Epidermal growth factor receptor activation is localized within low-buoyant density, non-caveolar membrane domains

Mark G. WAUGH*, Durward LAWSON† and J. Justin HSUAN*‡[1]

*Ludwig Institute for Cancer Research, University College London Medical School, Courtauld Building, 91 Riding House Street, London W1P 8BT, U.K., †Department of Molecular Pathology, Windeyer Institute for Medical Science, University College London, 46 Cleveland Street, London W1P 6DB, U.K, and ‡Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K.

Increasing evidence for the organization of cell-surface proteins and lipids into different detergent-insoluble rafts led us to investigate epidermal growth factor (EGF) receptor activation in the plasma membranes of A431 carcinoma cells, using a combination of cell fractionation and immunoprecipitation techniques. Density-gradient centrifugation of sodium carbonate cell extracts revealed that the vast majority of both stimulated and unstimulated EGF receptors were concentrated in a caveolin-rich light membrane (CLM) fraction, with the biochemical characteristics of detergent-insoluble glycolipid-rich domains (DIGs). However, ultrastructural analysis of the CLM fraction revealed that it contained a heterogeneous collection of vesicles, some with sizes greater than that expected for individual caveolae. Experiments with detergent-solubilized cells and isolated CLMs indicated that, in contrast with caveolin, EGF receptors were unlikely to be localized to DIG domains. Furthermore, immunoisolation of caveolin from CLMs revealed that EGF receptor activation occurs in a compartment distinct from caveolae. Similarly, using an anti-(EGF receptor) antibody, the bulk of the cellular caveolin was not co-immunoprecipitated from CLMs, thereby confirming that these two proteins reside in separate membrane domains. The deduction that caveolar signalling and EGF receptor activation occur in separable rafts argues for a multiplicity of signal transduction compartments within the plasma membrane. In addition, by demonstrating that EGF receptor activation is compartmentalized within low-density, non-caveolar regions of the plasma membrane, it is also shown that the co-localization of proteins in a CLM fraction is insufficient to prove caveolar localization.

Key words: caveolae, growth factor signalling, membrane rafts.

INTRODUCTION

Caveolae were first identified in electron microscopy studies as flask-shaped invaginations of the plasma membrane [1,2], and have been shown to be enriched in a 22–24-kDa integral membrane protein called caveolin [3,4]. The caveolar signalling hypothesis [5,6] postulates that the presence of signalling molecules in caveolae facilitates the efficacious formation of signalling complexes in response to agonists. Much of the work that has supported this hypothesis has emerged primarily from experiments that have isolated putative caveolae on the basis of their insolubility in Triton X-100 [7,8], and their distinct low-buoyant density following isopynic centrifugation [9–11]. These low-buoyant density, caveolin-rich preparations have been shown to contain high concentrations of signalling molecules, including a variety of receptors [10,12–15], G-proteins [14,16,17], non-receptor tyrosine kinases [18,19] and lipid-signalling precursors, such as phosphatidylinositol [20,21]. Indeed, it has been proposed that domains of the caveolin protein itself might directly regulate a variety of signalling molecules, including endothelial nitric oxide synthase [22], epidermal growth factor (EGF) receptors [13], $p21^{ras}$ [11] and G-proteins [16].

However, concerns have been raised about the inference that caveolar localization can be assumed solely on the basis of these membrane preparations, since in the presence of detergent, glycosylphosphatidylinositol-linked proteins and lipids can form distinct microdomains [23,24], even in the absence of any caveolin protein [25,26]. Moreover, there is some evidence to suggest that caveolae prepared in the absence of detergent might also be contaminated by non-caveolar membrane fragments [9,27].

In the present study the distribution of the EGF receptor tyrosine kinase in the plasma membrane of A431 human epidermoid carcinoma cells has been investigated. Stimulation of EGF receptors is known to result in a plethora of signalling events that are instigated principally through the formation of signalling complexes and protein tyrosine phosphorylation, such as in the activation of the $p21^{ras}$ (reviewed in [28]), phospholipase C (PLC) and phosphoinositide 3-kinase pathways (reviewed in [29]). These signalling pathways can be regulated by numerous stimuli, in addition to EGF, and display heterogeneous subcellular distributions. Consequently, in order to identify the site of EGF receptor signalling, we have studied the subcellular localization of EGF receptor tyrosine autophosphorylation, which is an essential event in EGF receptor activation.

Evidence is presented that co-localization of receptors and caveolin in the caveolin-rich light membrane (CLM) fraction of a density gradient is, in itself, insufficient to substantiate caveolar localization. We show that EGF receptor activation occurs in discrete, low-density microdomains of the plasma membrane that co-purify with the CLM fraction, but are distinct from detergent-insoluble glycolipid-containing domains (DIGs) and caveolae. These results indicate that EGF receptors are highly organized within the plasma membrane of A431 cells.

EXPERIMENTAL

Materials

Protein G–Sepharose CL-4B, secondary antibodies, pre-stained molecular-mass markers and the enhanced chemiluminescence (ECL) Western-blotting detection system were purchased from

Abbreviations used: CLM, caveolin-rich light membrane; DIG, detergent-insoluble glycolipid-rich domain; DMEM, Dulbecco's modified Eagle medium; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; PKC, protein kinase C; PLC, phospholipase C.

[1]To whom correspondence should be addressed (e-mail justin@ludwig.ucl.ac.uk).

Amersham Pharmacia Biotech., Little Chalfont, Bucks., U.K. Monoclonal anti-(caveolin IgG) (mAb C060), anti-caveolin antiserum and a monoclonal antibody specific for the activated form of the EGF receptor were obtained from Transduction Laboratories, Lexington, KY, U.S.A. The monoclonal anti-(EGF receptor) antibody R1 was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A. The anti-(EGF receptor) peptide antiserum (anti-2E) has been described previously [30]. Cell culture reagents were from Life Technologies, Paisley, Renfrewshire, Scotland, U.K. Protein assay reagents were purchased from Bio-Rad (Hemel Hempstead, Herts., U.K.). Protease inhibitor cocktail tablets (COMPLETE) were from Roche Diagnostics (Welwyn Garden City, U.K.). OsO_4 was purchased from Fluka (Gillingham, Dorset, U.K.). All other reagents were obtained from Sigma–Aldrich (Poole, Dorset, U.K.).

Cell culture

A431 cells were maintained at 37 °C in a humidified incubator with 10 % CO_2. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing Glutamax, 10 % (v/v) fetal-calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin. In the case of EGF stimulation, cells were serum-starved for 24 h by culturing in DMEM–Glutamax supplemented with insulin (5 μg/ml) and transferrin (5 μg/ml), and then treated with EGF (100 nM) for 2 min at 37 °C.

Isolation of CLM domains

CLMs were prepared using a detergent-free method, as described previously [11]. All procedures were carried out at 4 °C and buffers contained 2 mM sodium orthovanadate and 1 mM sodium fluoride to inhibit protein phosphatases. A431 cell monolayers grown to 50–70 % confluence in 92-mm dishes were washed twice with PBS and scraped into 2 ml of 100 mM Na_2CO_3, pH 11.0 containing protease inhibitors. Cells were disrupted by Dounce homogenization (15 strokes), followed by sonication using a Soniprep 150 sonicator (MSE) on setting 10 (3×20 s bursts). Homogenate (2 ml) was mixed with 2 ml of 90 % (w/v) sucrose in MBS buffer (25 mM Mes/150 mM NaCl, pH 6.5) and placed in a 12-ml ultracentrifuge tube. A discontinuous 5–35 % (w/v) sucrose gradient was formed above the sample by layering on to 4 ml of 35 % (w/v) sucrose solution, followed by 4 ml of 5 % (w/v) sucrose solution. Both the 5 % and 35 % (w/v) sucrose solutions were made in MBS buffer containing 250 mM Na_2CO_3. The sample was then centrifuged at 39000 r.p.m. (175000 ***g***) for 16–18 h in a Beckman SW41 rotor. A light-scattering band was identified at the 5–35 % (w/v) sucrose interface that was enriched with caveolin, but excluded the bulk of the cellular protein. Fractions (1 ml) were collected from the top of each gradient. In some cases, samples were fractionated on a 5–35 % sucrose gradient that was formed using a pump and gradient-mixing system from a Pharmacia SMART separation unit. The protein content of each fraction was determined by using a Bradford assay (Bio-Rad).

Alternatively, Triton X-100-insoluble caveolae membranes were prepared as described by Brown and Rose [7,31]. The method was essentially the same as that described for the sodium carbonate method, with the exception that sodium carbonate was omitted from all steps and the initial extraction of the A431 cells was with 1 % (v/v) Triton X-100 in 25 mM Mes, pH 6.5.

Immunoblotting

Samples were mixed with an equal vol. of 2 × sample buffer and separated by SDS/PAGE. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) and probed with various antibodies. Bound antibody was detected using the ECL system.

In order to quantify the concentration of caveolin in various samples, immunoblots were analysed by two-dimensional densitometric analysis. The concentration of caveolin in the original cell homogenate was determined from immunoblots by constructing a standard curve of % volume (a measure of signal intensity) against protein concentration for serial dilutions of the cell lysate. This relationship was found to be essentially linear for protein concentrations < 0.6 mg/ml (results not shown).

Electron microscopy

Electron microscopic analysis of the CLM fraction was performed as described by Stan et al. [27]. CLMs were fixed in suspension in 1 % (w/v) OsO_4, pelleted by centrifugation for 30 min in a bench-top centrifuge at maximum setting and then stained with 2 % (v/v) uranyl acetate for 1 h at 4 °C in the dark. Subsequent processing of the samples for electron microscopy was performed as described previously [32].

Detergent-solubility experiments

A431 cell monolayers were solubilized with 1 % (v/v) Triton X-100 in PBS buffer, pH 7.4, with protease inhibitors on ice for 30 min. The entire contents of the plate were harvested using a cell scraper, and the volume brought up to 12 ml with 1 % (v/v) Triton X-100 buffer. Triton X-100-insoluble fractions were harvested by ultracentrifugation of the lysate for 1 h at 175000 ***g*** at 4 °C. The resulting Triton X-100-insoluble pellet was resuspended in 12 ml of 2 × SDS/PAGE sample buffer and boiled for 10 min to elute the associated proteins. The contents of the pellet and supernatant were then examined by immunoblotting.

A similar protocol was utilized for investigating the detergent-solubility profile of the CLM fraction isolated by the sodium carbonate method, with the exception that a 100 μl CLM aliquot was mixed with an equal vol. of each detergent made up at twice its final concentration in 20 mM Tris/HCl, pH 7.4, and left on ice for 30 min. The samples were then placed in the bottom of an ultracentrifuge tube, which was then topped up with ice-cold 20 mM Tris/HCl, pH 7.4, and centrifuged at 175000 ***g*** for 1 h at 4 °C.

Immunoprecipitation studies

For the isolation of caveolae under detergent-free conditions, CLM samples isolated by the carbonate method were precleared with 20 μl of Dynal 280 beads coated with sheep anti-(rabbit IgG). The cleared sample was incubated with anti-caveolin polyclonal antiserum (1 μg/ml) for 2 h at 4 °C, followed by the addition of Dynabeads for 30 min at 4 °C. Immunocomplexes were collected without centrifugation using magnetic separation, and washed four times in 20 mM Tris/HCl, pH 7.4. In other experiments, whole A431 cell lysates prepared in 100 mM Na_2CO_3 were sonicated and neutralized in MBS buffer containing 1 % (v/v) Tween 20. Immunocomplexes were collected on Dynabeads, and washed four times with 20 mM Tris/HCl, pH 7.4. Immunoprecipitated caveolin was detected by immunoblotting using a murine anti-caveolin monoclonal antibody. For anti-(EGF receptor) immunoprecipitations, CLMs were incubated with 10 μg/ml anti-(EGF receptor) antibody R1. Antibody-bound membranes were collected on Protein G–Sepharose CL-4B.

RESULTS

Preparation of a CLM fraction

A detergent-free method [11] was employed to isolate CLMs from A431 cells. Following density-gradient centrifugation of A431 cell homogenates extracted in sodium carbonate, caveolin, a 22-kDa integral membrane protein marker for caveolae, was enriched in fraction 5 corresponding to a region of the gradient situated at the interface of the 5% and 35% (w/v) sucrose layers (Figure 1A). Densitometric analysis of the caveolin immunoreactivity indicated that caveolin was purified 20–50-fold in the CLM fraction relative to the whole-cell lysate. In common with the findings of other groups [11,33], the CLM fractions were found to contain less than 2% of the total cellular protein, the bulk of which remained in the 45% (w/v) sucrose layer at the bottom of the ultracentrifuge tube, together with more than 90% of the total cellular 5′-nucleotidase activity (which acts as a plasma membrane marker; results not shown). These data are consistent with the CLM fraction containing discrete low-buoyant density, subdomains of the plasma membrane.

The distribution of EGF receptors in the gradient fractions was assessed by immunoblotting with an anti-(EGF receptor) serum. The results showed that EGF receptors were concentrated in the CLM fraction (Figure 1B). Similarly, when CLMs were prepared from EGF (100 nM)-stimulated A431 cells, immunoblotting of the resulting cell fractions with an antibody specific for the tyrosine-phosphorylated form of the EGF receptor revealed that activated EGF receptors were also concentrated in the CLM fraction (Figure 1C). When these experiments were performed on a 5–35% continuous sucrose gradient, it was found that both EGF receptor and caveolin immunoreactivity co-fractionated and peaked within the 23–28% region of the gradient (results not shown). Such a co-localization of receptors and caveolin in a CLM fraction has previously been interpreted as implying the caveolar localization of receptor activation [13].

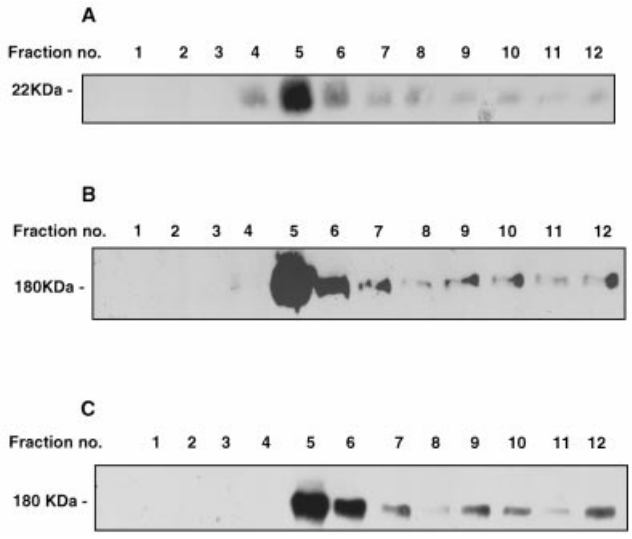

Figure 1 Isolation of CLMs by density-gradient centrifugation

(**A**) A431 cells were sonicated in the presence of 100 mM sodium carbonate, pH 11.0, and the homogenate was centrifuged overnight in a 5–35% (w/v) discontinuous sucrose density gradient. Fractions were collected and their protein content was analysed by SDS/PAGE (10% gels). Proteins were electroblotted and probed with an anti-caveolin antibody. (**B**) Distribution of EGF receptor in the gradient fractions, as determined by immunoblotting with the anti-(EGF receptor) serum, anti-2E. (**C**) A431 cell monolayers were stimulated for 2 min with EGF (100 nm); the cells were then harvested in sodium carbonate and CLMs were prepared by density-gradient centrifugation. Activated EGF receptors were detected using an antibody that specifically recognizes the tyrosine-phosphorylated form of the EGF receptor.

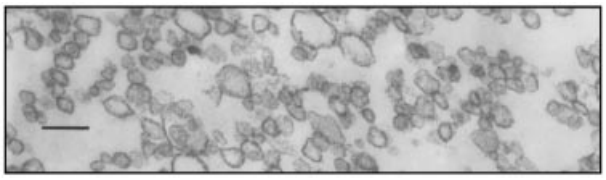

Figure 2 Electron micrograph of CLMs

CLMs were prepared by using the sodium carbonate method, fixed and analysed by electron microscopy. The results show that the CLM consists of vesicles ranging in diameter from approx. 40–250 nm. Bar = 200 nm.

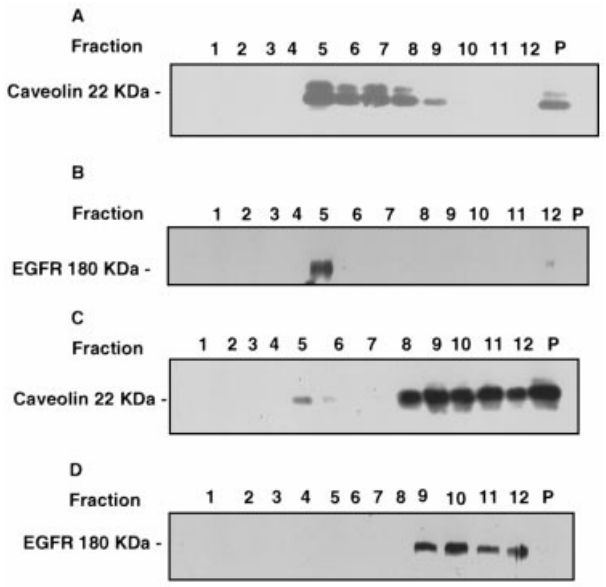

Figure 3 Preparation of DIG domains

A431 cell monolayers were scraped into ice-cold 1% (v/v) Triton X-100 in Mes buffer, pH 6.5. The cells were disrupted by Dounce homogenization, and caveolar and DIG membranes were isolated by density-gradient centrifugation. Immunoblots showing distribution of (**A**) caveolin and (**B**) EGF receptor immunoreactivity in the gradient fractions prepared subsequent to homogenization of the detergent-solubilized cell extracts. Distribution of (**C**) caveolin and (**D**) EGF receptors in gradient fractions from 1% (v/v) Triton X-100-solubilized cell extracts that were not subjected to Dounce homogenization.

Electron microscopy studies of the CLM fraction

When CLM membranes were fixed and examined by electron microscopy, it was clear that this fraction contained vesicles of heterogeneous size (Figure 2). Many had diameters in the range 50–100 nm, which is consistent with the size of purified caveolae, but vesicles with diameters in the range 100–300 nm were also abundant. These observations show that a proportion of the membrane vesicles in the CLM fraction are too large to be pure caveolae and might consist of caveolae fused to adjacent areas of non-caveolar plasma membrane, or could be quite unrelated to caveolae. The latter possibility is more probable, since the larger vesicles are depleted selectively following immunopurification of caveolae from the CLM fraction [34].

Triton X-100 extraction of caveolin and EGF receptor-containing membranes

Insolubility in 1% (v/v) Triton X-100 is a characteristic of DIGs, of which caveolae may be considered a subclass [35]. We found that when A431 cells were homogenized in the presence of 1% (v/v) Triton X-100 and the cell lysates subjected to sucrose density-gradient centrifugation, caveolin and EGF receptors co-fractionated to a low-buoyant density region of the gradient (Figures 3A and 3B) that could be visualized as a light-scattering band. These results are again consistent with the presence of EGF receptors in caveolae.

In the absence of homogenization, EGF receptors remained in the dense sucrose cushion at the bottom of the gradient and only a proportion of the cellular caveolin floated up the gradient (Figures 3C and 3D). Hence, in the absence of homogenization, it is clear that EGF receptors are not located in low-density, detergent-insoluble membranes. In addition, a substantial amount of the caveolin, but few of the EGF receptors, were found in the pellet.

Taken together, these data indicate that differences in the distribution of EGF receptors and caveolin are most evident when the Triton X-100 cell extracts are not subjected to homogenization.

EGF receptor solubility in detergent

The question of whether or not the EGF receptor exists within a detergent-insoluble membrane domain has important implications for the study of receptor-signalling complexes by co-immunoprecipitation. Consequently, ultracentrifugation was used to pellet insoluble membrane proteins from Triton X-100 cell lysates prepared under conditions similar to those used in the preparation of DIGs. Caveolin was found in the pellet and soluble fractions as observed previously [36], whereas the receptor was exclusively in the supernatant (Figure 4A). SDS/PAGE revealed that the supernatant contained approx. 10-fold more protein than the pellet (results not shown), which, combined with the immunoblot data obtained with the anti-caveolin serum, demonstrated that there was an approx. 10-fold enrichment of caveolin in the pellet relative to the Triton X-100-soluble fraction. Note that homogenization was found not to be required for EGF receptor solubilization in 1% (v/v) Triton X-100. These results illustrate that, unlike caveolin, EGF receptors are solubilized by 1% (v/v) Triton X-100.

We also examined the detergent solubility of caveolin and EGF receptors in the CLM fraction isolated by the sodium carbonate method (Figure 4, lower two panels). We found that CLM-associated EGF receptors were pelleted following centrifugation, but were completely solubilized following treatment of the CLMs with either Triton X-100 (1%), Nonidet P40 (1%), CHAPS (30 mM) or 2-octyl glucoside (120 mM). These data indicate that EGF receptors are either not localized to DIGs or are extracted from DIGs by detergent. In contrast with EGF receptors, CLM caveolin was relatively resistant to solubilization in Triton X-100 (1%), and partly insoluble in each of the other detergents tested (Figure 4B). These results are similar to previous observations on the detergent insolubility of caveolin-containing rafts [9] and indicate that EGF receptors are excluded from these DIGs by detergent.

Immunoprecipitation of caveolae

Unlike the cell-fractionation experiments performed in the presence of Triton X-100, the results from the sodium-carbonate-based method for isolating CLMs indicated that EGF receptors

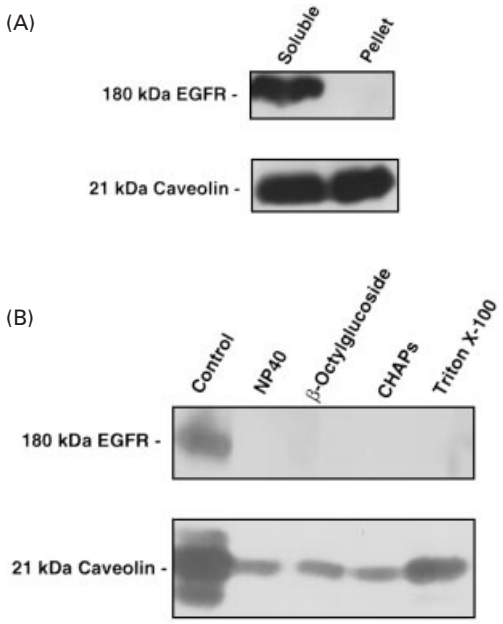

Figure 4 Caveolin and EGF receptors are differentially sensitive to detergent extraction

(**A**) A431 cells were solubilized in 1% (v/v) Triton X-100 in PBS, pH 7.4, at 4 °C. The cell lysate was homogenized and centrifuged at 179000 ***g*** for 1 h at 4 °C. The supernatant was collected and resuspended in an equal vol. of 2 × SDS/PAGE sample buffer. The Triton X-100-insoluble pellet was washed twice with ice-cold water, resuspended in 12 ml of 2 × SDS/PAGE sample buffer and boiled. The protein content of the supernatant and pellet fractions was analysed by immunoblotting with anti-caveolin and anti-(EGF receptor) sera. (**B**) Aliquots of CLMs isolated by using the sodium carboante method were solubilized in the indicated detergent prepared in 20 mM Tris/HCl, pH 7.4, at 4 °C and membrane pellets were collected by centrifugation of the samples at 179000 ***g*** for 1 h at 4 °C. The pellets were boiled in 2 × SDS/PAGE sample buffer for 5 min and their respective caveolin and EGF receptor contents were assessed by immmunoblotting with the appropriate antisera.

are localized to and activated within caveolar membranes. To test further this deduction, intact caveolae were immunoisolated from CLMs using a polyclonal anti-caveolin serum and the immunocomplexes were collected using secondary-antibody-covered magnetic Dynabeads [27]. Using this technique, 80–100% of the caveolin in the CLM fraction could be immunoprecipitated (Figure 5). Notably, only a small proportion (amounting to not $> 10\%$) of the EGF receptors present within the CLM fraction were occasionally co-immunoprecipitated with caveolin (Figure 5). Co-immunoprecipitation of EGF receptors with caveolin was not reproducible, indicating that EGF receptors in A431 cells are not concentrated in caveolae, but in light membrane rafts that appear to represent a distinct subcompartment of the plasma membrane. These findings are significant, since localization in CLMs has previously been interpreted as implying localization in caveolae.

Nevertheless, the observation that a small proportion of the CLM-associated EGF receptors occasionally co-immunoprecipitated with caveolin (e.g. compare the first two lanes in Figure 5) warranted further investigation, especially since overexpression of EGF receptors on A431 cells might give rise to a large pool of redundant receptors, such that only a small fraction of the total receptors, e.g. the putative pool of caveolar EGF receptors, need to be activated to generate a maximal cellular response.

Consequently, in order to examine the relationship between EGF receptor activation and receptor compartmentation, we

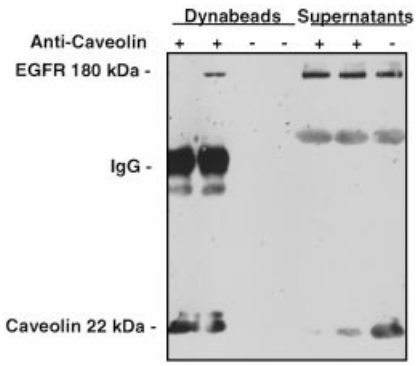

Figure 5 Caveolin and a small pool of EGF receptors irreproducibly co-immunoprecipitate

CLMs from A431 cells were prepared by density-gradient centrifugation and subjected to immunoprecipitation with an anti-caveolin antiserum. Immunoprecipitated proteins and the corresponding supernatants were electroblotted on to PVDF. The top half of the blot was probed with anti-(EGF receptor) antiserum (anti-2E) and the bottom half of the blot was probed with anti-caveolin monoclonal antibody. In order to improve the detection of immunoprecipitated receptor, the proportion of each immunoprecipitate loaded on the gel was twice that of each supernatant.

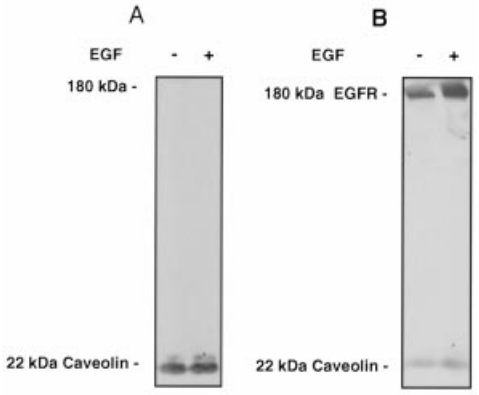

Figure 6 EGF receptor activation occurs in a membrane compartment distinct from caveolae

A431 cells were stimulated with EGF or vehicle for 2 min, lysed in ice-cold Na_2CO_3 buffer, sonicated and precleared with sheep anti-(rabbit IgG)-coupled Dynabeads. Caveolae were immunoisolated with an anti-caveolin antiserum. (**A**) Caveolin immunoprecipitates were probed with anti-caveolin and anti-(activated EGF receptor) antibodies. Note that no activated EGF receptors were detected in the caveolin immunoprecipitates. (**B**) The cell lysates post-immunoprecipitation were probed with anti-(activated EGF receptor) and anti-caveolin antibodies.

investigated the distribution of autophosphorylated receptors. Unlike many of the downstream responses to EGF, such as the activation of both mitogen-activated protein kinase and PLC, ligand-induced EGF receptor autophosphorylation provides an index of receptor activation not complicated by further compartmentation of downstream components of signalling cascades. Addition of EGF evoked a 5–10-fold increase in EGF receptor activation, as determined by a monoclonal antibody specific for the autophosphorylated EGF receptor (Figure 6). Furthermore, in sodium-carbonate-extracted and sonicated whole-cell lysates, it was possible to immunoisolate caveolin (Figure 6) while the activated EGF receptors remained in the caveolin-depleted supernatant (Figure 6). No activated EGF receptors could be detected in these anti-caveolin immunoprecipitates.

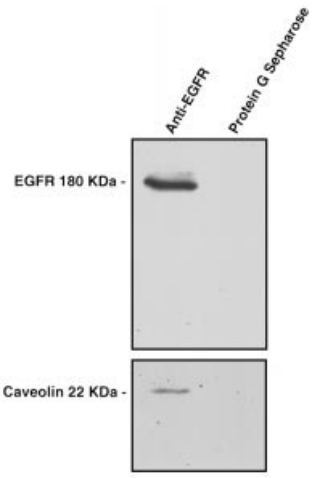

Figure 7 Immunoisolation of EGF receptor-containing rafts from the CLM fraction

Anti-(EGF receptor) antibody was used to isolate EGF receptor-rich vesicles from CLMs on Protein G–Sepharose. Vesicular proteins were separated by SDS/PAGE. Parallel immunoblots were probed for the presence of EGF receptor and caveolin.

Immunoprecipitation of EGF receptor-containing domains from the CLM fraction

EGF receptor-containing membranes were isolated by immunoprecipitation from CLMs prepared by the sodium carbonate method. All washing steps were carried out in the absence of detergents and at neutral pH to preserve the vesicles present in the samples. Using a monoclonal anti-(EGF receptor) antibody it was possible to isolate approx. 20–30 % of the EGF receptors from the CLM fraction (Figure 7). Immunoblotting for caveolin revealed that a small pool, amounting to not more than 1–2 % of the CLM-associated caveolin, was present in the EGF receptor-containing vesicles (Figure 7). These data concur with the results obtained by immunodepleting caveolae from the CLM preparation, and indicate that only a small proportion of the cellular EGF receptors are localized to caveolin-containing membrane domains. Furthermore, these data are consistent with the vast bulk of the cellular EGF receptors and caveolin being localized to distinct regions of the plasma membrane with similar buoyant densities, but with differing protein compositions.

DISCUSSION

Through a combination of cell fractionation and immunoprecipitation, the present study identifies an EGF receptor-rich compartment of the plasma membrane. Membrane fragments derived from this compartment possess similar buoyant density to caveolae, but are soluble in detergent. These findings demonstrate that the isolation of caveolar membranes solely on the basis of their buoyant density does not adequately purify caveolae. The problem of defining purity is compounded by the observation that many vesicles in the CLM fraction fall into a 50–100 nm size range that others have accepted to be indicative of purified caveolae, e.g. see [37]. Consequently, vesicle size alone cannot be used as a measure of caveolae purity.

The EGF receptor-rich plasma membrane fraction is clearly biochemically distinct from caveolae, as immunoisolation of caveolin-containing vesicles does not result in co-immunoprecipitation of the bulk of the CLM-associated EGF receptors either

before or after stimulation with EGF, demonstrating that this fraction also corresponds to the site on the cell surface where at least early intracellular signalling takes place. Previous reports have led to the conclusion that EGF receptors are localized within caveolae [10,13,38]; however, our results indicate that incomplete purification of caveolae from associated low-buoyant density membranes might have given rise to an overestimation of the role of caveolae in receptor-dependent signalling.

Co-immunoprecipitation of caveolin and receptors has also been taken as evidence that signal transduction occurs in caveolae [13,39,40]. We found that EGF receptors occasionally co-immunoprecipitated with caveolin from A431 CLMs. However, this small fraction of EGF receptors was not reproducibly detected by immunoblotting, indicating that small pieces of EGF receptor-rich, non-caveolar membrane occasionally fuse with, or do not fully separate from, caveolae during sonication. The complete absence of any phosphorylated EGF receptors co-immunoprecipitating with caveolin argues further against the notion that caveolae constitute a signalling compartment.

As caveolae have been implicated in a variety of receptor vesicle-trafficking events between the *trans*-Golgi network and the plasma membrane [41–43], it is possible that a small number of newly synthesized EGF receptors might be transported to the plasma membrane by caveolae. In this context, it is interesting to note that caveolin can directly inhibit EGF receptor autophosphorylation [13]. This would be consistent with our results indicating that caveolae contain no activated EGF receptors. On the other hand, the apical targeting of caveolin [42,44] seems to be at odds with the basolateral distribution of EGF receptors in polarized epithelial cells (reviewed in [45]).

Caveolin-rich and EGF receptor-containing membrane compartments were found to be differentially susceptible to solubilization in 1% (v/v) Triton X-100. Unlike caveolae, EGF receptor-containing membranes were soluble in Triton X-100. When DIGs were prepared from A431 cells, EGF receptors and caveolin co-fractionated with buoyant DIGs. However, in the absence of homogenization most of the caveolin and EGF receptors did not rise up the sucrose gradient and had different distributions. In contrast with caveolae, the inability to pellet EGF receptors from Triton-solubilized samples suggested that the EGF-receptor compartment was readily solubilized by detergent. These findings indicate that, in common with rhodopsin receptors [46], EGF receptors within detergent micelles are buoyant. It is possible that homogenization might affect the supramolecular structure of detergent micelles and DIGs by disrupting their respective interactions with high-buoyant density structures, such as the cytoskeleton. Ultrastructural evidence has shown that, in the absence of homogenization, plasma membrane contacts with the actin cytoskeleton are preserved [47,48], and therefore shear-induced disruption of such interactions might underlie the homogenization-dependent differences in buoyant density observed both for EGF receptor-containing micelles and caveolae. In support of this view, there are detailed reports of both caveolae [8] and EGF receptors [48] associating with the actin cytoskeleton in Triton-solubilized cells. Furthermore, it is well established that the cytoskeleton remains intact following treatment in 1% (v/v) Triton X-100 alone [47,48].

The complexities in interpreting protein localization to DIG domains have been partly addressed by previous studies. In particular, Parkin et al. [49] demonstrated that the partitioning of proteins into DIGs is affected by calcium-induced annexin interactions that might induce the formation of DIG aggregates. Mayor and Maxfield [50] have also investigated the basis of DIG formation, and have shown that the addition of Triton X-100 to cells creates holes in their plasma membranes and results in some proteins that were formerly diffusely distributed to aggregate in the remaining surface-membrane patches. Additionally, the idea that caveolin is found exclusively in membrane compartments has been recently challenged by the observation that up to 10–15% of the cellular caveolin is present in a soluble cytosolic form [51]. Furthermore, in agreement with our findings with non-homogenized Triton X-100 lysates of A431 cells, Lipardi et al. [36] found that in Madin–Darby canine kidney cells and caveolin-transfected Fischer rat thyroid cells, the bulk of the cellular caveolin was excluded from the low-density region of the density gradient. All these data suggest that the partitioning of proteins, including caveolin, into a low-buoyant density fraction in the presence of Triton X-100 is not an accurate measure of caveolar or DIG localization.

In conclusion, although ultrastructural studies have found that A431 cell plasma membranes contain numerous caveolae [25,53] and robustly express caveolin, using a detergent-free fractionation procedure, we have demonstrated that EGF receptor-rich rafts can be separated from caveolae. A recent report on the plasma membrane organization of G-proteins [54] demonstrated that, whereas $G_i\alpha$ and $G_0\alpha$ co-fractionated with caveolin on density-gradient centrifugation, only a minute proportion of these G-proteins were localized in caveolae. A report describing the immunoisolation of caveolae from rat lung microvasculature also questioned the caveolar-signalling hypothesis by demonstrating that several signalling proteins, such as c-src and heterotrimeric G-proteins, were not concentrated in caveolae, but in other regions of the plasma membrane [27]. In addition, non-caveolar, low-density putative signalling domains have been identified in neuroblastomas [26], platelets [52] and lymphocyte cell lines [25]. However, these structures were isolated on the basis of their insolubility in Triton X-100, and are subsequently DIGs. The detergent-soluble, EGF receptor-rich rafts that we describe are therefore neither caveolar nor non-caveolar DIG-signalling domains. Future experiments will elucidate the importance of these novel plasma membrane signalling domains.

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