Q1.

a. G protein does not have the GEF activity. I think the binding of GTPγs to G protein is based on thermodynamic instability and exchange. Binding to the receptor, which acts as a guanyl nucleotide exchange factor to catalyze GDP release from Gα, will help the GDP to GTP exchange and accelerate the Gα- GTPγs formation. Ligand bound to the receptor will dramatically increase the GEF activity of the GPCR and promote the GTPγs binding.

b. GTPγs cannot be hydrolyzed by GTPase. GTPγs binds to the Gα, and forms a constitutive activated Gα- GTPγs complex. Usually, GTPγs in the reaction solution is more than enough, but the amount of Gα is restricted. When all the Gα in the solution form the complex, the value becomes max and the curve reaches a plateau. Without GPCR and ligand, the dissociation of heterotrimeric G-proteins and the formation of Gα- GTPγs are much slower, so in the limited time (30 min here) you cannot see the plateau.

c. The intrinsic GTPase activity of G protein α subunit, which allows GTP hydrolysis and subsequent effector release, is very small. The receptor has the GEF activity and the ligand binding will increase the GEF activity, both of which are helpful to GTP hydrolysis by accelerating the GTPase cycle. RGS protein, which has the GTPase activity and controls the G protein signaling, can help hydrolyze GTP. Therefore, G protein by itself hydrolyzes GTP slowest.

d. Adding the receptor and the ligand will help the GDP to GTP exchange and sequentially increase the GTP hydrolysis a little by accelerating the GTPase cycle. The activation of RGS's GAP activity needs the stimulation of agonist. Without the receptor and the ligand, RGS's GAP activity cannot be fully activated. Both treatments increase GTP hydrolysis by only a small amount.

e. When add both the receptor/ligand and RGS, the GTP hydrolysis will be accelerated, not only by accelerating the GTPase cycle from the receptor's GEF activity, but also by directly promoting from RGS's GAP activity.

f. The experiment in panel A uses GTPγs as substrate, which cannot be hydrolyzed and form a stable Gα- GTPγs complex. When the reaction uses up all the Gα in the solution, the curve reaches plateaus. The experiment in panel A measures the GTP binding of G protein with single time. The experiment in panel B use GTP as substrate, and reaction can go on and on, which means after the GTP hydrolyzed, the GDP- Gα will be recycled to form GTP- Gα by GEF activity then hydrolyzed again, until the reaction uses up all the GTP in the solution. Usually, GTP in the reaction solution is enough, so there is no plateau in the curve.

g. The activity of RGS is GTPase activity, which helps GTP hydrolysis and sequentially helps the GTP binding by accelerating the GTPase cycle. GTPγs cannot be hydrolyzed and the only method to enhance the GTPγs binding is the GEF activity. As RGS does not have the GEF activity, the addition of RGS has no effect on GTPγs binding.

Q2.

a. In general, ErbB1 and ErbB4 can be regulated by epiregulin (EPR). ErbB2, which is ligand independent and transactivated by heterodimerization, appears to have a special role in cell motility, as cells lack ErbB2 fail to migrate simply and ErbB2 overexpression potentiates tumor cell motility and invasion.

In total, I think ErbB1 and ErbB4 homodimers, ErbB1/ErbB2, ErbB4/ErbB2 and ErbB1/ErbB4 heterodimers can be regulated by EPR.

b. There are three methods below can be used to investigate my hypothesis.

1. We can use RNAi technique to knock down endogenous expression of specific member of EGFR family, and then add EPR into the media to check whether cell motility is changed or not (using wound closure assay etc.). If ErbB1 is regulated in EPR stimulated cell motility, knocking down ErbB1 will cause decrease in cell motility.

2. EGFR family has been studied for decades, and different mutants of EGFR have been identified. We can express constitutively activated EGFR, and check whether the cell motility increases or not. If ErbB2 is involved in this response, expression of constitutively activated ErbB2 will dramatically increase cell motility.

3. We can express mutant EGFR, which will function as dominant negative in vivo, to test whether the cell motility is influenced or not. EGFR family proteins have been studied for years, and there are many different types of mutant have been identified. I prefer to use "kinase dead" EGFR for dominant negative experiments. You can transfect the cells with dominant negative construct, and see whether the cell motility is changed or not. Or you can use in vitro would healing assay: after scratching cell monolayer to initiate cell migration, microinject the cells at the leading edge of either side of the wound with dominant negative constructs (adding some fluorescent label), trace these cell for a long time to see whether they can remain at the front of the migrating edge or not. Considering the possible damage from microinjection, if the ErbB1 is involved, expression of dominant negative ErbB1 construct will decrease the cell motility definitely.

c. I was illuminated by a paper and decided to use "function directed cell selection" to concentrated cells, which is more mobile under EPR stimulation, and then check the expression spectrum to find out which members of EGFR family are involved.

I will use a modified Boyden chamber, containing polycarbonate membrane; place it into the lower chamber containing media plus appropriate concentration of EPR. Add cells to the upper chamber; allow them to migrate to the underside of the chamber. Remove the "nonmigratory cells" (less mobile) by a cotton swab; collect the "migratory cells", which are attached to the bottom surface of the membrane, by trypsin treatment. Culture cells until enough and repeat the selection process. I will change the EPR concentration or duration for migration to accelerate the selection of EPR sensitive cells. Later, when get the cells that are highly sensitive to EPR stimulation, I will use immune blotting or RT-PCR to test the expression levels of members of EGFR family. Compare to normal cells and find out what EGFR is involved. You can also use gene chip or protein array to resolve the expression spectrum and do some systematic analysis about EPR induced response.

The expression enhanced EGFR members from this experiment will be responsible for EPR.

Q4.

Hypothesis: At the leading edge, integrin signaling promotes phosphorylation of FAK, which inhibits Rho activity and stimulates adhesion turnover and nascent adhesion formation; at the front part not the leading edge, high Rho activity stabilizes adhesion and help its maturation; at the rear, tractional force induces FAK phosphorylation and promotes adhesion disassembly. [Some notes are available at the end of this answer.]

Explanations to the results in 1992 paper:

1. About Fig.1 C/D and E/F, "pTyr is more prominent in the peripheral adhesions than the central ones." Phosphorylation of FAK, which inhibits Rho activity and accelerate the focal adhesion turnover, mainly happen in the peripheral.

2. About Fig.2/3/4, "attaching to the ECM induces elevated levels of pTyr." The ECM attachment induces cell spreading and focal adhesion formation, which is based on focal adhesion turnover and phosphorylation of FAK.

3. About the herbimycin A inhibition experiments. Suspension cells, which have no focal adhesions, treated with inhibitor, blocked the activation of FAK. While high Rho activity will stabilize the cytoskeleton and inhibit the turnover, which means no focal adhesion will form. Then the inhibitor is rinsed and tyrosine kinase activity will be recovered and the Rho activity will be partially inhibited by FAK phosphorylation, which will restore the focal adhesion formation.

Actually, in 1992 paper, they just studied the cell adhesion induced tyrosine phosphorylation (pTyr) and suggested that the pTyr was critical to the cytoskeletal assembly. They did not distinguish the mature focal adhesions from nascent ones. As the inhibition of focal adhesion turnover has the same phenomena as the promotion of adhesion formation, the data in 1992 paper also can be perfectly interpreted by the hypothesis that pTyr inhibits Rho and promotes focal adhesion turnover.

Experiments:

There are many evidences show that Rho family small GTPases and FAK function together in focal adhesion dynamics, but limited by the available technique none has give a vivid model. In my hypothesis, FAK and Rho are the key molecules in focal adhesion dynamics. I would like to use various biosensors to show the dynamics (including specific spatial and temporal distributions) of FAK and Rho in live cells, combined with focal adhesion dynamics (exhibiting by fluorescent adhesion components or some unique microscope techniques such as TIRF), to build the model.

A paper, published in 2000 by Klaus M Hahn, shown a biosensor to activated Rac based on FRET technique. I hear from a seminar that Rho biosensor is available too. How about the FAK biosensor? My thinking is that activated FAK has Y397 phosphorylated, which is recognized by SH2 domain of Src and stimulates downstream signaling. We can design the FAK biosensor based on Src's SH2 domain. Actually, in a 2004 *Nat Cell Bio* paper, Donna J. Webb *et al* use GFP and dsRFP tagged paxillin to show the turnover of focal adhesion sites, use a phosphorylation site-specific antibody to show the location of activated FAK, and make the conclusion that "FAK–Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly", which supports my model. They also tried expression of dominant negative or constitutively activated Rho, both of which decrease focal adhesion turnover. It is strange according to my model. I think that the total Rho level in cell is different from the

Rho status at the focal adhesion sites, and we need to have a closer look then make the conclusion. Usage of biosensors and advanced microscopy techniques will progress this study.

NOTES: There are many proteins will be phosphorylated during focal adhesion formation, such as paxillin and talin, which are substrates of the FAK-Src complex and function as adaptor molecules for signaling and structural proteins for adhesion. FAK and Src, which are tyrosine kinases, are required for efficient adhesion turnover at the cell front and mediate adhesion disassembly at the rear. To simplify the model, I will just talk about FAK here. Activation of FAK recruits a number of SH2-domian- and SH3-domain- containing proteins, which mediate to several downstream pathways and function in focal adhesion formation and disassembly. Some results implicate that ERK and MLCK are key targets of FAK signaling that promote adhesion turnover, other effectors might also contribute to this process. It is very convinced that α-actinin is present in stable adhesions that do not turnover, while paxillin is present in nascent focal adhesions and tyrosine phosphorylation of paxillin promotes its disassembly. However, intracellular calcium level is implicated in the disassembly of adhesions, both protein kinases and phosphatases (PTEN etc.) appear to be central to the regulation of adhesion turnover and stability.

Rho family small GTPases are pivotal regulators of actin dynamics: Rac stimulates lamellipodial extension; Cdc42 induces filopodia; Rho functions in stress fiber formation. Rho, which activity is low at the leading edge and high at the rear and side, is involved in focal adhesion formation; while localized activation of Rac and Cdc42 decreases Rho activity. Rho and Rac come into the center of actin cytoskeleton, and regulates focal adhesion. Although there are many other proteins such as PAK and CAS/Crk involved in focal adhesion, based on what I know I am sure that focal adhesion regulation is a complicated network and has many crosstalks. My model is too simple to exhibit the magnificent scene, and I know that there are many interesting things to be investigated too.

Q5.

a. As PI3K is required for FAK-promoted cell migration, I think PI3K is definitely required for chemotaxis in response to growth factors. It is possible that there are some redundant pathways exist, but if PI3K functions in chemotaxis, any perturbation to PI3K will influence the chemotactic response. Based on this criterion, I decide to use these approaches to study the problem.

1. PI3K inhibitor treatment. For example, treat cell with wortmannin (50nM~100nM) or LY294002 (25~75nM) for 30 min will inhibit almost all the PI3Ks activity, then analyze their chemotactic ability. If the response is inhibited, PI3K is involved. You can also use more specific inhibitors to study different PI3K family members' role in chemotaxis.

2. In vitro kinase assay. If PI3K is involved in chemotaxis, it should be activated and have kinase activity. You can use immunoprecipitate to get the PI3K from growth factor stimulated cells, and use in vitro system to analyze its ability to phosphorylate substrate. Compare to the activity of PI3K from control cells, you will have an idea whether PI3K is involved. It may not work when the recognition site for the antibody is the catalytic domain of PI3K.

3. Constitutively activate PI3K. If express constitutively activated mutant (mutant that can be constitutively phosphorylated in the absence of ligands) of PI3K in cell will generate some chemotaxis like morphology, my interpretation will be that PI3K is involved in chemotactic response.

4. Add products of PI3K to the media. If PI3K is involved in chemotaxis, it is very possible that the product generated by PI3K such as PtdIns(3,4,5)P_3 functions in the related response. After adding PIP_3 to the media (without the agonist), if you can get chemotactic phenomenon, it is very possible that PI3K functions in chemotaxis.

5. RNAi. Based on the sequence of PI3K, design siRNA to knockdown the expression level of some specific PI3K family member to see whether the chemotactic response is interfered or not.

b. Specifically block the interaction between EGFR-PI3K or FAK-PI3K is the approach I want to use. I know that FAK D395A mutant loses the ability to bind to PI3K but do not other FAK functions. We can express this mutant in cell to disrupt the interaction between FAK and PI3K. If there is a redundant between EGFR signaling and FAK signaling, the cells are still able to response to growth factors by EGFR signaling. Similarly, I will also try the EGFR mutant, which only loses the ability to bind PI3K.

I think we can also use small molecule EGFR tyrosine kinase inhibitor or FAK specific inhibitor to block one signaling pathway from the origin to see whether the chemotactic response changes or not. Dominant negative constructs of FAK or EGFR can be requisition too. I am not sure whether there is a competition between EGFR and FAK signaling to PI3K or not. Suppose there is a competition, then you can overexpress one signaling protein, and make the other signaling protein inhibit. By analyzing in vitro protein activity or activated protein distribution, you can get the answer for the question.