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Review

# Growth factor receptors, lipid rafts and caveolae: An evolving story

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**Abstract**

Growth factor receptors have been shown to be localized to lipid rafts and caveolae. Consistent with a role for these cholesterol-enriched membrane domains in growth factor receptor function, the binding and kinase activities of growth factor receptors are susceptible to regulation by changes in cholesterol content. Furthermore, knockouts of caveolin-1, the structural protein of caveolae, have confirmed that this protein, and by implication caveolae, modulate the ability of growth factor receptors to signal. This article reviews the findings pertinent to the relationship between growth factor receptors, lipid rafts and caveolae and presents a model for understanding the disparate observations regarding the role of membrane microdomains in the regulation of growth factor receptor function.


*Keywords:* Lipid raft; Caveola; Cholesterol; Growth factor; Insulin receptor; EGF receptor

Lipid rafts are small cholesterol- and sphingolipid-enriched domains present in the membranes of most cells. Due to interactions between the cholesterol and sphingolipids as well as the presence of phospholipids containing saturated fatty acyl chains, lipid rafts appear to represent a more ordered lipid environment than the surrounding bulk membrane, [1]. This tightly packed, highly ordered construction gives rise to the principle defining feature of lipid rafts—their resistance to solubilization with detergents [2]. Such domains also appear to have a relatively higher lipid to protein ratio than bulk membranes. This gives rise to the second characteristic property of these protein/lipid complexes—low density.

Lipid rafts have been physically isolated based on their combination of low density and insolubility in detergent. The typical method involves extraction of cells in 1% Triton X-100 followed by centrifugation in a linear 5 to 30% sucrose gradient [2]. More recently, other detergents, including NP-40, octylglucoside, CHAPS, Brij 98 and Lubrol, have been used to extract cells and isolate low density, detergent-resistant membranes [3–5]. In all cases, the lipid rafts distribute towards the low density regions of the gradient and are separated from bulk plasma membrane marker proteins. Detergent-free methods have also been developed to isolate lipid rafts [6,7]. The advantage of the detergent-free methods is that they are unlikely to promote membrane fusion that could generate mixed raft domains that do not exist in the intact cell.

It was eventually recognized that there were at least two kinds of "low density, detergent-resistant membranes"—caveolae and lipid rafts. Caveolae is a term that is used to describe flask-shaped invaginations that are enriched in cholesterol and surrounded by a coat of the structural protein, caveolin-1 [8]. Lipid rafts are flat domains in the membrane that lack caveolin-1 but nonetheless are enriched in cholesterol and are resistant to extraction with detergents.

Beyond this simple dichotomy, a significant amount of recent evidence has suggested that lipid rafts are heterogeneous both in terms of their lipid and their protein composition. For example, Madore et al. studied the relationship between rafts that contained the two GPI-anchored proteins, prion protein and Thy-1. These workers used immunoaffinity purification to show that a subpopulation of prion protein-containing rafts could be separated from the bulk of the Thy-1-containing detergent-resistant membranes [9]. Thus, while these GPI-anchored proteins are both localized to lipid rafts, they do not appear to be

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localized to the same lipid raft. In a more direct approach, Gomez-Mouton et al. [10] and Bagnat and Simons [11] used immunofluorescence to show that proteins and lipids that were recovered together in detergent-resistant membrane fractions were present in distinctly different locations in intact cells. Thus, recovery in a common low density raft fraction is not an indication of co-localization in the intact cell.

Lipid rafts have been implicated in a variety of cellular processes including protein and lipid trafficking [12], viral entry [13] and signal transduction [14]. Many proteins involved in cell signaling, including receptors, low molecular weight G proteins and heterotrimeric G proteins, have been shown to be enriched in lipid rafts (for reviews see [15,16]). The finding that signal transduction is often altered when lipid rafts are disrupted by depletion of cellular cholesterol [16] suggests that these cholesterol-rich domains participate in the control of cell signaling.

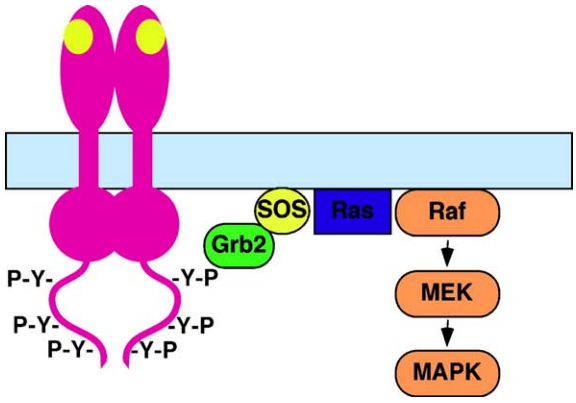


Fig. 2. Activation of the MAP kinase signaling pathway downstream of a receptor tyrosine kinase. Sites of autophosphorylation on the growth factor receptor act as a binding site for the adapter protein Grb2 which is in complex with SOS, a Ras guanine nucleotide exchange factor. Recruitment of SOS to the membrane allows it to activate Ras which activates the Raf serine–threonine kinase. Raf phosphorylates and activates MEK which phosphorylates and activates MAP kinase.

## 1. Lipid rafts and receptor tyrosine kinases

Receptor tyrosine kinases are a prominent example of the proteins involved in cell signaling that are enriched in lipid rafts. The EGF receptor, the insulin receptor, the PDGF receptor, the VEGF receptor and the NGF receptor among others have been shown to be localized to low density, cholesterol-rich membrane domains. In all cases, signaling by these receptors is modulated by changes in cellular cholesterol content. Thus, raft localization appears to be of functional importance to the receptors.

The EGF receptor is a typical example of a receptor tyrosine kinase (Fig. 1). This receptor is composed of three domains: a 620 amino acid extracellular domain that recognizes and binds EGF; a 24 amino acid transmembrane domain that passes through the membrane once in the form

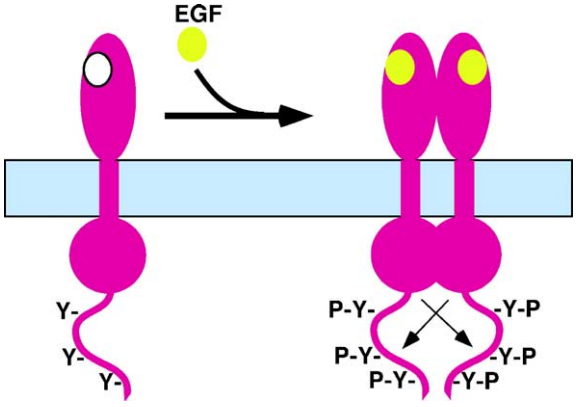


Fig. 1. Dimerization and activation of the EGF receptor. The EGF receptor is present in the membrane as a monomer. Upon binding of EGF, the receptor dimerizes, leading to the activation of its intracellular tyrosine kinase activity. The receptors autophosphorylate in trans, generating phosphotyrosine residues that serve as binding sites for SH2 and PTB domain-containing proteins which mediate the assembly of signaling complexes.

of an alpha helix; and a cytoplasmic domain that contains the tyrosine kinase module [17]. Under basal conditions, the EGF receptor exists as a monomer. However, upon binding EGF, the receptor dimerizes. This induces activation of the intracellular tyrosine kinase which autophosphorylates the receptor in trans in the C-terminal region [18,19].

The phosphorylated tyrosine residues serve as binding sites for a variety of SH2 or PTB domain-containing proteins that are involved in the process of signal transduction (Fig. 2). For example, the SH2 domain-containing Grb2 protein binds to the autophosphorylated EGF receptor bringing the guanine nucleotide exchange factor, SOS, to the membrane where it induces the exchange of GDP for GTP on membrane-bound Ras. This leads to the activation of Ras which activates Raf, a serine–threonine protein kinase. Raf phosphorylates and activates MEK and MEK phosphorylates and activates MAP kinase. Thus, in a process that begins with a protein–protein interaction that depends on the phosphorylation of a tyrosine residue, stimulation of the EGF receptor leads to the activation of MAP kinase, a serine–threonine kinase that mediates some of the intracellular actions of EGF.

With a few variations on the theme, this paradigm holds true for all receptor tyrosine kinases. For example, the insulin receptor is composed of two different subunits that nonetheless derive from a single precursor protein. The alpha subunit contains the insulin binding site and is disulfide-bonded to the beta subunit which contains the tyrosine kinase domain. The receptor is made up of two disulfide-bonded alpha-beta units and hence is present in a dimeric form prior to ligand binding. Presumably ligand binding alters the conformation within this heterotetrameric structure leading to the activation of its tyrosine kinase activity (for review, see [20,21]).

Both the insulin and FGF signaling systems contain an extra component termed IRS (insulin receptor substrate) or

FRS (FGF receptor substrate) that is lacking in the EGF receptor system. IRS [22–24] and FRS2 proteins [25–27] are phosphorylated on tyrosine residues by their respective receptors and serve as adapter proteins for the formation of the protein–protein complexes necessary for signal transduction. Like the carboxy-terminal tail of the EGF receptor, tyrosine phosphorylated IRS or FRS is bound by proteins containing SH2 or PTB domains and this begins the process of signal transduction that leads to the activation of down stream signaling molecules.

## 2. Lipid rafts or caveolae?

In general, receptor tyrosine kinases are not recovered in Triton X-100-resistant membranes [28,29]. This has been confirmed by multiple proteomic analyses of Triton-resistant membranes, none of which identify a receptor tyrosine kinase among the detergent-insoluble raft proteins [30–34]. However, when detergent-free methods are used to prepare lipid rafts, the receptors have been found in low density membrane fractions. On average, about half of the total population of growth factor receptors are recovered in the low density fraction after detergent-free fractionation of cells [7,35–38]. Thus, while the receptors are clearly present in the raft/caveolar fraction, they do not appear to be exclusively localized to this compartment.

Early studies of the localization of growth factor receptors to lipid rafts were confounded by the failure to realize that caveolae and lipid rafts represented two distinct cholesterol-rich membrane fractions. Since most of the early localization work relied on the co-fractionation of raft markers with the tyrosine kinase receptors in density gradients and because caveolin was the best known marker for the low density membrane fraction, most early reports state that growth factor receptors are enriched in caveolae [36,39].

Subsequently, when it became apparent that caveolae and lipid rafts were distinguishable compartments, additional studies were undertaken to specifically determine into which of these two classes of low density membrane domains the receptors actually partitioned. Because lipid rafts and caveolae (<100 nm diameter) are smaller than the resolution of the light microscope (~300 nm), fluorescence microscopy does not provide an unequivocal answer to the question of co-localization. Furthermore, because of the problems inherent in many cell fractionation procedures and the fact that most do not separate caveolae from lipid rafts, this methodology is also imperfect. The most definitive answer is obtained through the use of electron microscopy. Thus, the studies cited here focus on those that used electron microscopy to determine receptor localization.

Most electron microscopy studies label the growth factor receptor of interest with gold particles and then examine thin sections of cells or cell bottoms fixed on grids to determine whether the target receptor is present within the caveolar invaginations or whether it resides on the flat surface of the membrane. With one notable exception, the EGF receptor, most receptors that have been localized by electron microscopy appear to be localized to caveolae.

The EGF receptor was one of the first receptors shown to be enriched in the caveolin-containing, low density fraction of cells [36,39]. Nonetheless, it was ultimately shown by electron microscopy to be present in flat rafts, not in caveolar invaginations [40]. Consistent with this conclusion, the distribution of EGF receptors on the plasma membrane is distinct from that of caveolae. Using immunofluorescence, EGF receptors could be seen to be diffusely distributed on the plasma membrane whereas caveolin was present in a punctate distribution [41]. Similarly, Matveev and Smart [42] used immunoprecipitation to show that under non-stimulated conditions the EGF receptor could be separated from caveolin-containing membrane domains. These data indicate that the EGF receptor partitions into a non-caveolar, lipid raft compartment.

Stimulation of cells with EGF leads to the loss of EGF receptors from the low density raft compartment [36]. EGF is known to induce the clathrin-dependent internalization of the EGF receptor. Thus, this observation can be understood as the EGF receptor moving out of rafts and into coated pits where it is internalized. Indeed, EGF receptor mutants that do not internalize also fail to exit rafts after treatment with EGF [36]. Interestingly, after treatment with EGF for 60 min, Matveev and Smart [42] showed that the EGF receptor could be immunoprecipitated with caveolin, suggesting the possibility that the EGF receptor may also be internalized through a caveolae-dependent pathway.

Unlike the EGF receptor, the insulin receptor appears to be largely localized to caveolae. Immunogold electron microscopy demonstrated that insulin receptors are essentially restricted to caveolae in both 3T3-L1 adipocytes [28] and in primary human adipocytes [43]. However, electron microscopy in rat adipocytes indicated that insulin receptors were localized to the planar surface of the membrane. Thus, there may be some species-specific differences in insulin receptor localization [44].

In addition to adipocytes, liver is a major target for insulin action. The human hepatoma cell line, HuH7, expresses insulin receptors but lacks detectable caveolin-1 and hence the cells have no caveolae. Under these conditions, the unstimulated insulin receptor was found in the Triton X-100-soluble portion of a density gradient. However, after insulin stimulation, a portion of the receptor was recovered in the low density, detergent-resistant fraction suggesting that agonist induces the recruitment of the insulin receptor into rafts in cells that lack caveolae [45]. Consistent with this conclusion is the observation that insulin stimulation led to the enrichment of rat adipocyte insulin receptors in a low density membrane fraction [44]. These findings suggest that insulin stimulation enhances the affinity of the insulin receptor for raft-like domains.

Like the insulin receptor, the PDGF receptor appears to localize to caveolae rather than flat rafts. Immunoelectron microscopy demonstrated the presence of PDGF receptors in caveolar invaginations in normal human fibroblasts [46]. Similar to the EGF receptor, treatment with PDGF led to the slow loss of the receptor from the low density raft fraction of cells, consistent with slow internalization of the receptor via coated pits. Confirmation of the caveolar localization of the PDGF receptor was provided by Liu et al. [35] who used fractionation of silica-coated endothelial cell plasma membranes to show that PDGF receptors fractionated with caveolar vesicles rather than with non-caveolar but Triton-insoluble lipid raft markers. By contrast, Matveev and Smart [42] failed to immunoprecipitate PDGF receptors with caveolin in Swiss 3T3 cells, leading them to conclude that PDGF receptors were in rafts rather than in caveolae. Whether this represents a difference due to cell type or methodology is not clear.

Deep etch and immunogold techniques were used to demonstrate that the high affinity NGF receptor, TrkA, was present in caveolae [47]. Thus, TrkA appears to be similar to the insulin receptor and the PDGF receptor in terms of its localization.

The FGF receptor has not been shown to be present in either lipid rafts or caveolae. Negative findings with Triton X-100-extracted membranes [48] might suggest that this receptor is not in rafts or caveolae. However, because many receptor tyrosine kinases are extracted from cells by 1% Triton X-100, Triton resistance is not a definitive test for raft localization of this class of proteins. Interestingly, FGF is bound by heparan sulphate proteoglycans in a Triton X-100-resistant membrane compartment [49] and FGF has been shown to be internalized via a endocytic process that involves caveolae [50]. In addition, FRS2, the adaptor protein for the FGF receptor, is apparently present in Triton-resistant membranes [51]. Thus, indirect evidence would suggest that the FGF receptor is likely to be present in some type of low density, cholesterol-enriched membrane domain.

From the above discussion, it is clear that the majority of growth factor receptors are present in caveolae. It is therefore a conundrum as to why caveolin-1, the structural protein of caveolae, is always found in the low density, Triton-insoluble fraction of cells while growth factor receptors are almost never reported to be present in such membrane preparations. Clearly, the association of growth factor receptors with caveolae is different from that of caveolin-1. The observation that many receptors move into or out of caveolae following hormone stimulation suggests that their association with this compartment is not permanent and is subject to regulation. It is likely that the association of receptor tyrosine kinases with rafts or caveolae is an equilibrium process that can be shifted in one direction or another based on external conditions. Detergent extraction may promote the migration of receptor tyrosine kinases out of caveolae and result in their loss from this membrane fraction. This could be due to alteration in the lipid composition of detergent-extracted rafts that make them unable to accommodate transmembrane receptors. Alternatively, changes in the structure of the caveolin coat or in other raft proteins may force the migration of receptor tyrosine kinases out of these domains.

## 3. Localizing receptors to rafts/caveolae

Lipid rafts contain only a subset of proteins that are found in the plasma membrane. Thus, proteins must selectively partition into rafts to give rise to this non-equilibrium distribution. Proteins that contain a glycosyl-phosphatidylinositol anchor were among the first to be shown to be enriched in lipid rafts [2]. Subsequently, proteins modified by acyl groups, typically myristate and/or palmitate, were also shown to preferentially partition into rafts [52]. Thus, attachment of a lipid group, particularly one that contains a saturated fatty acyl chain, appears to facilitate interaction with the liquid-ordered phase and leads to the localization of the modified protein to lipid rafts.

Receptor tyrosine kinases are transmembrane proteins and the mechanisms through which transmembrane proteins are localized to rafts are varied and less well understood. For some transmembrane proteins, the membrane-spanning domain appears to mediate the partitioning of the protein into cholesterol-enriched membrane domains. The influenza virus hemagglutinin protein (HA) is present in lipid rafts while the VSV-G protein is not. Replacement of the transmembrane domain of the HA protein with that of the VSV-G protein results in the failure of the chimera to localize to lipid rafts [53]. Surveys of the behavior of other transmembrane proteins have suggested that the association of proteins with cholesterol-enriched domains is influenced by the length of their transmembrane domains [54]. Extracellular carbohydrate-containing motifs have also been implicated in directing the association of transmembrane proteins with cholesterol-enriched domains [55,56].

For the $Ca^{2+}$-sensitive adenylate cyclase, raft localization appears to be a function of its cytosolic domain [57]. Studies on chimeras made from the raft-targeted adenylate cyclase 5 and the non-raft targeted adenylate cyclase 7 indicated that the tandem 6 transmembrane domains of adenylate cyclase 5 were not required for raft localization. However, the two non-conserved cytoplasmic domains were responsible for raft targeting. Because these domains do not interact directly with the membrane lipids, this finding suggests that that the cytoplasmic domains may bind to a resident raft protein or lipid to direct the protein to that compartment. Consistent with this notion is the finding that a so-called SoHo domain (sorbin homology domain) is present in several cytoplasmic proteins and is responsible for targeting them to lipid rafts through binding to the raft protein, flotillin [58].

Of the growth factor receptors, only the EGF receptor has been examined for the presence of targeting sequences that would direct the receptor to lipid rafts. EGF receptors

lacking the entire cytoplasmic domain as well as EGF receptors in which the transmembrane domain was exchanged with that of the LDL receptor still localized to lipid rafts. By contrast, a receptor in which the entire extracellular domain had been deleted failed to be target to rafts [59]. The targeting signal was further localized to a 60 amino acid membrane proximal sequence. However, when just this sequence was deleted from the extracellular domain of the EGF receptor, the receptor still localized to rafts suggesting that multiple raft localization signals may be present. Like the data on the adenylate cyclase, these findings suggest that the EGF receptor may become enriched in lipid rafts by binding to a resident raft lipid or protein.

A caveolin-interacting motif that is thought to mediate the association of caveolin with a variety of proteins has been identified [60]. This motif, ψXψXXXXψ or ψXXXXψXXψ where ψ is a hydrophobic amino acid, is sufficiently loose in specificity so that many proteins can be identified as containing one of these motifs. The insulin receptor, the PDGF receptor and the EGF receptor all contain such a motif and these sequences have been proposed to mediate the effects of caveolin on the function of these receptors [61] (see below). This sequence might also potentially mediate the localization of the receptors to caveolae. However, as might be expected from the hydrophobic nature of these sequences, they are located in the internal core of the protein kinase domain in all three proteins and would be unavailable for interaction with caveolin. Thus, it is unlikely that these promiscuous motifs either interact with caveolin or serve to target receptor tyrosine kinases to caveolae.

## 4. Regulation of receptor tyrosine kinase function by cholesterol

Cholesterol is a major component of lipid rafts, accounting for ~50% of the lipid present in these domains. Because the interaction between cholesterol and sphingolipids is the primary organizing principle for lipid rafts, removal of cholesterol from cells is thought to disrupt the structure of rafts and caveolae and release their protein components into the bulk plasma membrane. This is typically visualized as a shift of proteins out of the low density region of a density gradient and into the higher density fractions. The ability to shift raft/caveolar proteins out of this compartment by depleting cells of cholesterol provides the opportunity to compare the function of receptor tyrosine kinases when they are in rafts or caveolae and when they are in non-raft membranes. This approach has been used to define the effect of raft/caveolar localization on receptor tyrosine kinase function.

The most common method used for acute depletion of cellular cholesterol involves the use of methyl-β-cyclodextrin. Cyclodextrins are a series of cyclic compounds consisting of a variable number of α-(1→4)-linked D-glucopyranose units. Beta-cyclodextrins contain seven such sugars that can be substituted with various groups to enhance the specificity of the compound for particular hydrophobic compounds. β-Cyclodextrins desorb cholesterol from the surface of cells and bind it in their hydrophobic cavity [62]. Typically half or more of the total cellular cholesterol can be removed in 30 min by treatment of cells with methyl-β-cyclodextrin. Filipin is a polyene antibiotic that forms a complex with cholesterol and appears to disrupt caveolae and lipid rafts [63]. Because it complexes, rather than removes cholesterol, it is used less widely than cyclodextrin to test the involvement of cholesterol in cellular processes. However, its fluorescence properties allow its use as a stain to detect and localize cholesterol in intact cells.

An alternative approach to cholesterol depletion is through the use of progesterone. Progesterone impairs cholesterol trafficking in cells and leads to a depletion of cholesterol at the plasma membrane. Thus, treatment with this steroid for ~1 h can be used to selectively reduce the amount of cholesterol present at the plasma membrane [64]. Similarly, U18666A, which inhibits cholesterol biosynthesis and trafficking, induces a redistribution of cholesterol to the late endosomes leading to a reduction in plasma membrane cholesterol [65]. Chronic treatment with HMG-CoA reductase inhibitors, such as lovastatin can also be used to deplete cholesterol. This approach requires long-term growth of the cells in the presence of the inhibitor (>24 h) and the use of delipidated serum.

Of all the growth factor receptor tyrosine kinases shown to reside in lipid rafts or caveolae, the EGF receptor and the insulin receptor have been the best characterized with respect to the effects of cholesterol on their function. A few studies have examined the effect of changes in cholesterol levels on PDGF and TrkA function. In most cases, cholesterol depletion has been found to impair receptor function and down stream signaling, consistent with a role of caveolae/rafts in organizing signaling components. The lone exception to this rule is the EGF receptor for which it is clear that cholesterol depletion leads to an increase in receptor binding and kinase activities as well as the enhancement of downstream signaling pathways. The fact that the EGF receptor is alone among growth factor receptors in being localized to lipid rafts rather than caveolae, offers a possible clue as to why such a discrepancy exists among an otherwise similar group of signaling molecules. However, how raft vs. caveolar localization should give rise to opposing effects of cholesterol on receptor function is an open question.

### *4.1. The EGF receptor*

Using cholesterol depletion as a tool to investigate the role of lipid raft localization in regulating EGF receptor function, several groups have demonstrated significant

cholesterol-dependent changes in receptor activity. Depletion of cholesterol consistently induces an increase in the amount of $^{125}$I-EGF bound by cells [40,41,66]. Conversely, cholesterol loading of cells leads to a reduction in the binding of EGF [40]. The increased binding of $^{125}$I-EGF is not due to changes in ligand affinity but rather is associated with an ~50% increase in the total number of cell surface EGF receptors [66]. This increase does not appear to be due to externalization of receptor from an internal pool since cell surface biotinylation studies indicate no difference in the amount of cell surface EGF receptor before and after cholesterol depletion. In addition, the size of the intracellular pool of receptor was too small to account for the observed increase in EGF binding [66]. These studies suggest that localization to lipid rafts impairs the ability of EGF to bind to its receptor, possibly due to steric constraints on the receptor when it is present in this domain.

In addition to altering EGF binding, cholesterol also modulates the ability of EGF to induce receptor dimer formation, the first step in the activation of its tyrosine kinase activity. Cholesterol depletion was shown to enhance EGF-induced receptor dimer formation whereas cholesterol loading was found to inhibit EGF-induced dimer formation [40]. These findings suggest that changes in cholesterol levels affect not only receptor binding but also aspects of receptor signaling.

Consistent with the observed changes in receptor dimer formation, the tyrosine kinase activity of the EGF receptor was also shown to be susceptible to regulation by cholesterol depletion. Depletion of cholesterol with methyl-β-cyclodextrin or U18666A led to the enhancement of both basal and EGF-stimulated autophosphorylation [40,66,67]. Cholesterol loading led to a reduction in EGF receptor autophosphorylation [40]. The increase in receptor autophosphorylation following cholesterol depletion was not due to a change in the rate of receptor dephosphorylation as there was no difference in the rate at which phosphotyrosine was lost from the receptor in control and cholesterol-depleted cells [66]. In vitro studies on isolated membranes further demonstrated that EGF stimulated a higher level of phosphorylation of an exogenous tyrosine-containing peptide in membranes derived from cholesterol-depleted cells than in membranes derived from control cells. Thus, not only receptor autophosphorylation but also receptor kinase activity directed against other substrates was enhanced following treatment with methyl-β-cyclodextrin.

Interestingly, not all autophosphorylation sites on the EGF receptor are equally affected by cholesterol depletion [67]. Phosphorylation at Tyr-992 and Tyr-1173 was enhanced following cholesterol depletion while phosphorylation at Tyr-1045 and Tyr-1068 was unchanged. Because different autophosphorylation sites serve as docking sites for different downstream signaling molecules [68–70], these findings suggest that cholesterol depletion may selectively activate some signaling pathways while leaving others unaffected.

In addition to the changes of phosphorylation at receptor autophosphorylation sites, cholesterol depletion also induced an increase in the phosphorylation of the EGF receptor at Tyr-845, a site phosphorylated by pp60$^{src}$ [67]. This phosphorylation is known to enhance the intrinsic kinase activity of the EGF receptor and hence cholesterol-dependent increases in phosphorylation of this site could contribute to the observed increase in receptor kinase activity following cholesterol depletion [71].

Consistent with the effects of cholesterol depletion on receptor binding and kinase activity, the ability of EGF to stimulate the activation of MAP kinase is enhanced in cells treated with methyl-β-cyclodextrin [64]. This is somewhat surprising since the EGF-stimulated interaction of Ras and Raf-1 appears to occur in caveolae/rafts [39] and disruption of this compartment might therefore be expected to inhibit the ability of EGF to stimulate MAP kinase activity. The observation that MAP kinase activation is not impaired under conditions when rafts are disrupted suggests that lipid rafts may not be necessary for EGF stimulation of this particular signaling pathway or alternatively, that cholesterol depletion does not entirely disrupt lipid rafts.

A dual specificity MAP kinase phosphatase that is inhibited by cholesterol depletion of cells has been reported [72]. It is possible that the inhibition of this enzyme that accompanies cholesterol depletion contributes to the enhancement of EGF-stimulated MAP kinase activation under these conditions.

Like the Ras/Raf interaction, phosphatidylinositol 4,5-bisphosphate turnover appears to be localized to lipid rafts [29]. However, in contrast to the activation of MAP kinase, cholesterol depletion results in the abrogation of EGF-stimulated PI turnover [73]. Repletion of cholesterol reverses the effect. These data suggest that unlike MAP kinase activation, PI turnover is dependent on the integrity of lipid rafts. This could be due to a requirement for $PIP_2$ clustering in lipid rafts to facilitate the processive hydrolysis of this lipid or it may arise from a need to present the $PIP_2$ in a particular conformation that is more readily achieved within the context of a lipid raft.

The fact that cholesterol depletion has opposing effects on signaling pathways downstream of the EGF receptor indicates that not all EGF receptor-mediated signaling has the same dependency on lipid rafts. A logical corollary of this is that changes in cellular cholesterol levels are likely to differentially effect certain signaling pathways leading to the enhancement of some and the impairment of others. As the ratio of signaling through various pathway changes, this could give rise to alterations in the effects of EGF on metabolism, proliferation or differentiation.

### 4.2. Insulin receptor

As noted above, the insulin receptor clearly localizes to caveolae [28,43]. Depletion of cholesterol with methyl-β-

cyclodextrin leads to the flattening of caveolae and the apparent disappearance of these invaginations from the cell surface [74]. Despite the loss of obvious invaginations, caveolin remains clustered, indicating that the protein does not become randomly distributed in the membrane when cholesterol is removed. Likewise, the insulin receptor remains associated with the residual elements of caveolae [75] and is not released into the bulk plasma membrane. Thus, it would appear that cholesterol depletion results in the formation of a 'caveolar remnant' that perhaps retains some, but not all, of the functions of normal caveolae.

Although cholesterol depletion results in a significant morphological change in cavolae, it does not induce a change in $^{125}$I-insulin binding. Scatchard analyses indicated no change in either the number or affinity of insulin receptors in 3T3-L1 adipocytes treated with methyl-β-cyclodextrin [75]. Furthermore, there was no change in the ability of insulin to stimulate receptor autophosphorylation. Thus, unlike the EGF receptor, the intrinsic binding and kinase activities of the insulin receptor appear to function normally in the face of cholesterol depletion. Interestingly, in liver cells that lack caveolae, cholesterol depletion *does* lead to an inhibition of receptor autophosphorylation [45]. This suggests that caveolae/caveolin may exert a positive effect on the function of the insulin receptor. As caveolin is known to be a cholesterol binding protein [76], this could be through the concentration of residual cholesterol in the caveolar remnant or through direct interaction with the receptor itself.

While receptor binding and kinase activities appear to be largely retained following cholesterol depletion of caveolin-containing cells, downstream signaling is almost uniformly impaired. Phosphorylation of IRS-1, the insulin receptor substrate protein that serves as an adaptor protein in the transduction of insulin receptor-mediated signaling, is impaired in both 3T3-L1 adipocytes [75] and liver cells [45] but not in primary human adipocytes [43]. And phosphorylation of ATP citrate lyase, a downstream target of insulin signaling, is also reduced [28]. Activation of Akt, a surrogate for PI 3-kinase activation, is reduced [43,75] and glucose uptake is inhibited [28,43,75]. MAP kinase activation is impaired in primary adipocytes [43] but not in 3T3-L1 adipocytes [75], a difference that may be the result of differential expression of a cholesterol-dependent MAP kinase phosphatase [72]. These findings suggest that cholesterol, and by inference caveolae, plays a significant role in organizing and promoting signaling via the insulin receptor. While physical co-localization of signaling components would appear to be the simplest explanation for the requirement for caveolae in insulin signaling, other possibilities such as a need for cholesterol to maintain membrane integrity necessary for lipid-based signaling cannot be ruled out.

The mechanism of insulin-stimulated glucose uptake has been extensively studied and the role for lipid rafts in this pathway has been demonstrated (for review, see [77]). Glucose transport appears to require activation of two separate signaling pathways: (i) a PI 3-kinase-dependent pathway; and (ii) a PI 3-kinase-independent pathway (for review, see [78]). It is the PI 3-kinase-independent pathway that is known to involve lipid rafts.

Binding of insulin stimulates autophosphorylation of the receptor and leads to the recruitment of APS, an adapter protein that contains PH and SH2 domains, to the insulin receptor [79]. APS is then phosphorylated by the insulin receptor on a carboxy-terminal tyrosine which in turn serves as a binding site for the SH2 domain of c-Cbl [79]. c-Cbl is a proto-oncogene with E3 ubiquitin protein ligase activity [70,80] that is present in cells in a complex with CAP, Cbl associated protein. The Cbl–CAP complex is recruited to the insulin receptor whereupon c-Cbl is tyrosine phosphorylated [79,81]. Following phosphorylation of c-Cbl, the Cbl–CAP complex is released from the insulin receptor and is recruited to lipid rafts by formation of a tertiary complex with flotillin [82]. This complex formation appears to be mediated by the SoHo domain present on CAP [58]. Tyrosine phosphorylated c-Cbl recruits the SH2-containing adaptor protein, CrkII along with the guanine nucleotide exchange factor, C3G, to lipid rafts [83]. C3G then activates the Rho family GTPase, TC10 [83].

TC10 is localized to lipid rafts due to post-translational modification with both farnsyl and palmitoyl groups [84]. Both activation and raft localization of TC10 appear to be critical for insulin-stimulated translocation of the GLUT4 glucose transporter to the plasma membrane. Dominant negative mutants of TC10 inhibit insulin-stimulated glucose uptake [83]. And if either raft integrity or raft localization of TC10 is impaired, insulin fails to stimulate glucose uptake [85]. Rho family GTPases, such as TC10, are known to regulate the actin cytoskeleton [86] and this may provide the basis for the insulin-stimulated externalization of Glut4 to the plasma membrane.

The role of the pathway in glucose uptake has recently come into question due to the finding that siRNA-mediated knockdown of Cbl, CAP and CrkII did not result in the impair of insulin-stimulated glucose uptake [87]. Nonetheless, these findings demonstrate that lipid rafts function in the signaling network downstream of the insulin receptor.

### 4.3. Other receptor tyrosine kinases

Substantially less evidence exists for the involvement of cholesterol and rafts or caveolae in the function other receptor tyrosine kinases. As noted above, the PDGF receptor appears to be localized to caveolae rather than flat rafts [35]. Treatment of cells with PDGF leads to an increase in the tyrosine phosphorylation of proteins recovered in the caveolar fraction but not in non-caveolar membranes [35,46]. This suggests that the effects of PDGF on protein phosphorylation are largely restricted to the caveolar compartment, at least during early times

after activation. Consistent with this observation, stimulation of isolated caveolae with PDGF leads to the activation of MAP kinase present in the membrane preparation indicating that all the components necessary for growth factor-stimulated MAP kinase activation can be found, at least to some extent, in isolated caveolae [88].

Treatment of endothelial cells with filipin to sequester cholesterol resulted in the inhibition of PDGF receptor autophosphorylation as well as a reduction in the phosphorylation of other cellular proteins [35]. However, treatment of Swiss 3T3 cells with filipin or methyl-β-cyclodextrin failed to inhibit PDGF receptor phosphorylation [42]. Similarly, oxidation of cholesterol to cholest-4-en-3-one by treatment of cells with cholesterol oxidase led to an uncoupling of receptor autophosphorylation from phosphorylation of other cellular proteins indicating that intact caveolae may be required for phosphorylation of cellular proteins but not for autophosphorylation of the PDGF receptor itself. These findings are similar to observations with the insulin receptor and suggest that, in general, receptor function is retained following cholesterol depletion but downstream signaling is impaired. Consistent with this notion, cholesterol depletion by treatment with lovastatin led to an inhibition of PDGF-stimulated PI 3-kinase activity [89].

Although nothing is known regarding the localization of the FGF receptor to rafts or caveolae, FGF-stimulated phosphorylation of its substrate protein, FRS-2 has been shown to occur in the caveolar/raft fraction, suggesting that like the PDGF receptor, signaling via this growth factor may well be compartmentalized [48,51]. Results with the NGF receptor, TrkA, indicate that treatment with filipin blocks both NGF-stimulated TrkA autophosphorylation [38] and MAP kinase activation [47]. These findings suggest that TrkA raft biology may more closely mimic that of the insulin receptor and the PDGF receptor rather than the EGF receptor. This distinction is interesting given that NGF and EGF both stimulate tyrosine phosphorylation in PC12 cells, but EGF stimulates proliferation whereas NGF stimulates differentiation. The difference in effects has been attributed to transient stimulation of tyrosine phosphorylation by EGF but prolonged stimulation of tyrosine phosphorylation by NGF [90,91]. Interestingly, transient MAP kinase activation by the EGF receptor appears to be due to assembly of a short-lived complex on the EGF receptor itself that contains Crk, C3G, Rap1 and B-Raf. Prolonged activation of MAP kinase by NGF is associated with phosphorylation of the caveolar protein, FRS2 which then scaffolds the assembly of a more stable complex of Crk, C3G, Rap1 and B-Raf [92]. It is tempting to speculate that differences in the localization of EGF and NGF receptors to rafts and caveolae lead to differences in the nature of the signaling complexes formed and may contribute to the known distinction between these two growth factors with respect to PC12 differentiation.

## 5. Regulation of receptor tyrosine kinase function by caveolin

Caveolin-1 is the first member of a family of homologous molecules that are localized to cholesterol-enriched, low density membrane domains. Caveolin-1 appears to be the structural component of caveolae. It is present in the striated coat that is seen surrounding caveolae in the electron microscope [8] and de novo expression of caveolin-1 leads to the formation of morphologically identifiable caveolae in cells that did not previously exhibit these invaginations [93]. Caveolin-2 is most abundantly expressed in adipocytes but is also present in lung, heart and diaphragm [94]. In the absence of caveolin-1, caveolin-2 is retained in the Golgi but redistributes to caveolae when caveolin-1 is expressed [95,96]. These data suggest that caveolin-2 by itself cannot induce formation of a caveolar coat but it can interact with caveolin-1 to generate a caveolar coat. Caveolin-3 is the muscle-specific member of the caveolin family and is found in skeletal muscle, cardiac muscle and smooth muscle [97]. Caveolin-3 appears to be important in the development of T-tubules [98].

Early studies on the effects of caveolin-1 on growth factor receptor activity generally involved co-immunoprecipitation of receptors with caveolin-1 or the use of recombinant forms of caveolin or peptides derived from caveolin to modulate receptor function. With the exception of the insulin receptor which appears to be stimulated by caveolin-1 [99], these studies demonstrated that caveolin-1 bound to and inhibited growth factor receptor kinase activity [61,100,101]. Caveolin or caveolin-derived peptides were also reported to bind and be inhibitors of protein kinase C [102], protein kinase A [103], G protein-coupled receptor kinases [104], src family kinases [105], heterotrimeric G proteins [60,105,106], ras proteins [105,107] and adenylyl cyclase [108]. This led to the hypothesis that caveolin served as a scaffold for the formation of complexes involved in cell signaling [105].

Given the number of signaling molecules that caveolin was reported to bind and regulate, it therefore came as a surprise that caveolin-1 knockout mutants were viable with no obvious developmental abnormalities [109,110]. In retrospect, it is apparent that many of these early studies were done in vitro using either recombinant caveolin-1 or caveolin-derived peptides. As neither of these reagents are water-soluble, many effects specifically attributed to caveolin may, in fact, have been due to non-specific hydrophobic interactions with proteins. As noted above, the so-called caveolin binding motif said to mediate the interaction of proteins with caveolin is itself hydrophobic in nature and, at least in receptor tyrosine kinases, is located in the hydrophobic core of the protein kinase domain where it is unlikely to be available for interacting with caveolin. Indeed, mutation of this sequence produced either poorly expressed, kinase-impaired receptors or receptors that showed no difference in their ability to localize to low-

density membrane domains [111,112]. Thus, it seems unlikely that direct binding of this plethora of signaling molecules by caveolin-1 represents the mechanism whereby caveolin modulates signal transduction.

However, a number of studies were done using ectopic expression of caveolin-1 in cells and in these cases caveolin did modulate signal transduction [99,100]. Furthermore, studies on caveolin-1 knockout mice have demonstrated a variety of phenotypes consistent with alterations in hormone signaling. For example, caveolin-1 null mice show defects in nitric oxide and calcium signaling [110], cardiac hypertrophy [113], cardiomyopathy and pulmonary hypertension [114], insulin resistance and decreased insulin receptor expression in adipose tissue [115], hypertriglyceridemia [116] and a reduction in life span [117]. Knockout of caveolin-3 yielded mice with insulin-resistant skeletal muscle [118]. Furthermore, knockdown of caveolin-1 through siRNA led to an enhancement of basal as well as sphingosine-1-phosphate- and VEGF-induced activation of Akt [119]. Finally, caveolin levels tend to be reduced in oncogenically transformed cells [120], whereas recombinant expression of caveolin-1 blocks anchorage-independent cell growth [121].

These in vivo studies confirm the importance of caveolins in signaling and growth regulation. However, in several of these studies, the role of caveolin was specifically observed to be independent of its ability to localize signaling molecules to lipid rafts. Insulin receptors still localized to the low density membrane fraction in soleus muscle from caveolin-3 knockout mice [118]. In addition, the distribution of endothelial nitric oxide synthase, src, rac, flotillin, Gq and the insulin receptor in density gradients was not affected by siRNA-mediated knockdown of caveolin-1 [119]. These observations indicate that caveolin is not necessary for the localization of signaling proteins to lipid rafts However, there are several other mechanisms through which caveolin could exert an effect on growth factor receptor function.

One possible mechanism derives from the ability of caveolin to bind and traffic cholesterol. Caveolin-1 has been shown to bind cholesterol [76] and fatty acids [122]. The rate of cholesterol efflux from cells is proportional to the levels of caveolin-1 [123] and caveolin mRNA levels are up-regulated by free cholesterol [124]. In addition, caveolin-1 appears to be involved in trafficking newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane [125,126]. A link between the effects of caveolin on cholesterol trafficking and cell signaling was provided by experiments using dominant-negative mutants of caveolin. These amino terminally truncated forms of caveolin-3 inhibited H-ras function by mediating the depletion of cholesterol from the plasma membrane [127,128]. The effects of the dominant-negative caveolin-3 on Ras signaling could be reversed by replenishing plasma membrane cholesterol demonstrating that cholesterol depletion was the mechanism through which this mutant affected signaling [127].

An alternative, and not mutually exclusive possibility, relates to the ability of caveolins to undergo growth factor-stimulated phosphorylation. Both EGF [129,130] and insulin [131] induce the tyrosine phosphorylation of caveolin-1. In the case of the EGF receptor, the phosphorylation appears to be dependent on expression of $pp60^{src}$ [132]. However, for the insulin receptor, phosphorylation may be mediated by the src family kinase, fyn [133] or by the insulin receptor itself [134]. These differences in the mechanism through which caveolin-1 is tyrosine phosphorylated may account for the differences in the characteristics of its phosphorylation when stimulated by different growth factors. The phosphorylation of caveolin-1 in response to EGF occurs only in cells expressing mutant or high levels of the EGF receptor [129,132]. By contrast, caveolin-1 phosphorylation in response to insulin occurs in cells expressing normal levels of insulin receptors and tends to be persistent [131,133,134].

Caveolin was first discovered as a substrate for phosphorylation by $pp60^{src}$ [135] and thus its ability to undergo phosphorylation had long been recognized. The $pp60^{src}$ phosphorylation occurs on tyrosine-14 near its amino terminus [136] and this appears to be the site phosphorylated in response to insulin and EGF as well [130,132,134]. Phosphorylation of caveolin-1 leads to the co-immunoprecipitation of several tyrosine-phosphorylated proteins with caveolin-1[129–131]. Two of these proteins have been identified and found to bind to caveolin-1 only in its tyrosine phosphorylated state. They are the SH2 domain containing proteins: (i) Csk, the kinase that phosphorylates and inactivates $pp60^{src}$ [137]; and (ii) Grb7 (growth factor receptor binding protein 7) [130]. These findings suggest the possibility that caveolin-1 serves a function akin to that of IRS-1, forming SH2- or PTB domain-mediated complexes with potential substrates or regulatory proteins for growth factor receptors and localizing them to caveolae where the majority of growth factor receptor appear to reside. This scenario would be consistent with the ability of caveolin-1 to enhance insulin receptor signaling since the insulin receptor is specifically localized to caveolae. How this might lead to the observed caveolin-dependent inhibition of EGF receptor-mediated signaling [138] is more difficult to explain since EGF receptors are not localized to caveolae.

A final role for caveolin-1 with respect to growth factor signaling may be related to the role of caveolae in endocytosis of proteins and lipids. In caveolin-1 null mice, the level of insulin receptors in adipose tissue was reduced by ~90% [115]. Expression of recombinant caveolin-1 in the cells reversed the effect on insulin receptor levels. These findings were interpreted as indicating that caveolin-1 acts as a molecular chaperone for the insulin receptor and that it is necessary to stabilize the receptor in adipocytes. An alternative hypothesis derives from the work of Le et al. [139]

who showed that expression of caveolin-1 reduced caveolae-mediated endocytosis by stabilizing the caveolar invaginations at the plasma membrane. Thus, by reducing the rate of insulin receptor internalization and down-regulation, caveolin-1 could help to preserve high levels of insulin receptors on the cell surface, thereby enhancing insulin signaling. Consistent with this possibility, caveolin-1 deficient endothelial cells show a reduction in the uptake of albumin which is known to be mediated by caveolae [140].

## 6. A general model of the role of lipid rafts and caveolae in growth factor receptor function

Although it is clear that rafts and caveolae are important for growth factor signaling, our understanding of the system is incomplete. Fig. 3 depicts a scheme through which some of the differences in the behavior of various growth factor receptors can be understood. It is based on a distinction between receptors that are localized to lipid rafts (e.g., the EGF receptor) and those that are targeted to caveolae (e.g., the insulin receptor).

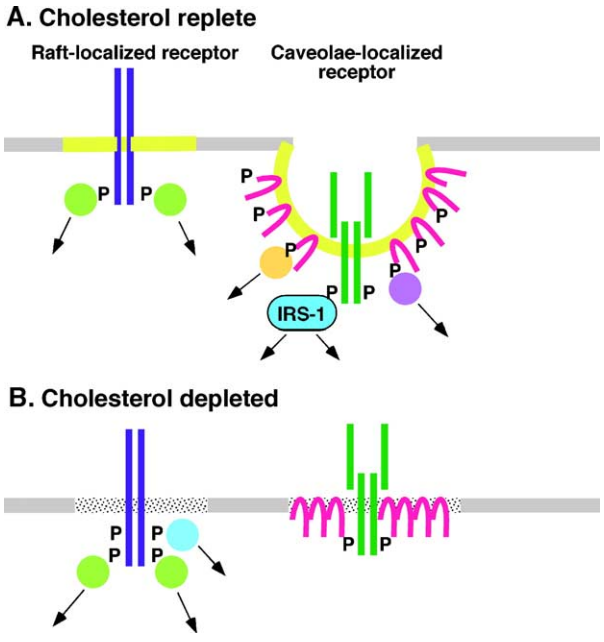


Fig. 3. Model for the role of lipid rafts and caveolae in growth factor receptor function. (Panel A) Receptors are localized to either flat lipid rafts (blue receptor) or invaginated caveolae (green receptor). Signaling complexes formed by receptors in rafts are relatively simple and provide only a transient signal. Signaling complexes formed by receptors in caveolae are more complex and more stable and thus generate a more prolonged signal. (Panel B) Upon cholesterol depletion, receptors in rafts increase function due to the release of constraints on receptor activity. Signaling complexes are more elaborate due to phosphorylation of additional sites. Receptors in caveolae are retained in the residual elements of the caveolar coat. While receptor function is normal, downstream signaling is blocked due to steric inhibition and the inability to assemble a signaling complex.

For the raft-localized growth factor receptor, its function is suppressed by the raft environment, either due to effects of fluidity, membrane thickness or the clustering of receptors or other proteins. Binding of the ligand leads to receptor autophosphorylation and signaling is mediated through complex formation. The complex is simple and transient, and signaling is down-regulated by rapid receptor internalization.

For the caveolae-localized receptor, the invaginated structure provides an optimal environment for receptor function, possibly due to lipid composition, membrane curvature or the clustering of receptors. Within this environment, the growth factor receptor can form a more complicated, more stable signaling complex. This is due to the ability of phosphorylated caveolin to bind some receptor substrates and the ability of IRS-1 (or other similar molecules) to scaffold other substrates. The entire structure is stabilized by the caveolar coat, a function that results in reduced endocytosis and a more prolonged signal.

Upon cholesterol depletion, the raft-localized receptor is relieved of the inhibitory effects of the raft environment and signals more effectively, partially due to enhanced kinase activity and partially due to an increase in the sites that are phosphorylated. By contrast, the caveolar receptor is now localized in a ‘caveolar remnant’ that contains caveolin but is not in an appropriate configuration to scaffold a signaling complex. Receptor function is normal but access of the receptor to exogenous substrates is limited due to steric factors. Thus, all down stream signaling is impaired.

## 7. Future directions

The precise role played by lipid rafts and caveolae in growth factor receptor-mediated signal transduction is far from clear. While these receptor tyrosine kinases are clearly localized to low density, cholesterol-rich membrane domains, it is not yet known exactly what targets the receptors to these domains and why some appear to localize to caveolae while others are specifically enriched in the less stable lipid rafts. While changes in cholesterol content modulate the activity of the growth factor receptors, a definitive understanding of the molecular basis for this effect is lacking. And while caveolins clearly affect signal transduction, the mechanism through which this occurs has not been elucidated. These are questions that will need to be addressed to clarify the role of these membrane microdomains in the regulation of growth factor receptor function.

Studies of lipid rafts and caveolae have been hampered by the lack of suitable physical methods for visualizing these microdomains in intact cells. Future progress in this area will depend on the development of such methods so that the behavior of single receptor molecules or other signaling components can be examined in the context of the cell membrane and the effects of cholesterol and caveolin on their behavior assessed.

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