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# Dynamic Visualization of Expressed Gene Networks

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Cellular biochemical machineries, what we call pathways, consist of dynamically assembling and disassembling macromolecular complexes. While our models for the organization of biochemical machines are derived largely from in vitro experiments, do they reflect their organization in living cells? We have developed a general experimental strategy that addresses this question by allowing the quantitative probing of molecular interactions in intact living cells. The experimental strategy is based on protein fragment complementation assays (PCA), a method whereby protein interactions are coupled to refolding of enzymes from cognate fragments where reconstitution of enzyme activity acts as the detector of a protein interaction. A biochemical machine or pathway is defined by grouping interacting proteins into those that are perturbed in the same way by common factors (hormones, metabolites, enzyme inhibitors, etc). In this review, we describe how we go from descriptive to quantitative representations of biochemical networks at an individual to whole genome level and how our approach will lead ultimately to better descriptions of the biochemical machineries that underlie living processes.
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Technology and scale are themes that define all things "omic" and the emerging offspring of the genomics revolution variously called proteomics, functional genomics, or systems biology can be attributed an overall aim: as only a fraction of gene functions can be inferred from primary gene sequences, we need to develop strategies to define gene function that are not conducted at the level of a classical gene-by-gene approach, but that aim at characterizing the totality of genes or large subsets thereof. The question then is: by what approaches do we meaningfully ascribe function to genes and more so, address the problems that genomics has traditionally sought to address, such as establishing common and unique traits to determine phylogenic and evolutionary relationships among organisms? In the broadest and most ambitious sense, those of us working at the frontiers beyond the analysis of DNA sequence data hope that our efforts will result in a deeper appreciation of the biochemical organization of living cells and the molecular schemes that all living things share as well as those things that make individual cells and organisms unique. In this review, we describe a general strategy that goes directly to the heart of this problem: physically mapping biochemical pathways in living cells.

## LARGE-SCALE EXPRESSION CLONING PROBLEM

Before a discussion of pathway mapping, we have to face the fact that we really do not know the function of most genes in any genome and so we must answer an essential question: what does it mean to ascribe function to genes products and how can this be done on a large scale? Although many proteins have been identified by functional cloning of novel genes, such "expression cloning" remains a significant experimental challenge. Many ingenious strategies have been devised to simultaneously screen cDNA libraries in the context of assays that allow both selection of clones and validation of their biological relevance (Aruffo and Seed, 1987; D'Andrea et al., 1989; Lin et al., 1992; Sako et al., 1993). However, in the absence of an obvious functional assay that can be combined with cDNA library screening, researchers have turned to strategies that use as readout some general functional properties of proteins. A powerful experimental approach to obtain clues about gene function would entail both the ability to establish how proteins and other biological molecules interact in living cells and simultaneously, the ability to validate the biological relevance of the interactions using the same assay system. A first step in defining the function of a novel gene is to determine its interactions with other gene products; that is, since proteins make specific interactions with other proteins as part of functional assemblies, an appropriate way to examine the function of the product of a novel gene is to determine its physical relationships with the products of other genes. This is the basis of the highly successful yeast two-hybrid system, which has been demonstrated to be effective in genome-wide screening for interacting proteins (Drees, 1999; Vidal and Legrain, 1999; Uetz et al., 2000;

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Walhout et al., 2000). The central problem with two-hybrid screening is that detection of protein–protein interactions occurs in a fixed context, the nucleus of *Saccharomyces cerevisiae* and the results of a screening must be validated as biologically relevant using other assays in appropriate cell, tissue, or organism models. While this would be true for any screening strategy, it would be advantageous if one could combine cDNA or defined array library screening with tests for biological relevance into a single strategy, thus tentatively validating a detected protein as biologically relevant and eliminating false-positive interactions immediately. This is both an intellectual and technical challenge all life scientists are facing. This is a challenge that we took up some years ago and our solution is described in the next section.

## PROTEIN FRAGMENT COMPLEMENTATION STRATEGIES FOR LARGE-SCALE EXPRESSION CLONING

It would be advantageous if one could combine screening of protein–protein interactions with tests for biological relevance into a single strategy, thus validating detected protein interactions as biologically relevant and eliminating spurious interactions immediately. It was with this goal in mind that we developed a strategy called protein fragment complementation assays (PCA) to detect protein–protein interactions in living cells. The first assay, we have developed is based on protein interaction-induced folding and reassembly of the enzyme *murine* dihydrofolate reductase (DHFR) (Pelletier et al., 1998; Remy and Michnick, 1999; Remy et al., 1999). The gene for DHFR is rationally dissected into two fragments called F[1,2] and F[3]. Any two proteins that are thought to bind to each other are fused to either of the two DHFR fragments. Folding and reassembly of DHFR from its fragments is induced by the binding of the test proteins to each other and is detected as reconstitution of enzyme activity. Reconstitution of enzyme activity can be monitored in vivo by cell survival in the absence of nucleotides or by fluorescence detection of fluorescein-conjugated methotrexate (fMTX) binding to reconstituted DHFR (F[1,2] + F[3]) (Fig. 1) (Remy and Michnick, 1999). The DHFR PCA allows for rapid detection of interactions between full-length proteins, even at very low expression levels, to measure in vivo the effects of specific stimuli and inhibitors on particular interactions and to determine the physical location of the interacting partners in the cell (Remy and Michnick, 2001). Further, protein interactions can be studied in

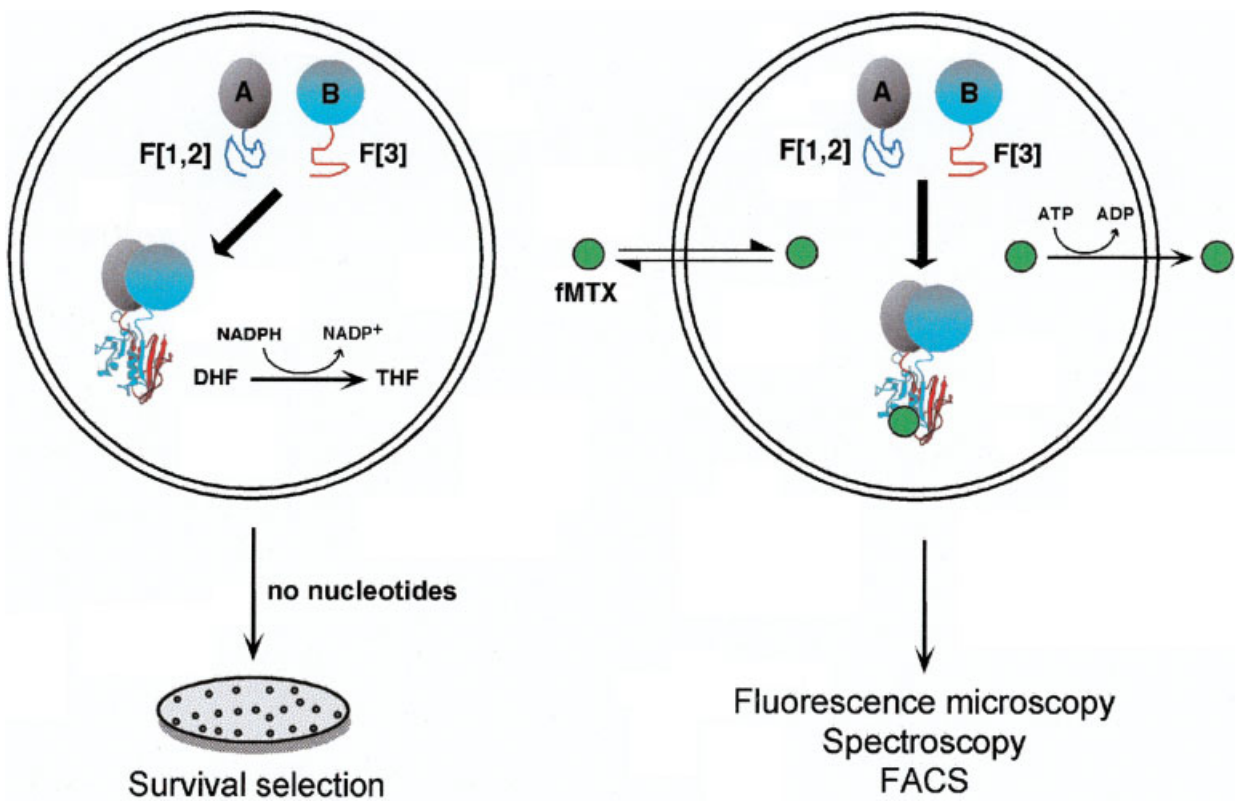


Fig. 1. Schematic representation of the strategy used to study protein–protein interactions in mammalian cells with the dihydrofolate reductase (DHFR) protein fragment complementation assays (PCA). Left part: Interacting proteins A and B are fused to the complementary fragments of murine DHFR (F[1,2] and F[3]) to generate A–F[1,2] and B–F[3] fusions. A physical interaction between proteins A and B drives the reconstitution of DHFR from its fragments (F[1,2] + F[3]), allowing DHFR-negative cells expressing these constructs to grow in media lacking nucleotides. DHFR-positive cells can also be used in a recessive selection strategy (see text). Right part: The fluorescence assay is based on high affinity binding of the specific DHFR inhibitor fluorescein-methotrexate (fMTX) to reconstituted DHFR. fMTX passively crosses the cell membrane and binds to reconstituted DHFR (F[1,2] + F[3]) and is thus retained in the cell. Unbound fMTX is rapidly released from the cells by active transport. Detection of bound and retained fMTX can then be detected by fluorescence microscopy, FACS or fluorescence spectroscopy.

the specific compartment of the cell where they function and in the context of other proteins that participate in biochemical pathways and networks.

In addition to the specific capabilities of PCA described above, are special features of this approach that make it appropriate for genomic screening of molecular interactions, including: (1) PCAs are not a single assay but a series of assays; an assay can be chosen because it works in a specific cell type appropriate for studying interactions of some class of proteins; (2) PCAs are inexpensive, requiring no specialized reagents beyond those necessary for a particular assay and off the shelf materials and technology; (3) PCAs can be automated and high-throughput screening could be done; (4) PCAs are designed at the level of the atomic structure of the enzymes used; because of this, there is additional flexibility in designing the probe fragments to control the sensitivity and stringencies of the assays; (5) PCAs can be based on enzymes for which the detection of protein–protein interactions can be determined differently including by dominant selection or production of a fluorescent or colored product. We have already developed five PCAs based on dominant-selection, colorimetric, or fluorescent outputs (Remy et al., 2001). Here, we will discuss the most well developed PCA, based on the enzyme murine dihydrofolate reductase (mDHFR).

## "MAPPING" BIOCHEMICAL PATHWAYS

The advent of DNA microarray technologies has changed the manner in which we view biochemical pathways (Chu et al., 1998; Holstege et al., 1998; Spellman et al., 1998; Hughes et al., 2000a; Roberts et al., 2000; Ideker et al., 2001). The practical monitoring of changes in expression of complete genomes or large subsets of genes has allowed researchers to begin to scrutinize in some detail the evolution of genetic programs and sometimes, by inference, the underlying biochemical pathways that control these programs. The most well described analyses of gene expression have been performed for the bakers yeast *S. cerevisiae*, for which the entire genome has been sequenced and microarrays representing all predicted genes have been available for some time (Lashkari et al., 1997). Complete analyses of the cell cycle and responses of the organism to a number of perturbations have been explored (Chu et al., 1998; Holstege et al., 1998; Spellman et al., 1998; Hardwick et al., 1999; Hughes et al., 2000a,b; Roberts et al., 2000; Ideker et al., 2001). In combination with other data and a complete set of knockouts, it is becoming possible to conceive of mapping the output of a genetic program (gene expression changes) back to the organization of the biochemical pathways underlying these changes (Chu et al., 1998; Holstege et al., 1998; Hardwick et al., 1999; Roberts et al., 2000; Causton et al., 2001; Ideker et al., 2001). A particularly important recent effort was described by (Ideker et al., 2001), in an attempt to define a comprehensive strategy to map the biochemical response of yeast to changing the primary carbon source from glucose to galactose. The strategy consisted of four steps: (1) generate a comprehensive model of the underlying biochemical pathways involved based on literature with addition available data on protein–protein interactions from large scale two-hybrid screening studies; (2) perturb each pathway component through a series of genetic gene deletions or over expressions or environmental (e.g., changes in growth conditions or temperature) manipulations; (3) detect and quantify the corresponding global cellular response to each perturbation with technologies for large-scale mRNA and protein-expression measurement; (4) integrate the observed mRNA and protein responses with the current pathway specific model and with the global network of protein–protein, protein–DNA, and other known physical interactions; (5) formulate new hypotheses to explain the incorporation of new data. They performed an analysis of gene expression at the mRNA and protein levels in responses to: (i) switch from glucose to galactose utilization and (ii) deletion in yeasts strains for individual genes in the GAL pathway. In this way, they were able to propose modifications to existing models for the GAL pathway and identify some novel proteins as being implicated in the GAL response.

The "global" integrated approach to mapping pathways described above does not help us to side-step a problem which should be obvious to any engineering undergraduate, let alone an experienced biologist: if you do not know the details of the underlying machinery leading to specific outputs of a system, then any new insights from analyzing outputs remain inferences that must still be tested directly. The bottleneck to understanding biochemical networks is not opened by examining more and more complex and detailed outputs without going to the heart of what created the outputs in the first place. Recently, we have proposed a way to address this problem head-on and performed a proof-of-principle study, demonstrating that a PCA-based analysis of biochemical networks not only affords an approach to mapping biochemical pathways but also reveals detail of such pathways that are not obvious (Remy and Michnick, 2001). Further such analyses are performed in living cells in which the pathways under study are probed.

## GETTING TO THE HEART OF BIOCHEMICAL MACHINERY

As we pointed out above, protein–protein interactions can be used as a basic readout for linking a protein of unknown function to proteins that are known to be involved in a known biochemical pathway. In this way, as Roger Brent has described it, a protein function can be inferred, to a first approximation, as "guilt by association." The genius of yeast two-hybrid screens is this fact. The studies, we describe below both remind us of a basic problem in biology as well as provide us with a powerful new tool to explore this problem. Biochemical processes are mediated by non-covalently associated multienzyme complexes (Reed, 1974). Cellular machineries for transcription, translation, and metabolic or signal transduction pathways are examples of processes mediated by multiprotein complexes. The formation of multiprotein complexes produce the most efficient chemical machinery, in which the substrates and products of a series of steps are transferred from one active site to another over minimal distance, with minimal diffusional loss of intermediates, and in chemical environments suited to stabilizing reactive intermediates. Further, physical coupling of enzymes can allow for allosteric regulation of different steps in a chain of reactions (Perham, 1975).

Much of modern biological research is concerned with identifying proteins involved in cellular processes, determining their functions and how, when, and where they interact with other proteins involved in specific pathways. For instance, signal transduction "pathways" in eukaryotes have been shown to in fact consist of both constitutive and transient macro-complexes organized by modular protein domains (Pawson and Nash, 2000). Bluntly worded: biochemical "pathways" are networks of dynamically assembling and disassembling protein complexes and therefore, a meaningful representation of a biochemical pathway in a living cell would be a step-by-step analysis of the dynamics of individual protein–protein interactions in response to perturbations that impinge upon the pathway under study and the time and spatial distribution of these interactions.

Thus a strategy for genome-wide mapping of biochemical pathways using PCA would entail first, a screening step; a simple assay to detect protein–protein interactions among potential partner proteins, followed by the generation of a functional validation profile (Fig. 2A). Such a profile would consist of two types of data. First, a biochemical network of interest should be perturbed by specific stimuli or inhibitors, for example, hormones, drugs, or nutrients. Consequently, interactions between component proteins of the pathway should be perturbed by these reagents and a pattern of responses or "pharmacological profile" observed by PCA should be consistent with the response of the pathway or network under study. We have previously demonstrated that we could measure the direct induction of protein–protein interactions (Remy and Michnick, 1999; Remy et al., 1999) and the perturbations of protein interactions by drugs or hormones acting at steps remote from the interactions studied (Remy and Michnick, 2001). Second, interactions of protein components of a network should take place in specific subcellular compartments or locations consistent with the function of the pathway. The combined pharmacological profiles and subcellular interaction patterns would then form the basis of a functional validation profile to be used to annotate novel gene products and to describe the biochemical pathway or network.

## PROOF OF PRINCIPLE: MAPPING A BIOCHEMICAL NETWORK THAT CONTROLS TRANSLATION-INITIATION IN MAMMALIAN CELLS

To validate the PCA strategy for genome-wide functional annotation, a clear application of the approach to mapping a known biochemical network needed to be chosen. Signal transduction pathways have proven useful models for examining biochemical processes on a genome-wide scale. For example, gene microarray expression analyses have been used to study cellular responses to general stimuli, pharmacological responses to drugs acting on convergent targets and to testing hypotheses concerning the hierarchical organization of

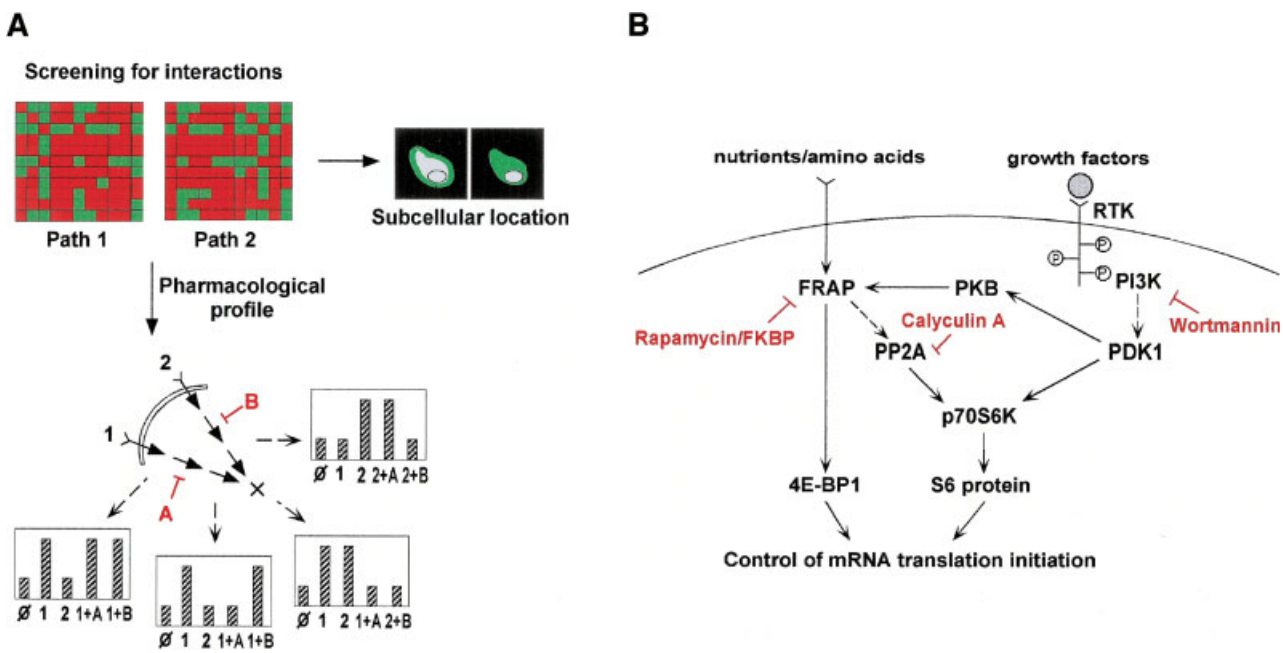


Fig. 2. **A**: Schematic representation of the strategy for generating a functional validation profile of a biochemical network using the DHFR PCA. Positive clones are detected with the DHFR survival-selection assay. They correspond to interacting component proteins of two convergent signal transduction pathways (Path 1 and Path 2). An interaction matrix (upper left) represents all positive (green) and negative (red) interacting pairs observed in the survival-selection assay. Positive clones from survival selection are propagated and subjected to two functional analyses: (1) using the DHFR fluorescence assay, interactions are probed with pathway specific stimulators (1 and 2) and inhibitors (A and B). Pharmacological profiles are established based on the pattern of response of individual interactions to stimulators and inhibitors, represented in the histograms (ordinate axis represent fluorescence intensity). For example, stimulation of pathway 1 will augment all the interactions composing that pathway. The inhibitor A will inhibit protein interactions downstream, but not upstream of its site of action in pathway 1. (2) Cellular locations of the interactions are determined by fluorescence microscopy, also using the DHFR fluorescence assay. **B**: Well established connections within RTK (growth factor activated) and FRAP mediated pathways that control translation-initiation and sites of action of inhibitors of these pathways. Broken lines indicate that actions are indirect.

signal transduction networks (Marton et al., 1998; Fambrough et al., 1999; Iyer et al., 1999). For these reasons, we chose to apply the PCA strategy to two convergent signal transduction pathways involved in insulin, growth factor, and amino acid-activated control of translation-initiation (Fig. 2B). The two pathways have been implicated in activation of the 70 kDa S6 ribosomal protein serine/threonine kinase (p70S6K) and phosphorylation of eIF-4E binding protein (4EBP1, also known as PHASI), events involved in the control of the initiation of protein synthesis. The first pathway implicates phosphatidylinositol-3-kinases (PI3K), enzymes activated by insulin and many growth factor receptor tyrosine kinases (RTK). PI3K phosphorylates phosphatidyl inositol (4,5) diphosphate ($PIP_2$) to produce phosphatidyl inositol (3,4,5) triphosphate ($PIP_3$). $PIP_3$ acts as a receptor for pleckstrin homology (PH) domains of a number of protein kinases, directing their localization to the plasma membrane. Inhibitors of PI3Ks, including wortmannin and LY294002, prevent insulin and growth factor mediated phosphorylation of p70S6K. The immunosupressent drug rapamycin also causes dephosphorylation of p70S6K, independent of wortmannin, defining a second pathway. Rapamycin binds to a soluble receptor called FKBP12 and the FKBP12–rapamycin complex binds to the serine/threonine kinase FRAP (FKBP12–rapamycin associating protein) also known as mTOR (mammalian target of rapamycin) or Raft1 (Brown et al., 1994; Sabatini et al., 1994). It has been suggested that the action of FRAP on p70S6K is mediated indirectly, through the serine/threonine phosphatase PP2A (Di Como and Arndt, 1996; Jiang and Broach, 1999; Peterson et al., 1999).

The strategy for mapping the RTK/FRAP signaling network can be summarized as follows (Fig. 2A). First, we used the survival assay based on DHFR PCA to determine which protein–protein interaction occur and to select positive clones for further studies. The principle of the survival assay is that cells simultaneously expressing complementary fragments of DHFR fused to interacting proteins or peptides will survive in media depleted of nucleotides, only if the proteins interact and then bring the complementary fragments of DHFR into proximity where they can fold and reassemble into active enzyme (Remy and Michnick, 1999). It is obviously more convenient to use a DHFR-negative cell line and therefore, perform a dominant selection for DHFR activity. However, DHFR-positive cell lines can be used in a recessive selection strategy. The DHFR used in our studies contains a methotrexate resistance mutation that nonetheless is capable of binding fluorescein-methotrexate sufficiently to perform fluorescence assays. However, DHFR-positive cells grown in the presence of methotrexate will only survive if complemented with the DHFR PCA (unpublished data). The survival selection assay is extremely sensitive, we have previously demonstrated that only 25–100 molecules of reconstituted DHFR per cell are necessary for cell survival (Remy and Michnick, 1999). We performed essential controls, including tests for orientation specificity and for interactions that were not probable, to establish whether false positives are observed. First, except in specific cases, we tested the same interactions with fusions of the test proteins at either the N or C terminus of DHFR fragments. We tested these variants because, not knowing the structures of these proteins, we would not be able to predict the optimal orientation of the protein fusions to bring the complementary DHFR fragments into proximity to fold/reassemble. In addition to that, we inserted a flexible linker peptide of 10 amino acids between the test protein and the DHFR fragment in the fusion, allowing us to probe interactions across distances of 80 Å ($\approx 4$ Å per peptide bond $\times$ 10 aa $\times$ 2 linkers: 1 per fusion). Second, as an additional control, we tested interacting pairs of proteins in which the DHFR fragments were swapped. We reasoned that an observed interaction should occur regardless of which fragment either of the proteins was attached to. Finally, we reasoned that interactions among kinases and their substrates could occur exclusively through the catalytic site of the kinase and then be too transient to be detected by PCA. Thus in some cases we also tested "kinase dead" mutants that are thought to bind with higher affinity to their substrates. However, we observed interactions with both wild type and kinase-dead forms. To functionally validate the protein–protein interactions identified in the survival screen, pharmacological profiles were established using the in vivo quantitative fluorescence assay based on DHFR PCA (detection of fMTX binding to reconstituted DHFR), to assess the responses of the various protein–protein interactions to insulin or serum stimulation and pathway-specific inhibitors (wortmannin and rapamycin) (Remy and Michnick, 2001). Insulin- and serum-activated signal transduction pathways have been studied in detail in CHO cells (Mamounas et al., 1989; Ruderman et al., 1990; Mamounas et al., 1991) and analysis of the p70S6K pathway is well documented (for review, see Proud and Denton, 1997; Avruch, 1998). These studies have largely been performed in cell lines in which the insulin receptor is overexpressed (CHO-IR cells, Mamounas et al., 1989). However, we found in preliminary studies that insulin/serum induction and drug inhibition of protein–protein interactions could be easily detected by the DHFR fluorescence assay, without overexpression of insulin receptors, and that the degree of induction of protein interactions were consistent with increases in interactions or enzyme activity over background observed previously in in vitro and in vivo studies (Alessi et al., 1998). Finally, we performed fluorescence microscopy to establish the physical locations of individual interactions within cells.

A total of 148 combinations of 35 different protein–protein interactions in the RTK/FRAP signal transduction pathways were tested against each other (Fig. 3). In all cases, full-length proteins were used. Of the 35 interactions tested, 14 corresponded to interacting partners of which five have not been previously reported. No false positive interactions were observed among the protein pairs tested, based on pharmacological responses and cellular location analysis described above. Four distinct types of pharmacological profiles and two physical locations of interacting proteins were immediately evident (Fig. 4). These patterns reflect a hierarchical organization of the RTK/FRAP pathways with RTK pathway components sensitive to wortmannin and FRAP pathway components sensitive to rapamycin, and protein pairs involved in early steps are exclusively

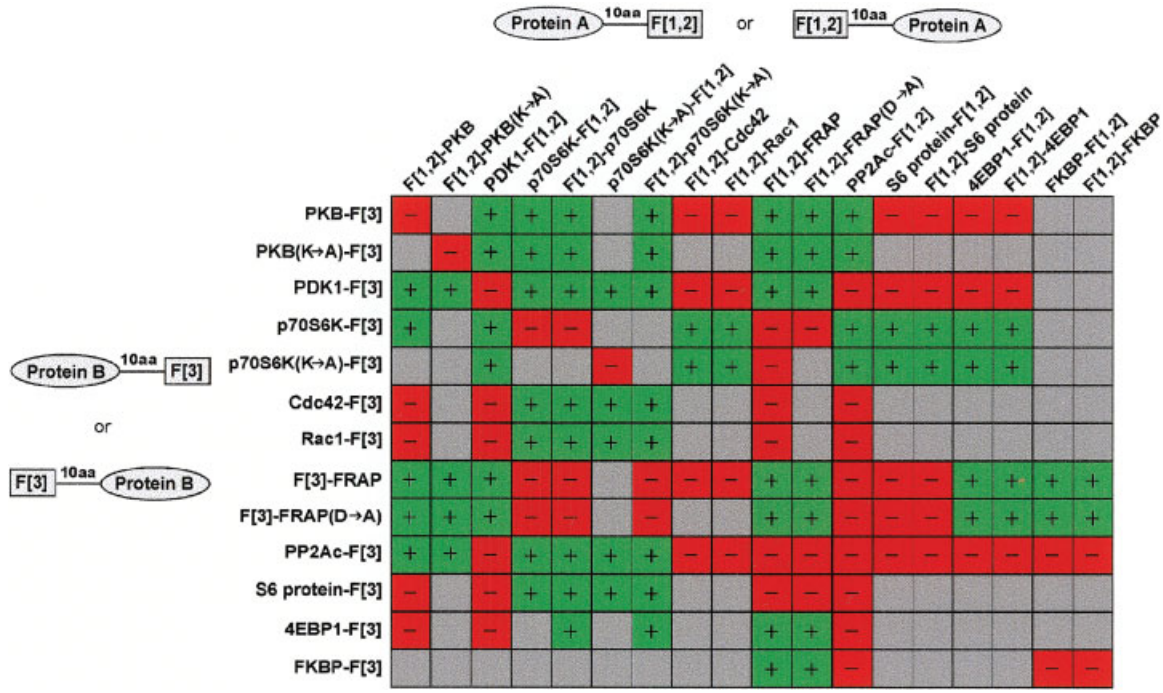


Fig. 3. Summary of the results obtained for the different protein–protein interactions tested in the RTK/FRAP network with the DHFR survival selection assay in CHO DUKX-B11 ($DHFR^-$) cells. On the X-axis are the fusions to DHFR[1,2] fragment and on the Y-axis the fusions to DHFR[3] fragment. The orientations of the fusions (N-terminal or C-terminal) are also indicated. Positive interactions: green (+), absence of interaction: red (−), not tested: gray squares.

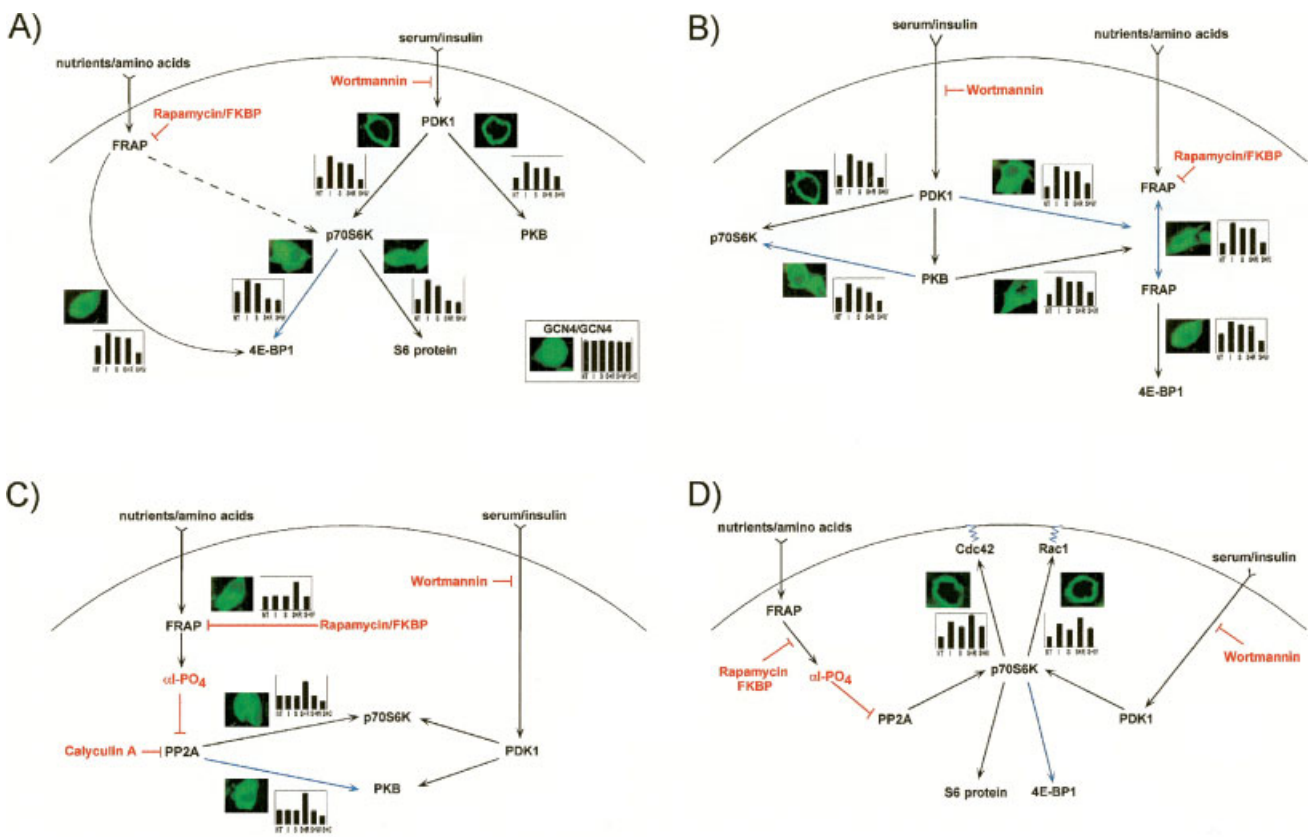


Fig. 4.

at the plasma membrane (such as PDK1/PKB and PDK1/p70S6K) while later occurring interactions are in the cytosol. Below we describe the individual pathways, but as will become immediately evident, even studying a limited number of genes a number of new and intriguing relationships were revealed.

## MAPPING THE RTK PATHWAY

The membrane to cytosol hierarchical organization of the RTK pathway can be clearly demonstrated by the fluorescence DHFR PCA (Fig. 4A). Near the top of this hierarchy, we observed a direct interaction between PDK1 and PKB. PDK1 has been identified as a specific PKB kinase (for review see Belham et al., 1999; Vanhaesebroeck and Alessi, 2000). PKB is activated, in part, by phosphorylation by PDK1 on Thr 308 in the kinase domain activation loop and perhaps also in another crucial C-terminal site, Ser 473 (PDK2 activity). It has been proposed that membrane localization of both enzymes is required for PKB phosphorylation by PDK1, via binding to $PIP_3$ through PH domains. We showed that the interaction between PDK1 and PKB occurs exclusively at the plasma membrane and is inhibited by wortmannin (Fig. 4A), which inhibits PI3K and thus prevents the synthesis of $PIP_3$. The association of PKB with $PIP_3$ then appears to be an obligatory step in PKB activation.

The kinase p70S6K, like PKB, is a member of the AGC class of protein serine/threonine kinases and both have homologous crucial phosphorylation sites in the activation loop and C-terminus. It was demonstrated that PDK1 phosphorylated this homologous site in p70S6K (Alessi et al., 1998; Pullen et al., 1998). We observed that the cellular location and the pattern of stimuli/inhibitor-responses of the PDK1/p70S6K interaction were identical to those of the PDK1/PKB interaction (Fig. 4A). Surprisingly, a novel direct interaction between PKB and p70S6K showed the same pattern of stimulus/inhibitor-response but with a cytosolic distribution (Fig. 4B). This interaction has been suspected but never demonstrated before and PKB has not been shown to act as a p70S6K kinase in vitro (Dufner et al., 1999).

## PATHWAY CONVERGENCE

The only step of the wortmannin/rapamycin-sensitive pathways that is inhibited by both drugs is the endpoint downstream interaction of p70S6K/S6 protein (Fig. 4A). This is an example of pathway convergence, represented by an "X" in Figure 2A. However, we also observed a novel interaction between p70S6K and 4EBP1, which has the same pharmacological profile and occurs in the cytosol as does p70S6K/S6 interaction. There is no evidence that 4EBP1 is a substrate of p70S6K in vitro (Diggle et al., 1996). However, it is possible that in vivo there is an obligatory first step, perhaps phosphorylation at another site on 4EBP1 that is necessary prior to phosphorylation by p70S6K. 4EBP1 has been shown to be phosphorylated on multiple residues in vivo and some of these sites are sensitive to rapamycin (Diggle et al., 1996; Fadden et al., 1997; Gingras et al., 1998; Heesom et al., 1998). Further, it has been shown that rapamycin can augment the activity of PP2A against 4EBP1, and that PP2A directly interacts with and dephosphorylate p70S6K (Peterson et al., 1999). As discussed below, we showed that the PP2A/p70S6K interaction occurs but we did not observe an interaction between PP2A and 4EBP1, nor has this interaction been reported in the literature. We propose, therefore, that the inhibitory effect of PP2A on 4EBP1 could occur via dephosphorylation of p70S6K.

## RTK TO FRAP PATHWAY CROSS-TALK

One thing that has become obvious from both direct analysis of signaling pathways and transcriptional output from such pathways is that they are far more ramified than previously imagined (Fambrough et al., 1999). Our analysis of the RTK and FRAP pathways dramatically illustrates this, with suspected as well as completely novel evidence of cross-talk. The first clue was our observation that the interaction between FRAP and 4EBP1 was wortmannin sensitive, but rapamycin resistant (Fig. 4A). Previous studies have shown that FRAP can directly phosphorylate 4EBP1 in vitro (Brunn et al., 1997; Burnett et al., 1998; Gingras et al., 1999). What is surprising with this picture is that the profile we observed would put FRAP downstream from the RTK pathway as opposed to being part of a completely parallel path. How could this happen? We observed a direct interaction between PKB and FRAP, but also a novel interaction between PDK1 and FRAP (Fig. 4B). Both are inducible by serum and insulin, inhibited by wortmannin, but are rapamycin insensitive. Direct phosphorylation of FRAP by PKB on Ser2448 in vitro has been reported (Nave et al., 1999). Because we have shown that the interactions between FRAP and 4EBP1, and between PDK1 and PKB with FRAP, are sensitive to

Fig. 4. Fluorometric and microscopic analysis of the interacting protein pairs fused to the complementary fragments of DHFR. The pharmacological profiles are represented by the histograms. Cells were treated with stimulants and inhibitors as indicated (X-axis: NT, no treatment; I, insulin; S, serum; R, rapamycin; W, wortmannin; C, calyculin A). Fluorescence intensity is given in relative fluorescence units (Y-axis). The background fluorescence intensity corresponding to non-transfected cells was subtracted from the fluorescence intensities of all the samples. Error bars represent standard errors for the mean calculated from at least three independent experiments. Fluorescence microscopy images revealing patterns of cellular location are also presented. The constitutive dimerization of GCN4 leucine zipper (GCN4/GCN4) is used as a control. Blue arrows indicate new protein–protein interactions. **A**: PDK1/PKB and PDK1/p70S6K interactions occur at the plasma membrane, FRAP/4E-BP1, p70S6K/4E-BP1, and p70S6K/S6 protein interactions are cytosolic. Pharmacological profiles for the first three interactions are consistent with rapamycin resistant, wortmannin sensitive pathways. The serum/insulin-stimulated and wortmannin/rapamycin-inhibited profiles of the p70S6K/4EBP1 and p70S6K/S6 interactions place them at a convergent point downstream of both wortmannin- and rapamycin-sensitive pathways. **B**: Analysis of pharmacological profiles reveals novel ramifications of wortmannin and rapamycin-sensitive pathways including serum/insulin stimulated and wortmanin-sensitive association of FRAP, placing FRAP as a downstream target of PDK1 and PKB. **C**: Regulation of p70S6K and PKB by FRAP (through PP2A) and PDK1. αI-PO4 represents a regulatory subunit of the phosphatase PP2A, regulated via its phosphorylation by FRAP. The FRAP/FKBP, PP2A/p70S6K, and PP2A/PKB interactions are serum/insulin-insensitive but rapamycin-induced. The interactions between PP2A/PKB and PP2A/p70S6K are also inhibited by the PP2A phosphatase inhibitor calyculin A. All of these interactions occur in the cytosol. **D**: Positive/negative regulation of p70S6K in the RTK/FRAP network. The serum/insulin/rapamycin-induced interactions of p70S6K/Cdc42 and p70S6K/Rac1 occur at the plasma membrane, suggesting that p70S6K is recruited at the membrane via the two GTPases.

wortmannin but not to rapamycin, we suggest a direct role of PDK1 and/or PKB in regulating the function of FRAP. We also observed insulin- and serum-induced homodimerization of FRAP, consistent with evidence that FRAP autophosphorylates (Peterson et al., 2000), and this is blocked by wortmannin but not by rapamycin (Fig. 4B). Induction of FRAP homodimerization may also, therefore, depend on its phosphorylation by PDK1 and/or PKB.

## MAPPING THE FRAP PATHWAY

The precise role of FRAP in mediating p70S6K and 4EBP1 phosphorylation and how rapamycin/FKBP12 modulates these effects has been the subject of considerable revision recently. In vivo studies have demonstrated that FRAP kinase activity is insensitive to rapamycin (Peterson et al., 2000). Evidence from genetic and biochemical studies in yeast and mammalian cells, suggests that FRAP actions are mediated indirectly through the general serine/threonine phosphatase PP2A (Di Como and Arndt, 1996; Murata et al., 1997; Chen et al., 1998; Jiang and Broach, 1999). We observed a rapamycin-induced cytosolic interaction between FKBP and FRAP (Fig. 4C), as previously demonstrated (Sabatini et al., 1994; Lorenz and Heitman, 1995). We did not observe a direct interaction between FRAP and full-length p70S6K, supporting the argument that FRAP actions on this enzyme are indirect. In contrast, we were able to clearly demonstrate a rapamycin-induced PP2A/p70S6K complex (Fig. 4C). Further, the complex was inhibited by the PP2A-specific inhibitor calyculin A, suggesting that the interaction occurs, at least in part, between the catalytic site of PP2A and substrate sites on p70S6K. We also observed a rapamycin-induced and calyculin A-sensitive direct interaction between PP2A and PKB (Fig. 4C). A direct interaction between these two proteins has not been previously demonstrated in vitro or in vivo, but indirect in vitro evidence suggests that PKB is a substrate of PP2A (Andjelkovic et al., 1996). The pattern of stimulatory and inhibitory responses and cytosolic location of PP2A/PKB were identical to those for PP2A/p70S6K, suggesting similar mechanisms of induced interaction of PP2A/PKB and PP2A/p70S6K. The fact that rapamycin induces this interaction provides evidence of a negative feedback circuit to PKB, via FRAP activation of PP2A. These results present a paradox, as rapamycin would be predicted to inhibit PKB in a similar manner to wortmannin. In a few cases such inhibition has been observed (Halse et al., 1999; Li et al., 1999). Further, rapamycin has been shown to induce apoptosis in some cancer cell lines, possibly via this mechanism (Muthukkumar et al., 1995; Shi et al., 1995; Hosoi et al., 1999). However, in most cells, it is likely that there is a compensatory mechanism by which PKB is rephosphorylated and reactivated.

## EXAMINING MECHANISTIC PARADOXES

In Figure 4A, we show that p70S6K interacts with PDK1 at the cellular membrane; no particular surprise as the most well known substrate of PDK1, PKB, also interact at the membrane. However, how does p70S6K get to the membrane? Both PDK1 and PKB contain PH domains that interact with phosphoinositides; p70S6K does not have a PH, nor any other recognized membrane localization domain. Candidate membrane anchoring proteins for p70S6K have been suggested to be the Rho family GTPases Rac1 and Cdc42 (Chou and Blenis, 1996). We examined interactions of p70S6K with both Cdc42 and Rac1. We were able to detect both interactions, inducible by serum and insulin stimulation, and show that these interactions occur at the plasma membrane (Fig. 4D). The pharmacological profiles were identical for both interactions; rapamycin enhanced serum-induced association whereas wortmannin had no effect. Our results can be interpreted in the same way as for rapamycin effects on the p70S6K/PP2A interaction. In the presence of rapamycin, PP2A is activated, resulting in an increase in the quantity of hypophosphorylated p70S6K. Since Rac1 and Cdc42 only interact with this form (Chou and Blenis, 1996), we see an enhancement. Wortmannin has no effect on Rac1/Cdc42/p70S6K interaction. As PI3K likely plays a role in the activation of Rac1 and Cdc42 (Bishop and Hall, 2000), this result could be interpreted as contradictory. However, because inhibition of PI3K also prevents membrane translocation of PDK1, an increase in the quantity of hypophosphorylated p70S6K and therefore, an increase in the number of Rac1/Cdc42/p70S6K complexes would be predicted. Thus, in vivo, a potential decrease in available activated Rac1 or Cdc42 may be compensated for by an increase in available deactivated p70S6K.

## SUMMARY AND PERSPECTIVES

The results presented here demonstrate that the PCA strategy has the features necessary for a general gene function annotation strategy. Further, such analysis is not limited to a specific cell type; we have already demonstrated the utility of PCA strategies in bacteria and mammalian cells and more recently in plant cells (Pelletier et al., 1998; Remy and Michnick, 1999, 2001; Remy et al., 1999; Subramaniam et al., 2001). We have demonstrated that pharmacological perturbations of interactions can be observed, even if the site of action of the perturbant is distant from the interaction being studied. The pharmacological profiles and subcellular locations of interactions, we observed allowed us to "place" each gene product at its relevant point in the pathways. It should also be noted that the direct probing of biochemical networks in living cells has not been achieved on this scale by any other approach. Further, while specific inhibitors such as those used in this study may not be available for other pathways, other perturbants could be used to generate a functional profile, including dominant-negative forms of enzymes, receptor- or enzyme-specific peptides or antisense RNA. The ability to monitor the network in living cells containing all of the components of the network studied revealed hidden connections, not observed before, inspite of intense scrutiny of this network. From the results of our analysis, a map of the organization of the RTK/FRAP network emerges. Figure 5 summarizes the results. Two activation–deactivation cycles can be defined for PKB and p70S6K, in which the dephosphorylated/deactivated kinases are localized to the plasma membrane; PKB via its N-terminal PH domain to PIP3 and p70S6K, via association to activated Rac1 and Cdc42. At the

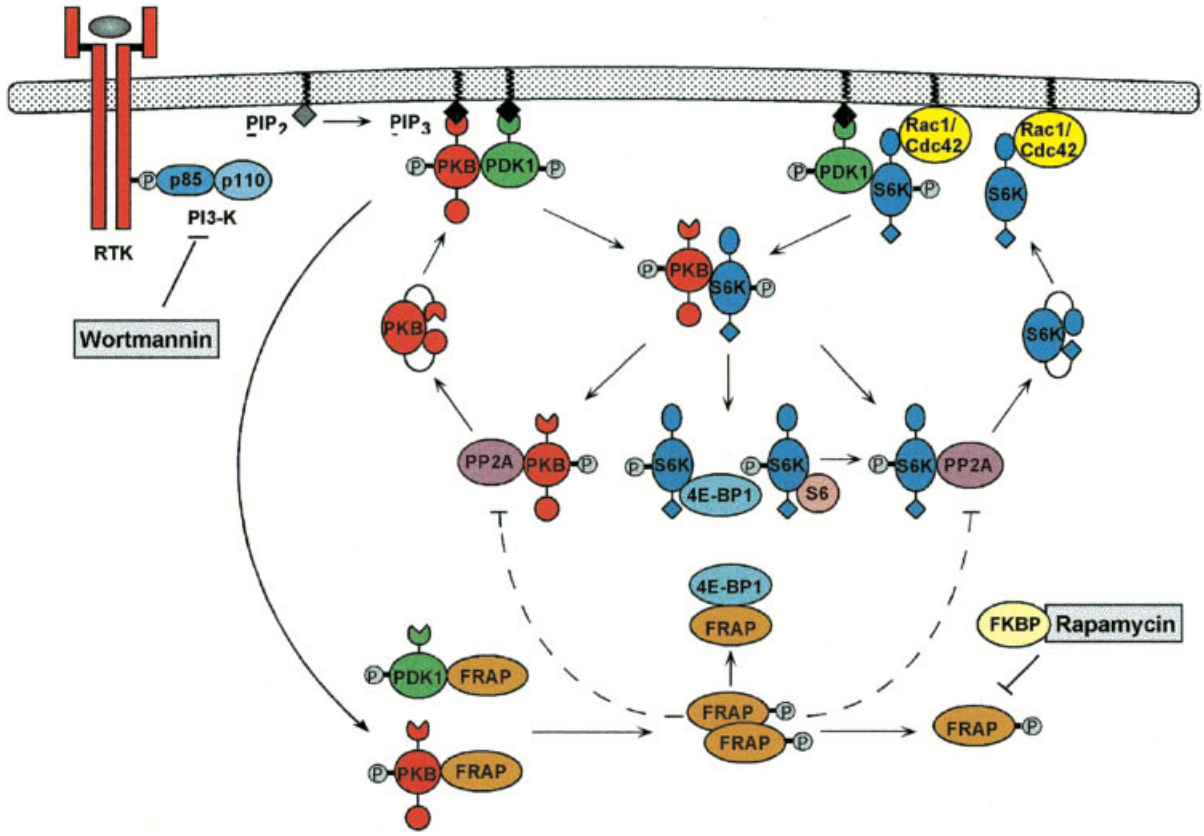


Fig. 5. Proposed model for the RTK/FRAP signaling network. Regulation of translation of mRNA is controlled by FRAP and p70S6K, leading to the phosphorylation of 4EBP1 and the ribosomal protein S6. Growth factor-mediated PI3K activation results in the production of the lipid second messenger $PIP_3$, which stimulates the translocation of PKB to the plasma membrane through its PH domain. This translocation displaces the inhibitory PH domain of PKB, rendering the phosphorylation sites accessible for phosphorylation by PDK1, which is also anchored to the membrane via a PH domain. p70S6K is recruited to the plasma membrane through the GTPases Rac1/Cdc42 and is then phosphorylated by PDK1. We have also shown that PKB interacts with p70S6K. The phosphorylated form of p70S6K is released in the cytosol where it can interact with its substrates S6 protein and 4EBP1. The activated PDK1 and PKB translocate to the cytosol to phosphorylate FRAP, inducing its homodimerization. FRAP phosphorylates 4EBP1 in the cytosol. The phosphatase PP2A inactivates p70S6K and PKB by dephosphorylation. These last interactions are stimulated by rapamycin and are proposed to be regulated via FRAP. The hypophosphorylated form of p70S6K is then recruited at the plasma membrane by Rac1/Cdc42 completing a cycle of stimulation/activation/deactivation and finally, re-recruitment to the membrane.

membrane, both kinases are phosphorylated and activated by PDK1. These early events were shown to occur unambiguously at the plasma membrane, whereas downstream target interactions all occurred in the cytosol. We suggest that FRAP is a point of integration for growth factor-mediated pathways. FRAP is modulated by the RTK pathway via its direct interactions with PKB and PDK1 but likewise, FRAP feeds back on both p70S6K and PKB by modulating the activity of PP2A.

Functional mapping of biochemical networks by PCA would be complementary to other approaches for genome-wide probing of cell function. For example, as noted above, recent and dramatic evidence of highly ramified integration of signal transduction pathways has been suggested by studies on the induction of early genes (IEG) by activation of parallel pathways emanating from a receptor tyrosine kinase (Fambrough et al., 1999). The results of these studies suggested that IEGs are activated in a concerted way by networks of interconnected pathways. However, while these results strongly support the idea that pathways are highly ramified, they do not provide direct evidence of the organization of signaling networks. A pair-wise analysis of all known interactions in ramified signaling pathways as performed here would provide the essential evidence. Finally, an effort to standardize functional annotation of known or emerging genomes is underway. The organization of information in this "Gene Ontology" database is based on a vocabulary describing biological processes, molecular function and cellular component ontologies (Ashburner et al., 2000; Rubin et al., 2000). The data derived from PCA detection of interactions among members of a biochemical network, pharmacological profiles, and subcellular locations can be directly translated into the language of gene ontologies.

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