Review

A Random Walk in Oncogene Space: The Quest for Targets

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Introduction

Oncogenes code for components of cellular growth-regulatory signals (1, 2). Such signals typically start at the cell surface, traverse the cytoplasm, and then terminate in the nucleus. They are propagated by protein-protein interactions between modular recognition domains and controlled by conformational changes and protein phosphorylation. The oncogenic potential of these growth-regulatory signals is linked to a mutational gain of function in one of the signaling components. While genetic analyses have identified the specific biochemical activities of these mutated signal components, structural studies have assigned these activities to distinct protein domains.

As components of signaling pathways, oncogenes are bound to interact with each other. Conceptually, these interactions define a genetic space with hierarchical order that reflects the inherent directionality of signaling: each upstream mediator feeds its signal to downstream targets. Exploring oncogene space is essentially a quest for targets.

The first part of this review will explore the oncogenic determinants of a common cytoplasmic signaling pathway. The results show that whereas a growth regulator can be oncogenic in one cell type, it can enhance differentiation in another.

The second part of this review will focus on nuclear effects of growth signals. Signaling networks are branched trees with cross-talk between different components that ultimately regulate a transcription factor. The transcription factor then converts the signal into a program of gene expression that affects the specific phenotype of the cell. Oncogenic signals corrupt normal programs of gene regulation, and, as a result, the patterns of gene expression in the neoplastic cell differ from those of its normal progenitor. Genes that are differentially expressed in the cancer cell control the malignant properties of that cell. An understanding of the neoplastic cellular phenotype requires the identification of these differentially expressed genes that are targets of oncogenes and of tumor suppressor genes. The ultimate goal of the search for targets is to isolate a few genes that, when differentially expressed, will transform a normal cell into a cancer cell. This is an attainable goal if the number of these oncogenic effector genes is small. The aim becomes unrealistic if the number of genes required to induce and maintain the neoplastic phenotype is large.

Oncogenic Versions of PI 3-Kinase6 and of the Serine-Threonine Kinase Akt (Protein Kinase B) Are Membrane-bound and Constitutively Active

The recently cloned genome of avian sarcoma virus 16 contains a cell-derived oncogene, p3k, that is homologous to the gene encoding the catalytic subunit p110α of PI 3-kinase (3). The viral oncoprotein carries several mutations, including an NH2-terminal deletion, and is fused to sequences that code viral structural (Gag) proteins. This viral form of p110α is highly oncogenic, whereas overexpression of the cellular gene coding for the catalytic subunit of PI 3-kinase does not transform cells. Rare transformation events induced by the cellular gene result from activating mutations in that gene.7

A downstream target of PI 3-kinase is the serine-threonine kinase Akt, also known as protein kinase B (4–6). Akt is activated by two phosphatidylinositol-3-phosphate-dependent kinases, PDK1 and PDK2, which phosphorylate Akt on threonine 308 and serine 473 (7–9). The activation is facilitated by an NH2-terminal pleckstrin homology domain that directs Akt to cellular membranes, the sites of PDK1 and PDK2 activity (10–12). Like P3k, a viral form of Akt functions as an oncogene, whereas overexpression of cellular Akt fails to induce transformation (12, 13). Therefore, one or several of the genetic changes suffered by P3k or Akt during their sojourn in a retrovirus must be responsible for activating a latent oncogenic potential.

To identify the functions and domains that activate and are required for oncogenicity, mutational analyses of P3k and of Akt were carried out. The results for the two kinases are

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6 The abbreviations used are: PI 3-kinase, phosphoinositol 3-kinase; PDK, phosphoinositol-dependent kinase; LEF, lymphocyte-enhancing factor; TCF, T-cell factor; TAF, transactivation function; ER, estrogen receptor; AP-1, activator protein 1; HB-EGF, heparin-binding epidermal growth factor-like growth factor; GSK-3β, glycogen synthase kinase 3β.

remarkably similar. The cellular P3k protein can be made oncogenic by adding a membrane anchor: a myristylation signal, a farnesylation signal, or fusion to Gag sequences that have an affinity for cellular membranes. Oncogenic versions of P3k do not need to interact with the regulatory subunit p85 of PI 3-kinase. All these transforming mutants of P3k have acquired NH2-terminal Gag sequences that can direct the protein to the plasma membrane. Membrane localization mediated by a myristylation signal or a farnesylation signal also activates the oncogenic potential of P3k. A 72-amino acid NH2-terminal deletion that prevents binding of myristylated P3k to regulatory subunit p85 of PI 3-kinase also inactivates the oncogenic potential, despite a functional membrane address. A point mutation in the kinase domain has the same effect.

Wild-type P3k does not induce oncogenic transformation; however, during replication in the retroviral vector RCAS, it mutates and becomes oncogenic. All these transforming mutants of P3k have acquired NH2-terminal Gag sequences that can direct the protein to the plasma membrane. Membrane localization mediated by a myristylation signal or a farnesylation signal also activates the oncogenic potential of P3k. A 72-amino acid NH2-terminal deletion that prevents binding of myristylated P3k to regulatory subunit p85 of PI 3-kinase also inactivates the oncogenic potential, despite a functional membrane address. A point mutation in the kinase domain has the same effect. B, oncogenic transformation induced by Akt (protein kinase B). Wild-type cellular Akt is marginally transforming; membrane localization mediated by retroviral Gag sequences, myristylation signal, or a gain of function mutation in the pleckstrin-homology domain (PH domain) activates the transforming potential. Inactivation of kinase activity, either by mutating PDK1 and PDK2 phosphorylation sites or by a loss of function mutation in the kinase domain, abolishes transforming potential.

**Fig. 1.** A, Oncogenic transformation induced by the retroviral oncogene v-p3k. v-p3k codes for a protein, v-P3k, that is homologous to the catalytic subunit p110 of PI 3-kinase, here labeled c-P3k. v-P3k differs from c-P3k by an NH2-terminal 13-amino acid deletion, fusion to viral Gag sequences, and several point mutations. Wild-type c-P3k does not induce oncogenic transformation; however, during replication in the retroviral vector RCAS, it mutates and becomes oncogenic. All these transforming mutants of c-P3k have acquired NH2-terminal Gag sequences that can direct the protein to the plasma membrane. Membrane localization mediated by a myristylation signal or a farnesylation signal also activates the oncogenic potential of c-P3k. A 72-amino acid NH2-terminal deletion that prevents binding of myristylated P3k to regulatory subunit p85 of PI 3-kinase also inactivates the oncogenic potential, despite a functional membrane address. A point mutation in the kinase domain has the same effect. **B**, oncogenic transformation induced by Akt (protein kinase B). Wild-type cellular Akt is marginally transforming; membrane localization mediated by retroviral Gag sequences, myristylation signal, or a gain of function mutation in the pleckstrin-homology domain (PH domain) activates the transforming potential. Inactivation of kinase activity, either by mutating PDK1 and PDK2 phosphorylation sites or by a loss of function mutation in the kinase domain, abolishes transforming potential.
The connection between PI 3-kinase and Akt is evident in the correlation between transforming activity of P3k mutants and phosphorylation of Akt. All oncogenic mutants of P3k efficiently induce phosphorylation of Akt. The nononcogenic mutants of P3k do not cause Akt phosphorylation and therefore fail to activate the Akt kinase. Constitutively active P3k induces PDK1- and PDK2-dependent phosphorylation of Akt and thus activates Akt. Akt appears to function as an essential downstream effector of P3k-induced transformation. A transdominant mutant of Akt can induce resistance to transformation by P3k. Both constitutively active Akt and constitutively active P3k induce hemangiosarcomas in young chickens. The two kinases, P3k and Akt, are components of the same signaling pathway, and a gain of function in either one can result in oncogenic transformation.

The Same Signal Produces Opposite Responses in Different Cell Types

As a general rule, oncogenes interfere with terminal differentiation. They keep cells dividing and inhibit the expression of tissue-specific proteins. A differentiation system that has been extensively studied with diverse oncogenes is myogenesis in cultures of chicken embryo myoblasts. Over the course of a few days, these potential muscle cells exit the cell cycle, fuse into myotubes, and turn on muscle-specific proteins. The myotubes may even show spontaneous contractions. Expression of some oncogenes such as src, myc, or jun interferes with this differentiation process (14–22). Surprisingly, the oncogenic forms of P3k and of Akt strongly stimulate myogenic differentiation (Refs. 23 and 24; Fig. 2). They stimulate the fusion of myoblasts into nondividing, multinucleated myotubes and elevate expression of the muscle-specific proteins desmin, myosin heavy chain, creatine kinase, and MyoD. Constitutively active PI 3-kinase and Akt kinase not only stimulate muscle differentiation, but they are also essential for myogenesis. Transdominant negative mutants of PI 3-kinase and of Akt inhibit spontaneous muscle differentiation in myoblast cultures. An inhibitor of PI 3-kinase, LY294002, also interferes with myogenesis in a dose-dependent fashion. The connectedness of PI 3-kinase and Akt is again revealed by the fact that the inhibition of muscle differentiation by the PI 3-kinase inhibitor LY294002 can be abrogated by constitutively active Akt. In muscle differentiation, as in oncogenesis, Akt functions as a downstream mediator of the PI 3-kinase signal.

A gain of function in the PI 3-kinase and Akt kinase pathway elicits opposite responses in the two cell types, fibroblasts and myoblasts. In fibroblasts, it induces oncogenic transformation characterized by enhanced cell proliferation and anchorage-independent growth. In myoblasts, constitutively active PI 3-kinase and Akt induce terminal differentiation.

PI 3-kinase and Akt are essential for myogenesis and may be rate limiting components of myogenic signals. An increase in their activity would then be expected to stimulate muscle differentiation. The oncogenic consequences of constitutively active PI 3-kinase and Akt are less readily explained. They suggest that the target spectra of PI 3-kinase in myoblasts and fibroblasts are not identical. It will be interesting to identify those differences in target spectra that are responsible for the contrasting effects of PI 3-kinase signals in fibroblasts and muscle cells.

LEF-1 Can Use Diverse Transactivation Domains to Become Oncogenic

A downstream target of Akt is GSK-3β, which is negatively regulated by Akt-dependent phosphorylation (25–27). GSK-3β is a component of the Wnt-1-LEF-1/TCF signaling pathway. It is found in a cytoplasmic multiprotein complex together with the adenomatous polyposis coli protein APC with axin, conductin, and β-catenin (28–32). The complex transduces the Wnt-1 signal by controlling the level of free cytoplasmic β-catenin (33, 34). GSK-3β phosphorylates β-catenin bound to the APC-containing complex and marks β-catenin for ubiquitin-dependent degradation. Mutations in various components of this cytoplasmic complex lead to accumulation of β-catenin in the cytoplasm followed by translocation into the nucleus, where β-catenin serves as transactivation domain of the LEF-1/TCF DNA-binding proteins (19, 35–39). Enhanced LEF/TCF-dependent transcription has been implicated in oncogenic transformation. Human cancer can result from elevated β-catenin levels due to either inactivating mutations in APC or stabilizing mutations in β-catenin (40–42). To learn more about the downstream effects of Wnt signaling, it was decided to look at the end point of that pathway, the transcriptional regulators LEF/TCF. Fusion constructs were designed that covalently link...
β-catenin to LEF-1 to make Wnt signaling constitutive (Fig. 3). Additional fusion constructs with LEF-1 used the transactivation domains of the herpes simplex virus protein VP16 and, separately, the hormone-binding domain of the human ER (43). The latter also contains a weak transactivation domain, TAF-2, that is active only in the presence of hormone. These constructs were tested in transient transfections together with a reporter plasmid that contains four LEF-1 binding sites. The LEF-1-β-catenin and LEF-1-VP16 fusions functioned as strong constitutive transcriptional activators in these tests. The LEF-1-ER constructs activated transcription of the reporter only in the presence of estrogen. These results show that LEF-1 can cooperate with diverse transcriptional activation domains. In the case of the LEF-1-ER constructs, LEF-1-dependent transcription becomes tightly hormone regulatable and is mediated by the transactivation domain contained in the hormone-binding region. All three types of constructs also induce oncogenic changes in stably transfected chicken embryo fibroblasts. They confer the ability of anchorage-independent growth (Fig. 4), and the LEF-1-ER constructs also induce distinct foci of morphologically altered cells in monolayer cultures. Like its transactivation function, the oncogenicity of LEF-1-ER is hormone dependent. The estrogen antagonist tamoxifen, which induces nuclear translocation of the LEF-1-ER construct but fails to enable the TAF-2 domain, did not elicit transformation by LEF-1-ER. These results suggest that LEF-1 is indeed an essential target of oncogenic signaling from Wnt/β-catenin and that activation of LEF-1 target genes is sufficient for oncogenic transformation. The LEF-1 constructs will be particularly useful in the identification of target genes involved in oncogenesis. The most important targets will be those that are differentially expressed in cells transformed by the fusion constructs as well as in human cancer cells with mutations in the Wnt signaling pathway.

A Jun Target That Induces Partial Transformation of Chicken Embryo Fibroblasts

Jun is the quintessential oncogenic transcription factor. Originally isolated as the product of the cell-derived oncogene in the avian retrovirus avian sarcoma virus 17, it belongs to the structural class of BZip proteins and is a member of the AP-1 complex of transcriptional regulators (44–46). AP-1 proteins control transcription as dimers. Jun can homodimerize, but it more effectively heterodimerizes with Fos, Fos-related antigens, and some members of the ATF transcription factor family (47–49). Jun has three principal functional domains, an NH2-terminal transactivation domain, a COOH-terminal dimerization domain (the leucine zipper), and an adjacent basic region forming the DNA contact surface. The oncogenic viral version of Jun shows several mutations, including two amino acid substitutions that remove regulatory sites, and a 27-amino acid deletion that prevents interaction with the regulatory Jun kinase (45, 46). These mutations act in concert to liberate viral Jun from normal cellular controls (50–52). However, oncogenic Jun is not merely a gain of function mutant of its cellular counterpart. A comparison of various Jun mutants finds no correlation between transcriptional activation from the consensus AP-1 site TGAATCA and oncogenic activity. Some poor transactivators are potently oncogenic, and highly transactivating mutants may be poor transformers (53–55). However, transcriptional activation appears to be vitally important in transformation. If the transactivation domain is deleted from Jun, the protein be-
The efficiency of transformation as measured by focus titers of transformed cell foci and in anchorage-independent growth. The conversion of estrogen to the culture medium results in the formation of blasts when expressed in the absence of estrogen. The ER-Jun chimera does not transform chicken embryo fibroblasts with the RCAS retroviral vector. Cells overexpressing this target formed morphologically transformed foci in monolayer cultures, characterized by multilayering and increased cellular refractility (Fig. 6). These morphological indicators of transformation were not as pronounced in HB-EGF-expressing cells as those seen with viral Jun. However, focus titers per unit of DNA were comparable to those achieved with RCAS-expressing v-Jun. HB-EGF also induced anchorage-independent growth. The colonies were markedly smaller than those induced by Jun, and the efficiency of colony formation was about 20% that of Jun. In contrast to v-Jun, HB-EGF did not induce tumors in chicken embryos. Its oncogenic activity must therefore be considered partial. Complete oncogenic transformation probably depends on the differential expression of several Jun target genes. The crucial question here is whether HB-EGF plays an essential or an ancillary role in transformation. Antisera reacting with the chicken HB-EGF are currently being prepared and should provide data on this point. If an autocrine loop of estrogen to the culture medium results in the formation of transformed cell foci and in anchorage-independent growth. The efficiency of transformation as measured by focus titers achieved per unit DNA is identical or slightly higher than that of viral Jun.

The utility of this ER-Jun construct is best illustrated with a presumptive viral Jun target gene, VJT-6, that is overexpressed in Jun-transformed cells (61). The partial clone of this target isolated by directional tag PCR subtraction was used to obtain a full-length cDNA from a chicken cDNA library. The sequence of this clone shows close homology to the membrane-bound precursor of the mammalian HB-EGF. The clone encompasses the coding domain for the mature growth factor as well as signal and propeptide and COOH-terminal transmembrane and cytoplasmic domains. HB-EGF is a mitogen for fibroblasts and epithelial cells. It binds to the epidermal growth factor receptor, and this interaction is modulated by binding to heparin-like molecules. HB-EGF is overexpressed in murine cells transformed by the raf oncogene and in some human tumors, e.g., hepatocellular, gastric, and pancreatic carcinomas, gliomas, and glioblastomas (63–65). In chicken embryo fibroblasts that express hormone-responsive Jun, the addition of estrogen leads to an up-regulation of HB-EGF within an hour. Maximum levels of HB-EGF RNA are reached after 12 h of hormone treatment. For comparison, morphological transformation of 50% of the cells can be seen by 42 h. Removal of the hormone turns off HB-EGF transcription and also leads to a reversion of the transformed phenotype.

There are several mutants of viral and cellular Jun that differ in their transactivation and oncogenic potentials. Up-regulation of HB-EGF in these mutant transfected cells is roughly correlated with transforming potential, but not with transactivation potential. For instance, mutant CJ3-34, a poor transactivator but a strong transformer, affects HB-EGF to the same extent as does viral Jun. HB-EGF is also differentially expressed in cells transformed by the BZip transcription factors Fos and Maf, but is much less, if at all, up-regulated in cells transformed by several other oncogenes including src, myc, or ras.

Transformation-relevant targets must affect the cellular phenotype, either alone or in combination with other target genes. HB-EGF was introduced into normal chick embryo fibroblasts with the RCAS retroviral vector. Cells overexpressing this target formed morphologically transformed foci in monolayer cultures, characterized by multilayering and increased cellular refractility (Fig. 6). These morphological indicators of transformation were not as pronounced in HB-EGF-expressing cells as those seen with viral Jun. However, focus titers per unit of DNA were comparable to those achieved with RCAS-expressing v-Jun. HB-EGF also induced anchorage-independent growth. The colonies were markedly smaller than those induced by Jun, and the efficiency of colony formation was about 20% that of Jun. In contrast to v-Jun, HB-EGF did not induce tumors in chicken embryos. Its oncogenic activity must therefore be considered partial. Complete oncogenic transformation probably depends on the differential expression of several Jun target genes. The crucial question here is whether HB-EGF plays an essential or an ancillary role in transformation. Antisera reacting with the chicken HB-EGF are currently being prepared and should provide data on this point. If an autocrine loop...
including HB-EGF contributes to transformation, a neutralizing antibody will change the oncogenic phenotype of the cell or lead to complete reversion of transformation. HB-EGF is probably a direct target of Jun because of its rapid response, and because the promoter of the mouse HB-EGF gene contains a combined AP-1/Ets-binding site that is required for the up-regulation of HB-EGF in Raf-transformed cells. The mouse HB-EGF promoter was also used to drive a reporter gene in human choriocarcinoma cells, JEG-3 and proved responsive to expression of c-Jun or v-Jun. Similar studies with the promoter of the chicken gene are in progress.

As investigations into the mechanism of transformation by oncogenes continue, the search for targets has become a central preoccupation of the field. Although new DNA chip-based technologies speed up the process of identifying differentially expressed genes and make the search for such genes much more comprehensive, they cannot provide the functional information that is needed to relate targets to the process of inducing and maintaining the neoplastic phenotype. Identifying targets essential in transformation and determining their specific role in oncogenesis is likely to be a much more challenging task than compiling a list of differentially regulated genes. Genetic screens may be of help in weeding out irrelevant targets that are innocent bystanders in the transformation process, but imaginative new techniques will also be needed to get to the hopefully few mediators of transformation.

Cellular oncogenes are activated by genetic change: viral capture; chromosomal translocation; or point mutation. The process of oncogene discovery makes use of these genetic changes. Although there is an element of randomness in discovery, the pursuit of targets has systematically uncovered cellular signaling networks with increasing details. Even random explorations of these networks reveal relationships of broad significance. In cytoplasmic signaling cascades, one oncogenic protein interacts with another protein of oncogenic potential. The P3k-Akt and the Ras-Raf pairs are prime examples of such oncprotein interactions. However, the ultimate recipients of all cellular signaling cascades are transcriptional targets. The phase transition from cytoplasmic signal to a program of gene expression is best illustrated by Jun. Similar events take place in Wnt signaling, where the path of β-catenin leads all the way from the cell surface to the nucleus; the ultimate targets are transcriptional. Transcriptional target genes that are important in oncogenesis probably code for weak growth stimulators, and several of them will have to act in concert to bring about frank transformation. The discovery of a Jun target that provides a partial growth advantage is a hopeful sign for future target searches. Research into the signaling generated by the differential expression of this mitogen may lead to a deeper understanding of the mechanism of oncogenic transformation.

References


