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Compartmentalization of Epidermal Growth Factor Receptor in Liver Plasma Membrane

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ABSTRACT

We have investigated epidermal growth factor (EGF)-induced compartmentalization and activation of the EGF receptor (EGFR) in rat liver plasma membrane (PM) raft subfractions prepared by three different biochemical methods previously developed to characterize the composition of membrane rafts. Only detergent-resistant membranes (DRMs) possessed the basic characteristics attributed to membrane rafts. Following the administration of a low dose of EGF (1 μg/100 g BW) the content of EGFR in PM–DRMs did not change significantly; whereas after a higher dose of EGF (5 μg/100 g BW) we observed a rapid and marked disappearance of EGFR (around 80%) from both PM and DRM fractions. Interestingly, following the administration of either a low or high dose of EGF, the pool of EGFR in the PM–DRM fraction became highly Tyr-phosphorylated. In accordance with the higher level of EGFR Tyr-Phosphorylation, EGF induced an augmented recruitment of Grb2 and Shc proteins to PM–DRMs compared with whole PM. Furthermore neither high nor low doses of EGF affected the caveolin content in DRMs and PM. These observations suggest that EGFR located in DRMs are competent for signaling, and non-caveolae PM rafts are involved in the compartmentalization and internalization of the EGFR. J. Cell. Biochem. 107: 96–103, 2009.

KEY WORDS: EGFR; COMPARTMENTALIZATION; SIGNALING; DRM; RAFTS

Membrane rafts are microdomains enriched in cholesterol, sphingolipids, and phospholipids with saturated acyl chains [Brown and London, 1998]. Caveolae are flask-shaped plasma membrane invaginations, a proportion of which appear to constitute a subset of membrane rafts [Anderson, 1998; Balbis et al., 2004]. These highly ordered lipid domains have been shown to be involved in modulating membrane trafficking and signal transduction [Simons and Ikonen, 1997; Simons and Toomre, 2000; Parton and Richards, 2003].

Extraction of cellular membranes in cold 1% Triton X-100 followed by density gradient centrifugation allow the separation of detergent-insoluble, low-density membranes (DRMs) with a lipid composition characteristic of rafts [Brown and London, 1998]. Although DRMs do not represent physically intact rafts, it is recognized that proteins co-purifying with DRM have a high affinity for these lipid domains in vivo [Shogomori and Brown, 2003]. Because of the artificial nature of DRMs, detergent-free methods have also been developed for the isolation of rafts [Smart et al., 1995; Song et al., 1996; Macdonald and Pike, 2005].

The localization of many tyrosine kinase receptors in membrane rafts, including the IRK and epidermal growth factor receptor (EGFR), seems important for the regulation of downstream signaling [Pike, 2005]. It has been reported that disruption of membrane rafts by cholesterol depletion increased the number of EGFRs accessible to epidermal growth factor (EGF) and consequent downstream signaling [Furuchi and Anderson, 1998; Pike and Casey, 2002; Ringerike et al., 2002; Roepstorff et al., 2002; Pike, 2005]. These data indicate that EGFR signaling is suppressed in lipid rafts. Using a detergent-free method to isolate rafts/caveolae Mineo et al. concluded that, following EGF stimulation, EGFRs rapidly leave rafts/caveolae, followed by clathrin-dependent internalization [Mineo et al., 1996, 1999; Pike, 2005]. Taken together these data suggest that movement of EGFR out of rafts in response to EGF is required for full activation of EGFRs and downstream signaling [Pike, 2005].

This model has recently been questioned by Puri et al. [2005] who, studying isolated DRMs and intact cells by immuno gold EM, reported EGF-induced recruitment of EGFR, signaling and

Abbreviations used: BW, body weight; EGFR, epidermal growth factor receptor; DRMs, detergent-resistant membranes; PM, plasma membrane; EM, electron microscopy; TfR, transferrin receptor.

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trafficking-related proteins to rafts. These data are comparable with our previous observations on EGFR activation in endosomal DRMs indicating that EGFR located in DRMs manifest heightened signaling competence [Balbis et al., 2007]. Puri et al. [2005] also showed that endocytosis of the EGFR occurs from non-caveolar lipid rafts within clathrin-coated pits. Furthermore Sigismund et al. [2005] observed the internalization of EGFR through both clathrin- and raft/caveolae-dependent mechanisms. These observations suggest that plasma membrane (PM) rafts are involved in the internalization of the EGFR.

It is possible that the different methodologies used in these studies (Triton X-100, detergent-free methods, EM) could explain, at least in part, the observed discrepancies. To clarify this point we compared three methods used to isolate membranes rafts from purified rat liver PM with respect to the content and signaling competence of EGFR in these fractions before and after EGF stimulation.

MATERIALS AND METHODS

ANIMALS

Female Sprague–Dawley rats, 10 week of age and 160–180 g body weight (BW), were purchased from Charles River Canada (St. Constant, PQ, Canada), and housed in an animal facility with 12:12 h light–dark cycles at 25°C. They were fed ad libitum on Purina chow, and fasted overnight (16–18 h) before use. All studies herein cited were performed with the approval of the McGill University Animal Care Committee.

MATERIALS

Antibodies against EGFR (sc-03), Rab5 (sc-309), Caveolin-1 (sc-894), Grb2 (sc-255), and phosphotyrosine (PY) proteins (PY99, sc-7020) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Shc (06-203) and Na/K ATPase (05-369) were from Upstate (Lake Placid, NY). An antibody against the transferrin receptor (13-6800) was from Zymed Laboratories (San Francisco, CA). An antibody against calnexin was kindly provided by Dr. J. J. M. Bergeron (McGill University, Montreal, Canada). Goat antirabbit and goat antimouse were labeled with $Na^{125}I$ as described [McConahey and Dixon, 1980] and were used as secondary antibodies for immunoblotting. Reagents for electrophoresis and for measuring the protein content of the liver fractions were from Bio-Rad (Richmond, CA). Polyvinylidene difluoride Immobilon-P transfer membranes were from Millipore Ltd (Mississauga, Ontario, Canada).

PREPARATION OF LIVER PLASMA MEMBRANES

Rats were anaesthetized and killed by decapitation after intra-jugular injections of low dose of EGF (1 μg/100 g BW) or high dose of EGF (5 μg/100 g BW) at the times indicated in the text. Livers were exsanguinated, rapidly excised, and minced at scissor-point in ice-cold buffer (5 mM Tris–HCl buffer, pH 7.4, containing 0.25 M sucrose, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM $MgCl_2$, 2 mM NaF, and 2 mM Na_3VO_4). PM was prepared as described previously [Khan et al., 1989].

PREPARATION OF DRMS

DRMs were isolated by a modification of the method of Liu and Anderson [1995]. PM was pelleted and mixed with 3 ml of ice-cold 1% Triton X-100 in buffer A (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM Na_3VO_4). The samples were homogenized (10 strokes in a glass homogenizer), incubated on ice for 1 h, adjusted to the same amount of protein, and diluted 1:1 with 80% sucrose in buffer B (50 mM Tris–HCl, pH 7.5, and 150 mM NaCl). The extract (4 ml, ~5 mg of protein) was loaded at the bottom of a 12 ml ultracentrifuge tube and overlaid with 4 ml each of 30% and 10% sucrose in buffer B. The gradient was centrifuged for 21 h at 13,000 rpm in a SW40 Ti rotor (Beckman Instruments), and 4 ml (corresponding to the middle of the 10–30% sucrose gradient), was collected and further centrifuged at 200,000*g* for 1 h. The resultant pellet, re-suspended in 0.5 ml of phosphate buffered saline (PBS), constitutes the DRM fraction. Samples (20 μg protein) were subsequently analyzed by SDS–PAGE and Western blotting.

PREPARATION OF MEMBRANE RAFTS/CAVEOLAE BY TWO DETERGENT-FREE METHODS

Lipid rafts/caveolae were isolated by the detergent-free method of Smart et al. [1995]. PM was briefly sonicated in buffer A (0.25 M sucrose, 1 mM EDTA, 2 mM Na_3VO_4, 20 mM Tricine, pH 7.8), then mixed with buffer C (50% (w/v) iodixanol in buffer B (0.25M sucrose, 6 mM EDTA, 120 mM Tricine, pH 7.8, plus 40 mM sucrose) to a final iodixanol concentration of 23% and overlaid with 6 ml of a linear gradient (10–20%) of iodixanol in buffer B. Samples were centrifuged at 53,000*g* for 90 min. The top 5 ml of the gradient was mixed with 4 ml of buffer C, overlaid with 1 ml of 15% (w/v) iodixanol in buffer B, followed by 0.5 ml of 5% (w/v) iodixanol in buffer B. The gradients were centrifuged at 53,000*g* for 90 min, and caveolae were collected from 5% to 15% interface. Samples (20 μg of protein) were subsequently analyzed by SDS–PAGE and Western blotting.

Rafts/caveolae were also prepared by the method of Song et al. [1996]. PM was mixed with 3 ml of ice-cold Na_2CO_3 (200 mM Na_2CO_3, 50 mM Tris–HCl, 50 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM Na_3VO_4, pH 11). Samples were homogenized (10 strokes in a glass homogenizer), sonicated (three times, 10 s each), and incubated on ice for 1 h. Thereafter, samples were mixed with the same volume of 80% sucrose, loaded at the bottom of a 12 ml ultracentrifuge tube and overlaid with 4 ml each of 30% and 10% of sucrose in buffer B. The gradient was centrifuged for 21 h at 13,000 rpm in a SW40 Ti rotor (Beckman Instruments), and 4 ml (corresponding to the middle of the 10–30% sucrose gradient), was collected and further centrifuged at 200,000*g* for 1 h. The resultant pellet was re-suspended in 2 ml of phosphate buffered saline (PBS). Samples (20 μg of protein) were subsequently analyzed by SDS–PAGE and Western blotting.

RESULTS

CHARACTERIZATION OF PM FRACTIONS

In this study we employed three different methods to isolate membrane rafts from rat liver PM (Fig. 1); and assessed EGF-induced

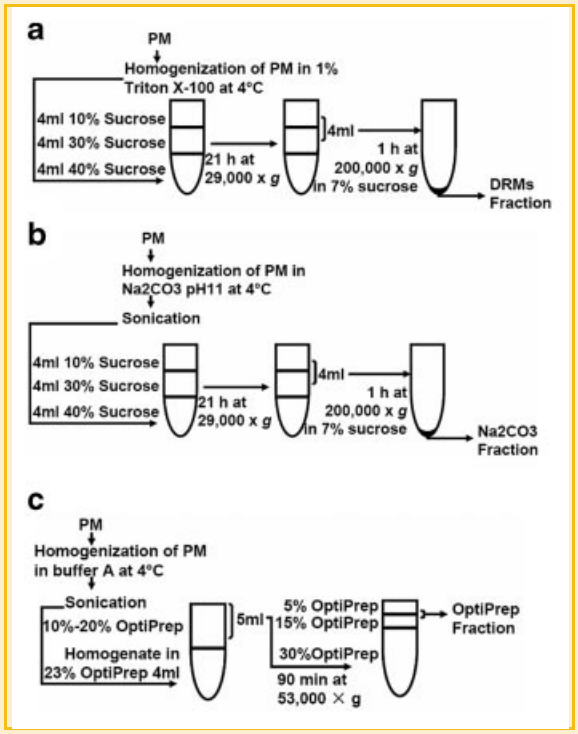

Fig. 1. Methods for preparing membrane rafts. The three schemes for preparing membrane rafts are: (a) extraction in 1% Triton X-100; (b) a detergent-free method based on Na_2CO_3 (pH 11) extraction; and (c) a detergent-free method based on using OptiPrep gradient centrifugation.

compartmentalization and signaling competence in each of the preparations. Our PM preparation was not unduly contaminated with other cellular compartments that could also contain EGFR (i.e., endosomes). Thus Figure 2 shows that the PM markers, actin and Na/K-ATPase are highly enriched in the PM fraction, whereas the endosomal marker Rab5, though concentrated in endosomes, was barely detectable in PM. The endoplasmic reticulum marker calnexin was found enriched in ER as compared with the other cellular fractions (Fig. 2).

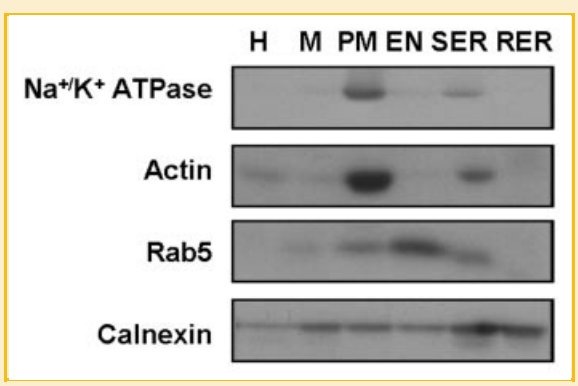

Fig. 2. Preparation of subcellular fractions. Homogenate (H), microsomes (M), plasma membrane (PM), endosomes (EN), smooth ER (SER), and rough ER (RER) were purified from rat liver as indicated in Materials and Methods Section. Samples (40 μg of protein) were subjected to SDS–PAGE followed by immunoblotting with antibodies specific to the Na+/K+ ATPase, actin, rab5, and calnexin.

COMPARISON OF RAFTS/CAVEOLAE PREPARED BY THREE DIFFERENT METHODS

We employed three different methods for preparing membrane rafts: (1) Isolation of DRMs by extraction with 1% Triton X-100 and sucrose gradient centrifugation [Brown and Rose, 1992]; (2) Sodium carbonate (pH 11) extraction followed by sucrose gradient centrifugation [Song et al., 1996]; and (3) A method based on OptiPrep gradient centrifugation [Mineo et al., 1996] (see Materials and Methods Section and Fig. 1). These fractions were characterized by measuring the content of caveolin (a raft marker) and the transferrin receptor (TfR; a non-raft marker). Since the high pH of Na_2CO_3 treatment removes peripheral membrane proteins it is possible that the enrichment of caveolin previously observed [Song et al., 1996] results from the relative enrichment of integral proteins (caveolin is an integral protein) and not due to the selective isolation of raft membranes. To test this possibility, the PM fraction treated with Na_2CO_3 (pH 11) was split into two aliquots; one subjected to sucrose gradient centrifugation (the presumed raft fraction) and the other concentrated by centrifugation (Na_2CO_3-P fraction) representing the total PM depleted of peripheral proteins. As seen in Figure 3, all the rafts fractions were enriched in caveolin compared to the starting PM fraction but caveolin was most highly enriched in DRMs (Fig. 3a). Interestingly the Na_2CO_3 and Na_2CO_3-P fractions contained similar levels of caveolin (Fig. 3a) consistent with a relative enrichment of integral membrane proteins and not the selective isolation of raft membranes. This interpretation is further supported by the observation that the TfR content in these two fractions was similar to that in the starting PM fraction (Fig. 3a). Whereas this was also true of the OptiPrep fraction TfR was essentially unmeasurable in DRMs (Fig. 3a). The distinctive nature of the DRM fraction is further supported by the very different protein pattern seen on SDS–PAGE/Commassie blue staining, compared with the starting PM fraction (Fig. 3b). In contrast the protein pattern of the Na_2CO_3, Na_2CO_3-pellet, and OptiPrep fractions were very similar and more closely resembled that of the starting PM preparation. From these observations we suggest that DRMs represent the fraction with the highest enrichment of raft-derived membranes proteins.

EFFECT OF LOW-DOSE EGF ON COMPARTMENTALIZATION OF EGFR IN PM FRACTIONS

We next investigated the impact of EGF administration (1 μg/100 g of BW) on the distribution of total and tyrosine-phosphorylated EGFR in liver PM and rafts fractions. This dose of EGF is known to saturate around 50% of the EGFR present at the PM [Lai et al., 1989]. Following 2 and 5 min of EGF administration we observed a decrease of approximately 20% in the EGFR content in PM, Na_2CO_3, and OptiPrep preparations (Fig. 4a–c). By 20 min, EGFR content returned to initial levels in all these fractions. This result is consistent with EGFR internalization and recycling as previously described by Lai et al. [1989]. However, changes in the EGFR content in DRMs following EGF stimulation were not statistically significant

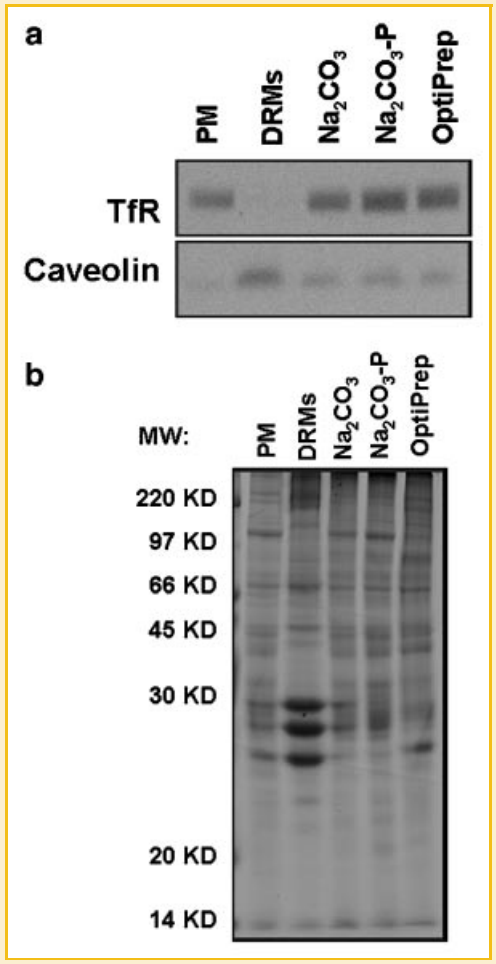

Fig. 3. Characterization of membrane rafts fractions. Isolation of membrane rafts from PM was performed as described in Materials and Methods Section. (a) DRMs, Na_2CO_3, Na_2CO_3-P, and OptiPrep fractions (20 μg of protein) were subjected to SDS–PAGE followed by: (a) immunoblotting with antibodies specific to the TfR and caveolin; and (b) Coomassie blue staining.

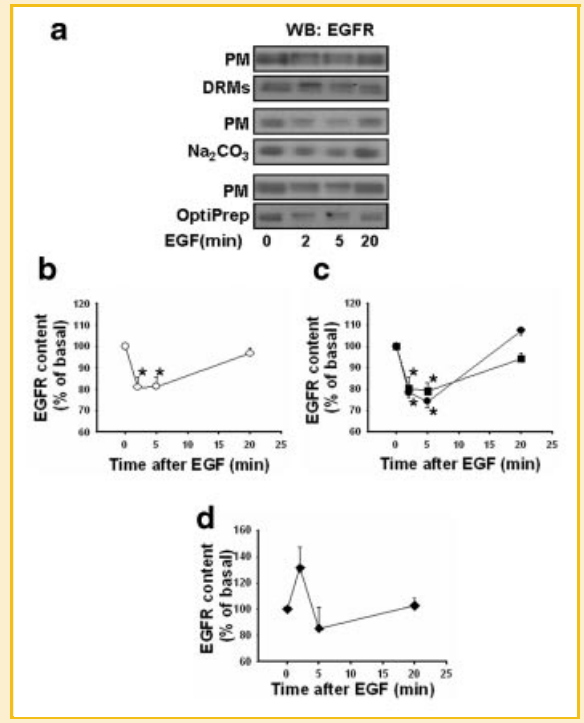

Fig. 4. Compartmentalization of EGFR in PM rafts. After an overnight fast, rats received a single dose of EGF (1 μg/100 g BW) and were killed at the noted times thereafter. Livers were removed and PM, DRMs, Na_2CO_3, and OptiPrep fractions were prepared and subjected to SDS–PAGE as described in Materials and Methods Section. a: Fractions were analyzed by Western Blotting with antibodies against EGFR. b–c: The level of EGFR in PM fractions was quantified using scanning densitometry and the data were expressed as a percent of the basal value in each experiment—(b) EGFR content in PM preparations; (c) EGFR content in Na_2CO_3 (●) and Optiprep fractions (■); and (d) EGFR content in DRM preparations. Each bar is the mean ± SEM of three independent experiments. $^*P < 0.05$ versus basal state.

(Fig. 4d). It is interesting that the phospho-tyrosine content of the EGFR in DRMs was threefold enriched compared to PM and the other raft fractions (Fig. 5a,b). The presence of highly activated EGFR in DRMs is further supported by the augmented EGF-induced association of Shc and Grb2 with this fraction compared to PM (Fig. 5c). Taken together, these results indicate that, of the three raft fractions investigated, only DRMs contain a distinctive population of activated EGFRs.

EFFECT OF HIGH-DOSE EGF ON COMPARTMENTALIZATION OF EGFR IN PM FRACTIONS

It was recently suggested that physiological levels of EGF effect receptor internalization mainly via clathrin-coated pits, while pharmacologic levels result in a substantial fraction of EGFR being internalized via a caveolae-dependent, clathrin-independent mechanism [Sigismund et al., 2005]. We thus sought to evaluate the impact of high-dose EGF on EGFR distribution in PM. It is noteworthy that at the high dose of EGF, highly Tyr-phosphorylated EGFR and signaling proteins were recruited to DRMs (Fig. 6), as observed with the lower dose of EGF.

To investigate the hypothesis that, at a high dose of EGF, internalization involves caveolar structures, we administered EGF (5 μg/100 g BW) known to provoke prolonged downregulation of PM receptors [Lai et al., 1989]. At this dose of EGF 80% of the pool of EGFR located at the PM was internalized by 5 min (Fig. 7a); nor did the level of EGFR at the PM recover by 20 min, consistent with the targeting of internalized EGFR to late endosomal structures (Fig. 7a). As well EGFR disappeared from DRMs with the same kinetic as that observed in the PM fraction (Fig. 7a), suggesting internalization of EGFR from rafts/caveolae.

We next examined the impact of EGF on the caveolin content in whole PM and the DRM subfraction. Following either a low or high dose of EGF there was no significant change in the caveolin content of either whole PM or the DRM subfraction (Fig. 7b). This observation suggests that, in response to EGF, caveolin did not undergo internalization from the PM as observed for the EGFR, nor was there re-distribution of EGFR between PM raft and non-raft domains. The lack of any change in the content of caveolin in DRMs at 5 min after a high dose of EGF (Fig. 7b) contrasts with the 80%

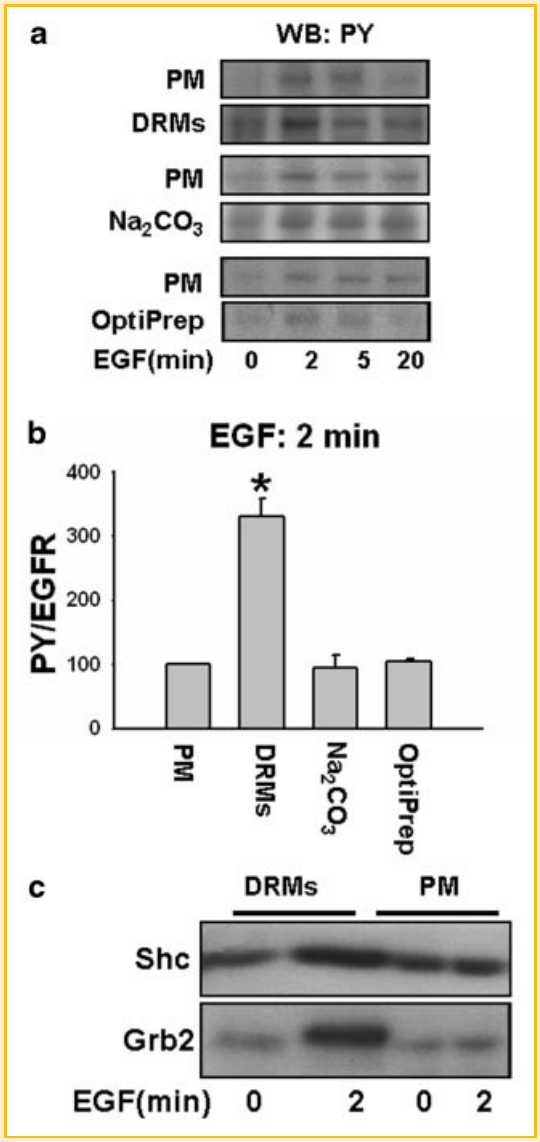

Fig. 5. DRMs are enriched in highly Tyr-phosphorylated EGFR and signaling proteins. After an overnight fast, rats received a single dose of EGF (1 μg/100 g BW) and were killed at the noted times thereafter. Livers were removed and PM, DRMs, Na_2CO_3, and OptiPrep fractions were prepared and subjected to SDS–PAGE as described in Materials and Methods Section. a: Fractions were analyzed by Western blotting (WB) with antibodies against phosphotyrosine (PY). b: The ratio of tyrosine-phosphorylated EGFR/EGFR (PY/EGFR) was determined. Each bar is the mean ± SEM of three independent experiments. $^*P < 0.05$. c: PM and DRMs fractions were analyzed by Western blotting with antibodies against: Shc and Grb2. WB, Western blotting.

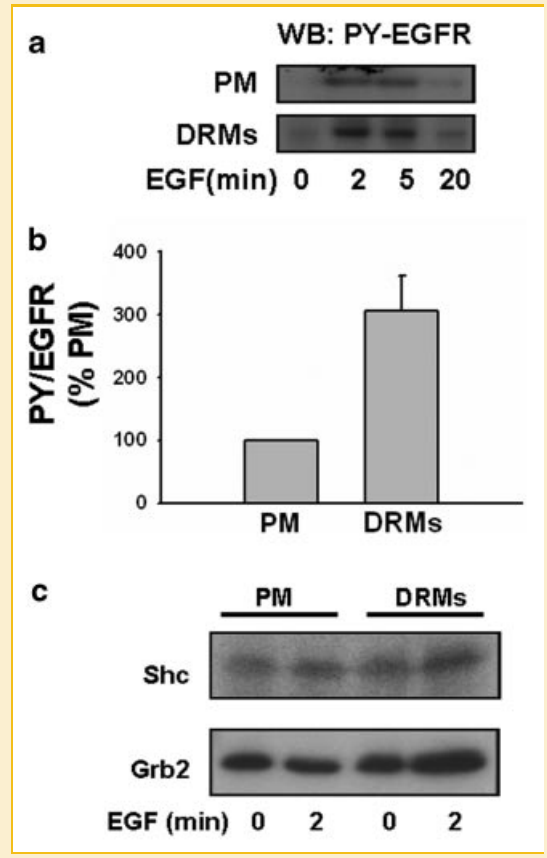

Fig. 6. Effect of high-dose EGF on Tyr-phosphorylated EGFR and signaling proteins in PM and PM–DRMs. After an overnight fast, rats received a high dose (5 μg/100 g BW) of EGF and were killed at the noted times thereafter. Livers were removed and PM and DRMs fractions were prepared and subjected to SDS–PAGE and analyzed by Western blotting with antibodies to PY, Shc, and Grb2 as described in Materials and Methods Section. a: The level of PY-EGFR was assessed in PM and DRMs; (b) the ratio of PY-EGFR/EGFR (PY/EGFR) at 5 min after EGF is plotted (each bar is the mean ± half of the difference of two independent experiments); and (c) Western blots of PM and DRMs with antibodies to Shc and Grb2 are depicted.

decrease of the EGFR (Fig. 7a). These data support the view that non-caveolar PM rafts are involved in the compartmentalization and internalization of the EGFR.

DISCUSSION

COMPARISON OF PREPARATION METHODS

Previous work, examining the localization of EGFR to rafts/caveolae upon ligand-activation, has yielded variable results. Thus activated EGFRs were found to move out of rafts/caveolae by Mineo et al. [1999] but to be recruited to these structures byPuri et al. [2005]. In view of this controversy we have attempted to define the compartmentalization of activated EGFR in membrane rafts/caveolae derived from a highly purified rat liver PM preparation. We compared the results obtained by applying three different biochemical methodologies for preparing membrane rafts to purified rat liver PMs.

DRMs were shown from the beginning to reflect artificial structures (large sheets and vesicles) induced by detergent-extraction [Shogomori and Brown, 2003] but nevertheless appeared to reflect the composition of lipid rafts and thus the lipid and protein entities selectively concentrated in rafts [Shogomori and Brown, 2003]. Subsequently, to circumvent the use of detergents other methods for purifying lipid rafts/caveolae were developed. Song et al. sonicated lysates of whole cells in a Na_2CO_3 buffer (pH 11) followed by centrifugation on a discontinuous sucrose gradient yielding a caveolin-enriched preparation at the 5/35% sucrose

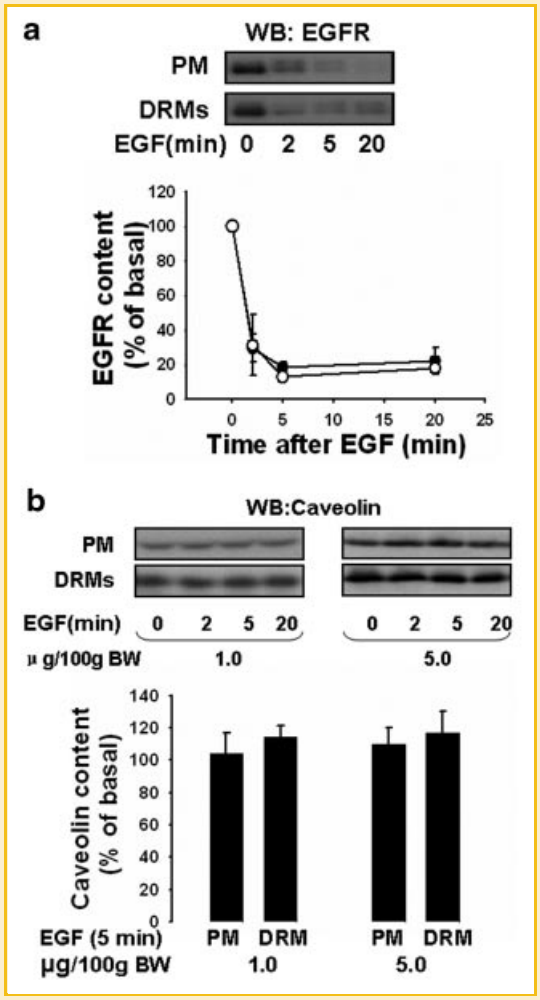

Fig. 7. Dynamics of EGF-induced compartmentalization of EGFR and caveolin at the PM following a high dose of EGF. After an overnight fast, rats received a single dose of EGF (5 μg/100 g BW) and were killed at the noted times thereafter. Livers were removed and PM and DRMs were prepared and subjected to SDS–PAGE as described in Materials and Methods Section. The fractions were analyzed by Western blotting (WB) with antibodies against: (a) EGFR and (b) Caveolin. a: The level of EGFR in DRMs (●) and PM (○) was quantified using scanning densitometry and the data were expressed as a percent of the basal value in each experiment. b: The caveolin content in PM and DRMs is noted at 5 min after the administration of a low (1.0 μg/100 g BW) or high (5.0 μg/100 g BW) dose of EGF.

interface. Enrichment of caveolin was expressed relative to whole cell homogenate, both in this and other studies employing this technique [Song et al., 1996; Yamamoto et al., 1998; Waugh et al., 1999]. Thus Waugh et al. [1999] found caveolin to be purified 20- to 50-fold relative to whole-cell lysate; but whether it was enriched relative to PM was unclear. Furthermore, although many signaling proteins have been found in this fraction (i.e., Ras, G protein, Src [Song et al., 1996], IRK [Yamamoto et al., 1998], and EGFR [Waugh et al., 1999]) there were observations that it contained significant ER and golgi components [Macdonald and Pike, 2005]. A second detergent-free method used a sequential gradient technique employing OptiPrep and sonication to isolate membranes reportedly highly enriched over PM in respect to caveolin [Smart et al., 1995]. However others [Matveev and Smart, 2002] observed caveolin enrichment to be markedly less than that originally reported. Furthermore Macdonald and Pike [2005] found that the raft/caveolae fraction, obtained by the sequential gradient technique, contained a relatively small proportion of PM caveolin; and noted considerable day-to-day variability in recovery of raft markers in this fraction.

In our study we started with highly purified PM and compared DRMs [Brown and Rose, 1992] with fractions prepared using Na_2CO_3 (pH 11) [Song et al., 1996] and OptiPrep methods [Mineo et al., 1996], all of which have been previously used to investigate the role of membrane rafts in respect to EGFR compartmentalization and signaling [Mineo et al., 1996; Waugh et al., 1999; Puri et al., 2005]. We found that the Na_2CO_3 and OptiPrep methods yielded fractions minimally enriched in caveolin, and contained TfRs equal to or greater than that in the starting PM (Fig. 3). Indeed the Na_2CO_3-P fraction, which represents PM stripped of peripheral membrane proteins, did not appear to differ from the Na_2CO_3 "raft" fraction to any significant degree. In addition the Na_2CO_3 preparation showed a protein pattern on SDS–PAGE similar to that of the starting PM, whereas the OptiPrep fraction, though displaying a somewhat less similar pattern, contained a comparably high proportion of integral membrane proteins (Fig. 3). In contrast DRMs reflected more closely the basic features of membrane rafts in that this fraction was enriched in caveolin, free of TfRs, and displayed a very different distribution of protein as assessed by SDS–PAGE (Fig. 3). Although non-detergent methods applied to whole cell preparations have resulted in fractions enriched in rafts/caveolae their use with purified PM yields little if any enrichment. In contrast the DRM preparation achieves substantial enrichment and reflects more closely the properties attributable to rafts/caveolae.

EGFR INTERNALIZATION FROM RAFTS/CAVEOLAE

Internalization of caveolae is well documented in work on the uptake of simian virus 40 (SV40) [Pelkmans et al., 2001, 2002, 2004]. Recently, Di Guglielmo et al. [2003] reported that TGF-β is internalized by a clathrin-dependent mechanism leading to signaling, as well as by a caveolar-dependent pathway leading to receptor degradation and turnover. Sigismund et al. [2005] reported that while clathrin-dependent uptake occurred at low concentrations of EGF, at high-ligand concentrations the EGFR was additionally endocytosed via caveolae. However, in contrast to the SV40 virus studies, these studies on TGF-β and EGF did not document that caveolae ever leave the PM, nor did they deal with kinetic aspects of caveolar downregulation. It should be noted that the presence of a receptor in caveolae as well as in caveosome-like intracellular structures does not necessarily mean that caveolae have been internalized and delivered along with their cargo to an intracellular compartment. In this regard a recent study of Kazazic et al. [2006] demonstrated that EGF-induced activation of the EGFR did not trigger mobilization of caveolae both at high and low concentration. This observation is in accordance with our current results in which, following a low or high dose of EGF, we did not detect any significant change in the caveolin concentration of either whole PM or PM–DRMs.

In previous work we found that, following the administration of low dose EGF (1 μg/100 g BW), EGFRs, rapidly internalized to endosomal DRMs, were approximately threefold more tyrosine-phosphorylated than EGFRs in whole endosomes [Balbis et al., 2007]. This similarity between EGFRs in both PM (Figs. 5 and 6) and endosomal DRMs suggests that those in endosomes are derived from PM raft domains. However, the EGFR content of PM–DRMs, following low-dose EGF, showed no significant change. A possible explanation may be deduced from the observations of Puri et al. [2005] who showed that, following EGF stimulation, EGFR is recruited to PM-rafts followed by internalization from this compartment. If the kinetics of EGFR-induced recruitment to and internalization from PM-rafts are similar, changes in the content of EGFR in PM-rafts would be difficult to detect.

Our findings agree with those of Sigismund et al. [2005] who showed significant internalization of the EGFR via PM-rafts only at high concentrations of EGF (Fig. 7a). However in contrast to Sigismund et al. we found, in both the present and previous work [Balbis et al., 2007], that the raft compartment involved in the internalization and trafficking of EGFR is non-caveolar in nature. Our findings are compatible with those of Puri et al. [2005] where endocytosis of the EGFR occurs from non-caveolar lipid rafts within clathrin-coated pits.

EGFR SIGNALING FROM RAFTS

The functional significance of rafts in signaling has been intensely debated. It has been postulated that PM rafts are privileged sites for signal transduction by various receptors including the EGFR [Cheng et al., 1999; Krauss and Altevogt, 1999; Drevot et al., 2002; del Pozo et al., 2004]. However several groups have generated data indicating that EGFR signaling is suppressed in lipid rafts [Furuchi and Anderson, 1998; Pike and Casey, 2002; Ringerike et al., 2002; Roepstorff et al., 2002]. Using methyl-β-cyclodextrin, a cholesterol-depleting reagent, to disrupt rafts, it was demonstrated that EGFRs in lipid rafts display reduced ligand-binding and EGFR activation [Pike and Casey, 2002; Ringerike et al., 2002; Roepstorff et al., 2002]; as well as blunted EGF dependent activation of MAP kinase [Furuchi and Anderson, 1998]. In contrast, a recent study by Puri et al. [2005] reported EGF-induced recruitment of signaling and trafficking-related proteins to EGFRs in rafts. This accords with our results in which, following EGF treatment, the pool of EGFR in PM–DRMs became approximately threefold more highly Tyr-phosphorylated than EGFRs in the starting PM fraction. Furthermore we found augmented recruitment of EGFR-related signaling proteins to PM–DRMs indicating that EGFR located in DRMs manifest heightened signaling competence. These data are comparable to our previous observations on EGFR activation in endosomal DRMs [Balbis et al., 2007] suggesting that the latter may derive from a selected population of EGFRs concentrated in cell surface rafts. The discrepant observations on the role of membrane rafts in signaling could be partially explained by the effects of cholesterol depletion which not only affects the integrity of lipid rafts but also the physical properties of cellular membranes such as permeability and fluidity thus influencing cell surface protein–protein interactions [Kurzchalia and Ward, 2003; Munro, 2003].

In summary, the results in this study, taken together with our previous work [Balbis et al., 2007], suggest that in response to EGF a distinctive pool of EGFR, that is competent for signaling, is recruited to PM DRMs and subsequently internalized into endosomal DRMs.

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