Cell signaling and cancer

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During the course of tumor progression, cancer cells acquire a number of characteristic alterations. These include the capacities to proliferate independently of exogenous growth-promoting or growth-inhibitory signals, to invade surrounding tissues and metastasize to distant sites, to elicit an angiogenic response, and to evade mechanisms that limit cell proliferation, such as apoptosis and replicative senescence. These properties reflect alterations in the cellular signaling pathways that in normal cells control cell proliferation, motility, and survival. Many of the proteins currently under investigation as possible targets for cancer therapy are signaling proteins that are components of these pathways. The nature of these signaling pathways and their roles in tumorigenesis were the subject of a recent Beatson International Cancer Conference.

Cellular signaling pathways are not isolated from each other but are interconnected to form complex signaling networks. Cells receive information from many different growth factor receptors and from cell-matrix and cell-cell contacts. They must then integrate this information to regulate diverse processes, such as protein synthesis and cell growth, motility, cell architecture and polarity, differentiation, and programmed cell death. The same signaling molecules are used to control different processes within different signaling complexes or at different intracellular locations. Moreover, signaling pathways are subject to developmental regulation and generate different outcomes in different cell types; the activation of a signaling molecule may have distinct consequences, depending on the cellular context. Understanding how these extraordinarily complex signaling networks function in vivo and how they are altered in cancer cells represents a major intellectual challenge.

The ability of intracellular signaling networks to integrate and distribute regulatory information requires that individual signaling proteins must act as nodes, responding to multiple inputs and regulating multiple effector outputs. One of the major advances in the last decade has been the recognition that many signaling proteins contain modular protein domains that mediate protein-protein interactions. These interaction modules serve to target signaling proteins to their substrates or to specific intracellular locations, to respond to posttranslational modifications, such as phosphorylation, acetylation and methylation, and to link polypeptides into multiprotein signaling complexes and pathways (Pawson and Nash, 2003). The same protein modules can also mediate intramolecular interactions that regulate signaling function, and a frequent theme is that upstream regulators may act by promoting or disrupting these intramolecular interactions. Thus, to understand the overall architecture of the signaling network, we will ultimately need to identify all of these inter- and intramolecular interactions.

The intricacy of cellular signaling networks has major implications for our understanding of tumor cell behavior and for our ability to use this knowledge for cancer therapy. Cell proliferation, motility, and survival are regulated by multiple pathways, and the changes that occur in cancer cells are the result of multiple alterations in cellular signaling machinery. Cancer cells are genetically unstable, undergo multiple genetic and epigenetic changes, and continuously evolve in response to selective pressures. Even if a mutationally activated pathway can be blocked by an inhibitor, tumor cells may evade the inhibitor by activating other pathways. Thus, even though early stage malignancies may respond to a single inhibitor—the success of Gleevec (STI-571) being a prime example—effective therapies for more advanced malignancies may require combinations of signaling inhibitors, or signaling inhibitors in conjunction with traditional DNA-damaging chemotherapeutic reagents.

The complex architecture of signaling networks and the consequences of this complexity for possible cancer therapeutics were recurrent themes at the Beatson International Conference on Cell Signaling and Cancer, held July 6–9, 2003 in Glasgow, Scotland. The meeting was sponsored by Cancer Research UK and the Association for International Cancer Research and was organized by the Beatson Institute for Cancer Research. Some of the signaling pathways discussed at the meeting are illustrated in Figure 1.

The roles of Src and FAK in cell motility and invasion

The cellular *src* gene was the first protooncogene to be discovered in the vertebrate genome. In the last few years, there has been increasing evidence that Src plays an important role in tumor cell invasion, in particular through its interaction with FAK (focal adhesion kinase). Src and FAK are nonreceptor tyrosine kinases that are localized to cell-matrix adhesions and mediate integrin signaling. Following integrin engagement, FAK undergoes autophosphorylation at Tyr 397 and Src becomes recruited to activated FAK via an interaction between the SH2 domain of Src and FAK pTyr397. Src then phosphorylates FAK at a number of tyrosine residues, creating docking sites for SH2 domain-containing signaling proteins, such as the adaptor Grb2. Genetic inactivation of Src family kinases or of FAK causes defects in cell motility, associated with an inability to turnover focal adhesions. In addition, Src and FAK are found in membrane ruffles and in podosomes (also known as invadopodia), dynamic protrusions that are prominent in certain cancer cells and which are involved in the degradation of the extracellular matrix. Both Src and FAK exhibit elevated expression in a number of different epithelial tumors, especially in invasive cancers. These observations have provided circumstantial evidence for a role of Src and FAK in tumor cell motility and invasion.

Margaret Frame (Beatson Institute, Glasgow) described the use of several different model systems to study the role of FAK in carcinogenesis. In the two-stage skin carcinogenesis model, mouse skin is initially painted with dimethylbenzanthracene (DMBA), which causes Ha-Ras mutations and the growth of

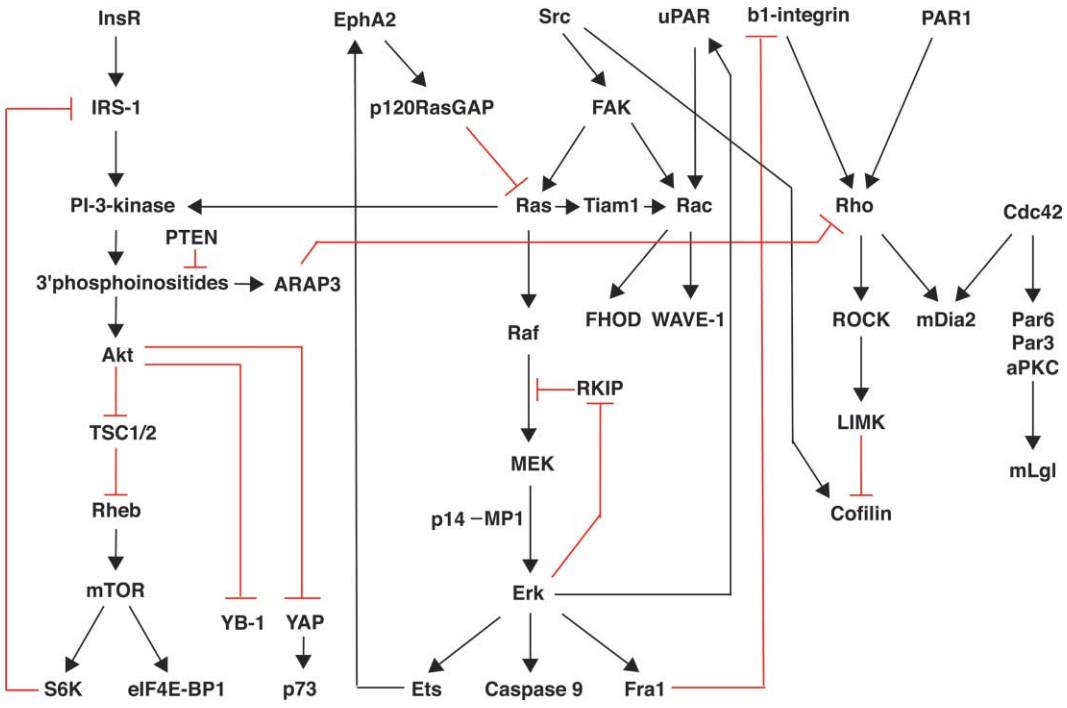

Figure 1. Cellular signaling pathways involved in cancer

This figure illustrates some of the signaling pathways involved in malignant transformation and tumor cell proliferation that were discussed at the meeting. Arrows (black) indicate activation or induction, T-bars (red) indicate inhibition. See text for details.

papillomas. Subsequent treatment with the tumor promoter tetradecanoyl phorbol acetate (TPA) causes progression of the papillomas to malignant carcinomas. Experiments using *fak* knockout heterozygotes or a conditional *fak* allele indicate that the level of FAK protein appears to be a determinant of both initiation and progression. Reconstitution experiments with $FAK^{-/-}$ fibroblasts and mutant forms of FAK suggested that tyrosine phosphorylation of FAK is critical for cell motility. Moreover, when activated Src was introduced into noninvasive colon cancer cells, they were converted from an epithelial to a more invasive, mesenchymal phenotype, associated with a change from cadherin-dependent cell contacts to integrin-dependent matrix adhesions (Avizienyte et al., 2002). This alteration was also correlated with tyrosine phosphorylation of FAK. Thus, FAK activation and phosphorylation of FAK by Src appear to be important determinants of tumor cell motility and invasiveness.

Another mechanism by which the interaction between Src and FAK enhances tumor cell invasion was described by David Schlaepfer (Scripps Institute, La Jolla, California). Transformation of FAK null cells by v-Src yields transformants that are noninvasive in Matrigel assays. The invasive properties of these transformants were restored by reexpression of wild-type FAK but not by reexpression of FAK mutated at the Src docking site (Tyr397). Reconstitution experiments linked cell invasion to the formation of a signaling complex involving Src, FAK, the docking protein Cas, the adaptor Crk, and a guanine nucleotide exchange factor, Dock180, which activates the small GTPase Rac. Activation of Rac was shown in turn to activate the Jun-kinase (JNK) cascade, which leads to the activation and secretion of matrix metalloproteinases such as MMP-2 and MMP-9 (Hsia et al., 2003). Thus a Src-FAK-Rac pathway appears to promote the invasiveness of Src-transformed cells. In addition, although expression of FAK does not promote the growth of Src-transformed cells in vitro, it does promote their growth as tumors in vivo, and this is correlated with increased expression of VEGF and increased tumor angiogenesis. Furthermore, these effects of FAK are not restricted to cells transformed by Src in vitro, because a dominant-negative form of FAK (FAK-related non kinase or FRNK) blocks the growth of breast carcinoma cells as tumors in vivo. Finally, FAK plays still other roles in Src-induced focal adhesion turnover. Neil Carragher (Beatson Institute, Glasgow) reported that the protease calpain is activated in v-Src-transformed cells and that its recruitment to focal adhesions promotes focal adhesion turnover and is dependent on FAK (Carragher et al., 2002).

Another Src substrate, FISH, may also be involved in the induction of cell invasion by activated Src. This adaptor protein was identified by Sara Courtneidge (Van Andel Institute, Grand Rapids, Michigan) and so named because it has *five SH3* domains. The N terminus of FISH contains a PX domain that targets tyrosine phosphorylated FISH to podosomes/invadopodia. FISH in turn binds via one of its SH3 domains (SH3 #5) to ADAMs family metalloproteinases, in particular ADAMs 12, 15, and 19 (Abram et al., 2003). Thus Src appears to be involved in the recruitment of FISH and associated metalloproteinases to podosomes.

Yet other pathways are involved in morphological transformation by activated Src. Morphological transformation and increased cell motility are generally associated with loss of stress fibers. Stress fiber formation is induced by the small GTPase RhoA. Since activated RhoA can suppress morphological transformation by v-Src and since Src can activate the Rho

GTPase-activating protein p190RhoGAP, it had been suggested that Src decreases Rho activity. However, results from our own laboratory communicated at the meeting reported that activated Src does not decrease the level of Rho[GTP] and can actually increase Rho[GTP] levels (G.S.M., unpublished results). RhoA promotes stress fiber formation in part via the Rho-dependent kinase (ROCK)-LIM kinase pathway, which results in the phosphorylation and inactivation of the actin-severing protein cofilin. As first reported by Pawlak and Helfman (2002), Src transformation can result in the dephosphorylation of cofilin. We reported that the kinase activity of Src activates a MEK-independent pathway that results in dephosphorylation and activation of cofilin.

Thus Src promotes cell motility and tumor cell growth by multiple mechanisms. Many of these are dependent on FAK and involve the local activation of Ras or Rac, or the recruitment of calpain, while others, such as the phosphorylation of FISH and the dephosphorylation of cofilin, may be FAK independent. Clearly Src and FAK are promising targets for inhibitors of tumor cell invasion.

The Ras/MAP kinase pathway: Targets and scaffolds

Ras is mutationally activated in a significant fraction of human cancers. It interacts with multiple effectors, probably more than twenty. One of these, the serine/threonine kinase Raf, activates a MAP kinase pathway, the Raf-MEK-ERK pathway, which is required for transformation by oncogenic Ras in a number of systems. The ERK MAP kinases themselves have many substrates, and the identity of these substrates and their roles in tumorigenesis remain central questions. The pathway is regulated and organized by scaffolding proteins, in ways that are not yet fully understood. Finally, as discussed at the end of this report, components of this pathway are under intense investigation as possible targets for anti-cancer therapeutics.

The MAP kinase pathway activates Ets family transcription factors that in turn regulate the expression of multiple genes, including an ephrin receptor, the receptor-tyrosine kinase EphA2 (Frank McCormick, University of California, San Francisco). EphA2 activates p120RasGAP (Miao et al., 2001), and so downregulates wild-type Ras but not mutationally activated Ras. Thus, EphA2 forms a component of a negative feedback loop, in which the Ras-MAP kinase pathway stimulates EphA2 expression, which in turn downregulates Ras. McCormick reported that breast cancer lines fall into two classes. More differentiated epithelial lines expressing wild-type Ras also express the EGF-related receptor-tyrosine kinase ErbB3 and the EphA2 ligand ephrin, but do not express EphA2. The reason that EphA2 is not expressed in this class of breast cancer cell is partly because there is selection against EphA2 expression (since this downregulates wild-type Ras) and partly because ephrin itself downregulates EphA2. In contrast, the second class of breast cancer lines are more invasive and mesenchymal in phenotype and express both EphA2 and, in some cases, mutationally activated Ras. The invasive phenotype of this second class can be decreased by expression of ephrin.

Activation of MAP kinase can exert antiapoptotic effects that are important in tumor cell survival. Lindsey Allan (University of Dundee) described a novel target of MAP kinase signaling, caspase 9. This is a caspase that mediates mitochondrion-dependent apoptosis by activating caspase 3 in response to cytochrome c release. ERK MAP kinases phosphorylate caspase 9 at Thr125, and this phosphorylation inhibits proteolytic processing and activation of caspase 9. Okadaic acid, which inhibits protein phosphatases 1 and 2A, allows the accumulation of the phosphorylated form and also inhibits caspase 9 activation (Allan et al., 2003). Thus, MAP kinase phosphorylation of caspase 9 may represent one of the events by which oncogenic Ras promotes tumor cell survival.

The Raf-MEK-MAP kinase pathway is organized by a variety of scaffold proteins, including Ksr, MP1, and 14-3-3 proteins. These scaffold proteins may regulate the activity of the pathway (positively or negatively), localize its output, increase the efficiency of signal transmission, or isolate the pathway from crosstalk by other MAP kinase pathways. Two MAP kinase pathway scaffolds were discussed. One of these, identified by Walter Kolch (Beatson Institute, Glasgow), is termed the Raf kinase inhibitor protein or RKIP. This protein negatively regulates the pathway by blocking the interaction of Raf with MEK (Yeung et al., 1999). However, RKIP is itself phosphorylated by MAP kinase, reducing its affinity for Raf and causing its dissociation from Raf in vitro and in vivo. This appears to result in a positive feedback loop, in that MAP kinase activity inhibits an inhibitor of the pathway, and Kolch proposed that this loop may function to convert the pathway from a graded to a more switch-like response. This represents another example of a phenomenon that is common to a number of signaling systems, namely the use of positive or double-negative feedback loops to create a switch-like "bistable" system (Ferrell, 2002). As an inhibitor of the Raf-MAP kinase pathway, RKIP may function as a tumor suppressor, and indeed RKIP expression is downregulated in metastatic breast and prostate cancers. Thus, this scaffold appears to serve primarily a regulatory function. A different function appears to be exerted by the protein p14, which was described by David Teis (University of Innsbruck). This is an endosomal protein that interacts with the MAP kinase scaffold MP1, which in turn interacts with both MEK and ERK kinases. p14 is required both for the endosomal localization of MAP kinases and for their activation on endosomes in response to EGF. It appears that the EGF receptor and Ras both traffic to endosomes and can activate the MAP kinase cascade at this intracellular site (Teis et al., 2002). Thus, p14 is an endosomal adaptor for the MAP kinase scaffold MP1 and serves to localize MAP kinase activation to endosomes in response to EGF. It will be of interest to identify endosomal substrates of MAP kinase and to determine whether the endosomally localized enzyme has a distinct function.

PI 3-kinases and the regulation of cell growth, motility, and survival

The lipid kinase phosphatidylinositol 3-kinase (PI 3-kinase) generates 3′-phosphoinositides that recruit proteins containing lipid-recognition domains (PH domains, FYVE domains) to the membrane. PI 3-kinase signaling regulates cell growth, motility, and survival. PTEN, a phosphatase that dephosphorylates 3′-phosphoinositides, is a tumor suppressor, and the gene encoding p110α, the catalytic subunit of a type IA PI 3-kinase, is amplified in human cancers. Moreover, many downstream targets of PI 3-kinase signaling, such as the protein kinase Akt and the translation initiation factor eIF4E, are transforming in cell culture. These and other observations indicate that PI 3-kinase signaling is important in tumorigenesis.

In recent years, there has been increasing interest in the regulation of cell growth and cell size, stimulated in part by genetic analyses in *Drosophila*. The 3′-phosphoinositide-

dependent kinases PDK1 and Akt (also known as PKB) initiate a kinase cascade that plays a key role in growth regulation. PDK1 activates Akt by phosphorylation of a Thr residue in the activation loop, although full activation requires phosphorylation of a Ser in a C-terminal hydrophobic motif. Akt in turn activates another kinase, the mammalian target of rapamycin, mTOR. mTOR activates translation in two ways. It activates the ribosomal S6 protein kinase, which has been implicated in the increased translation of 5′-terminal oligopyrimidine tract (TOP) mRNAs that encode ribosomal proteins and other components of the translational machinery. mTOR also inactivates 4EBP-1, an inhibitor of the initiation factor eIF4E. In this way, PI 3-kinase signaling regulates the translational machinery and thus cell growth. Peter Vogt (Scripps Institute, La Jolla) described the role of mTOR in transformation by avian sarcoma viruses encoding activated and membrane targeted forms of PI 3-kinase or Akt. Transformation by these viruses is inhibited by rapamycin, indicating that mTOR activation is necessary for transformation (Aoki et al., 2001). Akt and PI 3-kinase downregulate an RNA and DNA binding protein, the Y box binding protein YB-1. Overexpression of YB-1 produces resistance to transformation by Akt, without affecting Akt-dependent phosphorylation of 4EBP-1. YB-1 inhibits both cap- and IRES-dependent translation. A mutant of YB-1 that is defective in RNA binding localizes to the nucleus, rather than the cytoplasm, and does not block transformation. Taken together, these observations suggest that the ability of YB-1 to block transformation is related to its ability to inhibit translation and that downregulation of YB-1 represents one of the pathways responsible for the transforming activity of Akt.

Recent observations indicate that mTOR is regulated by the tuberous sclerosis proteins, hamartin (TSC-1) and tuberin (TSC-2). The tuberous sclerosis proteins are so called because they are growth inhibitors and tumor suppressors, and mutations in the TSC-1 and TSC-2 genes cause tubers in the brain or hardened (sclerotic) tumors in other organs. Ernst Hafen (University of Zurich) described a genetic and biochemical analysis of the relationship between TSC-1/2, TOR, and a small GTPase named Rheb (*R*as *h*omolog *e*nriched in *b*rain) (Garami et al., 2003; Stocker et al., 2003). Mutations in the *Drosophila* Rheb gene inhibit growth, while overexpression of Rheb promotes cell growth. Epistasis tests indicate that Rheb functions downstream of TSC-1/2 and upstream of TOR and S6K. Indeed it appears that TSC-1/2 are GTPase-activating proteins for Rheb. So PI 3-kinase and Akt signaling appear to activate TOR by inhibiting TSC-1/2 and thereby activating Rheb. If this is the case, however, and inhibition of TSC1/2 mediates the effects of PI 3-kinase signaling on growth, why do mutations in TSC-1/2 produce only tumors and overgrowths, while mutations in PTEN also result in cancer susceptibility? A possible answer was provided by Richard Lamb (Institute of Cancer Research, London). Loss of TSC-1/2 not only activates TOR and S6K signaling, but also impairs PI 3-kinase signaling and Akt activation. This negative feedback loop is mediated in part by S6K phosphorylation of the insulin receptor substrate IRS-1, which impairs its interaction with receptor-tyrosine kinases, and in part by S6K- and TOR-mediated inhibition of IRS-1 transcription. Thus in tuberous sclerosis, loss of TSC1/2 leads to increased S6K and TOR signaling and increased growth, but the IRS-1-mediated negative feedback loop decreases PI 3-kinase signaling, blocking cancer development. These observations therefore provide an explanation for the different effects of loss of TSC-1/2 and inactivation of PTEN.

In addition to its effects on cell growth, the PI 3-kinase-Akt pathway can also promote malignant progression by enhancing survival signaling. The PI 3-kinase/Akt pathway promotes cell survival by Akt-dependent activation of mTOR, Iκ-kinase (IKK) and Mdm2, and by inactivation of pro-apoptotic Bcl-2 family members such as Bax and Bad and of pro-apoptotic transcription factors such as FKHR. Julian Downward (Cancer Research UK London Laboratories) described the use of 14-3-3 proteins, which bind to a motif closely related to the phosphorylated Akt substrate motif, as affinity reagents to purify and identify novel Akt substrates. One of the Akt substrates so identified is YAP, an activator of the p53-related transcription factor p73. YAP enhances p73-induced transcription of the pro-apoptotic Bax protein, and Akt reverses this effect, apparently by inducing cytoplasmic translocation of YAP. Another novel Akt substrate, Mad3, was identified by two-dimensional PAGE analysis of phosphoproteins recognized by an Akt substrate anti-phosphoepitope antibody. Mad3 is a component of the Myc/Max/Mad transcription network that inhibits Myc-dependent transcription. Akt appears to promote Myc function by phosphorylating Mad3 and sequestering it away from the Myc partner, Max. Paradoxically, Downward reported, PI 3-kinase and its downstream effector S6 kinase also mediate Ras-induced cellular senescence, a phenomenon observed in primary cells that is believed to act as a tumor suppressor mechanism. Thus, PI 3-kinase signaling not only promotes tumor cell growth and survival but also elicits this protective checkpoint mechanism.

PI 3-kinase signaling also regulates cell motility and the actin cytoskeleton. A PtdIns(3,4,5)P3 binding protein which may mediate some of these effects was described by Len Stephens (Babraham Institute, Cambridge). This protein, ARAP3, was identified by virtue of its ability to bind to matrices carrying 3′-phosphoinositides (Krugmann et al., 2002). ARAP3 has five PH domains, of which one is required for translocation to membrane ruffles following PDGF treatment. Overexpression or RNAi knockdown of ARAP3 results in changes in cell spreading and in the ruffling response to PDGF, suggesting that ARAP3 plays some role in the regulation of the actin cytoskeleton by 3′-phosphoinositides. ARAP3 contains a 3′-phosphoinositide-stimulated GAP domain that is specific for Arf6, a small GTPase involved in delivery of an endosomal compartment to the lamellipodium, and the Arf6 GAP activity of ARAP3 blocks Arf6 transport to the cell surface. ARAP3 also contains a Rho GAP domain, which is stimulated by the Ras-related GTPase, Rap. Both Arf GAP and Rho GAP domains are required to mediate PI 3-kinase-dependent rearrangements in the actin cytoskeleton and cell shape. Thus, the GAP domains of ARAP3 appear to transduce signals from PI 3-kinase to the actin cytoskeleton and cellular trafficking machinery.

Although it is convenient to refer to PI 3-kinase activity generically as though it were a single enzyme, there are multiple isoforms of PI 3-kinase, differing in modes of regulation and pattern of expression. Class IA PI 3-kinases are regulated by recruitment to tyrosine-phosphorylated receptors or docking proteins and by direct interaction of the catalytic subunit with small GTPases such as Ras, while class IB PI 3-kinases are regulated by the Gβγ subunits of heterotrimeric G proteins. The class IA PI 3-kinases contain one of three catalytic subunits (p110α, p110β, and p110δ), plus one of five SH2 domain-containing regulatory subunits. Do these different isoforms have specific signaling mechanisms or physiological functions?

Attempts to answer this question with knockout mice have been bedeviled by compensatory changes in the remaining isoforms. Bart Vanhaesebroeck (Ludwig Institute, London) described the generation of knockin mutations that render the molecule catalytically inactive and the use of these mutants to study isoform-specific function (Okkenhaug and Vanhaesebroeck, 2003). Whereas p110δ-deficient mice are viable but display defects in T and B cell signaling, p110α-deficient mice exhibit embryonic lethality at E10–11, and p110α-deficient fibroblasts do not proliferate. These results imply that PI 3-kinases have isoform-specific functions; since they all carry out the same catalytic function, perhaps they interact with different regulators or function at different intracellular locations. It will be important to determine whether different isoforms play different roles in different cancers.

Activators and effectors of Rho family GTPases

In normal cells, Rho family GTPases regulate cell motility and proliferation. The levels of various Rho family members are elevated in different cancers, and Rho, Rac, and Cdc42 are required for transformation by Ras. However, whereas in human cancers Ras is activated primarily by mutations that block GTP hydrolysis, Rho family members are activated by changes in upstream regulators. Indeed, Rho family GEFs are oncogenes in vitro. RhoGEFS have Dbl homology (DH) domains paired with adjacent PH domains, plus additional protein modules that link the GEFs to upstream signals. For example, as Channing Der (University of North Carolina at Chapel Hill) described, the Rac GEF Tiam1 contains a Ras binding domain. Tiam1-deficient mice are resistant to Ras-induced Rac activation and to Ras-initiated tumorigenesis (Lambert et al., 2002; Malliri et al., 2002). Moreover the anchorage-independent growth of pancreatic cancer cells carrying activated (G12V) K-Ras is dependent on the continued expression of both activated Ras and Tiam1. Thus, Ras transformation appears to be dependent on Tiam1-mediated activation of Rac. Another example of Rho-dependent transformation is provided by the seven-transmembrane G protein-coupled thrombin receptor, PAR1. PAR1 is an oncogene when overexpressed, and this transforming capacity is dependent on RhoA function. PAR1 internalization and degradation is impaired in invasive breast cancer cells, and antisense knockdown experiments indicate that the invasiveness of breast carcinoma lines is dependent on PAR1 function. PAR1-mediated invasion is dependent on Gαi (which activates Ras) and Gα12/13 (which activate RhoA). Thus, the activation of Rac in pancreatic cancers and the activation of Rho in breast cancers are both due to changes in upstream signaling.

Both integrin signaling and growth factor signaling are required for the proliferation of anchorage-dependent cells. One of the mechanisms by which integrin and growth factor signaling are coordinated is through the adhesion-dependent targeting of Rac to the membrane (del Pozo et al., 2000 #16). Martin Schwartz (Scripps Institute, La Jolla) described a mechanism by which integrin signaling modulates lipid rafts and caveolae at the plasma membrane, and as a consquence, Rac activity.

The multiplicity of biological effects of Rho family GTPases is reflected in the multiplicity of effectors with which they interact. Actin stress fiber assembly driven by RhoA is mediated by two primary RhoA targets. One class, the Diaphanous-related formins, promote the polymerization of actin filaments and microtubules and are discussed below. The other class, the Rho kinases (ROCKs), promote actomyosin contraction by enhancing myosin light chain phosphorylation and, as noted earlier, promote actin filament stability by inactivating the actin filament-severing protein cofilin. Chris Marshall (Institute of Cancer Research, London) reported that tumor cells exhibit two distinct modes of cell motility that differ in their requirement for Rho signaling through ROCK. The first mode of cell motility is the "classic" lamellipodial or mesenchymal motility driven by active Rac. This mode of motility requires downregulation of Rho-ROCK signaling. One way in which this can occur, as noted earlier for Src-transformed cells, is by dephosphorylation of cofilin. In cells transformed by activated Ras, downregulation of Rho-ROCK signaling is dependent on ERK MAP kinase activity (Vial et al., 2003). ERK activates the transcription and translation of the AP-1 component Fra-1, which inactivates integrin β1 signaling and thus downregulates RhoA. At the same time, ERK signaling upregulates the expression of uPAR (the receptor for urokinase plasminogen activator), which in turn activates Rac. In contrast to this lamellipodial, Rac-dependent type of movement, however, the invasiveness of some tumor cell types is actually dependent on Rho-ROCK signaling (Sahai and Marshall, 2003). These cells have a rounded "blebby" morphology and a polarized distribution of ezrin, and their ability to invade is independent of matrix metalloproteinase secretion. Some tumor cell types exhibit both the mesenchymal, protease-dependent mode of invasion and the protease-independent, Rho-ROCK-dependent mode of cell movement. In these instances, inhibition of both proteases and ROCK is required to block cell motility. This suggests that an effective therapeutic strategy to inhibit tumor cell metastasis might be to simultaneously target both matrix metalloproteinases and ROCK.

The Diaphanous-related formins, which function as active scaffolds that direct actin filament and microtubule assembly, represent another major class of effectors for Rho family GTPases (Waller and Albert, 2003). They are characterized by the presence of formin homology (FH) domains and regulatory domains thought to confer sensitivity to active Rho GTPases: an amino-terminal GTPase binding domain (GBD) and a carboxy-terminal Dia-autoregulatory domain (DAD). Interaction of the GBD with the cognate GTPase disrupts the intramolecular GBD-DAD interaction and promotes the scaffolding function of the protein. Different formins appear to interact with distinct spectra of GTPases, suggesting that different GTPase-formin pairs are functionally distinct. To approach this question, Art Alberts (Van Andel Institute, Grand Rapids, Michigan) has used FRET (fluorescence resonance energy transfer) to analyze the sites of formin-GTPase interactions in intact cells. In one example, Cdc42 specifically interacts with mDia2 at the leading edge and MTOC of cells migrating into a wound. Demonstrating an effector role for mDia2, blocking mDia2 function inhibits the generation of filopodia induced by activated Cdc42. To explain these observations, Alberts proposed a model in which Cdc42 activates mDia2 at the base of the nascent filopodium and thus stimulates actin filament assembly and filament protrusion at the leading edge of migrating cells. In contrast to Cdc42, FRET analyses indicate that RhoB interacts with mDia2 on endosomes and RhoA interacts with mDia2 across the plasma membrane. Despite their ability to interact in vitro, mDia2 and RhoC did not interact in intact cells, suggesting that cells confer an additional specificity on GTPase-formin pairs. In another example, another formin protein (FHOD1) binds Rac in vitro and in vivo interacts with Rac on ruffles. Though recent studies have shown that formins nucleate nonbranched actin filaments in

vitro, it appears that different formins participate in the formation of different cytoskeletal structures at discrete sites and in response to different GTPases. Surprisingly, however, it appears that the GTPase binding domain is not itself required for subcellular targeting. Thus, rather than the GTPase targeting the formin to the site at which it functions, it may be that formin localization precedes the activation of the GTPase. This is a different model than the general paradigm for small GTPase function, in which the GTPase is responsible for recruiting its effectors to their site of function.

The WAVE/Scar proteins (WAVE-1, -2, and -3 in mammals) represent another class of scaffolding protein that mediates the effects of Rho family GTPases on the actin cytoskeleton. These proteins link Rho family GTPases to the Arp2/3 complex, which nucleates actin filament assembly and actin filament branching. Rac promotes WAVE-1 activation, and signaling by Rac is terminated by a WAVE-1-associated GTPase-activating protein named WRP. John Scott (Vollum Institute, Oregon Health and Sciences University, Portland) described the properties of WAVE-1 knockout mice (Soderling et al., 2003). These animals are viable but exhibit a variety of sensorimotor and learning deficits that are remarkably similar to those associated with haploinsufficiency of WRP in humans. Thus, alterations in Rac-regulated actin filament assembly may underlie this form of mental retardation.

Expression of activated Rho family GTPases has global effects on gene expression and function. Natalie Ahn (University of Colorado, Boulder) described a functional proteomics approach to characterize these changes. Cells expressing activated mutants of RhoA, Cdc42, or Rac1 were analyzed by 2D-PAGE and spots identified by MALDI-qTOF peptide mass sequencing and peptide sequencing. The expression of some proteins was uniquely regulated by one specific GTPase, whereas others were regulated by more than one GTPase. The 2D-PAGE analysis also revealed covalent modifications induced by specific GTPases. For example, RhoA was found to inhibit PTP1B and thereby promote the phosphorylation of p130Cas. Since Cas phosphorylation promotes cell motility, it is conceivable that a Rho-PTP1B-Cas pathway might be involved in the Rho-dependent type of motility described earlier.

Novel and atypical protein kinase Cs in cell-matrix interactions and the control of cell polarity

Conventional isoforms of protein kinase C (cPKCs: α, β, and γ) are dependent on phospholipids, particularly phosphatidylserine, Ca^{2+}, and diacylglycerol. They have been the focus of intense interest since the discovery that they are targeted by tumor promoters such as TPA. The more recently identified novel PKCs (nPKCs: δ, ε, η, and θ) are dependent only on phospholipids and diacylglycerol, while the atypical PKCs (aPKCs: ι/λ and ζ) are dependent only on phospholipids. It now appears that the novel and atypical PKCs play critical roles in the response to cell-cell and cell-matrix interactions and in the development of cell polarity. Peter Parker (Cancer Research UK London Research Institute) described how the novel PKC PKCε integrates integrin and cytokine signaling (Ivaska et al., 2003). Integrin engagement regulates the formation of a complex between PKCε, protein phosphatase 2A, and PDK1, such that PKCε is fully phosphorylated and active only in attached cells. PKCε activity in turn regulates interferon γ-induced phosphorylation of STAT1 by Janus kinases (Jak1, Jak2) so that the interferon-γ pathway is only active in attached cells. In this way, PKCε acts as a signal integrator mediating integrin regulation of cytokine responses.

The atypical PKCs appear to play an important role in the response to cell polarity signals. As first demonstrated by genetic studies on asymmetric division in *C. elegans*, one of the major determinants of cell polarity is a complex of the GTPase Cdc42, an atypical PKC and two scaffolding proteins, Par3 and Par6. Tony Pawson (Samuel Lunenfeld Research Institute, Toronto) described how a series of interactions assemble this complex at tight junctions between epithelial cells and at the leading edge of migrating astrocytes. In mammalian cells, there are direct interactions between aPKC, Par6, and mLgl, the mammalian homolog of the *Drosophila* tumor suppressor Lethal (2) giant larvae (Plant et al., 2003). mLgl contains aPKC phosphorylation sites that are important for fibroblasts to polarize in response to wounding. Thus, aPKC is directed to its substrate by specific protein-protein interactions. These interactions are part of a much larger network of interactions that connect the Par3-Par6 cell polarity complex to other complexes that regulate cell polarity, vesicle trafficking, microtubule stability, cell junction formation, and cell proliferation.

Signaling inhibitors as cancer therapeutics

Transformation of fibroblasts by activated oncogenes such as Ras is dependent on multiple pathways, and in some instances, inhibition of a single one of these pathways can inhibit transformation. If this is a valid model for carcinogenesis, single signaling inhibitors might be effective cancer therapeutics. However there are also precedents for believing that effective inhibition of some aspects of transformation may require multiple inhibitors. The synergy between metalloproteinase inhibitors and ROCK inhibitors in inhibiting tumor cell invasion, as described by Chris Marshall (see above), represents one such precedent. Moreover, as argued earlier, because tumor cells are genetically unstable and continually evolving, they may be able to evade blockade of a single pathway so that effective therapies may require the use of multiple inhibitors, or the use of signaling inhibitors as supplements to therapy with conventional DNA-damaging agents.

Raf proteins are under active investigation as therapeutic targets. There are three isoforms of Raf in mammals, Raf-1, B-Raf, and A-Raf. Mutations in the activation loop of B-Raf that activate catalytic activity have recently been found to occur in some 66% of melanomas and at a lower frequency in other cancers (Davies et al., 2002). The most common mutation is V599E, and this mutant form of Raf is transforming for NIH-3T3 cells. Maria Karasarides (Institute of Cancer Research, Landon) reported that transformation can be blocked by RNAi downregulation of B-Raf or by MEK inhibitors. Furthermore, downregulation of B-Raf in melanoma cells results in caspase 3 activation and apoptosis. Frank McCormick (University of California, San Francisco) discussed the therapeutic potential of a Raf inhibitor developed at the Bayer Corporation. Starting with a lead structure with an IC50 for Raf inhibition of 17 μM, the Bayer group, using combinatorial chemistry, developed an orally active compound, BAY 43-9006, which has an IC50 for Raf inhibition of 12 nM. This inhibitor blocks MEK phosphorylation by mutant B-Raf. Phase I trials suggest that the inhibitor may induce a partial response or stabilize disease progression when administered as single agent in the treatment of renal cell carcinoma or when used in combination with carboplatin and paclitaxel for the treatment of melanomas. These exciting but still preliminary findings

suggest that Raf activity is a promising target for therapeutic intervention.

STI-571 (Gleevec) has proven extremely effective in the treatment of chronic myelogenous leukemia, in which Abl is activated by translocation, and is also effective in the treatment of gastrointestinal stromal tumors, in which there are mutations in either c-Kit or the PDGF receptor α. Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden) described other uses of STI-571 as a PDGF receptor antagonist (Pietras et al., 2003). One such use is in the treatment of dermatofibrosarcoma protuberans, a disease of intermediate malignancy. It results from a fusion of the collagen1A1 gene to the gene encoding the PDGF-B chain: the fusion gene product is processed to generate PDGF-B chain. STI-571 reduces the growth of subcutaneous dermatofibrosarcoma tumors in a xenograft model. Clinical trials suggest that STI-571 can induce tumor regression. Another use of STI-571 as a cancer therapeutic stems from its effect on tumor stromal cells, which frequently express PDGF receptors. Activation of stromal PDGF-R causes an increase in tumor interstitial fluid pressure, which reduces the uptake of chemotherapeutic drugs. Heldin demonstrated that STI-571 reduces tumor interstitial fluid pressure and thereby increases the uptake and efficacy of drugs such as taxol and 5-FU (Pietras et al., 2002).

The response of a tumor cell to an inhibitor or drug depends on its particular genetic and epigenetic status. Tumor cells acquire resistance to apoptosis during the course of tumor progression, and enhanced survival signaling may be important in promoting resistance to chemotherapeutic agents (Johnstone et al., 2002). For example, Scott Lowe (Cold Spring Harbor Laboratory, New York) reported that the introduction of various antiapoptotic lesions (e.g., p53 loss, or overexpression of Bcl-2 or Akt) in Eμ-Myc transgenic mice enhances lymphomagenesis and the chemoresistance of the lymphomas. Interestingly, the nature of the antiapoptotic lesion can have an impact on how the lymphoma responds to a combination of conventional and targeted agents. Thus, knowledge of apoptosis resistance mechanisms in cancer may allow the tailoring of therapies for individual patients. With this in mind, Lowe described the use of short-hairpin RNA libraries to identify genes that either sensitize or inhibit drug-induced apoptosis. In the same context, Margaret Frame and Caroline Dive (University of Manchester) reported that catalytically inactive mutants of Src sensitize metastatic colon cancer cells to oxaliplatin- and Fas-induced apoptosis. These src mutants might act as either adaptor proteins or dominant-negatives and might either inhibit an antiapoptotic pathway or promote a proapoptotic pathway.

The findings described at this meeting indicate that our understanding of signaling pathways has advanced to the point where specific targets for therapeutic intervention can be identified. However, we need to understand how whole signaling networks function within the context of the intact cell if we are to develop rational strategies based on the genetic alterations of individual cancers. Based on the pace of the progress reported at this meeting, it is safe to predict that the next few years should see exciting new developments in targeted cancer therapies.

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