottom of an ultracentrifuge tube. A 5-30% linear sucrose gradient (4 ml + 4 ml) in MES-saline lacking Triton X-100 and PMSF was formed above the lysates and centrifuged at 200,000 ***g*** for 17 hours in a SW41 rotor (Beckman, Palo Alto, CA); 1-ml fractions were collected from the top of the gradient. For immunoseparation experiments, 10 mM IAA was added to all buffers.

Western blot analysis

Cell lysates and/or gradient fractions were separated on 10% SDS-PAGE (Laemmli, 1970) slab gels and blotted to nitrocellulose membranes (Hybond C-Super, Amersham). Blots were saturated with Blotto (5% non-fat dried-milk in PBS containing 0.1% Tween-20). After incubation with the primary antibody in Blotto at the dilution recommended by the manufacturer, sheets were incubated with relevant biotinylated secondary antibodies (Amersham) diluted in the same solution and then with horseradish peroxidase (HRP)-streptavidin in PBS. For the analysis of immunoseparated fractions, (see below) secondary HRP-conjugated antibodies were used. The reaction was developed by ECL (Amersham). Biotinylated protein molecular size markers for ECL (range 14.3 to 97.4 kDa) were from Amersham. The reactivity of anti-FR and anti-HLA class I mAbs only was evaluated on unreduced samples.

Immunoprecipitation on TX-100 I fraction

Immunoprecipitation was carried out using agarose anti-phosphotyrosine (Santa Cruz; 20 μl/sample) and anti-lyn and anti-fyn antibodies bound to Protein A-Sepharose (Pharmacia Biotech AB, Uppsala, Sweden; 1 μg Ab/20 μl Protein A-Sepharose/sample). Beads were saturated with 0.25% gelatin in PBS prior to samples incubation overnight at 4°C with shaking. After removing unbound proteins by centrifugation, beads were washed twice with buffer containing 1 M NaCl, 0.02 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Triton X-100, 0.24 U/ml aprotinin, 1 mM PMSF, and once with the same buffer without NaCl. The precipitated molecules were extracted using SDS-PAGE sample buffer and boiling 5 minutes at 95°C, then analyzed by western blot as described above.

Immunoseparation of FR-containing membranes and kinase assay

The purification procedure was carried out essentially as described by Doyle et al. (1998). Briefly, goat anti-mouse and sheep anti-rabbit magnetic beads (Dynal AS, Oslo, Norway) were incubated for 2 hours at room temperature with mAbs (MOv18, MOv19, and anti-HLA class I) and polyclonal rabbit antisera (anti-uPAR, anti-lyn and anti-

fyn), respectively, 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 (4-6) fractions from sucrose gradient, diluted 1:2 with 50 mM Hepes buffer, pH 7.4, containing 0.75% TX-100, protease inhibitors and 10 mM IAA. BSA 1% (40 μl) was added to each sample during overnight incubation at 4°C in rotation. To separate bound from unbound material, beads were washed once with the above buffer, then 4 times with PBS containing 0.1% BSA, 10 mM IAA and protease inhibitors (10 minutes/wash). Eluted bound material was analyzed by 10% SDS-PAGE and western blot.

Alternatively, 10 μCi of [γ-^{32} P]ATP (spec. act. >5000 Ci/mmol; Amersham) were added to beads carrying the immunoisolated membranes, resuspended in 71 μl of Hepes buffer (see above), plus 8 μl of 10× kinase buffer (200 mM Hepes, pH 7.4, 100 mM $MgCl_2$, 200 μM ATP, 10 mM DTT) and incubated for 30 minutes at 30°C. The reaction was stopped by adding 80 μl of hot SDS-PAGE sample buffer to the beads and heating for 5 minutes at 95°C.

In re-immunoprecipitation experiments, after the kinase reaction, samples were boiled for 5 minutes in Hepes buffer with 2% SDS, then diluted 1:20 in the same buffer without SDS. A second round of immunoprecipitation was carried out for 3 hours at 4°C, with antibodies bound to magnetic beads, as before described. After 3 washings with PBS containing 0.1% BSA, bound material was eluted. All samples were analyzed by SDS-PAGE on 8% slab gels, using [^{14}C]methylated protein as molecular size markers (range from 14.3 to 200 kDa; Amersham). Proteins transferred onto nitrocellulose sheets were analyzed by exposure in a Phosphoimager and by immunoblot.

Northern blot analysis

Total RNA was purified from IGROV1 and A431 cell lines with RNeasy kit (Qiagen Gmbh, Hilden, Germany) essentially as described by the manufacturer. RNA (15 μg) was electrophoresed on 1% agarose-formaldeyde denaturing gel and transferred to a nylon membrane (Hybond N+, Amersham) by capillary blotting in 20× standard saline citrate (SSC). Both prehybridization and hybridization were carried out in 50% formamide, 5× Denhardt's solution, 1% SDS, 150 μg/ml sonicated salmon sperm and 5× SSC. Full-length caveolin cDNA (from A431 cell) and human β-actin cDNA were labeled with 5′-[α-^{32}P]dCTP by random priming (Boehringer). Filters were hybridized with the ^{32}P-labeled probes (2×10^6 cpm/ml) overnight at 42°C, washed with 0.1× SSC, 0.1% SDS at 65°C, and exposed to Hyperfilm MP (Amersham) with an intensifying screen at –80°C. The ratio of caveolin and human β-actin mRNA was evaluated by densitometry of relevant bands using Eagle Eye II Image Capture and Software analysis system (Stratagene, La Jolla, CA).

Analysis of FR distribution in cultured cells

To evaluate the FR distribution in TX-100 S and I fractions, cells were incubated 4 days in medium without FA containing 5% FCS, and incubated with 10 nM [^{3}H]FA (spec. act. 23 Ci/mmol, Amersham) in PBS containing 20 mM Hepes for 1 hour at 37°C. Cells were lysed in the presence of an excess (1 μM) of cold FA and TX-100 S and I fractions separated as described. Radioactivity contained in each fraction was measured by a β-counter.

To examine the effect of FR removal from the cell membrane, adherent cells were treated with 50 mU/ml of recombinant phosphatidyl-inositol specific phospholipase C (PI-PLC) from *Bacillus thuringensis* (GPI-02B; Oxford Glycosystem, Abingdon, UK) in L-RPMI medium for 1 hour at 37°C before detergent solubilization. Control cells were incubated for the same time and temperature in the absence of the enzyme (Alberti et al., 1990).

Changes in FR membrane distribution were evaluated on: (i) cells at different times of growth, (ii) subconfluent cells, starved overnight in folate- and serum-free L-RPMI medium and incubated for 10 minutes at 37° C with 10 μg/ml mAbs (MOv18, MOv19, CHI-19, anti-HLA class I). After two washings with ice-cold PBS, solubilization was carried out as described. The effect of anti-FR mAbs on internalization of 10 nM [5-*methyl*-^{3}H]tetrahydrofolate ([5-*methyl*-^{3}H] THF; spec. act. 33 Ci/mmole; Moravek Biochemicals, Brea, CA) was evaluated on cells incubated for 1 hour with the mAbs and then for 1 hour with the radiolabeled folate. The [5-*methyl*-^{3}H]THF internalized, acid-resistant fraction was evaluated by acid wash of the cells as described (Miotti et al., 1997). To discriminate between FR and RFC-mediated uptake, control cells were preincubated for 1 hour at 37°C with 1 μM cold FA, which inhibits the binding of radiolabeled 5-*methyl*-THF to FR.

RESULTS

Characterization of FR containing Triton-insoluble domains

The association of FR to lipid rafts resistant to 1% TX-100 solubilization at low temperature was evaluated in IGROV1 cells. Based on the [^{3}H]FA distribution in the soluble (S) and insoluble (I) fractions, 40 to 60% of FR was found in the TX-100 S fraction and the remaining radioactivity was almost completely solubilized by a second treatment with OG (TX-100 I fraction). Immunoblotting with specific anti-FR mAb confirmed nearly equal levels of FR in TX-100 S and I, while HLA class I antigen, used as a transmembrane control protein, was completely solubilized by the first detergent and detected only in the S fraction (Fig. 1a).

Western blot analysis using a panel of antibodies that recognize members of src-family NRTK (src, yes, lyn, fyn and lck), revealed a unique distribution of p53-56 lyn in the S and I fractions, whereas the other members were detected only in the S fraction or, in the case of fyn, not at all (Fig. 1a). Ultracentrifugation of the TX-100 cell lysates allowed us to detect in the pellet the presence of yes, whereas no changes in the distribution of the other kinases was observed. In particular the amount of lyn was not further increased in the TX-100 I fraction following ultracentrifugation (unshown results). The heterotrimeric G protein subunits G$\alpha_{i\text{-}3}$, α_{q11}, $\alpha_{i,o,z,t}$ and β were distributed in the S and I fractions, whereas no reactivity with the α_s subunit was observed. By western blot no reactivity at all was observed with anti-caveolin mAbs C060 and 2234 (data not shown).

Northern blot analysis of caveolin mRNA content in total RNA extracted from IGROV1 and A431 cells (Fig. 1b) indicated that IGROV1 cells produce only trace amounts of caveolin message (4.6% of the positive A431 control).

Analysis of phosphorylated protein content using anti-phosphotyrosine (PTyr) mAb revealed that while the TX-100 S fraction was enriched in many tyrosine-phosphorylated proteins (Fig. 2, lane 1), a very restricted pattern was present in the TX-100 I fraction (lane 2), including only three major bands of 53, 56 and 59 kDa, respectively. A faint 120 kDa band, more intense when cells were confluent (data not shown), was also observed. Immunoprecipitation of TX-100 I with anti-p53-56 lyn followed by immunoblotting with anti-PTyr (lane 4) demonstrated that the two 53 and 56 kDa bands correspond to this src-family NRTK. The 59 kDa band was immunoprecipitated by anti-PTyr (lane 3) but not by anti-lyn antibodies (lane 4), whereas no precipitation at all was observed with control anti-fyn antibodies. Immunoblotting

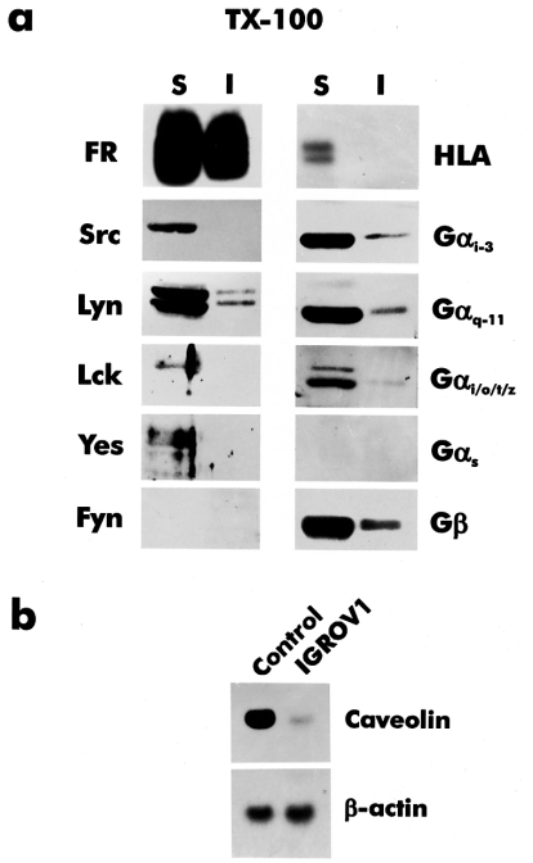

Fig. 1. Characterization of proteins associated with TX-100 insoluble fraction. (a) TX-100 S and I fractions were separated by 10% SDS-PAGE, blotted on nitrocellulose, and immunostained with different antibodies recognizing membrane proteins, such as FR and HLA class I, and a panel of cytoplasmic signaling molecules. For each lane, 30 μg of TX-100 S or a corresponding volume of TX-100 I fraction was loaded. (b) Northern blot analysis of caveolin mRNA was performed on RNA extracted from IGROV1 cells. A431 cells were used as positive control. Total RNA was separated on an agarose gel and probed for caveolin mRNA content. The amount of loaded RNA was standardized on β-actin mRNA.

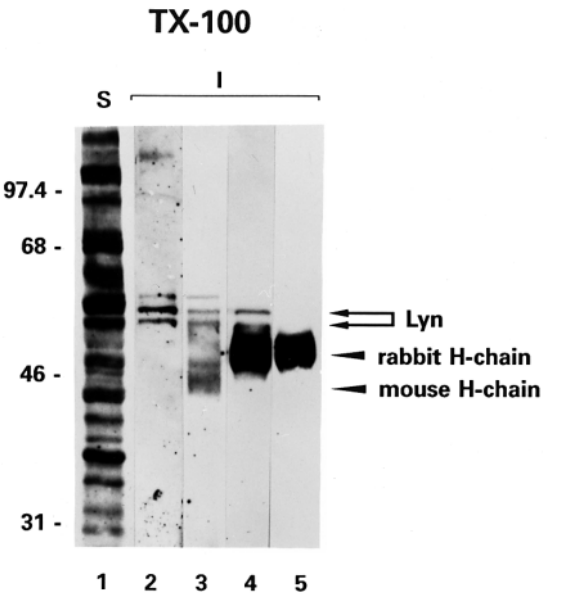

Fig. 2. Tyr-phosphorylation of proteins in TX-100 S and I fractions. IGROV1 cell were solubilized in the presence of 1 mM Na_3VO_4 and the pattern of Tyr-phosphorylated proteins was analyzed by western blot with anti-PTyr mAb. Samples of TX-100 S and I fractions were analyzed as total proteins (lanes 1 and 2, respectively); TX-100 I proteins were also immunoprecipitated with anti-PTyr mAb (lane 3), anti-lyn (lane 4) and, as a control, with anti-fyn antibodies (lane 5) before immunoblotting. H chains of antibodies used for immunoprecipitation and recognized by biotinylated goat anti-mouse Ig are indicated.

with anti-lyn antibodies showed reactivity only with the 53 and 56 kDa bands both in anti-PTyr and anti-lyn immunoprecipitates (data not shown).

FR and signaling molecule redistribution

In light of the changes observed in the relative solubility and distribution of FR in different experiments, we analyzed whether association of FR to TX-100 I lipid rafts might be modulated and whether such modulation might also affect the distribution of other associated molecules.

In proliferating cells, tested at different times of growth (Fig. 3a), total protein increased while the amount of FR present in the TX-100 I fraction decreased progressively from days 2 to 7, as did p53-56 lyn and, although less markedly, $G\alpha_{i-3}$ subunit (Fig. 3b).

Exploiting the IGROV1 cell FR-sensitivity to treatment with bacterial GPI-specific PLC (Alberti et al., 1990), we used this enzymatic digestion to establish whether an intact GPI-linkage is critical for FR interaction with cytoplasmic molecules. After enzyme treatment, the amount of FR was markedly reduced in both TX-100 S and I fractions (Fig. 4). By contrast, a clear decrease in lyn and $G\alpha_{i-3}$ content was observed only in the TX-100 I fraction while both were increased in the TX-100 S fraction.

The potential effect of ligand interaction on FR distribution was analyzed using two non-crossreacting anti-FR mAbs, MOv18 and MOv19 (Fig. 5a). FR localization in the TX-100 I fraction was slighty increased or not affected by MOv18 and anti-HLA class I mAb binding, respectively, whereas mAb MOv19, in both murine and chimeric form, induced mobilization and 30-50% reduction of the receptor in TX-100 I rafts (Fig. 5a). The same effects on FR redistribution were obtained by using another anti-FR mAb, LK26 (Garin-Chesa et al., 1993) that is partially cross-reacting with Mov19 (unshown results). The distribution of lyn and Tyr-phosphorylated proteins in the TX-100 I fraction was correspondingly increased by MOv18 and decreased by MOv19 interaction (Fig. 5b).

Similar effects on [5-*methyl*-^{3}H]THF internalization were also observed (Fig. 5c). FR-mediated internalization, which is completely inhibited by 1 μM FA, accounts for about 40% of the total FA internalized and an additional mechanism of folate transport is present on these cells (Miotti et al., 1997). MOv19 inhibited 50% of FR-mediated internalization, whereas no reduction in FR-mediated internalization followed the incubation with 5-*methyl* [^{3}H]THF in the presence of MOv18 or anti-HLA mAbs.

Together, these results indicate that FR distribution in particular areas of the membrane can be modulated by different

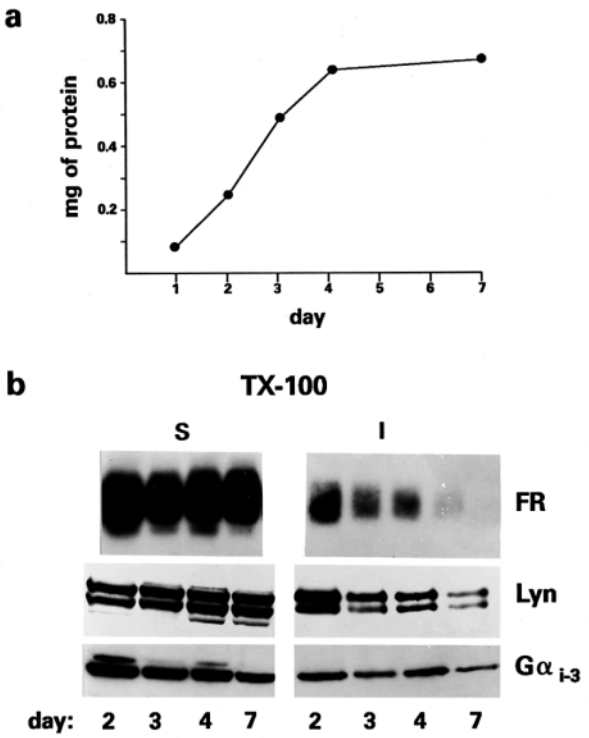

Fig. 3. Effect of cell proliferation on FR distribution. IGROV1 cells were grown for different time periods and solubilised as described to separate the TX-100 S and I fractions. (a) Total protein content of TX-100 S fraction from each day. (b) Western blot analysis of TX-100 S and I samples, separated by 10% SDS-PAGE and probed with different mAbs.

external stimuli, inducing comodulation of associated cytoplasmic proteins.

Immunoisolation of FR-enriched microdomains partitioned on a sucrose gradient

To further analyze the possible interaction between FR and signaling molecules, lipid rafts from IGROV1 cells were separated by sucrose gradient and immunopurified using magnetic beads and anti-FR antibodies. Since IAA reportedly stabilizes complexes of GPI-proteins and enhances the associated kinase activity (Solomon et al., 1998), all buffers were supplemented with 10 mM IAA.

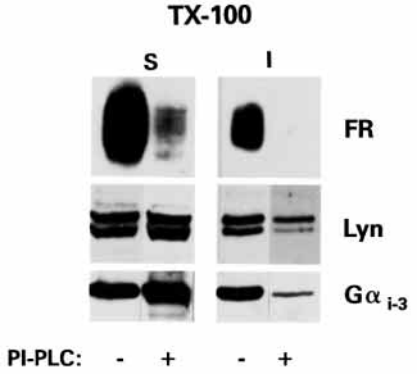

Fig. 4. Effect of PI-PLC-induced FR release on the distribution of TX-100 I associated molecules. Adherent cells were subjected to PI-PLC treatment (50 mU/ml) for 1 hour at 37°C. Treated and untreated control cells were solubilised and TX-100 S and I fractions were analyzed by immunoblotting.

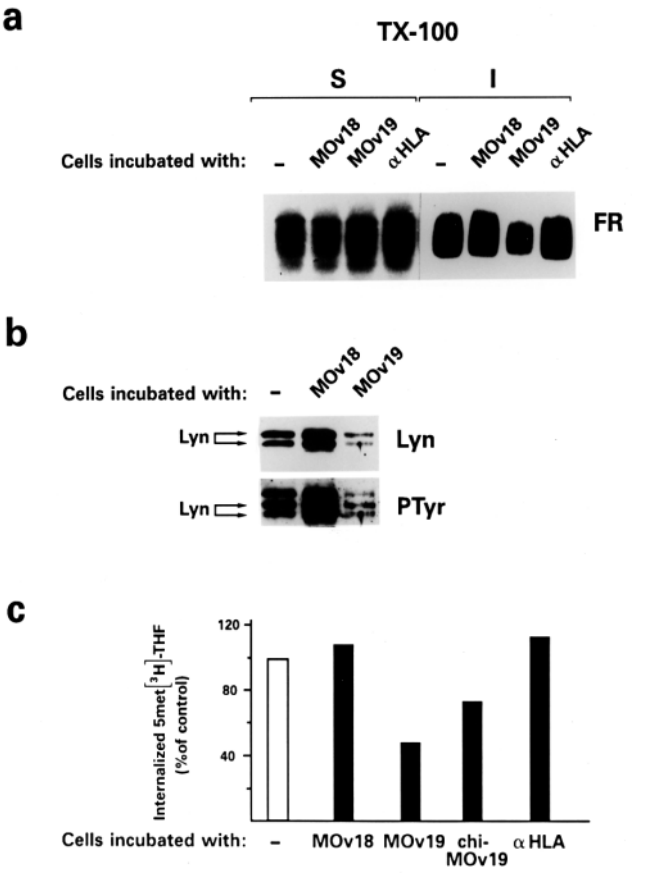

Fig. 5. Effect of ligand interaction on FR distribution and functional activity. IGROV1 cells, starved overnight in RPMI medium without FCS or FA, were incubated for 10 minutes at 37°C with anti-FR or anti-HLA mAbs (10 μg/ml). (a) Cells were solubilised and TX-100 S and I fractions, separated by 10% SDS-PAGE, and immunoblotted with anti-FR mAbs. (b) The effect of mAb MOv18 and MOv19 binding on the distribution of lyn and phosphorylated proteins in the TX-100 I fraction. (c) The effect of mAbs on 10 nM [5-*methyl*-^{3}H]THF internalization. Cells were incubated in the presence of the mAbs for 1 hour at 37°C, and further incubated with 10 nM [5-*methyl*-^{3}H]THF (final concentration) for 1 hour. The amount of internalized folate was evaluated as the acid-resistant fraction (see Materials and Methods). Other cell samples were incubated in the presence of 1 μM cold FA to evaluate the amount of folate uptake due to FR-mediated internalization. Results in c are the mean of two independent experiments.

Immunoblotting analysis of the sucrose gradient fractions for content of specific membrane and cytoplasmic proteins (Fig. 6), revealed FR distributed in both the top (fractions 4-6) and bottom (fractions 8-12) of the gradient, whereas another GPI-linked protein expressed on IGROV1 cells, the uPAR (Mirshahi et al., 1997), was found localized in the bottom fractions only. HLA class I, like β-actin, was homogeneously distributed in fractions 9-12. The distribution of p53-56 lyn and the $G\alpha_{i-3}$ subunit was similar to that of FR in the top fractions (4-6) but homogeneous in fractions 9-12, whereas yes was found essentially in the bottom fractions (8-12) with a peak in

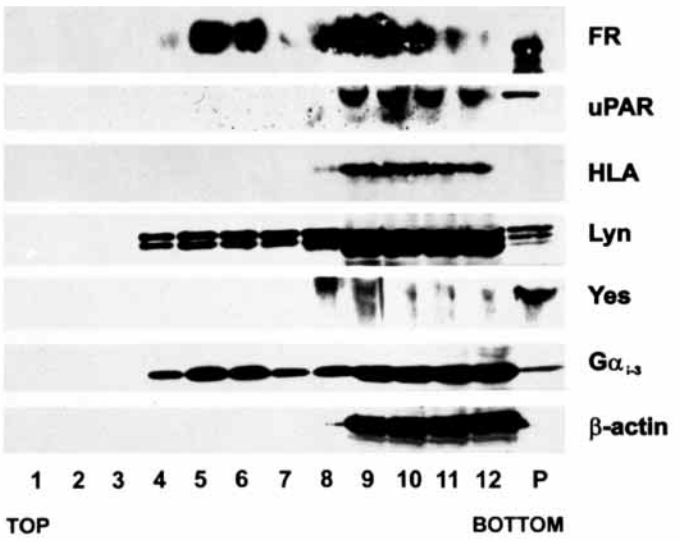

Fig. 6. Sucrose gradient separation of TX-100 lysate from IGROV1 cells. TX-100 lysate was separated on 5-30% linear sucrose gradient and 100 μl of each 1 ml collected fraction were analyzed by 10% SDS-PAGE. Nitrocellulose transferred proteins were analyzed by western blot for distribution of FR and another GPI-anchored protein (uPAR), cytoplasmic signaling molecules (lyn, yes and $G\alpha_{i\text{-}3}$). HLA class I and β-actin were tested as control of transmembrane and cytoplasmic proteins, respectively. P, pellet.

fraction 8. The pellet (P), containing high-density TX-100 insoluble material, showed reactivity with anti-FR, anti-uPAR, anti-lyn, anti-yes and anti-$G\alpha_{i\text{-}3}$ antibodies, but not with anti-HLA or anti-β-actin antibodies. In bottom fractions and in P, as well as in TX-100 S material, three bands of lyn were usually resolved. In top fractions, as well as in fraction 8, only two bands were present (see also TX-100 I in Fig. 1).

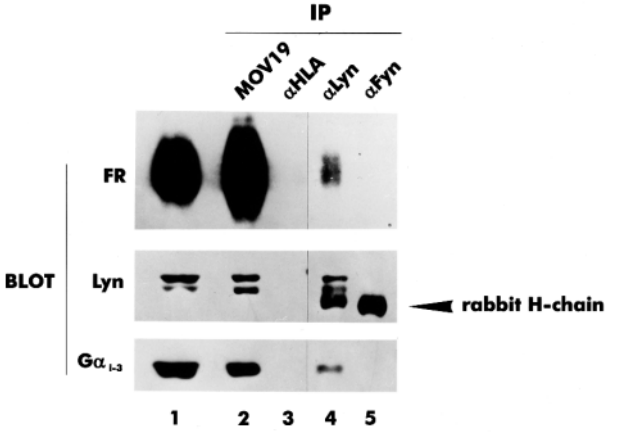

Fig. 7. Immunoseparation of FR containing microdomains isolated by sucrose gradient. Fractions 4-6 from the sucrose gradient were pooled and incubated with anti-FR mAb MOv19 (lane 2) and rabbit anti-lyn polyclonal antibodies (lane 4) bound to anti-mouse and anti-rabbit-conjugated magnetic beads, respectively. Anti-HLA class I mAb (lane 3) and rabbit anti-fyn (lane 5) were used as controls. Membranes immunoseparated with each antibody were analyzed by 10% SDS PAGE and western blot for the presence of FR, lyn and $G\alpha_{i\text{-}3}$. Total material before immunoprecipitation (lane 1) represents 1/10 of that used for immunoseparation.

Immunoseparation of FR-containing low-density membranes was carried out on 4-6 pooled fractions using mAb MOv19 bound to anti-mouse-congugated magnetic beads. Immunostaining of bound material following western blot revealed immunoprecipitation of FR and coprecipitation of p53-56 lyn and $G\alpha_{i\text{-}3}$ (Fig. 7). When a polyclonal anti-lyn antiserum was used for immunoprecipitation, FR was detected in lyn-selected microdomains. No immunoprecipitation using either unrelated antibodies was observed. Control immunoprecipitation by using the anti-uPAR did not show FR coprecipitation (unshown result).

To determine whether the anti-FR mAbs interaction with FR, which differentially influences FR distribution and functional activity (see Fig. 5), also affects signal transduction, an in vitro kinase assay with immunoisolated membranes bound to each anti-FR mAb (MOv18 and MOv19) was carried out in the presence of [γ-^{32}P]ATP (Fig. 8). The most prominent phosphorylated bands common to both the immunoprecipitates (lane 1 and 2) were 120-140, 63, 59 kDa and a strongly phosphorylated, diffuse band around 38-44 kDa. In addition, distinct bands specific for each antibody appeared to be phosphorylated, in particular a 50 kDa band after immunoprecipitation with MOv18 and a 53 kDa band after interaction with MOv19. Immunoblotting with anti-lyn on immunoprecipitates showed that the 53 kDa form of the molecule is better represented in the MOv19 immunoprecipitate after in vitro phosphorylation, as compared to that after immunoprecitation of cold material shown in Fig. 7. A second round of immunoprecipitation by anti-lyn (Fig. 8, lane 6) on dissociated MOv19-precipitate confirmed the presence of phosphorylated lyn in association with FR. This association was less evident in the MOv18 immunoprecipitate (data not shown), possibly due to quantitative differences in the precipitating capacity of the two anti-FR mAbs. Immunoprecipitated phosphorylated lyn (lane 4) was also associated with the prominent 38-44 kDa band.

Immunoblotting with antibodies directed against subunits of heterotrimeric G proteins found in the initial characterization (Fig. 1a) indicated coprecipitation of $G\alpha_{i\text{-}3}$ with both FR (lane 1 and 2) and lyn (lane 4) in association with Gβ subunit. Less intense reactivity of anti-$G\alpha_{\text{-}i/o/t/z}$ and $G\alpha_{q11}$ was also detected by immunoblotting (data not shown). Since $G\alpha_{i\text{-}3}$ has a molecular mass compatible with that of the prominently phosphorylated band found in all of the immunoprecipitates, a second round of immunoprecipitation on MOv19 immunoprecipitate was carried out with anti-$G\alpha_{i\text{-}3}$ (lane 10) and other antibodies recognizing proteins in the 38-44 kDa range (ERK1/2 and p38 MAPK; lane 8 and 9); specific immunoprecipitation was evident with anti-$G\alpha_{i\text{-}3}$ only.

DISCUSSION

In this study, we present evidence that the FR, overexpressed on IGROV1 ovarian carcinoma cells, distributes in low-density membrane microdomains in the absence of caveolin and demonstrate that the receptor is physically associated with the src-family member p53-56 lyn and the $G\alpha_{i\text{-}3}$ subunit of heterotrimeric G-proteins. Further, we find that FR and its associated signaling molecules are comodulated by different stimuli and that lyn and the $G\alpha_{i\text{-}3}$ become phosphorylated in

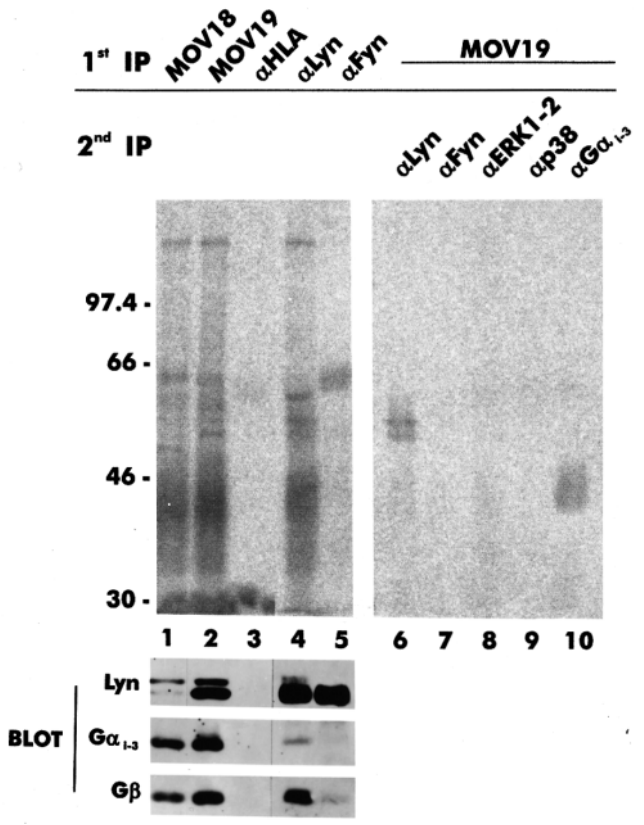

Fig. 8. Kinase assay on immunoseparated microdomains. Fractions 4-6 from the sucrose gradient were pooled and incubated with anti-FR mAb MOv18 (lane 1) and MOv19 (lane 2) or rabbit anti-lyn polyclonal antibodies (lane 4) bound to anti-mouse and anti-rabbit-conjugated magnetic beads, respectively. Anti-HLA class I mAb (lane 3) and rabbit anti-fyn (lane 5) were used as controls. Immunoseparated membranes were subjected to in vitro kinase assay in the presence of [γ-^{32}P]ATP. Phosphorylated proteins were analyzed by 8% SDS-PAGE (lanes 1 to 5) or dissociated from immunoprecipitating antibody MOv19 and subjected to a second round of immunoprecipitation before SDS-PAGE analysis (lanes 6 to 10). Proteins were transferred to nitrocellulose and phosphorylated proteins were analyzed by overnight exposure in a Phosphoimager. western blot with different antibodies was carried out on the same nitrocellulose sheet (lanes 1 to 5) to identify coimmunoprecipitated proteins. In lane 5, the band due to rabbit antibody H chain, present also in lane 6, partially masks the specific lyn bands.

anti-FR mAb immunoprecipitates. Together, these data indicate that all these molecules are part of a macromolecular complex in which FR can generate intracellular signals in ovary carcinoma cells.

Like other GPI-linked proteins (Hooper and Turner, 1988), FR was only partially solubilized by non-ionic detergents such as TX-100, and about one-half of the total amount of FR was recovered in the low-density fractions of the sucrose gradient, together with G$\alpha_{i\text{-}3}$ and lyn. The kinase lyn was found to participate in signal transduction events in association with different receptors on cells of hematopoietic origin (Tilbrook et al., 1997; Corey et al., 1993, 1994; Yamamoto et al., 1993; Campbell and Sefton, 1992; Cichowski et al., 1992; Torigoe et al., 1992; Yamanashi et al., 1992; Burkhardt et al., 1991) and only recently it was found abundantly expressed also on cells of endometrial origin (Couchman et al., 1997). The remaining FR was not homogeneously distributed in the bottom fractions, and the peak found at the 40% sucrose interface (fraction 8) suggests that, as reported for other GPI-linked proteins (Parkin et al., 1996), even the detergent-soluble portion of FR constitutes higher-density complexes. Interestingly, the yes kinase was found enriched in the same fraction.

FR association to Triton-insoluble microdomains requires GPI-anchorage of the protein since chimeric transmembrane receptors lose this property (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998); in addition, the nature of membrane anchorage (GPI vs transmembrane; Friedrichson and Kurzchalia, 1998) is relevant also for interaction with other molecules, including cytoplasmic kinases (Solomon et al., 1998). Several lines of indirect evidence suggest a link between FR and cytoplasmic signaling proteins found in the low-density microdomains. First, treatments that directly affected FR expression or distribution also modulated the distribution of the co-partitioned cytoplasmic molecules. Although PI-PLC treatment cannot be considered FR specific, however has to be taken in account that FR represent the main GPI-anchored protein in the low density fraction of ^{3}H-ethanolamine labelled cells, accounting for more than 90% of the radioactivity (unshown results). Removal of the FR protein domain by PI-PLC treatment might abrogate interactions with the extracellular domain of an as yet unidentified transmembrane protein(s) able to recruit intracellular signaling molecules. Second, we found that the amount of FR associated with insoluble domains was not constant during cell proliferation, decreasing progressively at cell confluence concomitant with a decrease in cytoplasmic signaling molecules. This decrease might reflect a reduced demand for signals originating from these membrane domains, or more specifically, for FR-mediated signals (folate intake or proliferation) in confluent cells.

Two recent reports agree on the natural occurrence of clusters of GPI-linked proteins, including FR, independent of antibody interaction (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998) whereas Mayor et al. (1994) reported that FR exists in an unclustered form when tested by direct immunofluorescence by mAb MOv19. In this context, our observation that a reduction of FR in TX-100-insoluble complexes is induced by interaction with Mov19 and another mAb having the same specificity (LK26) but not by Mov18, suggests that pre-existing clusters of FR can be disrupted affecting either FR internalization capability, which was reduced by 50%, and association with other molecules

It has been reported that Src-family members and G proteins can be immunoprecipitated from detergent-soluble macromolecular complexes in association with GPI-proteins other than FR (Solomon et al., 1996; Thomas and Samelson, 1992; Stefanova et al., 1991). Co-immunoprecipitation of complexes of FR and signalling molecules from the Triton-insoluble low-density fraction from sucrose gradient, solubilized by octyl-β-glucoside, failed to give positive results since this detergent reportedly dissociates lipid-based protein interactions (Solomon et al., 1998). For these reasons we choose to carry out immunoprecipitation directly on Triton-insoluble microdomains isolated by sucrose gradient. Using both anti-FR mAbs and anti-lyn antibodies we observed the specific coimmunoprecipitation of FR and cytoplasmic

kinases. This observation, together with the finding that the α and β subunits of G protein were also immunoprecipitated, suggest the existence of macromolar complexes including the membrane FR and cytoplasmic proteins. In vitro phosphorylation, carried out on anti-FR mAb immunoprecipitates, indicated coprecipitation of kinase activity; in particular, the 53 kDa form of lyn appeared to be enriched in the MOv19 immunoprecipitate. The exact reason for this preferential association remains unclear. Moreover, the nature of the 50 kDa band phosphorylated in the MOv18 immunoprecipitate and of the 120-140 kDa present in both anti-FR and anti-lyn immunoprecipitates is presently unknown. As found also by Solomon (1996), we identified $G\alpha_{i-3}$ as a component of the coprecipitated 38-44 kDa phosphorylated band. Although Gα free of β/γ subunits appears to be the preferred substrate of phosphorylation (Neer, 1995), we found that the β subunit also coprecipitated with the FR/lyn/Gα complex. Interestingly in *Dictyostelium discoideum*, binding of FA to the receptor activates mitogen-activated protein kinase via the $G\alpha_4$ subunit (Maeda and Firtel, 1997).

Finally, we isolated detergent-insoluble complexes from IGROV1 cells that lack caveolin, a finding consistent with reports indicating the loss of caveolin expression in strict association with tumor transformation (Lee et al., 1998; Engelman et al., 1997; Koleske et al., 1995). Several studies have demonstrated a role for caveolin in the recruitment of signaling proteins in their inactive form (Engelman et al., 1998; Couet et al., 1997; Song et al., 1996; Li et al., 1995), through a well-identified scaffolding binding domain (Li et al., 1996). Further analysis are needed to determine whether the lack of caveolin in IGROV1 cells underlies the constitutive activation of downstream molecules.

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