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Transit of Hormonal and EGF receptor-dependent Signals Through Cholesterol-rich Membranes

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Abstract

The functional consequences of changes in membrane lipid composition that coincide with malignant growth are poorly understood. Sufficient data have been acquired from studies of lipid binding proteins, post-translational modifications of signaling proteins, and biochemical inhibition of lipidogenic pathways to indicate that growth and survival pathways might be substantially re-directed by alterations in the lipid content of membranes. Cholesterol and glycosphingolipids segregate into membrane patches that exhibit a liquid-ordered state in comparison to membrane domains containing relatively lower amounts of these classes of lipids. These "lipid raft" structures, which may vary in size and stability in different cell types, both accumulate and exclude signaling proteins and have been implicated in signal transduction through a number of cancer-relevant pathways. In prostate cancer cells, signaling from epidermal growth factor receptor (EGFR) to the serine-threonine kinase Akt1, as well as from IL-6 to STAT3, have been demonstrated to be influenced by experimental interventions that target cholesterol homeostasis. The recent finding that classical steroid hormone receptors also reside in these microdomains, and thus may function within these structures in a signaling capacity independent of their role as nuclear factors, suggests a novel means of cross-talk between receptor tyrosine kinase-derived and steroidogenic signals. Potential points of intersection between components of the EGFR family of receptor tyrosine kinases and androgen receptor signaling pathways, which may be sensitive to disruptions in cholesterol metabolism, are discussed. Understanding the manner in which these pathways converge within cholesterol-rich membranes may present new avenues for therapeutic intervention in hormone-dependent cancers.

Cholesterol and Cancer

Extracellular signals influencing a tumor cell must cross a lipid barrier that is still poorly understood in terms of structure and regulatory function. This brief review focuses on the hypothesis that membrane cholesterol, through its capacity to mediate changes in lipid membrane structure, influences signal transduction mechanisms related to oncogenesis. Here, we specifically explore the concept that membrane cholesterol is a facilitator of cross-talk

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between members of the EGFR family of receptor tyrosine kinases and members of the nuclear hormone receptor family. Among other findings in a rapidly developing literature, this idea is based on recent evidence that nuclear receptors may signal independently from their transcription function and that they appear to inhabit cholesterol-rich membrane microdomains, generally referred to as “lipid rafts”[1,2].

Because tumor cell biologists have traditionally focused on proteins as mediators of oncogenesis, the role of lipids in tumor development and spread has received relatively limited attention. The importance of alterations in lipid metabolism to tumor development is clear, however. Many lipid modifying enzymes, including proteins that hydrolyze lipids, attach them covalently to other proteins, and translocate them across membranes, have been shown to play important roles in signaling mechanisms related to tumor formation. Certain lipid synthetic enzymes, such as fatty acid synthase[3], which is overexpressed in breast and prostate cancer, can be considered to be metabolic oncogenes. A wide variety of lipid binding motifs, some displaying a very high level of biochemical specificity, are components of proteins involved in signal transduction[4]. A role for disruption of lipid metabolism in cancer is also suggested by epidemiologic studies linking rates of certain types of cancer to Western and/or high-fat diets[5,6].

Cholesterol is a neutral lipid that plays an important role in the formation and stabilization of membrane topology. Together with sphingolipids and saturated phospholipids, cholesterol participates in the formation of “liquid-ordered” microdomains in membranes that can be distinguished from the less structured matrix of the bulk plasma membrane. These cholesterol-rich membrane patches are most commonly referred to as “lipid rafts” and can be envisioned as discrete membrane elements residing within a larger lipid “sea”[1,2]. Lipid rafts arise from a non-random lipid sorting mechanism, and may be either stable or dynamic depending on the subcellular context. The size of individual raft domains is also likely to vary widely in size (6-100 nm). Caveolae, which are invaginated membrane structures and cytosolic vesicles are a relatively large subtype of lipid raft (ca. 80 nm)[7]. Caveolae can be visualized by electron microscopy and their size determined by direct measurement. In contrast, non-caveolar rafts, which do not harbor the membrane deforming proteins, caveolin-1 or -3, and thus do not exhibit the distinct caveolar shape, are believed to be substantially more mobile than caveolar rafts [8,9]. These “flat” rafts are estimated to be much smaller (6-20 nm), more mobile and less stable than caveolae. The small size of the non-caveolar, flat type of lipid raft has made them more difficult to study and this has led to considerable controversy about the nature of these microdomains in the literature, even causing some to question their existence[10]. Concerns about whether raft-like structures actually exist in membranes, based on difficulties visualizing the non-caveolar form, were recently addressed in an excellent review[9].

In addition to its role in membrane structure and fluidity, cholesterol is able to regulate transcription and other signaling processes through interaction with sterol-sensing domains in multiple proteins. Caveolin-1, which is a major mediator of cholesterol transport to the plasma membrane, is negatively regulated by cholesterol binding to the sterol-sensing domain of sterol-regulatory element binding protein (SREBP)-cleavage activating protein (SCAP)[11]. SCAP activates the transcriptional regulatory function of SREBP, which inhibits *cav-1* gene expression at the chromatin level, while causing the upregulation of many genes involved in cholesterol synthesis and uptake. Cholesterol is also an essential component of the mechanism of protein trafficking to discrete subcellular regions and therefore is likely to affect subcellular protein distribution[12].

Over the past decade, extensive evidence has accumulated, particularly from biochemical studies of caveolin-containing membrane preparations, that lipid rafts sequester and exclude signaling proteins, and harbor pre-formed signal transduction complexes, suggesting that they

play an important role in cell signaling. Analysis of lipid raft preparations using biochemical methods indicates that raft-like structures are present on both the inner and outer leaflets of the plasma membrane[13]. Outer leaflet rafts sequester GPI-liked proteins, while inner leaflet rafts are enriched in acylated proteins (e.g. heterotrimeric G protein alpha subunits, and Src family tyrosine kinases). Recent molecular modeling studies have indicated that the smaller, non-caveolar rafts, which may actually be highly dynamic, transient structures that form and dissociate rapidly (in comparison to caveolae or other types of membrane domains), may be particularly effective at promoting intermolecular associations between proteins that migrate into them[14]. Rafts can capture proteins by encompassing a region of slower lateral diffusion than the liquid disordered component of the membrane, and also as a result of structural features on proteins, such as lipid modifications, that enhance protein affinities for rafts. Together with the regulated recruitment of signaling proteins to the plasma membrane, selective admission (and exclusion) of specific signaling components to the lipid raft platform(s) adds a separate layer of control to signaling events within the plasma membrane. An opportune area for investigation is experiments directed toward the elucidation of the nature of the lipid raft microdomain(s), the manner in which proteins transit into and out of these regions, and the characterization of the types of signals that pass through them. Current estimates indicate that proteins that can move laterally in the membrane (i.e., are not tethered to a larger structure) will reside within rafts for 25-60μs, depending on the size of the raft domain[14]. This view of raft organization and stability suggests that rafts that are not assembled into cytoskeletal or intercellular junctional complexes, form a platform where collision rates between cognate signaling components may be facilitated, thereby amplifying signal transduction through select pathways.

It was reported about a hundred years ago[15], and many times since in the biomedical literature [16,17], that cholesterol and other fatty deposits accumulate in solid tumors. Animal studies that have modeled the effect of increases in circulating cholesterol have shown that the growth of human tumor xenografts is stimulated in the high cholesterol environment, and that this effect coincides with changes in signal transduction mechanisms consistent with the promotion of an oncogenic state [18]. Increases in circulating cholesterol also increase the cholesterol content of lipid raft membranes, as demonstrated by direct biochemical analysis of tumors carried in rodent hosts[18]. Epidemiologic studies have suggested that prostate cancer may be one of several malignancies that demonstrates a sensitivity to circulating cholesterol levels, with higher levels promoting the disease[19] and lower levels, caused by pharmacologic manipulation, to be protective[20].

The lipid raft model of membrane organization is useful in conceptualizing the effect of increased levels of membrane cholesterol on oncogenic signaling. Expansion of the cholesterol-rich membrane compartment, within certain limits, is likely to promote the formation of raft-resident signaling complexes that promote cell proliferation and cell survival [18,21]. Expansion of the lipid raft compartment may also stimulate the production of tumor-derived microvesicles, which have been shown to contain a lipid composition similar to lipid rafts. These structures may transfer signaling complexes and other bioactive molecules between tumor cells and from tumor cells and inflammatory cells to various cell types in the tumor microenvironment. Experiments with isolated microvesicles indicate that these structures have a capacity to alter the behavior of tumor cells in a paracrine manner, and to alter the tumor microenvironment in ways relevant to cancer progression, including the stimulation of processes that promote angiogenesis and metastasis[22]. Consequently, increases in the extent of cholesterol-rich membranes may promote autocrine as well as paracrine mechanisms of signal transduction that affect not only tumor cell behavior but that can alter the tumor microenvironment and the capacity of tumor cells to metastasize to distant sites.

Our group has been studying the potential effect of cholesterol as a mediator of signal transduction using prostate cancer models. We have reported that EGFR signaling to the pro-survival serine-threonine kinase, Akt1[18,23], and from IL-6 to STAT3[24], are cholesterol-sensitive in human prostate cancer cells and that these signaling mechanisms can be disrupted by cholesterol targeting approaches. The cholesterol-sensitivity of EGFR/ErbB-dependent pathways is potentially provocative in the case of prostate cancer because a number of studies have linked the EGFR family of receptor tyrosine kinases to mechanisms of androgenic regulation, including progression to androgen independence[25-27].

The EGFR and lipid raft microdomains

The EGFR/ErbB1 is a receptor tyrosine kinase that signals by ligand-dependent and –independent dimerization with itself or with several paralogs within the EGFR family[28,29]. Within this family the proto-oncoprotein, HER2/ErbB2, is the preferred dimerization partner. Signaling through HER2 was recently implicated in androgen-independent activation of the androgen receptor (AR) in prostate cancer[26]. Studies from our group have suggested that signaling from EGFR may actually functionally inhibit the AR[30,31], suggesting that even at the level of the plasma membrane, signals originating from the EGFR and HER2 may be directed downstream through different pathways[25].

Both EGFR and HER2 have been found from biochemical studies to be associated with lipid rafts. Several groups have shown that depletion of plasma membrane cholesterol can result in EGFR activation[32-34], suggesting that rafts may serve to sequester the EGFR in a manner that allows rapid activation from upstream events, such as ligand binding. Tyrosine phosphorylation sites on the cytoplasmic domain of the EGFR are differentially sensitive to absorption of cholesterol from cell membranes, indicating that some signaling pathways affected by the EGFR are cholesterol-sensitive, while others may not be[34]. Phosphorylation on Tyr845 on the EGFR, a site within the activation loop of the kinase domain, is phosphorylated by pp60Src. Phosphorylation at this site, which correlates with increased kinase activity, is enhanced by cholesterol depletion[34]. The finding that the Src phosphorylation site on EGFR is cholesterol-sensitive potentially links the EGFR to the actin cytoskeleton, and other sites of action of Src, through a cholesterol-regulated pathway. In contrast to the Tyr845 site, the Tyr1045 phosphosite, which binds the c-Cbl ubiquitin ligase involved in down-regulation of the receptor, was not found to be cholesterol-sensitive[34]. These data suggest that proteins that reside within rafts can still coordinate signals from both cholesterol-sensitive and –insensitive pathways. The manner in which this occurs is not understood.

A recent study using single-particle tracking has identified cholesterol as a mediator of lateral mobility of EGFR and HER2 in living cells[35]. In that study, cholesterol depletion resulted in the restriction of mobility and confinement of EGFR and HER2 into nanoscale membrane domains, while cholesterol repletion restored lateral movement of both receptors. Notably, HER2 was more mobile that EGFR, a distinction that is potentially attributable to differences in the relative ability of the two receptors to interact with the cortical cytoskeleton. These findings are consistent with the view that signals from the two receptors diverge at the level of the plasma membrane. Applied to a cancer cell that accumulates cholesterol relative to corresponding normal cell types, the implications of these results are (1) that lateral diffusion of EGFR family members is increased with malignancy and (2) that cholesterol may alter the rate of formation and dissociation of receptor-associated signaling complexes. They also imply that different receptor tyrosine kinases may respond differently to changes in the cholesterol content of cell membranes.

EGFR activation may also change the raft environment by promoting the formation of oligomeric caveolin at the plasma membrane[36]. This would have the effect of drastically

increasing the potential binding sites for caveolin-associated proteins, with likely dramatic effects on downstream signaling. The EGFR appears to redirect cytosolic caveolin to the plasma membrane by a mechanism that involves phosphorylation of caveolin-1 on tyrosine 14 by Src[36], a well-known EGFR effector.

Nuclear receptors and lipid rafts

Evidence has accumulated that members of the classical nuclear receptor family of hormonal mediator proteins can mediate rapid responses to steroid hormones in a manner that does not directly involve their transcriptional regulatory function.

1,25 dihydroxy vitamin D3, (1,25-$(OH)_2D_3$; calcitriol) is the active form of vitamin D. 1,25-$(OH)_2D_3$ exerts its biological effects by binding to the Vitamin D receptor (VDR) which, as with the other nuclear hormone receptors, was thought to function exclusively as a nuclear receptor, expressed in a wide variety of tissues including muscle, adipose tissue, and bone. In the mid 1980's reports began to surface describing rapid responses (occurring in minutes to hours) to 1,25-$(OH)_2D_3$ that were too quick to be caused by transcriptional upregulation (reviewed in [37]). Recent evidence using direct binding analysis and VDR knockout mice have revealed that the classical VDR is the likely mediator of at least some of these rapid effects [38,39]. In addition, at least one other membrane-associated vitamin D binding protein has been described that mediates rapid cytosolic events: the 1,25D3-membrane associated rapid response steroid binding protein (1,25D3-MARRS) [40]. It is now clear that a fraction of the classical VDR is found in the plasma membrane of 1,25-$(OH)_2D_3$ cell targets and that it is concentrated in caveolae-enriched membrane fractions[39].

Rapid responses to estrogens that are not affected by translational or transcriptional inhibitors have also been reported in a wide variety of hormone sensitive tissue cells [41-44]. Thus began a search for the membrane localized estrogen receptor (ER). Here again there has been uncertainty as to whether the classical ERs were responsible for these effects or if another class of ER (e.g. G protein-coupled receptor (GPR)30) was involved[45-47]. Recent evidence using knockout mice, mass spectrometry and RNA interference has provided some clarity by demonstrating that in the tested cells and tissues only the classical ERs bind estradiol (E2) and signal in response to E2 stimulation, suggesting that nuclear and plasma membrane ERs are the same proteins [48].

Recent reports from several groups have shown ERα to be associated with lipid raft and/or caveolar fractions [49,50]. Razandi et al. (2002) demonstrated that ERα colocalized with caveolin in membranes isolated by a unique silica-coating technique performed on rat lung microvessels in situ, that ERα and caveolin-1 coimmunoprecipiated, and that caveolin-1 and ERα co-localized at membranes as assessed by immunofluorescence microscopy[51]. Interestingly, this report also demonstrates that E2 can regulate caveolin synthesis, and that caveolin-1 assists in the translocation of the ER to the plasma membrane, suggesting that estrogen signaling results in greater ER plasma membrane expression (i.e. a positive feedback loop). A study in which E2 was administered in vivo to newborn and middle-aged rats (13-14 months) demonstrated that caveolae and caveolin-1 expression was increased in the bladder sarcolemma, supporting the type of positive regulation of caveolin expression by E2 previously noted only in tissue culture[52].

Additional reports have provided insight into the apparent caveolin/ERα interaction. Mutation of serine 522 to alanine (S522A) reduced ERα membrane localization and colocalization with caveolin-1, but other S to A mutations affected neither[53]. Other analyses suggest that plasma membrane localization of the ERα is palmitoylation dependent. One study on the 46 kD isoform of the ER (this protein lacks the N terminus but is otherwise identical to ERα) first revealed an enrichment in caveolar fractions and colocalization with caveolin-1, as well as evidence

that the protein is palmitoylated[54]. Interestingly, two subsequent reports[55,56], applying mutational approaches to the study of the full length ERα, establish that mutation of cysteine 447 diminishes ERα palmitoylation, membrane localization and response to E2. Given the likely importance of palmitoylation for association with raft membranes, regulated S-acylation of cysteine residues in the ER might provide the basis for its nuclear vs. raft localization.

Mapping studies have indicated that residues 82-101 of caveolin-1 (the caveolin interaction domain) and residues 1-282 of ERα mediate the direct interaction between the two proteins [57], while serine 522 of ERα appears to be important for membrane translocation[58]. In comparison to murine $ER\alpha^{WT}$, the $ER\alpha^{S522A}$ mutant resulted in 62% fewer receptors expressed at the plasma membrane, with little influence on receptor affinity for ligand, and no effect on the ERα nuclear function. Additionally, the 522 mutant was shown to be much less effective at supporting E2-mediated ERK activation, cAMP generation, and stimulation of IP3. Other S to A substitutions (at amino acid residues 10 and 582) had no effect on either E2 binding to the membrane or signaling by E2, in comparison to $ER\alpha^{WT}$[58].

In contrast to the extensive reports describing the nongenomic function of ERα, the nongenomic signaling function of ERβ is not well described, and only a single report describes the localization of this protein to caveolae[59]. However, ERβ, along with the AR and Src, has been shown to rapidly assemble into a cytoplasmic complex in LNCaP cells following treatment with androgens or E2 and this complex appears to be involved in the control of cell proliferation in response to the hormonal signal[60].

The consequences of the ER localizing to, and functioning within, cholesterol-rich membrane microdomains may indeed explain some of the reported nongenomic signaling induced by E2. For example, an extensive literature describes the ability of E2 to activate eNOS[61,62], with evidence indicating roles for signaling intermediates such as Src family tyrosine kinases, MAP kinases, PI3 kinase and Akt[63-67]. There is also evidence that the ERα to eNOS signals are mediated by heterotrimeric G protein Giα subunits (i.e. pertussis toxin sensitive) [58,68,69]. An important observation is that eNOS[70,71] and heterotrimeric G protein α subunits [72-74] are known to preferentially localize to caveolae (and lipid rafts in general), thus providing a localization-based mechanism that might explain how E2 can rapidly promote NO production.

The literature on potential non-genomic actions of the androgen receptor (AR) is less robust than that of the ER; however, compelling evidence of similar non-genomic mechanisms of AR activation has been reported by several groups. Treatment of the LNCaP human prostate cancer cell line, or the MCF7 or T47D breast cancer cell lines, with either E2 or androgen results in the rapid formation of a cytosolic signaling complex containing ERα/ERβ the AR and the tyrosine kinase Src[60]. This multi-protein complex appears to be involved in the regulation of cell proliferation by these hormones. Src, through a physical interaction with the proline-rich region of the AR, seems to play an important role in this mechanism. Recently, the scaffolding protein, modulator of nongenomic activity of estrogen receptor (MNAR), was also identified as a component of this complex[75]. A recent report suggests that a nongenomic signaling pathway involving AR, Src and MNAR is upregulated constitutively in LNCaP variants that are androgen-independent, suggesting that this complex may play a role in progression to hormone-insensitive disease[76].

Like other nuclear receptors, the AR has been identified in caveolar/lipid raft biochemical fractions and AR has been shown to interact with caveolin-1. Using a mammalian two-hybrid system, AR domains required for interaction with caveolin-1 were mapped; they include the N-terminal AF1 domain and the ligand-binding domain[77]. AR contains a potential caveolin

scaffolding domain interaction sequence (YSWMGLMVFAMGWRSF), however the potential role of this region in AR interactions with caveolins has not yet been studied in detail.

Caveolin-1 has been identified as a tumor progression marker in prostate cancer and has been shown in animal models to be a potentially direct mediator of aggressive disease[78-80]. This situation appears to be different from the role of caveolin in the mammary gland, where it functions to inhibit oncogenic signaling[81]. A signaling function that has been identified for caveolin-1 in prostate cancer cells involves its ability to facilitate activation of the pro-survival kinase, Akt1, by interaction with, and inhibition of, the phosphatases PP1 and PP2A[79]. This study also demonstrated that overexpression of caveolin-1 augments androgenic signaling by facilitating translocation of phosphorylated AR to the nucleus.

Intersection of AR and EGFR signaling within lipid raft microdomains

Akt1 has been reported by several groups to be sensitive to manipulations in cholesterol level. Direct biochemical analysis has verified that a subpopulation of Akt1 molecules resides within lipid raft microdomains[18,82]. This is shown in Figure 1, where about 10% of endogenous Akt1 in the non-nuclear space (in the figure, cytoplasm + non-raft membrane (C+M) and lipid raft membrane (Raft) in LNCaP cells is shown to be present in the raft compartment. Rapid effects of androgen (R1881) on Akt1 phosphorylation can be seen from these data. In addition to interesting steady-state differences in immuno-reactivity with phosphosite-specific antibodies seen when the C+M and lipid raft fractions are compared, changes in phosphorylation state of Akt1 in response to androgen occur in the raft compartment within 15 min, and changes in the non-raft compartment are seen within 15-30 min. Interestingly, these changes are distinct and the results suggest that Akt1 in the raft and non-raft compartments are processing dissimilar signals. This pattern is in marked contrast to changes in phosphorylation state of Akt1 in response to EGFR activation with EGF. Although Akt1 phosphorylation changes are more rapid than those induced by R1881, the raft and non-raft compartments again are distinct from each other. Notably, the distinction between the pattern of phosphorylation seen with androgen and EGF is most evident when Akt1 is analyzed in the raft fraction. Another interesting feature of these results is that LNCaP cells do not express caveolin-1, and therefore the phenomena observed in the experiment shown in Figure 1 are independent of any influences from caveolin or caveolar components that are also not present in the flat form of lipid raft. We have verified that the AR is found in lipid raft membranes and can even be stabilized there in the presence of androgen (Cinar et al. in preparation). Studies on the functional role of lipid raft-resident AR are ongoing.

These data suggest the following interesting hypothesis: Non-genomic signals from androgen, and from EGFR activation, traverse the cholesterol-rich membrane subcellular compartments and, further, that signals elicited from the steroidal and peptide hormones within the raft compartment are distinct. Because both types of signals are believed to converge in prostate cancer cells to regulate proliferation and survival, the implication of this hypothesis is that the lipid raft membranes process both signals within a similar time frame. The data in Figure 1 also show that a prominent mediator of cell growth and survival signaling, the serine-threonine kinase Akt1, is a point of convergence of the two hormonal stimuli. We suggest that a potentially fruitful avenue of investigation to develop this hypothesis further is an analysis, by proteomic methods, of signaling complexes within lipid rafts that include Akt1 as a component.

Conclusions

Many studies over a long period have provided evidence that cholesterol, in association with sphingolipids and saturated phospholipids, participates in the formation of liquid-ordered membrane microdomains that are physically distinct from the larger liquid-disordered

membrane. The metaphor of a “raft” is useful in helping to imagine the coherence of these structures, and their potential for mobility, within the larger “lipid sea”. Because the bulk of intracellular cholesterol accumulates in the plasma membrane, lipid rafts may be a prominent type of microdomain at the interface between tumor cells and the tumor microenvironment. However, intracellular membranes also contain cholesterol and may similarly contain raft-like membrane microdomains that play important roles in signal transduction.

Biochemical analysis of lipid rafts, along with imaging studies, have provided evidence that these microdomains accumulate, as well as exclude, certain classes of proteins. The observations that EGFR family proteins are sensitive to disruptions in cholesterol synthesis and homeostasis, and that hormone receptors, such as the VDR, ERα/β, and AR can transit and potentially signal from these cholesterol-rich domains, provides an important new avenue for investigation about how hormone-sensitive cancers are regulated. These observations may also help us to understand the mechanisms behind diet-mediated effects on cancer progression. We believe that new opportunities for therapeutic intervention are likely to emerge from focused studies of signal transduction from the lipid raft signaling platform in prostate cancer and other malignancies.

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Abbreviations

1,25D3-MARRS
: 1,25D3-membrane associated rapid response steroid binding protein

Akt1
: serine threonine kinase (protein kinase Bα)

AR
: androgen receptor

E2
: estradiol

EGFR
: epidermal growth factor receptor

eNOS
: endothelial nitric oxide synthase

ER
: estrogen receptor

ERK
: extracellular-signal regulated kinase

GPI

glycosylphosphatidylinositol

GPR
G protein-coupled receptor

HER2/ErbB2
EGFR paralog receptor tyrosine kinase

IL-6
interleukin-6

IP3
inositol trisphosphate

MNAR
modulator of nongenomic activity of estrogen receptor

PI3-kinase
phosphoinositide 3-kinase

PP1 and PP2A
protein phosphatases 1 and 2A

SCAP
SREBP-cleavage activating protein

Src
non-receptor tyrosine kinase

SREBP
sterol-regulatory element binding protein

STAT3
signal transducer and activator of transcription-3

VDR
vitamin D receptor

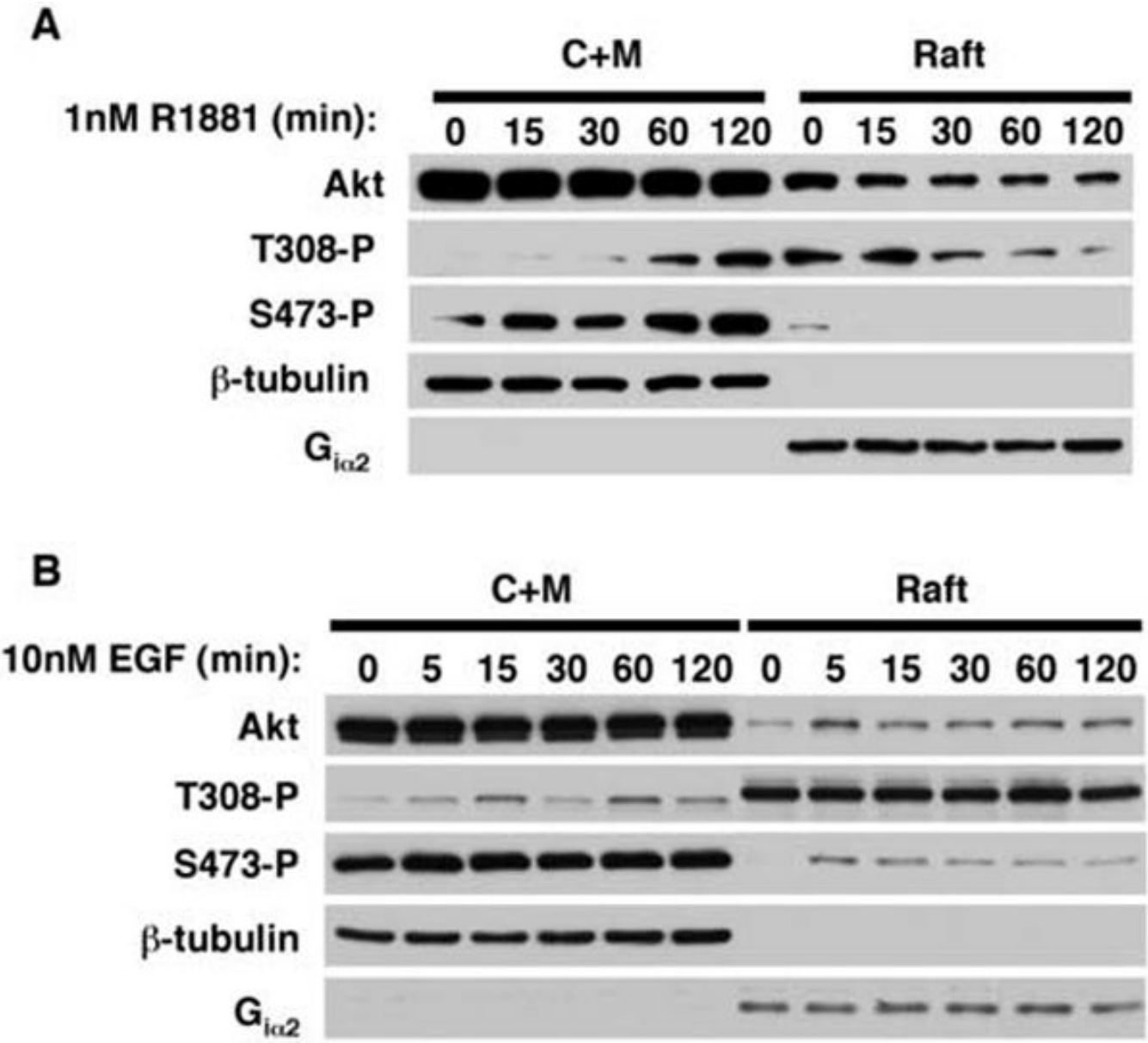

Figure 1. Androgen and EGF stimulate distinct changes in Akt phosphorylation state in lipid rafts isolated from LNCaP cells

A) Serum-depleted LNCaP cells were treated with 1nM R1881 or **B)** 10nM EGF for the indicated times and fractionated into cytoplasmic/Triton X100-soluble membrane (C+M) and raft fractions. Equal amounts of C+M and raft fractions were resolved by SDS-PAGE and immunoblotted with antibodies to total Akt1, Thr308-phosphorylated Akt1 (T308-P), and Ser473-phosphorylated Akt1 (S473-P). The fidelity of fractionation was confirmed by blotting for β-tubulin (cytosolic marker) and $G_{i\alpha 2}$ (raft marker).