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Minireview

Signaling Networks—Do All Roads Lead to the Same Genes?

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A significant issue in signal transduction is whether individual biochemical pathways activated in the cytoplasm induce unique, overlapping, or redundant alterations in the pattern of expressed genes. In an article published in this issue of *Cell*, Fambrough et al. (1999) explore this question by using oligonucleotide arrays to assess RNA transcripts induced by the β receptor for platelet-derived growth factor (βPDGFR) in mouse fibroblasts.

Following activation by ligand binding, a receptor tyrosine kinase (RTK) such as the βPDGFR undergoes autophosphorylation at Tyr residues that bind cytoplasmic targets with phosphotyrosine (pTyr) recognition modules, notably SH2 domains (Pawson, 1995). These receptor-binding proteins can be enzymes such as phospholipase C (PLC)-γ1, adaptor proteins that physically link the receptor to an enzyme, for example Grb2, which recruits the Ras guanine nucleotide exchange factor Sos, latent transcription factors (STATs), scaffolding proteins like Shc, or negative regulators such as Cbl. Since each SH2-containing protein binds preferentially to a distinct phosphorylated motif, it is possible to engineer mutant receptors in which specific Tyr docking residues are replaced by Phe, thereby selectively uncoupling the receptor from a particular pathway. In this way, the importance of a signaling pathway for a given biological response can be evaluated, either in vivo or in cultured cells.

Experiments of this sort have shown that docking sites for SH2 proteins confer an element of biological specificity. Met, the receptor for hepatocyte growth factor, has two C-terminal autophosphorylation sites that engage multiple SH2 proteins. Substitution of these two Tyr residues in the mouse gives the same embryonic lethal phenotype as a null *Met* mutation. In contrast, mice with a substitution that only blocks Grb2-binding to Met survive to birth, but show a selective defect in development of the limb musculature (Maina et al., 1996). Similarly, mutation of the PLC-γ1 binding site on the fibroblast growth factor (FGF) receptor 1 results in altered *Hox* gene expression and homeotic transformations in the mouse (Partanen et al., 1998). A detailed analysis has been made for LET23, the epidermal growth factor (EGF) receptor homolog in *Caenorhabditis elegans*, which has six potential SH2-binding sites that contribute to signaling (Lesa and Sternberg, 1997). Worms expressing a mutant receptor lacking all six of these Tyr residues are severely impaired for viability, formation of the vulva, and fertility. However, restoration of a single Tyr at position 1257 of LET23 confers full fertility but does not rescue viability or vulval induction. Conversely the presence of any one of three Tyr sites (1276, 1289, or 1311) with Grb2-binding motifs is sufficient for viability and vulval differentiation, but not fertility. These and related data have indicated that the SH2-binding sites of LET23 have distinct functions in vivo, possibly because they associate with tissue-specific effectors and negative regulators, but can also exhibit a degree of functional redundancy.

Multiple Pathways from Receptors to the Nucleus

There are several pathways through which pTyr signals can be relayed to the nucleus (Figure 1). STAT proteins form dimers through mutual SH2-pTyr interactions and consequently translocate to the nucleus, where they bind the promoters of cytokine-inducible genes. This pathway is the most direct and potentially the most selective route to the regulation of gene expression, since the SH2-containing target is the transcription factor itself. Other signals wend a more tortuous path to the nucleus, providing more opportunity for cross-talk and branching through the actions of docking proteins, phospholipid kinases and phospholipases, small GTPases, and cascades of protein kinases that regulate MAP kinase family members such as Erk and JNK/SAPK. Such pathways can be described in linear terms. Thus, binding of a Grb2-Sos complex to a receptor leads to activation of the Ras GTPase, which in turn stimulates the Raf, Mek, and Erk protein kinases. Erk phosphorylates ternary complex factors (TCFs), which associate with serum response factor (SRF) and initiate transcription through the serum response element of genes such as c-*fos* (Price et al., 1995). Phosphatidylinositol 3′-kinase (PI3K) is activated by direct binding of its SH2-containing subunit to receptors and catalyzes the formation of PI-3,4,5-P_3, which engages the PH domains of downstream targets such as the serine/threonine-specific protein kinases PKB/AKT and PDK1. PKB, once activated, can potentially regulate multiple transcription factors, including c-Jun, and members of the Forkhead family such as FKHRL1 and AFX that are retained in the cytoplasm upon phosphorylation by PKB (Brunet et al., 1999; Kops et al., 1999). PI3K can also stimulate the Rac GTPase, which has been implicated in JNK/SAPK activation and thereby in the positive phosphorylation of c-Jun and ATF2. In addition, the SH2 domain protein PLC-γ1, by hydrolyzing PI-4,5-P_2 to IP_3 and diacylglycerol, can potentially influence calcium-sensitive transcription factors such as NFAT isoforms and stimulate protein kinase C (PKC) signaling. Even this greatly simplified picture reveals several distinct means through which RTK signaling may either increase or repress transcription.

However, cytoplasmic signaling proteins are increasingly seen to form networks of interactions rather than simple linear pathways (Pawson, 1995). This may be observed at almost every step of the signaling process. The βPDGFR, for example, can bind the Grb2 SH2 domain either directly or indirectly through Shc or Shp2

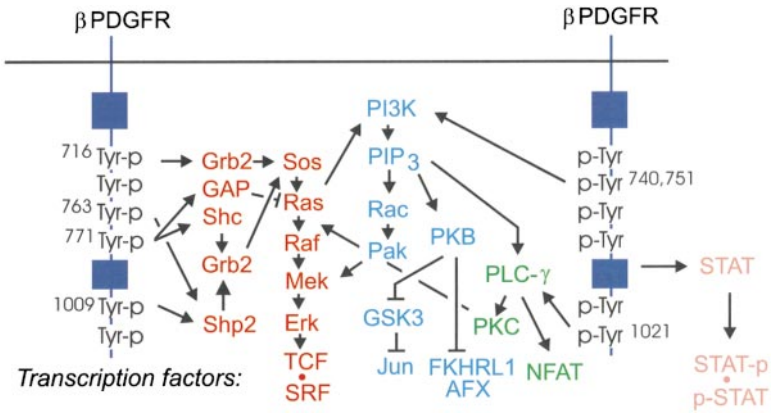

Figure 1. A Signaling Network from the Activated βPDGFR

The activated receptor is a dimer. Each receptor chain becomes phosphorylated on multiple sites, some of which are depicted here, and binds specific SH2-containing proteins. The receptor itself has redundant interactions, for example with Grb2. There are specific pathways leading from the receptor to the nucleus, each outlined in a different color. There are also numerous potential cross-connections between distinct pathways, some of which are shown. See the text for more detail. The figure is illustrative and by no means comprehensive; some binding partners and potential pathways, including Src family kinases, are not depicted.

(Heldin et al., 1998). There are also numerous cross-connections between signaling proteins more distal to the receptor. Ras can bind PI3K and potentiate its activation (Rodriguez-Viciana et al., 1994), while the Rac-dependent protein kinase PAK can phosphorylate Mek and thereby stabilize its association with Raf (Frost et al., 1997). PLC-γ1 has a PIP_3-sensitive PH domain (Falasca et al., 1998), and in some cells PKC can enhance the activation of Raf by Ras.GTP (Marais et al., 1998). As a consequence, the activation of distinct signaling pathways at the membrane may potentially converge on a related set of promoters.

Generic or Specific Transcriptional Responses to Receptor Signaling?

To explore the ability of different signaling pathways to modify gene expression, Fambrough et al. have selected a specific model system, namely the activation of immediate early genes (IEGs) by the βPGDFR in NIH 3T3 cells. IEGs represent direct nuclear targets of cytoplasmic signaling pathways, since their transcriptional response does not require novel protein synthesis. To circumvent endogenous βPDGFR, the authors have fused the extracellular region of the macrophage colony–stimulating factor (M-CSF) receptor to a cytoplasmic region containing either wild-type or mutant βPDGFR sequences. In mutant versions of this chimeric receptor, up to six Tyr residues that bind SH2-signaling proteins are altered, in combination, to Phe. The βPDGFR is remarkably well endowed with binding sites for SH2 proteins, including PLC-γ1, PI3K, Shp2, RasGAP (a negative regulator of the Ras GTPase), Src family tyrosine kinases, STAT5, and adaptors such as Grb2, Shc, Grb7, and Nck (Heldin et al., 1998; Rönnstrand et al., 1999). Mutants receptors with Tyr→Phe substitutions at five sites (5F; Tyr 740, 751, 771, 1009, and 1021), with an additional substitution at Tyr 716 (6F), or with single Tyr residues restored to the 5F mutant, were tested for their ability to induce IEG expression in NIH 3T3 cells. The novelty of the current work involves the use of oligonucleotide arrays to coordinately assess the response of 5,938 genes, representing between 5% and 10% of the total mouse genome.

DNA microarrays are a powerful tool for the comprehensive analysis of variations in RNA expression patterns (Brown and Botstein, 1999). In the approach employed by Fambrough et al., high density oligonucleotide arrays are probed with labeled cRNA pools from test or reference sources. The data therefore represent relative RNA message abundance based on a direct comparison of these two transcriptional states. This method gives the ability to globally assess gene expression and to identify sets of transcripts that respond in a similar fashion to a given stimulus or physiological state, and may therefore have a related function. Such experiments can yield unanticipated results, as shown by Iyer and colleagues who examined genes induced or repressed at various times after serum stimulation of primary fibroblasts using cDNA arrays (Iyer et al., 1999). Cluster analysis revealed the induction or repression of known IEGs, as well as genes for signaling proteins and cell cycle regulators. However, a large number of the induced genes proved to be involved in the physiology of wound repair, which in hindsight makes sense for a fibroblast exposed to serum.

In NIH 3T3 cells expressing the chimeric M-CSF/βPDGFR and stimulated with saturating amounts of M-CSF for up to 4 hr, Fambrough et al. initially classified 66 genes as IEGs based on an induction of approximately 3-fold by ligand. Reassuringly about half of these genes had previously been identified as IEGs. The question then is whether individual signaling pathways elicit unique subsets of IEGs, or stimulate a broad range of IEG expression. The answer seems to be that in NIH 3T3 cells distinct pathways can have surprisingly similar transcriptional outputs, but that some genes respond to specific signals. Of the 66 IEGs, 64 were induced by the 5F mutant receptor, which lacks the principal binding sites for PLC-γ1, Shp2, RasGAP and PI3K, to about half the level shown by the wild-type receptor; only *KC* and *chop-10* did not respond to M-CSF. Ablation of an additional SH2-docking site implicated in Grb2 binding in the 6F mutant caused a further reduction averaging 1.5-fold among 37 genes analyzed, but did not entirely eliminate IEG induction by ligand. The residual signaling of this 6F mutant may potentially be explained by the fact that even this somewhat threadbare receptor retains several pTyr/SH2-binding sites (Heldin et al., 1998). In addition, the kinase domain of the βPDGFR alone may transmit a signal by phosphorylating SH2-docking proteins, as shown for a truncated EGF receptor that retains the ability to phosphorylate Shc and transform cells (Soler et al., 1994).

In general terms, these results suggest that the distinct biochemical pathways activated by the receptor

can ultimately have related outputs at the level of IEG expression, and that each pathway has a quantitative rather than qualitative effect on transcriptional regulation. This is consistent with previous work from the Kazlauskas laboratory suggesting functional redundancy in mitogenic signaling by the βPDGFR, at least through the PI3K and PLC-γ1 pathways (Valius and Kazlauskas, 1993). The authors also show that FGF and PDGF induce a similar IEG response in NIH 3T3 cells, again suggesting that distinct signals can elicit a rather generic transcriptional output. However, the data also provide evidence for specificity. A 5F mutant receptor to which the Tyr-1021 PLC-γ1 binding site had been restored induced the expression of *KC* and *chop-10*, suggesting that these genes may be relatively specific targets of PLC-γ1 signaling. More strikingly, a 5F mutant with a restored Tyr-771 binding site for RasGAP induced a novel series of genes that are normally activated by interferon-γ, which signals through the STAT1 transcription factor. It is possible that recruitment of RasGAP to an impaired receptor unmasks STAT1 activity by attenuating a Ras signal that otherwise leads to inhibitory phosphorylation of STAT1 on serine. While the physiological relevance of this finding in the context of the βPDGFR is uncertain, it makes the point that an individual pTyr-initiated signal can induce a specific, as opposed to a global, transcriptional response.

There are several caveats to these experiments, as the authors themselves point out. One reservation is that the experiments were all performed at saturating concentrations of ligand, which is unlikely to be physiological and may emphasize the propensity of signaling pathways to cross-activate. For example, PI3K is activated by Ras only at higher concentrations of growth factor. Thus, it will be important to know whether specific biochemical pathways induce distinct subsets of IEGs when cells are stimulated with lower concentrations of ligand. Sensibly, the authors have simplified a complex task by focusing on IEGs that show significantly elevated expression. Obviously this does not take into account transcriptional repression, nor subsequent waves of gene induction. Further, although the authors have sampled a prodigious number of genes, it will be important to know what fraction of the remaining 90%–95% of promoters show pathway-specific responses. Another issue is the degree to which the data obtained for NIH 3T3 cells, which are highly adapted to life on plastic, can be generalized to other cells. Even more important will be to determine the roles of distinct signaling pathways activated by the βPDGFR (and the related αPDGF receptor) in vivo, by introducing the relevant Tyr→Phe mutations into the mouse germline, allowing the investigation of the nuances of gene expression in mice with such mutant receptors.

Signaling Proteins Are Organized into Networks

These cautions aside, the current data fit an emerging notion that a limited number of signaling proteins interact in a combinatorial fashion to build intracellular networks that allow diverse cellular responses. Thus, it is critical to understand how biological specificity can be generated through rather general intracellular signals. Cells with different histories, and therefore expressing different repertoires of transcription factors and coactivators, may form distinct complexes of preexisting and newly activated transcription factors, which target different promoters. The same signal may therefore induce distinct transcriptional readouts in different cells. It is also possible that within any one cell the same signal, or group of signals, may have different effects depending on the strength with which it is delivered. This may apply for signaling through the T cell antigen receptor (TCR). The nonpolymorphic signaling subunits of the TCR (the ζ and CD3 chains) contain an aggregate of 12 closely related tyrosine phosphorylation sites that act in pairs to engage the tandem SH2 domains of the ZAP-70 tyrosine kinase (Kersh et al., 1998). When an MHC/antigen complex engages the TCR, it induces a signal that can have markedly different outcomes on T cell development. For example, a cell with a TCR that binds with high affinity to self-antigen will be negatively selected, while cells with TCRs that bind antigen with moderate affinity will proliferate and undergo further development. Thus, quantitative differences in TCR signaling can potentially translate into qualitatively distinct biological reponses.

A possible advantage of a signaling network with multiple intersecting pathways is to direct a coherent response to numerous, potentially conflicting signals. In vivo a single cell will be exposed to multiple stimuli in the form of soluble hormones, cell surface proteins on adjacent cells, and extracellular matrix components. These different signals may act synergistically to enhance an internal pathway, as shown for growth factors and fibronectin in MAP kinase signaling, or may antagonize one another as found for interferon-γ, which inhibits TGFβ signaling by inducing expression of the inhibitory Smad7 (Ulloa et al., 1999). With the focus here on IEG expression, it is also important to consider that pathways leading to transcriptional regulation are also essential for other cellular responses (Heldin et al., 1998). As an example, PI3K regulation also plays an essential role in reorganization of the actin cytoskeleton and directed migration in response to PDGF. Furthermore, by controling signaling to p70 S6 kinase and 4EBP proteins, which together regulate protein synthesis, the PI3K pathway organizes cell growth. Through PKB, PI3K can also induce phosphorylation of the proapoptotic protein Bad in some cells, and thereby enhance survival. Thus, the same network of signaling proteins downstream of receptors must coordinate numerous cellular functions. Perhaps for this reason, eukaryotic cells have evolved scaffolding proteins that can simultaneously bind multiple components of a signaling pathway and thereby impose a degree of specificity and order on the highly interactive network of signaling proteins.

Subtle Differences in Gene Expression Can Have Important Biological Consequences

The virtues of signaling networks aside, minor differences in gene expression can potentially result in dramatically different developmental programs. To illustrate, the expression of single "selector genes," such as *Ubx* or *Tbx5*, distinguishes whether a wing or haltere will develop from a *Drosophila* imaginal disc or whether a forelimb or hindlimb will outgrow from the chick body wall (Weatherbee and Carroll, 1999). Elegant examples from *Drosophila* show that subtle differences in gene expression induced by a RTK can influence biological

output. The Torso receptor signals through the *Drosophila* Grb2 ortholog and the Ras pathway to induce the expression of tailless (*tll*) and huckebein (*hkb*), which specify terminal cell fate. The recruitment of Csw (the ortholog of the tyrosine phosphatase Shp2) to Torso mediates the transmission of receptor signals, as demonstrated by a Tyr→Phe substitution in Torso, which blocks this association and yields a loss-of-function phenotype. In contrast, mutation of the Torso-binding site for RasGAP gives a gain-of-function phenotype, as might be expected if RasGAP inhibits Ras signaling. An important effect of these competing regulators is to define the spatial boundaries of *tll/hkb* expression. Interestingly, substitution of the RasGAP-binding site restores signaling to a Torso mutant that cannot bind Csw, apparently because a major substrate for the Csw phosphatase is the pTyr motif that recruits RasGAP (Cleghon et al., 1998). However, these compensatory signals are still required for proper refinement of the Torso signal, as survival to adulthood is markedly reduced in flies expressing a Torso mutant lacking both Csw and RasGAP-binding sites.

Oligonucleotide and cDNA arrays are clearly changing the ways we think about cell biology. The paper by Fambrough et al. reveals both the power of this approach, and also our ignorance about cellular function. It will be fascinating in the future to extend the gene chip technology to a more comprehensive analysis of growth factor signaling. This approach may also be useful in resolving the role of other PDGFR binding partners, such as Src family kinases, in regulating the transcriptional response to PDGF. It may be particularly revealing to use inhibitors to proteins more proximal to the IEG promoters, such as components of the MAP kinase cassettes, to tease out whether there is increased specificity as one gets further down the signaling pathways. Regardless, Pandora's box has now been opened.

Selected Reading

Brown, P.O., and Botstein, D. (1999). Nat. Genet. *21*, 33–37.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blennis, J., and Greenberg, M.E. (1999). Cell *96*, 857–868.

Cleghon, V., Feldmann, P., Ghiglione, C., Copeland, T.D., Perrimon, N., Hughes, D.A., and Morrison, D.K. (1998). Mol. Cell *2*, 719–727.

Falasca, M., Logan, S.K., Lehto, V.P., Baccante, G., Lemmon, M.A., and Schlessinger, J. (1998). EMBO J. *17*, 414–422.

Fambrough, D., McClure, K., Kazlauskas, A., and Lander, E.S. (1999). Cell *97*, this issue, 727–741.

Frost, J.A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P.E., and Cobb, M.H. (1997). EMBO J. *16*, 6426–6438.

Heldin, C.-H., Ostman, A., and Ronnstrand, L. (1998). BBA Cancer Rev. *1378*, F79-F113.

Iyer, V.R., Eisen, M.B., Ross, D.T., Schuler, G., Moore, T., Lee, J.C.F., Trent, J.M., Staudt, L.M., Hudson, J., Jr., Boguski, M.S., et al. (1999). Science *283*, 83–87.

Kersh, E.N., Shaw, E.S., and Allen, P.M. (1998). Science *281*, 572–575.

Kops, G.J.P.L., de Ruiter, N.D., De Vries-Smith, A.M.M., Powell, D.R., Bos, J.L., and Burgering, B.M. (1999). Nature *398*, 630–634.

Lesa, M., and Sternberg, P.W. (1997). Mol. Biol. Cell. *8*, 779–793.

Maina, F., Casagranda, F., Audero, E., Simeone, A., Comoglio, P.M., Klein, R., and Ponzetto, C. (1996). Cell *87*, 531–542.

Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M.F., and Marshall, C.J. (1998). Science *280*, 109–112.

Partanen, J., Schwartz, L., and Rossant, J. (1998). Genes Dev. *12*, 2332–2344.

Pawson, T. (1995). Nature *373*, 573–580.

Price, M.A., Rogers, A.E., and Treisman, R. (1995). EMBO J. *14*, 2589–2601.

Rodriguez-Viciana, P., Warne, P.H., Dhand, R., Vanhaesbroek, B., Gout, I., Fry, M.J., Waterfield, M.D., and Downward, J. (1994). Nature *370*, 527–532.

Rönnstrand, L., Arvidsson, A.-K., Kallin, A., Rorsman, C., Hellman, U., Engström, U., Wernstedt, C., and Heldin, C.-H. (1999). Oncogene, in press.

Soler, C., Alvarez, C.V., Beguinot, L., and Carpenter, G. (1994). Oncogene *9*, 2207–2215.

Ulloa, L., Doody, J., and Massague, J. (1999). Nature *397*, 710–713.

Valius, M., and Kazlauskas, A. (1993). Cell *73*, 321–334.

Weatherbee, S.D., and Carroll, S.B. (1999). Cell *97*, 283–286.