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Regulation of Folate Receptor Internalization by Protein Kinase C α[†]

Hala Elnakat,[§] Mesfin Gonit,[§] Marcela D'Alincourt Salazar,[§] Juan Zhang,[§,#] Venkatesha Basrur,[‡] William Gunning,[§] Barton Kamen,[⊥] and Manohar Ratnam*[,§]

[§]*Department of Biochemistry and Cancer Biology, Medical University of Ohio, 3000 Arlington Avenue, Toledo, Ohio 43614,* [#]*Crown BioScience, Inc., Beijing, China,* [‡]*Division of Pathology Informatics, Department of Pathology, University of Michigan Medical School, 1301 Catherine Road, Ann Arbor, Michigan 48109, and* [⊥]*UMDNJ-R. W. Johnson Medical School, Cancer Institute of New Jersey, 195 Little Albany Street, New Brunswick, New Jersey 08901*

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ABSTRACT: The glycosyl-phosphatidylinositol anchored folate receptor (FR) mediates selective delivery of a broad range of experimental drugs to the receptor-rich tumors, but molecular mechanisms controlling FR internalization have not been adequately studied. FR quantitatively recycles between the cell surface and endocytic compartments via a Cdc42-dependent pinocytic pathway. Protein kinase C (PKC) activators including diacylglycerol and phorbol ester have previously been reported to increase the proportion of FR on the cell surface. Here we identify the α-subtype of PKC as the mediator of phorbol ester action on FR recycling and provide evidence that activated PKCα is recruited to FR-rich membrane microdomains where, in association with its receptor RACK1, it inhibits FR internalization; the activation state of Cdc42 remains unaltered. We also show that the PKC substrate, annexin II, is required for FR internalization. The studies clarify a molecular mechanism for the regulation of FR recycling through PKC which could potentially be exploited for effective drug delivery.

The human folate receptor (FR)[1] is a glycopolypeptide with a high affinity for folic acid and the circulating form of folate, (6S) N^5-methyltetrahydrofolate ($K_D < 10^{-9}$ M). Homologous FR isoforms are attached to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) membrane anchor (*1*−*4*). The membrane anchored FRs can mediate internalization of receptor bound (anti)folate compounds and folate conjugates(*5*−*10*). Since in most normal tissues, FR is absent, nonfunctional, or expressed on luminal surfaces that are inaccessible through the bloodstream (*11*), the receptor has been used as a route for the selective delivery of a broad range of experimental pharmacological agents to pathological tissues including malignant cells and activated macrophages (*6*, *8*−*19*). FR recycles between the cell surface and endocytic compartments via a clathrin-independent and Cdc42-dependent pinocytic pathway (*20*). The residence time within the endosomes is critical for the release of the receptor bound molecules for intracellular drug delivery and is also important for the activation of certain pro-drug conjugates (*5*, *21*). It has been suggested that at the high local concentration of (anti)folate molecules released by FR within the endosomes the molecules are transported into the cytosol by a proton-coupled membrane folate transporter (*22*). In order to improve FR-mediated drug delivery, it is therefore important to understand mechanisms that could influence the proportion of internalized FR at steady state.

It has been established, using MA104 (monkey kidney epithelial) cells, that FR quantitatively recycles within minutes between the cell surface and intracellular compartments (*23*−*25*). The recycling of FR was dependent upon its GPI anchor (*26*) as well as membrane cholesterol (*27*) and the actin cytoskeleton (*28*). The studies demonstrated that virtually every molecule of FR in the plasma membrane of MA104 cells is functional in this manner providing a compelling argument for the quantitative association of FR with functional membrane microdomains broadly defined as lipid rafts. Consistent with raft association, clustering of FR molecules was observed by using a monovalent, biotinylated folate affinity probe to label FR on live cells under transport permissive conditions prior to fixing for electron microscopy (*7*). Subsequently, FR clusters at the live cell surface were demonstrated by chemical cross-linking (*29*) and fluorescence resonance energy transfer (*30*). FR has been observed in endocytic compartments by cell fractionation, electron microscopy, and fluorescence microscopy (*31*−*33*). The recycling FR usually shows a 1:1 distribution between the cell surface and endocytic compartments in confluent cells (*23*−*25*).

It has previously been reported that the steady-state distribution of FR between the cell surface and intracellular compartments is altered by diacylglycerol or phorbol ester resulting in an increase in the proportion of the receptor molecules on the cell surface (*34*). This increase occurred due to inhibition of the internalization step of FR recycling, while the externalization step was unaffected. Since at the time of those studies, FR was believed to reside in caveolae, the effect of PKC activators on FR recycling was explained by the observed inhibition of the vesicularization of caveolae (*35*). It was further reported that the inhibition was associated with displacement of protein kinase C (PKC) α from caveolae inhibiting the phosphorylation of a 90-kDa protein (*36*). Since subsequently FR was established to occur in rafts distinct from caveolae, we undertook to investigate the molecular events underlying the effect of PKC signaling on FR

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*To whom correspondence should be addressed. Phone: 419-383-3862. Fax: 419-383-6228. E-mail: manohar.ratnam@utoledo.edu.

[1]Abbreviations: FR, folate receptor; PKC, protein kinase C; cPKC, classical protein kinase C; PMA, phorbol 12-myristate 13-acetate; RACK1, receptor for activated C kinase; Cdc42, cell division cycle 42.

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raft recycling and report a different mechanism than that reported for caveolae.

We initially identified putative components of functional FR complexes within a cold nonionic detergent resistant membrane fraction (DRM). DRM is enriched for GPI-anchored proteins (*37*−*39*), but experiments with artificial lipid vesicles suggest detergent treatment could cause artifact associations of proteins within complexes in this fraction (*40*−*42*). Therefore, we took advantage of our ability to use a folate ligand to further affinity purify FR-rich membrane complexes (*43*) to limit the number of candidate proteins that could then be tested for functional roles in FR internalization using the well-established MA104 monkey kidney epithelial cell model. In this manner, PKCα, the receptor for activated C kinase 1 (RACK1) and possibly the PKC substrate annexin II but not Cdc42 were established as key molecular players in the regulation of raft recycling by PKC signaling based on evidence for their physical and functional associations with FR.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Lipofectamine 2000, Geneticin, penicillin/streptomycin/L-glutamine, human recombinant purified PKC proteins (α, *β*, *γ*, *δ*, *ε*), fetal bovine serum (FBS), Coomassie Brilliant Blue, Opti-MEM I reduced serum medium, folate-free RPMI (FFRPMI), MEM and DMEM media were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). Monoclonal and affinity purified antibodies raised against PKCs α, *β*I, *β*II, *γ*, RACK1, karyopherin α2 and GAPDH were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Affinity purified rabbit antihuman annexin II was from BD Transduction laboratories (San Jose, CA). Polyclonal rabbit anti-14-3-3 antibody was purchased from Zymed laboratories Inc. (San Francisco, CA). Rabbit antiserum against FR was described previously (*3*). The reagents for RT-PCR and real-time PCR were purchased from Applied Biosystems (Branchburg, NJ). Hexadimethrine bromide "polybrene", 2-mercaptoethanol, puromycin dihydrochloride, nonessential amino acids, holotransferrin and protease inhibitors were purchased from Sigma (St Louis, MO). Bradford reagent was purchased from Bio-Rad Laboratories (Hercules, CA). Phorbol-12-Myristate-13-Acetate (PMA) was purchased from Calbiochem (La Jolla, CA). Liquid scintillation fluid "Ecoscint H" was purchased from National Diagnostics (Atlanta, GA). Streptavidin magnetic beads were purchased from Roche Diagnostics (Indianapolis, IN). [3′,5′,7,9-^{3}H]folic acid diammonium salt (43.2 Ci/mmol) and [^{125}I]-diferric transferrin (80.0 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA) and Perkin-Elmer (Boston, MA) respectively.

DNA Expression Plasmids. PKC α-EGFP and PKC *β*II-EGFP plasmids were purchased from Clontech (Mountain View, CA). PKC*β*I cloned into the pEF1 mammalian expression plasmid was a kind gift from Dr. Kevin Pan at the Medical University of Ohio. The constitutively active PKCα construct was kindly provided by Dr. D. Dartt (Harvard Medical School); in brief, the plasmid construct was made by adding the src myristoylation signal (MYPYDVPDYA) at the amino terminus of PKCα to target PKCα to the cell membrane for constitutive kinase activity (*44*). To generate the pSUPER-PKCα and the pSUPER-control constructs, the custom oligonucleotides (5′- gatccccGGATGTGGTGATTCAGGATttcaagaga ATCCTGAATCACCACATCCtttttggaaa-3′ and 3′-gggCCTACACCACTAAGTCCTAaagttctctTAGGACTTAGTGGTGTAGGaaaaaccttttcga -5′, position 1160 in the PKCα mRNA sequence accession number NM_002737) and (5′-gatccccGAGTGAGTTGCA GGTTAGTttcaagagaACTAACCTGCAACTCACTCtttttggaaa-3′ and 3′-gggCTCA CTCAACGTC CAATCAaagttctctTGATTGGACGTTGAGTGAGaaaaaccttttcga-5′) were respectively synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) (*45*). The 19 nucleotide PKCα target sequence and the control sequence are indicated in capital letters in the oligonucleotides. The annealed oligos were ligated into the pSUPER vector digested with *Bgl*II and *Hin*dIII and transformed using XL1-Blue competent cells. The DNA constructs were amplified using the Qiagen plasmid High-speed Maxiprep kit (Chatsworth, CA) and the sequences were verified by automated DNA sequence analysis.

Cell Culture. Unless otherwise mentioned, all the cell lines were grown in media supplemented with fetal bovine serum (10% v/v), penicillin (100 units/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM) at 37 °C in a 5% CO_2 cell culture incubator. MA104 cells and CHO cells purchased from American type Culture Collection (Rockville, MD) were cultured in DMEM and RPMI respectively. The recombinant CHO cell line stably expressing FR-*β* (CHO-FR*β*) (*7*) was maintained in RPMI supplemented with L-proline (14.5 mg/L).

The stable human-derived 293 GPG packaging cell line was cultured as previously described (*46*) in DMEM supplemented with heat-inactivated FBS (10%), puromycin (1 *μ*g/mL), doxycycline (2 *μ*g/mL), and Geneticin (300 *μ*g/mL). The 293 FT cells used for packaging lentiviral particles were kindly provided by Dr. Yeung (University of Toledo). These cells were maintained in DMEM, heat inactivated FBS (10%), Geneticin (500 *μ*g/mL), and nonessential amino acids (1%). HEK-293 cells were cultured in DMEM. All the cell lines used for packaging or generating viral particles were grown in FFRPMI three days before transfection or infection.

FR Recycling. MA104 cells were seeded at 9.5×10^4 cells per well in 6-well plates in FFRPMI and used for FR recycling experiments on day 6. At this time, the cells proliferated normally, indicating that they were supported by an adequate folate pool. Briefly, the cells were incubated with 27nM of [^{3}H] folic acid in serum-free FFRPMI for 1 h at 37 °C. After washing the cells twice with media at 37 °C to remove any unbound radioactivity, vehicle or 1 *μ*M PMA was added. At the end of an incubation period of 30 min at 37 °C, the media was aspirated and the cells were washed with 1 mL of acid buffer (10 mM sodium acetate, pH 3.5/150 mM NaCl) for 1 min on ice. The acid wash eluate was counted by liquid scintillation and represents the amount of external [^{3}H]folic acid bound. The cells were washed once with ice-cold PBS (137 mM NaCl, pH 7.4/27 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) and then lysed with 0.1 N NaOH to determine the amount of internalized [^{3}H]folic acid. MA104 cells were also incubated with a 20-fold excess of unlabeled folic acid, relative to the amount of [^{3}H]folic acid added, to ensure the specificity of uptake via FR. All samples were measured in triplicate.

FR Internalization and Externalization. MA104 cells were seeded in 6-well plates at 2.25×10^5 cells/well in FFRPMI and used for FR influx or efflux experiments on day 5. For FR internalization experiments, the cells were first incubated with vehicle or 1 *μ*M PMA in serum-free FFRPMI for 30 min at 37 °C. Then 27 nM of [^{3}H]folic acid was added in serum-free FFRPMI and incubated at 37 °C for different periods. At the end of the incubation period, the media was aspirated and the cells were

washed twice with ice-cold PBS and the amount of external or internal [^{3}H] folic acid was measured by liquid scintillation counting as specified above.

For FR externalization experiments, cells were incubated with 27 nM of [^{3}H]folic acid in serum free FFRPMI for 1 h at 37 °C. Then vehicle or 1 μM PMA was added and the cells were incubated for a further 30 min at 37 °C. The media was aspirated and the cells were washed once with ice-cold PBS and chilled on ice for 20 min. The cells were washed once with 1 mL of acid buffer for 1 min followed by two ice-cold PBS washes. At time 0, 37 °C FFRPMI was added to each well and incubated for different periods at 37 °C. At the end of each time point, the amount of external and internal [^{3}H]folic acid was measured as described under the "FR Recycling" section.

In all of the above experiments, samples were measured in triplicate and an excess of unlabeled folic acid was added to determine the nonspecific background binding.

Transferrin Receptor (TfR) Recycling. MA104 cells were seeded in 6-well plates as 2.25×10^5 cells/well in FFRPMI were used for TfR recycling experiments on day 5 as previously described (*47*). Essentially, the cells were incubated with vehicle or 1 μM PMA in serum-free FFRPMI for 30 min at 37 °C. Then the cells were washed once with ice-cold HEPES-buffer (20 mM HEPES, pH 7.2 containing 0.15 M NaCl, 0.5 mM $MgCl_2$, and 0.05 mM $CaCl_2$), chilled on ice for 20 min and incubated with 2 nM [^{125}I]transferrin (77 Ci/mmol) for 1 h on ice in HEPES-saline containing 0.3% (w/v) bovine serum albumin. The cells were washed twice with ice-cold HEPES-buffer and 37 °C serum free FFRPMI was added to each well. The cells were incubated at 37 °C for different periods, at the end of which the media was counted with a gamma counter to obtain the amount of TfR that recycled. Cell bound [^{125}I] transferrin was released by adding acidic HEPES-buffer (pH 2.0) for 4 min on ice. Finally, the cells were lysed with 1% TX-100 and 0.5% SDS and the amount of internalized [^{125}I] transferrin was measured. Nonspecific binding of [^{125}I] ransferrin was determined in identical assays by adding a 400-fold excess unlabeled holo-transferrin along with the labeled compound. All samples were obtained in triplicate and counted with a gamma counter.

Adenovirus-Mediated Gene Transfer to MA104 Cells. Replication-deficient adenoviruses expressing Ad5-type adenovirus vectors containing the cDNA of wild type PKCs (α, βII, δ, and ε) and kinase negative mutants (DN-PKCα and DN-PKCβII) were a kind gift from Dr. K. V. Chin (University of Toledo) (*48*, *49*). The constitutively active myristoylated PKCα (PKCα-CA) was a kind gift from Dr. D. Dartt (Harvard Medical School) (*44*). The adenovirus carrying the green fluorescent protein (GFP) gene was designated as GFP-Ad and used as a control virus to determine the infection efficiency. cDNA for wild type Annexin II, kindly provided by Dr. J. Vishwanatha (University of North Texas Health Science Center), was cloned into the adenovirus vector. The adenoviral stocks were amplified by infecting HEK-293 cells in 10 cm plates with $\sim 4 \times 10^{15}$ infectious particles in FFRPMI containing heated FBS and (8 μg/mL) of Polybrene. The cells were harvested 48 h postinfection when they displayed cytopathic effects, subjected to five consecutive freeze/thaw cycles to release adenoviuses trapped inside the cells, and centrifuged at 3000 rpm for 20 min. The supernatant was stored as aliquots at −80 °C. Finally, the adenoviral stocks were titered using the optical absorbance method (*50*).

MA104 cells were seeded in FFRPMI at 9.5×10^4 cells/well in a 6-well plate on day 0. On day 6, cells were infected for 1 h with 10^{15} infectious particles per well in 1 mL of serum free FFRPMI containing (8 μg/mL) of Polybrene, at the end of which, an equal volume of FFRPMI containing 20% heated FBS was added. The cells were cultured for a further 48 h before FR recycling experiments were conducted or cell lysates from the infected cells were analyzed by Western blot. Under these conditions, the adenoviruses infected most of the cells (>90%) based on the fluorescence of the GFP-Ad infected cells visualized with a fluorescence microscope.

Packaging of Recombinant Lentiviruses and Infection of Cells. 293 FT cells were transfected with MISSION shRNA lentiviral constructs in pLOK.1-puro for RACK1 and annexin II knockdown (Sigma-Aldrich) or a control sequence (Supporting Information Table 1) along with plasmids needed for lentiviral packaging (pMD2G, pMDLg/pRRE, and pRSV-Rev) using Lipofectamine 2000 according to the vendor's protocol. Viruses were harvested at 48 h and 72 h post-transfection. Essentially, the supernatant was centrifuged at 2500 rpm for 10 min at 4 °C and then filtered through a 0.45 μM sterile filter (Nalgene). MA104 cells were infected with lentivirus at 30% confluence. Polybrene (8 μg/mL) was added to increase the infection efficiency. Folate receptor recycling experiments were done 72 h postinfection of MA104 cells with the recombinant lentivirus.

Packaging of Recombinant Retrovirus and Infection of MA104 Cells. The stable human-derived 293 GPG packaging cells were transfected with pSuper constructs containing a control or a PKCα knockdown sequence (described under the "DNA Expression Plasmids" section) using Lipofectamine 2000 according to the vendor's protocol. The media was replaced 4 h post-transfection and the following day with fresh FFRPMI containing 10% heated FBS. The retroviral media was harvested at 48 h and 72 h post-transfection. Essentially, the virus was harvested by filtering the harvested media through a 0.45 μM sterile filter (Nalgene). MA104 cells split on day 0 as (0.095×10^6 cells/well) in FFRPMI containing heated FBS were infected on days 1 and 2 by adding the retroviral media and Polybrene (8 μg/mL) to increase the infection efficiency. On day 3, the media was replaced with fresh FFRPMI media. Folate receptor recycling experiments were conducted 72 h postinfection of MA104 cells with the retroviral media.

Membrane Preparations. Membranes from confluent CHO, CHO-FRβ, and MA104 cells were prepared as previously described (*43*). Essentially, cells were washed on ice with acid buffer to release any bound folates from FR followed by two PBS washes. The cells were lysed in lysis buffer (1 mM $NaHCO_3$, pH 7.2 containing 2 mM $CaCl_2$, 5 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) for 30 min on ice, homogenized with a dounce homogenizer and centrifuged for 10 min at 2000*g* at 4 °C. The supernatants were centrifuged at 30000*g* for 45 min at 4 °C, and the membrane pellet were washed with acid buffer followed by three PBS washes. Finally, the membranes were resuspended in 1× Hank's balanced salt solution (HBSS) containing protease inhibitors (1 mM PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin and 1 μg/mL pepstatin A).

Affinity Purification of FR Complexes Using Biotinylated Folic Acid. All the following steps were carried out on ice or at 4 °C unless specified otherwise. Streptavidin coated-magnetic particles (0.5 mg) from Roche Diagnostics (Indianapolis, IN) were washed twice with HBSS (containing 1% Triton X-100 and 1 mg/mL BSA). Membranes (50 μg) from CHOK1 or CHO-FRβ cells were incubated with biotin-SS-folate (*7*, *51*) (1 μM final concentration) for 30 min on a rotary

shaker. An equal volume of HBSS (containing 2% Triton X-100 and 2 mg/mL BSA) was added to each sample and the prewashed streptavidin particles and rotated for another 4 h. The samples were washed six times using a magnet stand with HBSS (containing 1% Triton X-100 and 0.5 M NaCl) followed by three washes with HBSS only. Finally, 10 mM DTT in HBSS (containing 0.5% Triton X-100) was added to the samples in order to cleave the disulfide bond in biotin-SS-folate thereby eluting the FR complex.

The same steps were followed to purify FR complexes from MA104 cells except that 200 μg of membranes were used instead of 50 μg. In addition, some samples were incubated with 1 mM folic acid for 30 min prior to the addition of biotin-SS-folate in order to determine nonspecific binding to the probe.

Mass Spectrometry. The FR complex affinity purified using biotinylated folic acid was electrophoresed on a 12% polyacrylamide−SDS gel and gel slices from each lane were digested with trypsin. The peptides were extracted with a solution of 60% acetonitrile/0.1%TFA followed by a reverse phase column separation step. The peptides were directly introduced into an ion-trap mass spectrometer equipped with a nanospray source. Collision induced dissociation spectra were either manually interpreted or searched against an appropriate nonredundant database using TurboSEQUEST.

Electron Microscopy. MA104 cells were washed with HBSS then incubated in solution A (1× HBSS containing 30 mM NaN_3 and 1% BSA) for 30 min at room temperature. The cells were then probed with rabbit anti-FR antibody or rabbit preimmune serum as negative control for 2 h, washed with solution A four times and then incubated with 12 nm colloidal gold-conjugated donkey-antirabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1.5 h at room temperature. The cells were washed again four times with solution A, fixed with 3% glutaraldehyde, washed with 0.2 M sodium cacodylate (pH 7.2), embedded in LR-White medium, and sectioned. The sections were first incubated in solution B (PBS containing 1% fish gelatin) for 15 min, then probed with goat anti-RACK1 or goat antikaryopherin α2 antibodies for 2.5 h. Then, the sections were washed five times with solution B, followed by the incubation with 6-nm colloidal gold-conjugated donkey-antigoat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1.5 h. Again, the sections were washed five times with s olution B, one high salt wash with 2.5 M NaCl (pH 8.5), and four distilled water washes. The grids were examined with a CM-10 Philips electron microscope. In order to better visualize the gold particles, uranyl acetate and lead citrate poststaining was avoided.

Immunoprecipitation. MA104 cells were seeded at 1×10^5 cells per well in 6-well plates in FFRPMI and used for FR immunoprecipitation on day 8. The cells were incubated with 27 nM [^{3}H] folic acid in serum free FFRPMI for 1 h at 37 °C. After washing the cells twice with PBS to remove any unbound [^{3}H] folic acid, cells were lysed with 0.5 mL of PBS containing 0.5% Triton 100 and the lysate was centrifuged to remove debris. An aliquot of the cell lysate (0.25 mL) was used to measure the total amount of cell associated [^{3}H] folic acid by liquid scintillation counting. The FR in the remaining lysate was incubated with 5 μL of rabbit anti-FR antiserum or normal control rabbit serum for 1 h at room temperature. The radioactivity was then precipitated using Protein A magnetic beads (New England Biolabs) for 1 h at room temperature on a rotary shaker. The magnetic beads were separated in a magnetic field and the radioactivity in the supernatant was counted by liquid scintillation. The reactions were conducted in triplicate.

RNA Isolation and Quantitative Real-Time RT-PCR. Total RNA from MA014 cells was prepared using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Reverse transcription PCR (RT-PCR) followed by quantitative real-time PCR was used to measure endogenous mRNA levels for PKCs α, βI, and βII and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Total RNA (400 ng/sample) was reverse transcribed with random primers using the "high-capacity cDNA archive kit" from Applied Biosystems. The reverse transcription product was measured by quantitative real-time PCR using the Real-Time PCR master mix (Applied Biosystems) in the 7500 Real-Time PCR System (Applied Biosystems). Essentially, 10 μL of the reverse transcribed RNA was mixed with 12.5 μL of PCR Mastermix (Applied Biosystems), 0.5 μL each of the forward and reverse primers (9 μM) and 0.5 μL of the TaqMan probe (2.5 μM). The primers and TaqMan probe for PKC α, PKC βI, and PKC βII (Supporting Information Table 2) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) as previously described (*52*). The primers and the TaqMan probe for the control GAPDH gene were purchased from Applied Biosystems. The PCR conditions were 2 min at 50 °C, followed by 10 min at 95 °C, then 40 cycles each of 15 s at 95 °C and 1 min at 60 °C. Fluorescence data generated were monitored and recorded on a 7500 Real-Time PCR sequence detection system (Applied Biosystems). All samples were measured in triplicate and normalized to GAPDH values. Different concentrations of PKC α-EGFP, PKC-βII-EGFP and PKC-βI plasmids were used as positive controls for the Taqman probes and to determine the relative level of mRNA of each of these isoforms in MA104 cells. Finally, mRNA without any reverse transcriptase added during the reverse transcription step was used as a negative control for quantitative Real-Time RT-PCR.

Cdc42 Activity Assay. MA104 cells treated with vehicle or 1 μM PMA for 30 min at 37 °C were assayed for Cdc42 activity in whole cell lysates using a GST-fusion protein containing the p21-binding domain (PBD) of human p21-activated kinase 1 (Pak1) to affinity pull-down active Cdc42. The assay was done following the manufacturer's protocol provided by Stressgen (BC, Canada). Briefly, the cells were washed once with ice-cold PBS and lysis buffer (freshly mixed with a cocktail of protease inhibitors) was added. The cells were immediately scraped off the plate, incubated for 5 min on ice, and centrifuged at 16000*g* for 15 min at 4 °C. The supernatant was added to a spin cup in which an immobilized glutathione disk and 20 μg of GST-Pak1-PBD were already mixed. The spin cup was vortexed, incubated for 1 h at 4 °C with gently rocking, then spun at 7200*g* for 30 s, and washed 3 times with the lysis/binding/wash buffer (provided in the kit). Finally, 50 μL of 2× SDS sample buffer with 5% fresh β-mercaptoethanol was added to the resin, boiled for 5 min at 100 °C, and centrifuged at 7200*g* for 2 min. For each of the cell lysates assayed a positive control was performed by adding 10 mM GTPγS, which is a nonhydrolyzable form of GTP that irreversibly binds to Cdc42, and 10 mM EDTA followed by an incubation of 15 min at 30 °C. The reaction was stopped by placing the samples on ice and adding $MgCl_2$ for a final concentration of 60 mM. All samples were then assayed for Cdc42 by Western blot using a mouse monoclonal anti-Cdc42 antibody.

Preparation of Cell Lysates and Western Blots. Cell pellets were resuspended in lysis buffer (PBS containing 1%

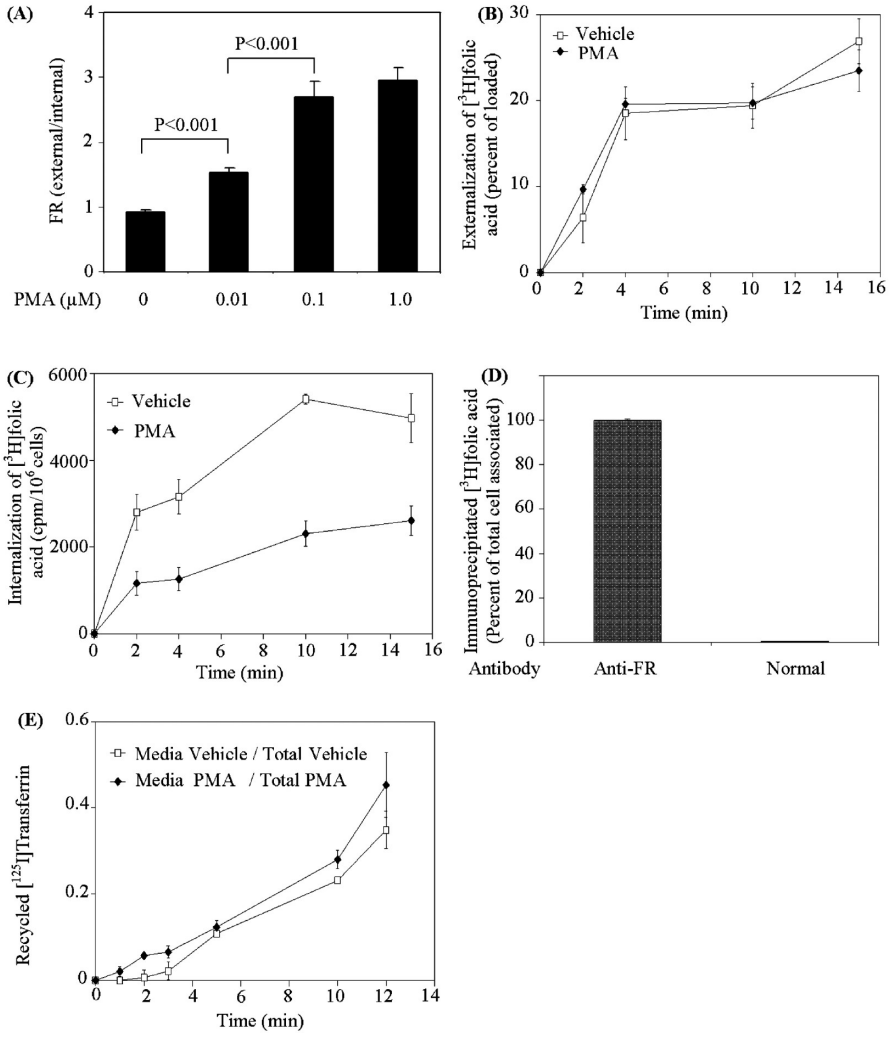


FIGURE 1: Effect of PMA on FR and transferrin receptor recycling. (A) MA104 cells were incubated with 27 nM [^{3}H]folic acid for 1 h at 37 °C, washed twice with 37 °C FFRPMI to remove any unbound radioactivity and incubated with vehicle or 1 μM PMA for 30 min at 37 °C. The cell surface bound radioactivity (representing external FR) was harvested by washing for 1 min using ice cold acid buffer, and the intracellular radioactivity (representing internal FR) was recovered by lysing the cell with 0.1N NaOH. (B) MA104 cells were incubated with 27 nM [^{3}H]folic acid for 1 h at 37 °C followed by the addition of vehicle or 1 μM PMA for 30 min at 37 °C. At the end of the incubation period, the cells were washed once with ice-cold PBS and chilled on ice for 20 min. Then the cells were washed once with acid buffer followed by two cold PBS washes. FFRPMI prewarmed to 37 °C was then added to each well and the cells were incubated at 37 °C for the indicated times. The amount of external [^{3}H] folic acid bound was removed with a 1 min cold acid buffer wash. (C) MA104 cells were treated with vehicle or 1 μM PMA for 30 min at 37 °C. The cells were incubated with 27 nM [^{3}H] folic acid at 37 °C for the indicated times. The cells were immediately washed twice with ice-cold PBS and the cell surface bound radioactivity harvested in acid buffer at different times as indicated. Whole cell lysates were prepared with 0.1 N NaOH to recover the intracellular radioactivity. (D) MA104 cells were incubated with 27 nM [^{3}H]folic acid for 1 h at 37 °C, washed twice with 37 °C FFRPMI to remove any unbound radioactivity and lysed with PBS containing 0.5% Triton. The radioactivity was counted in half of the lysate and the other half was subjected to immunoprecipitation using rabbit anti-FR antiserum or a control normal rabbit serum as described under Methods. The immunoprecipitated radioactivity is plotted as percent of the radioactivity in the lysate prior to immunoprecipitation. (E) MA104 cells were treated with vehicle or 1 μM PMA for 30 min at 37 °C. The cells were washed once with ice-cold HEPES-buffer and incubated with 2nM [^{125}I] transferrin for 1 h on ice in HEPES-saline containing 0.3% (w/v) bovine serum albumin. The cells were subsequently washed twice with ice-cold HEPES-buffer and further incubated in the presence of serum free FFRPMI at 37 °C for the indicated times. At the end of each time point, the media were harvested and radioactivities were counted to determine the amount of TfR recycled. Cell surface bound [^{125}I] transferrin was released by adding acidic HEPES-buffer for 4 min on ice. The internalized [^{125}I] transferrin was determined by lysing the cells. The total amount of TfR represents the sum of the amounts of [^{125}I] transferrin in the media, on the cell surface and inside the cells. Nonspecific binding of [^{125}I] transferrin was determined in identical assays by adding 400-fold excess unlabeled holo-transferrin along with the labeled compound (data not shown). All samples were counted with a gamma counter. In panels A, B and D, the total cell associated [^{3}H] folic acid was ~2 pmol/10^6 cells. All values are the average ± SD obtained from triplicate samples. *P* values for the differences noted the text were < 0.001.

Triton X-100 and a cocktail of protease inhibitors) and incubated on ice for 30 min. The cell lysates were centrifuged at 13000 rpm for 10 min. The protein concentration in the cell lysates was measured using the Bradford assay reagent. Cell lysates (10−100 μg) were mixed with 4× SDS loading buffer. Samples were resolved by electrophoresis on 8−12% polyacrylamide SDS gels and electrophoretically transferred to a nitrocellulose membrane. The blots were first probed overnight at 4 °C with the appropriate primary antibodies and then with goat antirabbit IgG or goat antimouse IgG conjugated to horseradish peroxidase. The bands were visualized by enhanced chemiluminescence. The same membranes were then similarly reprobed with a primary mouse anti-GAPDH antibody as loading control.

Data Analysis. The results are plotted as a mean of triplicate values ± standard deviation. The statistical difference between two different groups is represented by a P value indicated in the

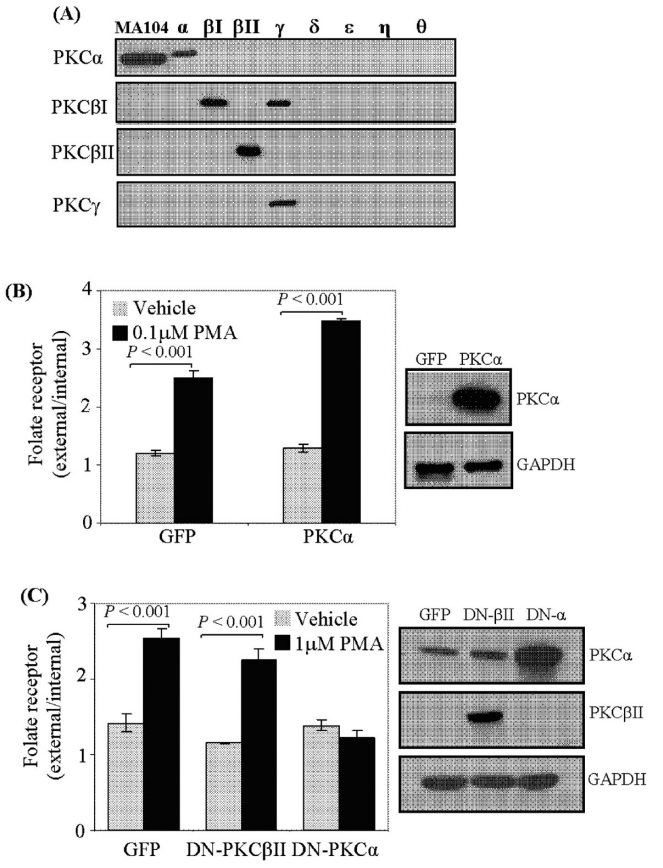


FIGURE 2: Effect of PKC expression on FR recycling. (A) Total cell lysates from MA104 cells (100 μg/well) and 5 ng of purified recombinant protein corresponding to each of the classical and novel PKCs α, βI, βII, γ, δ, ε, η, and θ were subjected to Western blot analysis using the cognate antibodies. (B) MA104 cells were infected with GFP-Adeno or PKCα-Adeno and FRα recycling was determined 48 h later. The cells were incubated with 27 nM [^{3}H]folic acid for 1 h at 37 °C, washed twice with 37 °C FFRPMI in order to remove any unbound radioactivity and further treated with vehicle or 0.1 μM PMA for 30 min at 37 °C (left panel). The cell surface bound radioactivity (representing external FR) was removed by incubation with ice-cold acid buffer and the intracellular radioactivity (representing internal FR) was recovered by the addition of 0.1 N NaOH. Cell lysates from the same experiment were also analyzed by Western blot using PKCα antibody, and the same blots were reprobed with a mouse anti-GAPDH antibody to ensure the uniformity of sample loading (right panel). The letters denote significant difference ($p < 0.005$). In (C), the same experiment was performed except that MA104 cells were infected with GFP-Adeno, DN-PKCβII, or DN-PKCα. The Western blots were additionally probed with PKCβII antibody. In all cases (B through C), the subtracted nonspecific association (< 2%) of [^{3}H]folic acid was determined using negative control samples that were treated with excess unlabeled folic acid (data not shown). In panels B and C, the total cell associated [^{3}H] folic acid was ~2 pmol/10^6 cells. All samples were counted with a gamma counter. All values are the average ± SD obtained from triplicate samples. *P* values for the differences noted the text were < 0.001.

figure legend where relevant. The P values were calculated using the ANOVA test and the software Statview.

RESULTS

The Effect of Phorbol Ester on FR Recycling Is Selective. The movement of FR molecules between the cell surface and intracellular compartments may be tracked using receptor bound [^{3}H]-labeled folic acid, the nonphysiologic form of the vitamin (*23*). This method takes advantage of the fact that once

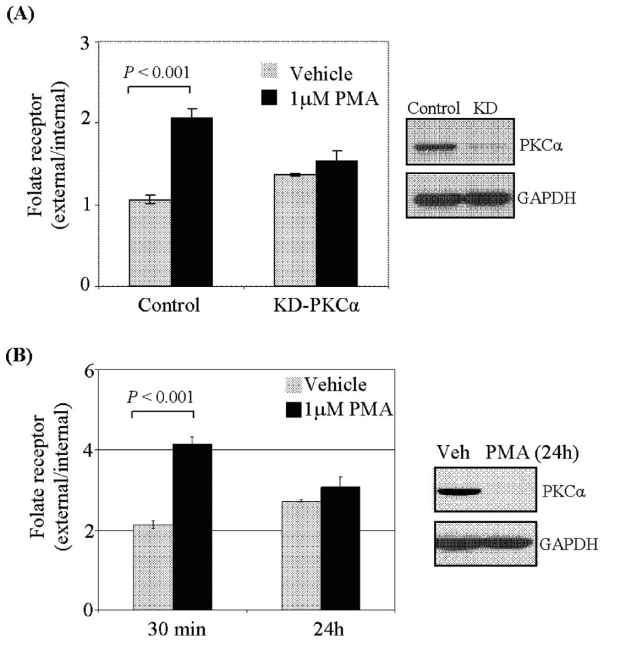
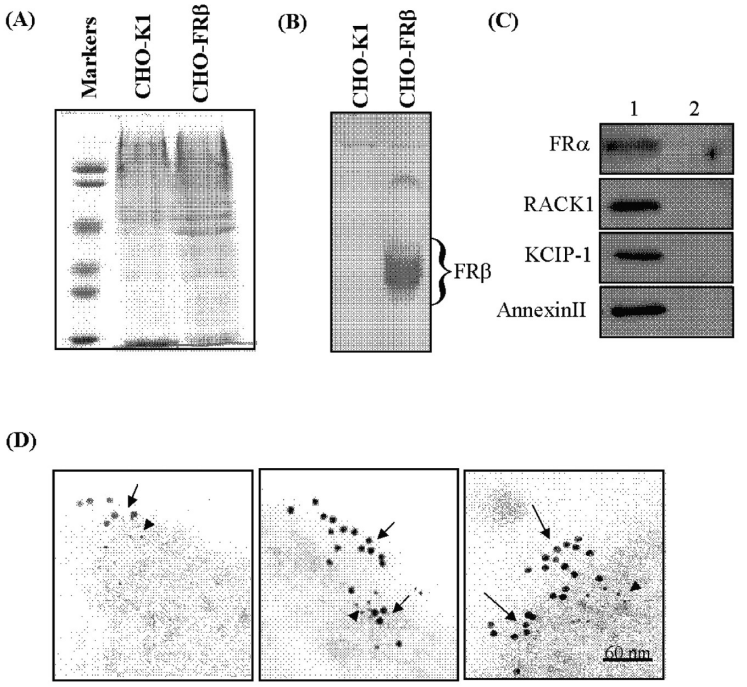


FIGURE 3: Effect of decreasing PKCα expression on FR recycling. (A) MA104 cells were infected with retrovirus containing non-target control or shRNA to knockdown PKCα. The recycling of FRα was determined 72 h postinfection. The cells were incubated with 27 nM [^{3}H]folic acid for 1 h at 37 °C, washed twice with 37 °C FFRPMI in order to remove any unbound radioactivity, and further treated with vehicle or 0.1 μM PMA for 30 min at 37 °C (left panel). The cell surface bound radioactivity (representing external FR) was removed by incubation with ice-cold acid buffer and the intracellular radioactivity (representing internal FR) was recovered by the addition of 0.1 N NaOH. Cell lysates from the same experiment were also analyzed by Western blot using PKCα antibody to confirm the knockdown (right panel). In (B), MA104 cells were split as 0.225 × 10^6 cells per well in FFRPMI. The cells were treated with vehicle or 1 μM PMA on day 2 and further cultured for another 48 h at the end of which FRα recycling was determined as described in (A). Cell lysate from the same experiment were also analyzed by Western blot using PKCα antibody and the same blots were reprobed with a mouse anti-GAPDH antibody to ensure the uniformity of sample loading (right panel). In all cases (A through B), the subtracted nonspecific association (< 2%) of [^{3}H]folic acid was determined using negative control samples that were treated with excess unlabeled folic acid (data not shown). All samples were counted with a gamma counter. In panels A and B, the total cell associated [^{3}H] folic acid was ~2 pmol/10^6 cells. All values are the average ± SD obtained from triplicate samples. *P* values for the differences noted the text were < 0.001.

bound, folic acid dissociates extremely slowly from the receptor and remains bound during the entire time that it recycles (in contrast to the physiological 5-methyl tetrahydrofolate, which is transported into the cytosol). It also takes advantage of the fact that the receptor molecules residing at the cell surface at any time may be identified by their ability to release the bound folic acid upon exposure to low extracellular pH at ~ 4 °C. Thus the ratio of the acid labile fraction of the receptor to the acid resistant fraction reflects the ratio of extracellular to intracellular FR. As previously reported, treatment of MA104 cells with PMA caused a dose-dependent increase in the extracellular fraction of FR reaching the maximal effect at 0.1 μM PMA (Figure 1A). PMA did not significantly alter the externalization rate of FR (Figure 1B) but did substantially decrease its internalization rate (Figure 1C). The decreased rate of internalization accounts quantitatively for the increase in the steady-state level of the cell surface fraction of FR. These results are in agreement with



RACK1 (6nm gold) associated with FR (12nm gold) **at ≤ 40nm**	17/31**(54.8%)**
Karyopherin α2 (6nm gold) associated with FR (12nm gold) **at ≤ 40nm**	2/34 **(5.8%)**
RACK1 (6nm gold) associated with FR (12nm gold) **at ≤ 10nm**	5/31 **(16.1%)**
Karyopherin α2 (6nm gold) associated with FR (12nm gold) **at ≤ 10nm**	0/34 **(0 %)**

FIGURE 4: Identification of proteins associated with FR rafts. (A) Proteins in DRMs were affinity purified from a detergent-insoluble membrane fraction prepared from CHO-K1 and CHO-FRβ cells, using Biotin-SS-folate attached to streptavidin-coated magnetic particles. The folate-bound complexes were specifically eluted from the beads using dithiothreitol. The proteins were resolved by SDS polyacrylamide gel electrophoresis. The figure shows a scan of a representative gel stained with Coomassie Brilliant Blue. In (B), the affinity purified DRMs from CHO-K1 and CHO-FRβ cells were analyzed by Western blot using anti-FR antibody. In (C), proteins in the affinity purified FR-rich DRMs obtained from MA104 cell membranes (lane 1) were analyzed by Western blot using antibodies for FR, RACK1, KCIP-1, and annexinII. As a negative control, the affinity purification was conducted after the samples were incubated with 1 mM folic acid for 30 min prior to the addition of biotin-SS-folate in order to determine nonspecific binding to the probe (lane 2). Equal loading in the Western blot was ensured by protein estimation. (D) Colocalization of FRα and RACK1 in MA104 cells. Cells were incubated in the presence of 30 mM NaN_3 with rabbit anti-FR antibody followed by 12-nm colloidal gold-conjugated donkey-antirabbit IgG. The cells were subsequently fixed with 1% glutaraldehyde, embedded in LR White medium, and sectioned. The sections were probed with goat anti-RACK1 antibody followed by 6-nm colloidal gold-conjugated donkey-antigoat IgG. The samples were then examined by electron microscopy. The arrows indicate clusters of 12-nm gold (FR) and the arrowheads indicate clusters of 6-nm gold. (RACK1). Three representative fields are shown in D. Panel D also shows quantitative analysis of associations between clusters of FR and RACK1 or FR and karyopherin on the cell surface of MA104 cells by electron microscopy.

previous studies of the kinetics of FR recycling which were designed to examine the effect of phorbol ester on caveolae (*34*). The total cell associated [^{3}H]folic acid probe at steady state (cell surface bound plus internalized) was specifically and quantitatively immunoprecipitated by antibody to FR (Figure 1D), confirming that in the above experiments the radiolabel did not associate significantly with any protein other than FR. The recycling of the transferrin receptor, monitored using [^{125}I]-labeled transferrin, was relatively unaffected by PMA (Figure 1E). Since the recycling transferrin receptor is localized in clathrin-coated pits, the results indicate that the effect of PMA on FR recycling is specific to its membrane microdomain in MA104 cells.

PKC α Is the Only Classical PKC Detected in MA104 Cells. PMA activates both classical and novel PKCs. Studies using PKC inhibitors (*5*) have suggested that the phorbol ester effect on FR recycling may be mediated by a classical PKC (cPKC) since this class of PKCs is typically Ca^{2+}-dependent. To directly test the role of individual cPKCs in the action of phorbol ester on FR recycling, it was first necessary to identify the possible candidate cPKCs in MA104 cells. Cell lysates together with purified recombinant classical and novel PKCs (antibody specificity controls) were probed on Western blots using antibodies against the individual cPKCs (Figure 2A). A strong band was obtained for PKCα in the cell lysate, whereas none of the cPKC isoforms including βI, βII, or γ could be detected. Since the antibody for PKCβI was cross-reactive with PKCγ, the absence of PKCβI/βII was further confirmed by real time RT-PCR which only revealed the expression of mRNA for PKCα (Supporing Information Table 3). It may be noted that the Taqman probes for PKCs βI and βII used (Supporting Information, Table 3) were >8-fold more sensitive to their respective target genes than the PKCα probe. On the basis of these results the likely isoform of PKC that mediates phorbol ester effects in MA104 cells is PKCα.

PKCα Regulates FR Recycling. Next, several experiments were conducted to examine a possible function for PKCα in regulating FR recycling. Overexpression of PKCα using an adenoviral vector resulted in an increase in PMA-induced FR externalization (Figure 2B); in contrast, a dominant-negative form of PKCα but not PKCβII abrogated the PMA effect (Figure 2C) similar to knocking down PKCα (Figure 3A).

Consistent with this finding, prolonged (48 h) exposure to PMA, which is known to downregulate PKCα, restored the original steady-state distribution of FR (Figure 3B).

FR-Rich DRMs Are Physically Associated with Cellular Effectors of PKC Action. To identify proteins with a known functional relationship with PKCα in FR rafts as well as candidate proteins involved in raft recycling, FR-rich DRMs were affinity purified using an immobilized biotinylated folate probe and the purified protein complexes were resolved by denaturing electrophoresis. Since the association of FR with lipid rafts has already been well established, proteins specifically associated with FR during affinity purification of FR from a detergent-insoluble membrane fraction would be testable raft proteins which could functionally interact with FR. Since this approach required an appropriate control to exclude background proteins that were not specifically associated with FR, initially parental FR-negative CHO cells (CHO-K1 cells) and recombinant CHO cells stably expressing FRβ (CHO-FRβ) were used. The purified complex contained FR (Figure 4A,B). All of the proteins in the preparations obtained from the parental and recombinant cells were subjected to mass spectrometry and the proteins that were specifically associated with FR were identified in this manner (Supporting Information Table 4). Most of the proteins identified in Supporting Information have previously been identified in association with lipid rafts and include cytoskeletal proteins (actin and tubulin) (*53*, *54*), heterotrimeric G-proteins (*55*, *56*), annexin II (*57*) and KCIP-1 (*53*). The apparent association of receptor for activated C kinase 1 (RACK1) as well as annexin II and KCIP-1 with FR-rich DRM aroused our interest because they represent either PKC binding proteins or PKC substrates. Though RACK1 is present in cells in both soluble and detergent-insoluble particulate forms this protein had not previously been reported to be associated with membrane rafts.

Since all the functional studies of FR recycling conducted in this study are in MA104 cells expressing endogenous FRα, the CHO cell experiments were extended to MA104 cells specifically to confirm the association of RACK1, annexin II and KCIP-1 with FR-rich DRM in these cells. Toward this end, FR-rich rafts were affinity purified using biotinylated folate from MA104 cell membranes and probed using antibodies to FR, RACK1, annexin II, and KCIP-1; all of the proteins were present in the FR-rich DRM isolated from MA104 cells (Figure 4C). However, these proteins were not detectable in preparations in which an excess amount of folic acid was used to block specific association of FR with the biotinylated folate during the purification (Figure 4C) indicating the specificity of the association of RACK1, KCIP-1, and annexin II with FR.

Additional Evidence for the Association of RACK1 with FR-Rich Membrane Domains. The association of RACK1 with FR-rich DRMs favors the notion that FR rafts may represent the physical site for membrane recruitment and action of PKCα in phorbol ester-induced regulation of their recycling. To more directly test whether RACK1 is associated with FR rafts, electron microscopy was used to visualize possible colocalization of FR and RACK1 in situ in MA104 cells (Figure 4D). FR rafts were first cross-linked with an FR-specific rabbit antibody to enable visualization of FR rafts in the form of large clusters of 12 nm colloidal gold particles conjugated to the secondary antibody. RACK1, which is intracellular, was visualized by probing with an anti-RACK1 antibody and 6 nm gold-conjugated secondary antibody in sections, postembedding. The

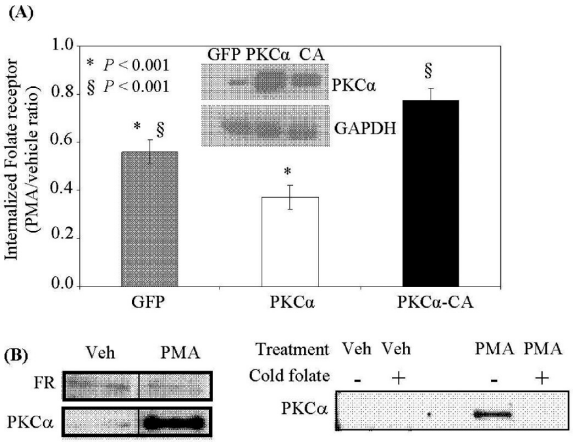


FIGURE 5: Membrane microdomain localization of PKCα in response to phorbol ester. In (A) MA104 cells in FFRPMI were infected with Adeno-GFP, Adeno-wild type PKCα, and Adeno-PKCα-CA (constitutively active PKCα). After 48 h of infection, the cells were treated with vehicle or 0.1 μM of PMA for 30 min and incubated with 27 nM [^{3}H] folic acid for 2 min at 37 °C. The cells were washed with PBS and the externally bound [^{3}H]folic acid was harvested using an acid buffer wash for 1 min on ice. The internalized [^{3}H]folic acid was collected by lysing the cells with 0.1 N NaOH. The radioactivity was measured using a liquid scintillation counter. Each bar represents the ratio of PMA to vehicle for the internalized [^{3}H]folic acid. The inset shows a Western blot of cell lysates probed with antibody to confirm the overexpression of PKCα or PKCα-CA. The total cell associated [^{3}H] folic acid was ~2 pmol/10^6 cells. All values are the average ± SD obtained from triplicate samples. *P* values for the differences noted the text were < 0.001. In (B), cell membranes (left panel) and folate affinity purified DRMs (right panel) were prepared from MA104 cells treated with vehicle or PMA (1 μM) as described under the Experimental Procedures section. Western blot analysis was performed using antibodies for FR and/or PKCα. The DRMs were also incubated with 1 mM folic acid for 30 min prior to the affinity purification using biotin-SS-folate in order to determine nonspecific binding to the probe (right panel).

use of LR White embedding medium (for antibody permeability) did not allow the use of reagents to clearly stain the cell membrane (*7*); however, the cell surface is visible in the electron micrographs (Figure 4D). RACK1 appeared to frequently associate with FR (Figure 4D). A much greater proportion of FR clusters colocalized with RACK1 compared to the control, karyopherin α2, which is a cytoplasmic/nuclear shuttle protein (Figure 4D).

Membrane Microdomain Localization of PKCα Is Crucial for the Phorbol Ester Effect on FR Recycling. To test whether a specific membrane microdomain localization is critical for activated PKCα to mediate the phorbol ester effect on FR recycling, a constitutively active mutant PKCα (PKCα-CA) was ectopically expressed in MA104 cells. Unlike the wild-type enzyme, the PKCα-CA is myristoylated due to the insertion of a signal peptide for myristoylation (see Experimental Procedures) and is consequently targeted in a ligand-independent manner to the plasma membrane but not specifically to lipid rafts (*44*). In contrast to the striking increase in the inhibition of FR influx by PMA due to overexpression of ectopic wild-type PKCα (Figure 5A), overexpression of an equivalent amount of PKCα-CA had the opposite (dominant negative) effect (Figure 5A). This result indicates that appropriate targeting of the activated PKCα to a specific membrane microdomain is necessary for it to modulate FR recycling.

As expected, activation by PMA resulted in the targeting of cytosolic PKCα to the plasma membrane in MA104 cells as

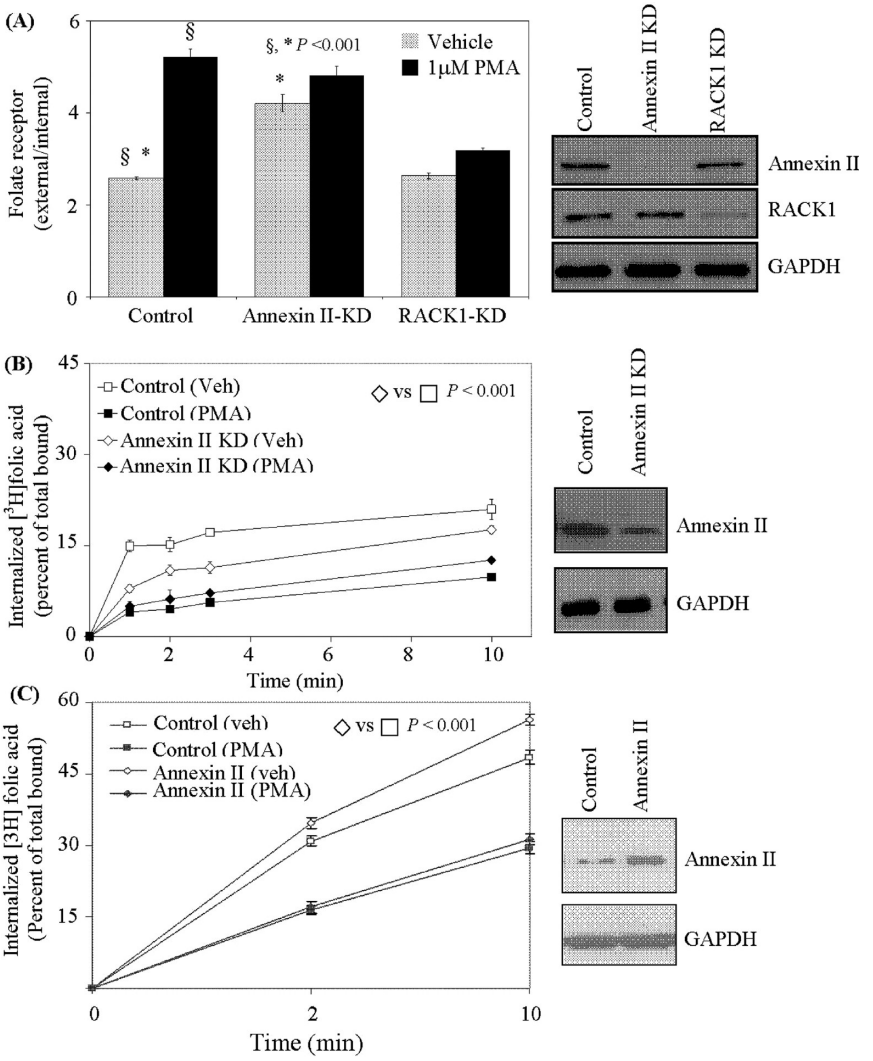


FIGURE 6: The role of annexin II or RACK1 on FR recycling in MA104 cells. (A) MA104 cells were infected with lentiviral particles containing a control shRNA or shRNA for the knockdown of RACK1 or annexin II. The next day, the media was replaced and the cells were cultured for another 48 h. The recycling of FRα was determined 72 h postinfection as previously described under the Experimental Procedures section (left panel). The total cell associated [^{3}H] folic acid was ~2 pmol/10^6 cells. Each bar represents mean ± SD of triplicate measurements. *P* values for the differences noted the text were < 0.001. Cell lysates from the same experiment were also analyzed by Western blot using annexin II and RACK1 antibodies, and the same blots were reprobed with a mouse anti-GAPDH antibody to ensure the uniformity of sample loading (right panel). In (B), MA104 cells plated as 2.25×10^5 cells per well in a 6-well plate were infected 48 h later with lentiviral particles containing a control shRNA or shRNA for the knockdown of annexin II. FR internalization was measured as described under the Experimental Procedures section (left panel). Cells were also lysed on day 6 and analyzed by Western blot using annexin II and GAPDH antibodies (right panel). The data are plotted as the percentage of [^{3}H]folic acid relative to the total radioactivity bound. All samples were measured in triplicates. The subtracted nonspecific association (< 2%) of [^{3}H]folic acid was determined using negative control samples that were treated with excess unlabeled folic acid. In (C) MA104 cells in FFRPMI were infected with Adeno-GFP and Adeno-annexinII. After 48 h of infection the cells were treated with vehicle or 0.1 μM of PMA for 30 min and incubated with 27 nM [^{3}H] folic acid for 0, 2, and 10 min at 37 °C. The cells were washed with PBS and the externally bound [^{3}H]folic acid was harvested using an acid buffer wash for 1 min on ice. The internalized [^{3}H]folic acid was collected by lysing the cells with 0.1 N NaOH. The radioactivity was measured using a liquid scintillation counter (left panel). Cells were also lysed on day 6 and analyzed by Western blot using annexin II and GAPDH antibodies (right panel). The data are plotted as the percentage of [^{3}H]folic acid relative to the total radioactivity bound. All samples were measured in triplicates. The subtracted nonspecific association (< 2%) of [^{3}H]folic acid was determined using negative control samples that were treated with excess unlabeled folic acid. All values are the average ± SD obtained from triplicate samples.

evident in the enrichment of the enzyme in the total membrane preparation (Figure 5B). A parallel physical recruitment of PKCα to FR-rich rafts induced by PMA was evident from the observation that upon PMA treatment affinity purified FR-rich DRMs were specifically and greatly enriched in PKCα (Figure 5B).

RACK1 Is Required for Redistribution of FR by Phorbol Ester. RACK1 is crucial for the activation of PKCα. The steady-state distribution of FR was unaltered by knocking down RACK1 (Figure 6A). However, knocking down RACK1 using siRNA essentially abrogated the PMA effect (Figure 6A). The results indicate that RACK1 is not involved in determining the steady-state distribution of FR but does mediate the PMA effect on FR recycling.

Annexin II Has a Functional Role in the Steady-State Recycling of FR. Annexin II is a substrate of PKCα that plays an important role in membrane fusion during vesicular transport. Therefore, the functionality of endogenous annexin II in FR recycling was tested. Knocking down annexin II using siRNA resulted in externalization of FR similar to the effect of PMA (Figure 6A). As predicted from this result, there was not an appreciable further externalization of FR by PMA when annexin

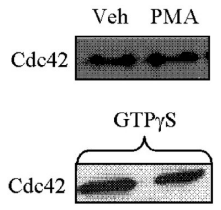


FIGURE 7: Effect of phorbol ester on Cdc42 activity in MA104 cells. MA104 cells were treated with vehicle or 1 μM PMA for 30 min at 37 °C and then assayed for Cdc42 activity. The cell lysates were centrifuged at 16,000 g for 15 min at 4 °C. The supernatant was added to a spin cup in which an immobilized glutathione disk and 20 μg of GST-Pak1-PBD were already mixed and incubated for 1 h at 4 °C with gently rocking then washed 3 times. Finally, 2× SDS sample buffer with 5% fresh β-merceptoethanol were added to the resin, boiled for 5 min and centrifuged. For each of the cell lysates assayed a positive control was performed in which both 10 mM EDTA and 10 mM GTPγS (a nonhydrolyzable form of GTP that irreversibly binds to Cdc42) were added for 15 min at 30 °C. The reaction was stopped by placing the samples on ice and adding $MgCl_2$ for a final concentration of 60 mM. All samples were then assayed for Cdc42 by Western blot using a mouse monoclonal anti-Cdc42 antibody.

II was knocked down (Figure 6A). A partial knockdown of annexin II (Figure 6B) resulted in a decrease in the influx rate of ~50% within 1 min of uptake of [^{3}H] folic acid (Figure 6B) indicating that annexin II is necessary to support influx of FR. The role of annexin II in the recycling of FR was further confirmed by the observation that ectopic annexin II increased the basal influx rate of FR without altering the influx rate after treatment with PMA (Figure 6C); the small though significant magnitude of this change in FR internalization in Figure 6C compared to the change resulting from knocking annexin II (Figure 6A,B) suggests that the endogenous level of annexin II may be close to optimal; alternatively, the rate of influx may be limited by factors unrelated to annexin II.

Inhibition of FR Internalization by PMA Is Not Associated with Inactivation of Cdc42. Since FR recycling has been shown to occur through a Cdc42-dependent pinocytic pathway, and since Cdc42 is a potential downstream substrate for phosphorylation by PKCα, it was of interest to test whether the effect of phorbol ester on FR recycling was mediated by inactivation of Cdc42. The activity of Cdc42 as determined by the whole cell lysate pull-down assay, performed using the Pak1-PBD binding domain, did not decrease upon phorbol ester treatment. Therefore, Cdc42 is not a likely mediator of the inhibition of FR raft internalization (Figure 7).

DISCUSSION

The results of this study offer a mechanistic link between PKC signaling and FR recycling. For the mechanistic studies described in this study phorbol ester, a stable agonist of cPKCs was used in lieu of diacylglycerol, their natural and relatively unstable agonist, to prolong the redistribution of FR for a duration that enabled methods designed to accurately measure the distribution of the receptor. The molecular mechanistic data described above for the effect of phorbol ester on FR distribution in the cell combined with known molecular interactions in PKC-mediated signaling may be used to put in perspective physiological processes involved in at least one mechanism controlling FR recycling. In this scheme, the second messenger diacylglycerol, generated due to a cell surface signal that activates membrane phospholipase C (PLC) (reviewed in ref *58*), activates PKCα, which is targeted to FR-rafts by RACK1. Intracellular Ca^{2+} that is mobilized by activation of PLC (reviewed in ref (*59*)) also contributes to the activation of PKCα. Whereas PKCα and RACK1 are not engaged in the steady-state recycling of FR-rafts, annexin II associated with the membrane is required for their influx. Phosphorylation of a FR-raft associated PKCα substrate, possibly annexin II (*60*−*62*), inhibits FR internalization to increase the receptor population on the cell surface. The activation of Cdc42, which is required for FR recycling (*20*) and is an indirect substrate for PKCα (*63*), is not diminished in this process and is not a likely mediator of the inhibition of FR internalization. The study also identified the PKC inhibitor KCIP-1 as an FR-raft associated protein but a possible functional role for it in FR recycling was not tested.

Curiously, the model supported by this study contrasts the molecular events that have been reported (*35*) in MA104 cells to mediate the effect of phorbol ester on caveolae, despite their compositional resemblance to GPI-protein-enriched membrane domains. Caveolae cycle through a process of invagination and vesicularization to form plasmalemmal vesicles followed by a return to the cell surface as a flattened membrane (*35*). In early studies, the kinetics of FR recycling were extended to the recycling of caveolae based on the notion that FR was primarily localized in caveolae and not in endocytic compartments (*25*, *64*). Subsequent studies have attributed the apparent colocalization of FR and caveolae to a technical artifact (*65*), and several complementary approaches have clearly established that FR is present in caveolin-free rafts that recycle through endosomes by a nonclassical pathway (*7*, *20*, *29*−*33*). Nevertheless, morphological studies have shown that the cycling of caveolae is associated with a cycle of phosphorylation of an apparent 90 kDa protein by PKCα that is constitutively associated with caveolae and its dephosphorylation by a resident phosphatase (*36*); in this model, phorbol ester or diacylglycerol causes dissociation of PKCα from the caveolae resulting in an arrest of vesicularization of caveolae. Dissociation of activated PKCα from caveolae is contrary to the well-known mobilization of the activated enzyme to the plasma membrane and to FR-rich lipid rafts observed in this study. Further, the unidentified 90 kDa PKCα substrate in caveolae cannot be related to annexin II since the latter protein has an apparent subunit molecular weight of 30−40 kDa. The mechanism of regulation by PKC signaling thus appears to represent a major point of functional divergence between caveolae and FR clusters in the membrane.

Regulation of FR recycling has clear implications in the receptor-mediated cellular uptake of reduced folate coenzyme and also in FR-targeted drug delivery. Certainly, the membrane microdomains enriched in FR are also likely associated with signaling molecules as previously suggested (*55*, *56*) and as confirmed in this study. Therefore, in the broader context, regulation of the population and residence time of FR-rafts at the cell surface could be related to their signaling function, and this may be regulated by endogenous signaling molecules and controlled by agents that specifically target this recycling pathway. Regardless of the physiological significance of regulation of FR recycling, the mechanism offers a potential means of controlling FR internalization for more effective delivery of cancer therapeutics. For example, the findings in this study may be useful in investigating the mechanism and significance of the observation that in a variety of malignant cell lines FR largely resides on the cell surface at steady state (*5*).

Future studies should investigate the potential use of agents that temporarily disrupt PKC α signaling to increase the

residence time of FR-rich endosomes, facilitating more efficient drug delivery through FR in tumors that commonly have dysregulated PKC signaling.

Whether the principles of regulation of FR raft recycling by phorbol ester elucidated in this study may be extended to the recycling of lipid rafts enriched in other GPI-anchored proteins remains to be tested. Such studies may have implications in therapeutic interventions to target raft recycling such as the development of immunomodulators and growth inhibitors or even drugs to disrupt the localization of a pathogenic protein such as the scrapie prion protein (*66*).

SUPPORTING INFORMATION AVAILABLE

Four tables. This material is available free of charge via the Internet at http://pubs.acs.org

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