COMMENTARY

Role of receptor tyrosine kinases in G-protein-coupled receptor regulation of Ras: transactivation or parallel pathways?

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Ras protein regulation by G-protein-coupled receptors has been thought to occur through transactivation of receptor tyrosine kinases. New evidence suggests that these two receptor types independently control different pathways leading to Ras activation in response to lysophosphatidic acid (LPA). Epidermal growth factor receptor function is needed for basal nucleotide exchange

on Ras, whereas the LPA receptor controls an inducible exchange activity.

Key words: epidermal-growth-factor receptor (EGFR), G-protein, guanine-nucleotide-exchange factor (GEF), lysophosphatidic acid (LPA), pertussis toxin, Ras.

Ras proteins are prototypic members of the superfamily of small guanine-nucleotide-binding proteins. They have profound effects on cell proliferation, survival and differentiation through their ability to interact with critical target enzymes or effectors, such as the protein kinase Raf and the lipid kinase phosphoinositide 3-kinase. Ras proteins act as signalling control switches that are active when bound to GTP and inactive when bound to GDP. Our enduring fascination with the details of exactly how the Ras switch works stems, at least in part, from the fact that a quarter of human tumours have constitutively activated Ras due to point mutations that inhibit its GTPase activity and many others may rely on elevated activity of wild-type Ras [1].

Normally, the activity of Ras proteins is tightly controlled by the co-ordinated actions of a number of regulatory proteins which act to influence the balance of GTP and GDP. The GAPs (GTPase-activating proteins) promote the intrinsic ability of Ras to hydrolyse GTP and hence become inactive. The GEFs (guanine-nucleotide-exchange factors) promote exchange of bound nucleotide (predominantly GDP due to the influence of GAPs) for free nucleotide (predominantly GTP) in the cytosol and hence are activators of Ras [2].

The study of the mechanisms involved in Ras activation by EGF (epidermal growth factor) led ten years ago to a consensus that activated RTKs (receptor tyrosine kinases) phosphorylate adaptor proteins, such as Shc, which in turn bind Grb2 and the GEF Sos [3]. This results in increased exchange activity at the plasma membrane where Ras is located. However, it was also known that many extracellular ligands that bind to other types of receptors, including the GPCR (G-protein-coupled-receptor) superfamily, can also efficiently activate Ras. The mechanism involved in this has been the subject of much debate: the new report from Rubio et al. [4] in this issue of the *Biochemical Journal* provides an important new twist to this that has significance for our understanding, not just of Ras activation, but also of the structure of signalling networks in general.

It has been known for many years that treatment of cells with ligands for GPCRs can result in the subsequent activation of RTKs, a phenomenon termed transactivation [5]. One of the most intensely studied pairs of receptors has been the GPCR for LPA (lysophosphatidic acid) and the RTK for EGF. Stimu-

lation of the LPA receptor has been found to lead to increased tyrosine phosphorylation, and hence activation, of the EGFR (EGF receptor) in several different cell types. A number of mechanisms have been proposed for this. Much attention has been paid to the ability of GPCR activation to stimulate the proteolytic processing of cell-surface-bound HB-EGF (heparin-binding EGF-like growth factor), resulting in the HB-EGF-induced activation of EGFR [6]. Alternative explanations have invoked the induction of reactive oxygen species which cause inhibition of tyrosine phosphatases and hence activation of RTKs [7].

The tacit assumption has been that LPA-induced Ras activation acts as a linear pathway, with LPA causing EGFR activation [via PTX (pertussis toxin)-sensitive heterotrimeric G-proteins of the $G_{i/o}$ type] and then EGFR activating Ras (via Shc/Grb2/Sos). The fact that both PTX and EGFR inhibitory drugs can block LPA-induced Ras activation appears to support this straightforward interpretation. However, a more careful analysis of the literature shows that there is very considerable variation in the effects of these inhibitors on LPA-induced Ras activation which is dependent on cell type and, in some cases, investigator [8–12]. Some cells with fully functional EGF signalling to Ras fail to use this pathway when treated with LPA, suggesting that other mechanisms must exist that connect LPA to Ras.

What might these mechanisms be? Rubio et al. [4] have analysed LPA-induced Ras activation in greater depth than before by actually measuring the rate at which guanine nucleotides turn over on Ras, in addition to looking at the more commonly studied overall ratio of bound GTP to total nucleotide, the activation state. Measuring turnover rate requires permeabilization of the cells and addition of labelled GTP, then determining how much labelled nucleotide (both GTP and GDP) is bound to Ras at a given time point. To do this, Ras is immunoprecipitated from lysates of the permeabilized cells using an antibody that does not displace bound nucleotide.

Using these methods, Rubio et al. [4] observed that both LPA and EGF treatment of COS-7 cells results in increased turnover of nucleotide on Ras, as well as increased activation state. Whereas the ability of LPA to increase the GTP/(GTP + GDP) ratio on Ras is blocked by both PTX and EGFR inhibitors, a very different picture is seen when nucleotide turnover is measured. PTX blocks

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LPA-induced, but not basal, nucleotide turnover on Ras, whereas the EGFR inhibitor blocks the basal rate of exchange, but leaves an LPA-induced component untouched.

The clear implication of these findings is that there are two distinct components to the guanine-nucleotide-exchange on Ras in LPA-treated cells. One is an LPA-activated component that requires the activity of the LPA-receptor-coupled $G_{i/o}$ proteins, but not EGFR. If this branch of the signalling pathway is blocked by PTX, no Ras activation will result from LPA treatment. The other component is not activated by LPA, at least not in the relatively short time course of up to 6 min studied here, but has a basal activity that is dependent on EGFR function. The blocking of this branch leaves the LPA-induced branch unable to push the exchange rate high enough to accumulate Ras in the activated state.

This is a fine illustration of the dangers of how inhibitors can mislead one's interpretation of signalling pathways if one makes unjustified assumptions that the pathway in question is linear. In genetics it has long been appreciated that inhibition of a given outcome can result from blocking a parallel signalling pathway that feeds in at some point downstream, but molecular cell biologists have been very slow to take this on board. Of course, it is tempting to assume that signalling systems such as this one are linear, particularly when there is evidence at some level that LPA receptor activation can lead to EGFR activation. However, it is very likely that the time course of HB-EGF-release-mediated activation of EGFR after LPA treatment is too slow to account for the major rapid peak of Ras activation by LPA.

The importance of the EGFR-dependent basal nucleotide exchange rate may vary considerably between cell types. It could well be affected by the basal level of activity of the EGFR, which is relatively high in COS-7 cells. It is likely that this basal activity is mediated by the familiar Shc/Grb2/Sos system. The identity of the $G_{i/o}$ -controlled Ras nucleotide exchange system remains unclear, although previously $G\beta\gamma$ subunits have been reported to control a different exchange factor, Ras-GRF [13].

The study by Rubio et al. [4] does raise important questions about the many examples in the literature of pathways in which a critical role for transactivated EGFR has been invoked, i.e. in G-protein and other signalling pathways. Were these systems really displaying linear activation of EGFR that was responsible for

downstream signalling, or was it the basal activity of the EGFR that was critical and any subsequent increased receptor activity incidental? Some critical re-analysis may be overdue!

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