COMMEMORATIVE LECTURE

Tyrosine phosphorylation in cell signaling and disease

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Let me begin by expressing my gratitude to the Keio Medical Science Fund for the award of the 2001 Keio Medical Science Prize. This is indeed a great honor.

I will start by discussing how the recent genomics revolution affects the way one thinks about cell signaling, particularly signaling through tyrosine phosphorylation. Then I will tell you three short stories about some recent work from members of my group on how tyrosine phosphorylation is used in signal transduction and how it can be used to regulate the actin cytoskeleton.

Protein Kinase Genomics

Almost exactly 15 years ago, I was rash enough to predict that the human genome might encode as many as 1,001 protein kinases.[1] That prediction was based on a graph I made in 1986, which showed that the total number of distinct protein kinases had been increasing almost exponentially, beginning in 1976 or 1977 when molecular cloning first became feasible. Extrapolating from this curve, I came up with a predicted total number of protein kinases that sounded outrageously high at the time, but one that actually has proved to be not so far from the truth.

As you know, a number of genomes have now been completely sequenced and one can scan the predicted open reading frames to identify protein kinases using the signature motifs that are present in all members of this superfamily of serine and tyrosine kinases. *S. cerevisiae* was the first eukaryotic genomic sequence to be completed and it has around 6,000 genes. Our analysis, done in collaboration with the bioinfomatics group at Sugen, has identified 116 protein kinase genes in budding yeast, which represents about 2% of all genes.[2] But interestingly, although there are a few dedicated tyrosine kinases, like Swe1p, which phosphorylates Cdc28 (Cdc2) on tyrosine, *bona fide* tyrosine kinases like those that belong to the large family found in metazoans are lacking. Indeed, it appears that the use of tyrosine phosphorylation as a signaling mechanism evolved hand-in-hand with multicellularity as a mechanism for intercellular communication. Interestingly, it has recently been shown that one species of choanoflagellate, which are unicellular organisms closely related to metazoa, has a tyrosine kinase receptor.[3] This suggests that tyrosine kinases evolved at about the same time as metazoa, and implies that the existence of a tyrosine kinase receptor may have been a prerequisite for the emergence of true metazoans which require coordinated intercellular communication. The emergence of tyrosine phosphorylation as a signaling mechanism also depended upon the evolution of other components of the current systems used to propagate signals generated by tyrosine phosphorylation, including SH2 domains, which bind to phosphorylated tyrosines, and protein-tyrosine phosphatases (PTPs).

The nematode *C. elegans*, a complex multicellular organism, has about three times as many total genes as budding yeast and about three times as many protein kinase genes, which again comprise about 2% of all genes. In this organism, tyrosine kinases now form a major distinct family, as shown by dendrogram analysis, and represent about a fifth of all protein kinases. Interestingly, *D. melanogaster*, even though it is a more complex organism, has fewer genes and fewer protein kinases than *C. elegans*, with relatively fewer tyrosine kinases, but in toto the protein kinases again comprise about $\sim$2% of all genes.[4]

So what about *H sapiens*? The total number of genes is somewhat in dispute. The numbers have ranged from

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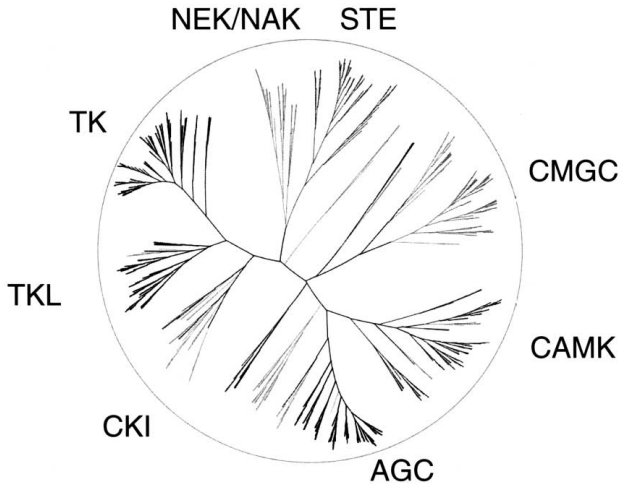

Fig. 1 Human protein kinase dendrogram.

30,000, based on gene predictions from genomic sequences, to as many as 150,000, based on cDNA sequencing, but I think it is probably going to be nearer to 30,000 genes. The analysis is still not yet complete, but our current estimate is that there are ~500 protein kinase genes, including 90 tyrosine kinase genes, and about 100 pseudogenes, which are recognizable copies of protein kinase genes that for one reason or another appear unlikely to be able to produce a functional product.[5] Because the sequence is not yet complete, and the assembly is not perfect, there could be a few missing protein kinase genes. Of course, the total number of distinct protein products derived from these genes is many more than 500, because a significant fraction express alternatively spliced products. If there are 30,000 genes, this means that about 2% of all human genes encode protein kinases, and about 20% of these protein kinases are tyrosine kinases. A dendrogram of the human protein kinases shows the prominence of the tyrosine kinase (TK) branch (Fig. 1).

As a sidelight, it is interesting to compare the human genome with that of *A. thaliana*, the favorite genetic organism for plant biologists. *Arabidopsis* has around 25,000 genes, but it has over a 1,000 protein kinase genes, which is relatively more than in humans, although, it lacks *bona fide* tyrosine kinase genes. The greater frequency of protein kinase genes may seem somewhat paradoxical, but it makes sense when one realizes that many of these protein kinases are used to respond to external stimuli and pathogens, which is particularly important for an organism that is sessile.

If we examine in more detail the complement of protein kinases encoded by the human genome – what we have called the kinome – out of the ~500 conventional protein kinases, there are 410 serine/threonine kinases and 90 tyrosine kinases. And among the 90 tyrosine kinases, 58 are receptor tyrosine kinases and 32 are non-receptor tyrosine kinases. For comparison, there are 42 predicted PTPs, which is about half the number of tyrosine kinases, 32 serine/threonine phosphatases and 46 phosphatases in the dual-specificity phosphatase family, which is distantly related to the tyrosine phosphatase family. In total, then, there are upwards of 600 human genes recognizably involved in the phosphorylation and dephosphorylation of proteins: the large number underscores the importance of this reversible protein modification for regulating protein function in eukaryotic cells.

Transmembrane Signaling by Tyrosine Phosphorylation

One of the major functions of tyrosine phosphorylation is in transmembrane signaling. There are two types of receptor system that use tyrosine phosphorylation. There are receptors with intrinsic tyrosine kinases activities, like the EGF/PDGF receptors, and there are binary or bimolecular receptors, where the surface receptor subunit lacks catalytic activity but associates with a non-receptor tyrosine kinase, which acts as a signaling subunit. Examples of this type are the interferon receptors and the T and B cell antigen receptors. Activation of both types of receptor tyrosine kinase occurs through ligand-induced dimerization, which juxtaposes the catalytic domains either in the receptors themselves or the associated non-receptor tyrosine kinase, resulting in transphosphorylation, initially in the activation loop and then subsequently in regions lying outside the catalytic domain, and in some receptors in an insert in the catalytic domain.

The phosphotyrosines generated by transphosphorylation then create binding sites for target proteins that have SH2 (Src homology 2), or PTB domains. Both of these are sequence specific phosphotyrosine (P.Tyr) binding domains; SH2 domains recognize P.Tyr and from 1–6 residues on its C terminal side, and PTB domains recognize P.Tyr and up 5 residues on its N terminal side. In addition to SH2 or PTB domains tyrosine kinase target proteins commonly have other protein or lipid interaction domains, such as SH3 and PH (pleckstrin homology) domains. Some of these targets act as adaptor proteins, such as the Grb2 SH2/SH3 proteins, and others have enzymatic activities, like phospholipase Cγ (PLCγ), Ras-GAP, and a subset of the PTPs. SH3 domains play a critical role in tyrosine phosphorylation based signaling, recognizing short proline-rich sequences, and increasingly important are the phospholipid binding domains. In many cases, these recognize 3′ phosphoinositides that are generated by PI-3′ kinases; such domains include the PH domain, the

recently identified PX domain, and the FYVE domain. A recent analysis of the numbers of these domains encoded by the human genome predicts there to be 126 SH2 domains, 264 SH3 domains and 223 PH domains.[6]

Activated receptor tyrosine kinases stimulate or trigger a plethora of signaling pathways through recruitment of SH2 or PTB-domain proteins to specific autophosphorylated residues. For example, in the case of the PDGFβ receptor, which has at least 9 identified autophosphorylation sites, PI-3′ kinase binds to two phosphorylated tyrosines in the kinase insert via the SH2 domains in its p85 regulatory subunit. Recruitment of the PI-3′ kinase complex to the plasma membrane puts it in apposition with its substrate, PIP_2, which it phosphorylates to yield PIP_3. PIP_3 can in turn be hydrolyzed to generate $PI_{34}P_2$; both PIP_3 and $PI_{34}P_2$ interact with a subset of PH domains, including those of the PDK1 and Akt/PKB protein kinases, which act in series to constitute a signaling pathway. Likewise, phosphorylated PLCγ can be recruited to the PDGFβ receptor at the membrane, and there it hydrolyzes PIP_2, generating DAG and IP_3, which releases Ca^{2+}; together DAG and Ca^{2+} stimulate protein kinase C (PKC). The Grb2 SH2/SH3 adaptor protein can also be recruited to the receptor, bringing along the Ras exchange factor Sos; Sos then activates Ras, which in turn activates the ERK MAP kinase pathway. The cellular response to PDGF reflects an integration of all of these pathways, many of which lead to the nucleus, and induce gene transcription.

Dysregulation of Tyrosine Phosphorylation and Disease

We are increasingly aware that mutations or changes in the expression of protein kinases and phosphatases play an important role in human disease. Many hereditary diseases are due to mutations in protein kinases and phosphatases. Many of the genetic changes in cancers that play a causal role in the cancer phenotype involve mutations of protein kinases and phosphatases. There has been a lot of progress in the development of specific inhibitors for protein kinases and phosphatases involved in disease.[7] And some of these inhibitors have been recently approved for the use in humans for treatments of cancer. I am sure that many more protein kinases and phosphatases inhibitors will become approved drugs in the next few years.

I would like to spend a little more time to illustrate some examples where mutations or amplification and overexpression of protein kinase genes, and tyrosine kinase genes in particular, are causal in human disease. Many of the tyrosine kinases, when overexpressed or activated by mutation play a role in cancer (Table 1A and B). For instance, ErbB2, is commonly amplified and overexpressed in breast cancer, as is the related EGF receptor, which is overexpressed in several types of cancer. A number of the receptor and non-receptor types of kinases are activated by chromosomal translocations, which generate chimeric fusion proteins. For instance, fusion of the PDGF receptor with TEL, an Ets family member, which provides a dimerization domain and elicits constitutive ligand independent activation, gives rise to chronic myelomonocytic leukemia.

The stem cell factor (SCF) receptor tyrosine kinase (KIT), which I will talk briefly about later, is activated by point mutations or small deletions in leukemia and gastrointestinal stromal tumors (GIST). Conversely, inactivation of KIT by germline mutations results in hereditary piebaldism. The FGF receptor tyrosine kinase family is interesting, because they are mutationally activated in hereditary syndromes that affect skull and bone development (craniosynostosis). FGF receptors are also activated sporadically in somatic cells by chromosomal translocations in some types of leukemia. Other receptor tyrosine kinases that are activated by chromosomal translocations in leukemia include ALK and TRK family members; in general these translocations result in gene fusions that encode chimeric proteins containing a dimerization domain from the fusion partner and the cytoplasmic domain, including the catalytic domain, from the receptor tyrosine kinase. Loss of function mutations also play a role in human disease such as in diabetes, where inactivating mutations in the insulin receptor are responsible; and in the case of RET, the GDNF receptor, loss of function mutations result in the hereditary intestinal disease, Hirschsprung's syndrome.

Non-receptor tyrosine kinases can also be either activated or inactivated by mutation in a variety of diseases (Table 1B). For instance, the SRC family kinases are commonly overexpressed in a wide range of carcinomas, and the ABL tyrosine kinase is activated by a chromosomal translocation which fuses it to BCR, creating the activated BCR-ABL protein that causes chronic myelogenous leukemia (CML). As we will hear later today, loss of function mutations in a non-receptor tyrosine kinase, such as ZAP70, are causal in human immunodeficiency syndromes.

There is also evidence that PTPs play a role in human disease, again largely based on mutational data (Table 1C). For example, loss of function mutations in CD45, a receptor-like PTP, causes severe combined immune deficiency disease. Interestingly, it has recently been reported that the PRL-3 dual-specificity phosphatase is specifically overexpressed in metastatic colon carcinoma, which suggests an important role for phosphatases in metastasis. The PTEN/MMAC-1 dual-specificity phosphatase, which dephosphorylates 3′ phosphoinositides, such as PIP_3, rather than phospho-

Table 1 Involvement of Tyrosine Kinases and Phosphatases in Human Disease

A. Receptor tyrosine kinases

EGF receptor	mutated, amplified and overexpressed in cancer
ERBB2/ERBB3/ERBB4	amplified and overexpressed in breast cancer
PDGF receptor β	activated by chromosomal translocation in leukemia
CSF-1 receptor	ectopic expression in cancer
KIT (SCF receptor)	inactivated by mutation in hereditary piebaldism activated by mutation in leukemia
FGF receptor 1	activated by mutation in craniosynostosis syndromes affecting skull/bone
FGF receptor 2	development (*e.g.* Crouzon/Pfeiffer syndromes, thanatophoric dysplasia)
FGF receptor 3	FGFR1/3 activated by chromosomal translocation in leukemia/myeloma
VEGF receptor 3	inactivated by mutation in hereditary lymphedema
MET (HGF receptor)	activated by mutation, and amplified/overexpressed in sporadic/hereditary cancer
AXL	overexpressed in myeloid leukemias
RSE/SKY	overexpressed in breast cancer
MER	inactivated in retinal dystrophy (defect in retinal pigment epithelial cell phagocytosis)
RET (GDNF receptor)	inactivated by mutation in hereditary intestinal disease activated by mutation in hereditary cancer syndromes
ALK	activated by chromosomal translocation in leukemia
TRK,TRKC (NGFR)	activated by chromosomal translocation in cancer
ROS	activated by chromosomal translocation in glioblastoma
TIE2 (angiopoietin receptor)	activated by mutation in hereditary vascular dysmorphogenesis disease
Insulin receptor	inactivated by mutation in non-insulin dependent diabetes

B. Nonreceptor tyrosine kinases

SRC	mutated, activated and overexpressed in colon and other cancers
YES	activated and overexpressed in cancer
LCK	activated by mutation and chromosomal translocation in leukemia
ABL	activated by chromosomal translocation to BCR (BCR-ABL) in chronic myelogenous leukemia (CML)
FAK	overexpressed in metastatic cancer
JAK2	activated by chromosomal translocation in leukemia
JAK3	inactivated by mutation in severe combined immune deficiency disease (SCID)
BTK	inactivated by mutation in hereditary agammaglobulinemia
ZAP70	inactivated by mutation in severe combined immune deficiency disease (SCID)

C. Protein-tyrosine phosphatases

Shp-1 (mouse)	SH2 domain PTP inactivated by mutation in immune hyperproliferative disease
SHP-2 (human)	SH2 domain PTP mutationally activated in Noonan syndrome
DEP-1/RPTPη	receptor PTP deleted in human thyroid cancer; suppresses thyroid cell growth
CD45	receptor PTP inactivated by mutation in severe combined immune deficiency disease (SCID)
KAP/Cdi1	dual-specificity protein phosphatase mutated in cancer
PRL-3	dual-specificity PTP overexpressed in metastatic colon carcinoma
PTEN/MMAC-1	3′ phosphoinositide (PIP_3) phosphatase inactivated by mutation in sporadic breast and prostate cancer and in Cowden's hamartoma hereditary cancer syndrome; may also dephosphorylate P.Tyr in protein substrates *e.g.* FAK
EPM2	dual-specificity phosphatase inactivated by mutation in myoclonus epilepsy (Lafora's disease)
myotubularin/MTM	3′ PI (PI-3P) phosphatase inactivated in X-linked myotubular myopathy
MTMR2	3′ PI (PI-3P) phosphatase inactivated in Charcot-Marie-Tooth disease 4B peripheral neuropathy
YopH1 (bacterium)	PTP encoded by the virulence plasmid of Yersinia (plague bacterium) that is required for pathogenesis – acts on FAK and p130Cas

proteins, is inactivated in sporadic cancers as well as in hereditary cancer, resulting in increased levels of PIP_3, which lead to persistent activation of downstream signaling.

The clear cut involvement of dysregulated tyrosine phosphorylation in disease has led to a major effort on the part of the pharmaceutical industry to develop inhibitors of tyrosine kinases and phosphatases. A number of TKIs – tyrosine kinases inhibitors – are in clinical trials for cancer therapy (Table 2).[8] Gleevec™, previously called STI571, an ABL/PDGFR/SCFR TKI has been approved by the US FDA, and is a very potent

Table 2 Tyrosine Kinase Inhibitor Drugs

EGF receptor inhibitors (ZD1839/Iressa™; OSI774/Tarceva™)	NSC lung/pancreatic cancer
EGFR/ErbB2 inhibitor (CI1033; EKB569; GW2016; PKI166)	NSC lung cancer
VEGF receptor inhibitors (ZK222584; ZD6474)	angiogenesis
VEGFR/FGFR/PDGFR inhibitors (SU6668; SU11248; PTK787)	angiogenesis
SCFR/Kit inhibitor (SU11248)	AML
NGF receptor (CEP2583)	prostate cancer
Abl/PDGFR/SCFR inhibitor (STI571/ Gleevec™)	CML, GIST
anti-EGF receptor MAbs (MAb225/ Erbitux™)	colorectal cancer
anti-ErbB2 MAbs (MAb4D5/Herceptin™)	breast cancer

Gleevec™ and Herceptin™ are approved for clinical use. The other drugs are in clinical trials.
Avastin™, an anti-VEGF MAb, is also in late stage clinical trials for breast cancer.

and effective treatment for CML and GIST.[9] A neutralizing anti-VEGF monoclonal antibody (MAb) is proving very effective as an anti-angiogenic agent in cancer clinical trials, and this MAb is likely to be the second MAb approved for clinical use that targets tyrosine kinase signaling (the first was Herceptin™, the anti-ErbB2 MAb).

There are several ongoing efforts to develop additional types of compound that inhibit or activate specific tyrosine kinases and phosphatases for treatment of other types of disease. Many companies are trying to find inhibitors of SRC family kinases, for instance to treat osteoporosis, or LCK, which is exclusively expressed in T cells; an LCK TKI could be used as an immunosuppressant. CD45 receptor PTP inhibitors are also being developed to act as immunosuppressants. JAK family kinases are very important in cytokine responses, and JAK TKIs could be used to repress undesirable cytokine responses. And then, for the treatment of type II diabetes, there are programs to develop inhibitors of the major insulin receptor PTP, PTP1B, with the goal of potentiating insulin receptor signaling. In fact, PTP1B inhibitors are already in clinical trials. An alternative insulin-independent method of enhancing insulin receptor signaling would be through insulin-mimetic dimerizers, and some success in this direction has been reported, with the development of a small membrane permeant molecule that activates the insulin receptor by artificially dimerizing the cytoplasmic catalytic domain. Already a large number of potentially important new drugs based on tyrosine kinases and phosphatases have been developed, and in the next few years we can look forward to many more such drugs becoming available for treatment of human disease.

Stem Cell Factor Receptor Signaling

Now I will tell you briefly about one project in my own group, where the goal has been to analyze the signaling functions of the SCF receptor/Kit, by making germ line mutations in the mouse. SCFR/Kit is a member of the PDGF-receptor family, having 5 immunoglobulin folds in its extracellular domain, and with a catalytic domain that is characterized by a large kinase insert.

The SCF receptor is encoded by the dominant white spotting (W) locus in the mouse, which was identified nearly 75 years ago now, through screens looking for overt genetic phenotypes. These mice have a white spot on their belly due to a pigmentation defect. The W alleles are all loss of function mutations in SCFR/Kit that decrease kinase activity. Analyses of these mice have shown that the SCFR/Kit is critical for the growth, survival and differentiation of stem cells in a series of lineages, including the hematopoietic, melanogenic, and gametogenic lineages. There are also loss of function mutations in the *KIT* gene in humans, which cause a condition known as piebaldism; piebald individuals are anemic and infertile, and have an obvious pigmentation defect. Interestingly, as I indicated, there are also gain-of-function mutations in the *KIT* gene, found in certain human leukemias. These include point mutations, which lie in the activation loop of the catalytic domain, particularly D816V/H that causes mastocytoma and myeloid leukemia through constitutive activation of the kinase (Fig. 2). Additionally, there are a series of small in-frame deletions in the cytoplasmic juxtamembrane domain that also constitutively activate the receptor and cause GIST in affected individuals.

Let me start by reviewing what is known about signaling downstream of the activated SCF receptor (Fig. 3). One of the major autophosphorylation sites is tyrosine 719, which recruits PI-3′ kinase and activates a number of downstream signal pathways, including one that indirectly leads to the activation of PKC, through phospholipase D-mediated generation of diacylglycerol. Activated PKC then phosphorylates serines 739 and 744 in the kinase insert, and this reduces SCFR/Kit signaling. On this basis we had proposed that PKC phosphorylation acts as a negative feedback loop to limit SCFR/Kit signaling in response to ligand. To study the physiological importance of these serine phosphorylations, Peter Blume-Jensen, a postdoctoral fellow in my group, developed a knock-in mutant mouse in which he converted the codons serines 739 and 744 to alanine in the c-*kit* gene in the mouse germline, using the same strategy we had used to make a Tyr719Phe mutant mouse.[10] If our model were correct, we might expect that this double mutation would lead to gain of function phenotypes, such as tumorigenesis.

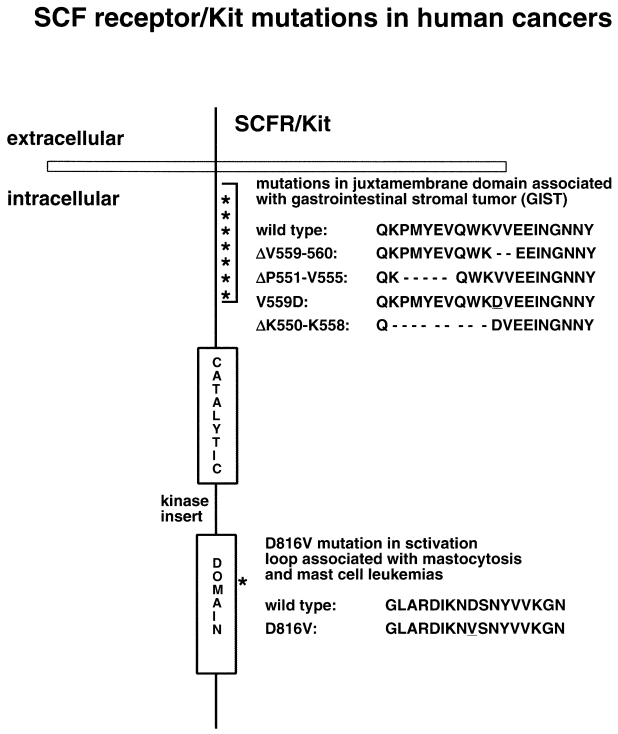

Fig. 2 SCF receptor/Kit mutations in human cancers.

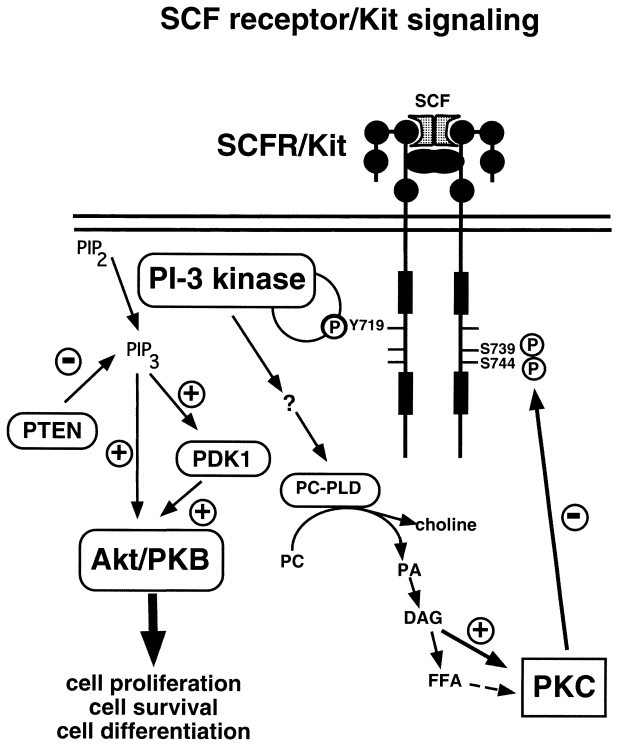

Fig. 3 SCF receptor/Kit signaling.

Homozygous S739/744A double mutant mice are viable, fertile, and show no obvious phenotypes at birth or when young. However, these mice begin to develop what appear to be gastrointestinal stromal tumors, with reasonable frequency between 12 and 18 months. Strikingly, this is the disease that humans with activating mutations in SCFR/Kit develop, and we conclude based on this that protein kinase C phosphorylation does exert a negative feedback which plays a role in inhibiting Kit function *in vivo*. These S739/744A mice could serve as a model for the human GIST syndrome. And we would predict, although we still need to do the experiment, that these mice might be cured by treatment with Gleevec. I think this underscores the importance of being able to develop mouse models for human disease, which one can then use for testing candidate drugs prior to embarking on full scale clinical trials.

Eph Receptor Tyrosine Kinase Signaling

The second story I have to tell you concerns the Eph receptor tyrosine kinase family. This is the largest of the receptor tyrosine kinase families, with 14 members. Eph receptor tyrosine kinases are conserved in evolution, with a single Eph gene being found in C. elegans. The ligands for Eph receptors are called ephrins; there are two main classes – A-type ephrins, which are GPI-linked, and B-type ephrins, which are transmembrane proteins. In this case, the ligands are presented on the surface of a neighboring cell, rather than being soluble, secreted molecules. A-type ephrins interact with EphA subfamily receptors, and B-type ephrins interact with the EphB subfamily receptors. Genetic analysis in many different organisms has shown that Eph/ephrin signaling is required for compartment boundary formation, as well as for cardiovasculogenesis, and is particularly important for axonal pathfinding in the CNS, where Eph/ephrin signaling provides a repulsive signal for axonal growth cones. Eph signaling is still rather poorly understood, but requires clustering of several Eph molecules – a dimer is not sufficient and a higher order oligomer is needed for a productive Eph signal. In addition, interaction with Eph allows the ephrins themselves, particularly the B-type ephrins, to generate signals in the ephrin-expressing cell, establishing a bidirectional signaling system. Autophosphorylation of Eph triggers the recruitment of a number of SH2-containing proteins, including Nck, RasGAP, and Shp2. In addition, Eph receptors interact with and can somehow regulate the FAK tyrosine kinase, p62Dok and ephexin, which is a Rho family GTP exchange factor (GEF). It was discovered early on that Eph signaling does not stimulate cell proliferation nor does it activate PI-3′ kinase, and more recently it has been shown to inhibit ERK MAP

kinase, possibly via p62Dok, which is the exact opposite of how a growth factor receptor signals.

The one agreed Eph signaling response is regulation of the actin cytoskeleton. Evidence from a number of groups shows that Eph activation causes a transient collapse of the neuronal growth cone, whose forward movement depends upon actin polymerization, and this potentially provides a repulsive mechanism.[11] Receptor tyrosine kinase induced assembly of the actin cytoskeleton generally requires activation of Rho family proteins, which promote F-actin assembly. Of relevance is the existence of an EphA-associated Rho family GEF called ephexin, which responds to ephrin binding. In PC3 carcinoma cells, Eph activation causes dephosphorylation of FAK, loss of F-actin and cell rounding. In endothelial cells, however, Eph stimulation activates integrin and promotes cell attachment and F-actin assembly.

Nigel Carter, a postdoctoral fellow in my group, became interested in studying ephrin-Eph signaling, and he chose to look at fibroblasts. Although fibroblasts are not normally thought of as a cell type that uses Eph signaling, many years ago when we first cloned EphA2 we found that mouse fibroblasts express high levels of this receptor tyrosine kinase. To study EphA2 signaling, we developed an assay in which recombinant ephrin A1 is coated onto a tissue culture dish, and then NIH3T3 mouse fibroblasts are plated onto the coated dish in the absence of serum.[12] NIH3T3 cells attach to the ephrin A1-coated dish, and begin to spread out, and by 60 minutes many of the cells are well spread. NIH3T3 cells plated onto polylysine-coated dishes attach but do not spread at all, whereas cells plated onto fibronectin, an extracellular matrix protein, attach and spread rapidly as expected. If one examines the actin cytoskeleton in these cells by staining with fluorescent phalloidin, in the cells plated on ephrin A1 the actin cytoskeleton shows an unusual morphology, with very strong circumferential staining of short actin bundles. That is in contrast to the cells plated on fibronectin, where there is some peripheral F-actin staining, but also bundles crossing the cell, which is typical. We also looked at other proteins that are implicated in the actin cytoskeleton and cell adhesion in this system. For instance, the focal adhesion protein paxillin is localized to discrete dots lying just beyond the F-actin ring – these could be proto-focal adhesion structures. One can also see p130Cas, activated FAK and phosphotyrosine staining in dots outside of the actin ring. A microtubule network forms between the F-actin ring and the nucleus. We conclude that plating mouse fibroblasts on ephrin A1 stimulates phosphotyrosine signaling at the leading edge of the spreading cell with concomitant formation of focal adhesions, and inside that zone the assembly of a very striking annulus of F-actin.

To test whether actin reorganization is directly due to EphA signaling, we generated an activated mutant form of EphA2, which was expressed stably in NIH3T3 cells following infection with a retrovirus vector. To make the activated mutant, we fused the 14 amino acids c-Src myristoylation sequence, which will dictate N-terminal myristoylation and plasma membrane association, followed by a constitutive dimerization domain, comprised of the GCN4 transcription factor leucine zipper, to the entire cytoplasmic domain of human EphA2. We expected that Myr-GLZ-EphA2 would localize to the plasma membrane, and dimerize through the GCN4 leucine zipper, which would promote transphosphorylation and lead to constitutive activation. As a control we made and expressed a kinase-dead Myr-GLZ-EphA2 mutant. The wild type but not the kinase-dead Myr-GLZ-EphA2 contains high levels of P.Tyr, consistent with it being constitutively active. When NIH3T3 cells expressing activated Myr-GLZ-EphA2 are plated on polylysine these cells attach, spread, and exhibit a peripheral actin ring similar to that seen in cells plated on ephrin A1, whereas cells expressing the kinase-dead Myr-GLZ-EphA2 do not. We conclude that EphA2 signaling per se can induce cell spreading and F-actin reorganization.

What sort of signaling events occur in ephrin A1-stimulated fibroblasts? When one analyzes tyrosine phosphorylation by blotting with anti-P.Tyr antibodies, phosphorylation of several proteins, including FAK and p130Cas, is stimulated when cells are plated on ephrin A1. In addition, p130Cas association with Crk is induced. To determine whether FAK and p130Cas are required for ephrin A1-stimulated cell spreading, we tested a panel of knockout mouse embryo fibroblasts (MEF) for their ability to spread on fibronectin and on ephrin A1. Wild type and FAK−/− MEFs spread on fibronectin. However, although wild type MEFs are well spread by 60 minutes on ephrin A1, FAK−/− MEFs attach but fail to spread. But, when FAK is reexpressed in the FAK−/− cells, they spread normally. Likewise, MEFs deficient for p130Cas spread on fibronectin but fail to spread on ephrin A1, unless p130Cas is reexpressed. This type of analysis has told us that FAK, Src family kinases and p130Cas are critical for spreading on ephrin A1, whereas they are not essential for spreading on fibronectin. Similar analysis showed that c-Abl/Arg are not required for spreading on ephrin A1, which will be important for what I will tell you next. Inhibitory antibodies to a number of integrins that are expressed on fibroblasts block spreading on ephrin A1, indicating a requirement for integrins. For instance, spreading of AG1523 human fibroblasts on ephrin A1 is inhibited by the RGDS peptide, which antagonizes most of the integrins, and also by anti-α4 and anti-α5 integrin subunit antibodies.

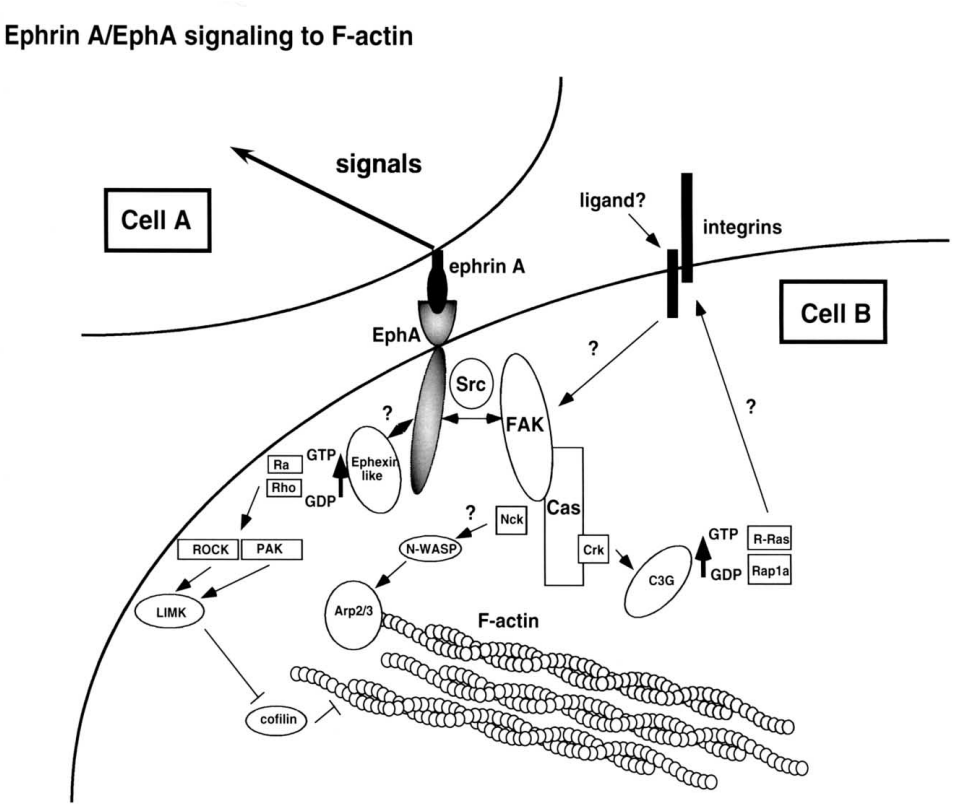

Fig. 4 Ephrin A/EphA signaling to the actin cytoskeleton.

We conclude that in fibroblasts ephrin A/EphA signaling promotes F-actin polymerization at the boundary of the spreading cell. Based on the results we obtained with the constitutively active form of EphA2, we deduce that F-actin organization is a direct consequence of EphA2 signaling. However, we do not yet have a precise model for how this happens (Fig. 4). With regard to the players downstream of EphA, we have learned that FAK, Src family kinases and p130Cas are essential. Integrins are somehow activated in this process and are required for spreading, but we do not know what ligand the integrins bind to, if anything. We know that ephrin A, which *in vivo* would be presented on an adjacent cell, causes clustering and activation of EphA, leading to FAK activation and processes downstream of FAK. We find that p130Cas becomes associated with Crk, and therefore integrin activation could be occurring through Crk-associated C3G, a GTP exchange factor for R-Ras, which is known to activate integrins. p62Dok bound to RasGAP may act downstream of EphA to inhibit ERK MAP kinase. In addition, EphA may provide more direct signals to actin through association with an ephexin-like Rho family GEF, which could activate Rac and Rho leading to activation of the Pak and ROCK kinases and increased F-actin formation. F-actin assembly may also be triggered through Nck, whose SH2 domain binds to p130Cas when it is tyrosine phosphorylated. Nck associates via its SH3 domains with N-WASP, which in turn binds to the Arp2/3 complex that initiates F-actin assembly and branching. Clearly, we still have a lot more to do to understand exactly what happens when EphA has been activated in fibroblasts.

On the face of it our results are in contrast to the current model for what occurs when a growth cone of an extending axon encounters an ephrin-expressing cell, where disassembly of F-actin is promoted and the growth cone stops or turns away. To reconcile this discrepancy we propose that ephrin/Eph signaling regulates local F-actin dynamics, and that depending on other factors, such as ephrin/Eph signal strength, and which Rho family GEFs are expressed, it can promote local assembly or disassembly of F-actin, and that the net consequence is reorganization of the actin cytoskeleton.

Abl Tyrosine Kinase and the Actin Ctoskeleton

Let me tell you one final story concerning the Abl tyrosine kinase. The domain structure of the N-terminal half of c-Abl and the closely related Arg tyrosine kinase is rather similar to that of the Src-family kinases, with SH3 and SH2 domains followed by the catalytic domain. But in contrast to Src family kinases, c-Abl/Arg

lack a regulatory tyrosine residue at the C terminus and have a very long C terminal extension, which contains several SH3 binding sites, a DNA-binding domain, and a G-actin binding domain, and at the very C-terminus an F-actin binding domain. c-Abl is present both in the nucleus and the cytoplasm. c-Abl has three NLS and a nuclear export signal, which allow shuttling of c-Abl between the cytoplasm and the nucleus. F-actin and G-actin can both bind to the C terminal region, and a number of SH3-containing proteins, including Abi, Crk, Grb2, and Nck interact with PXXP motifs in the proline-rich domain. The SH2-catalytic domain linker region is important in negative regulation of kinase activity, and very recent evidence suggests that the N terminus also plays a key role in negative regulation by binding back to the SH3 and SH2 domains.

A number of factors that regulate c-Abl kinase activity have been identified. Cell adhesion, which I am going to discuss, and growth factors like PDGF both activate cytoplasmic c-Abl. Indeed, there is evidence that c-Abl is required downstream for the PDGF mitogenic response. DNA damage activates nuclear c-Abl, and nuclear c-Abl is activated in the cell cycle in the S phase, when Rb is phosphorylated. Oncogenic constitutively active forms of Abl can be created by deleting the SH3 domain or by fusing the Bcr oligomerization domain in place of the N-terminal regulatory ''cap''. Knockout phenotypes have been quite informative: c-Abl knockout mice are runted, and most die postpartum; the survivors develop lymphoid defects. c-Abl/Arg double knockout mice die during embryogenesis at around E9-11 due to a neurulation defect. c-Abl is expressed ubiquitously and colocalizes with Arg and F-actin at the apical surface of the developing neuroepithelium.

I am going to focus on the interactions between c-Abl and F-actin. There is a lot of evidence connecting Abl and F-actin. For instance, c-Abl/Arg double knockout embryos have disruptions in the F-actin latticework of the neuroepithelium. And c-Abl knockout fibroblasts show delayed ruffling and F-actin ring formation in response to PDGF. PDGF also induces colocalization of WAVE1, one of the WASP family members, with c-Abl at ruffling cell membranes. Genetic evidence from *Drosophila* also provides a connection between F-actin and Abl. Enabled, a relative of vertebrate Mena and VASP, is a suppressor of loss of function D*abl* mutations, and negatively regulates cell motility and potentiates outgrowth of actin-rich structures.

Pam Woodring, a postdoctoral fellow in my group, has been trying to understand how c-Abl is regulated by adhesion and whether c-Abl in turn regulates adhesion. In particular, she has examined whether F-actin might regulate c-Abl, and conversely whether c-Abl modulates F-actin assembly. We started from the observation made over five years ago that when fibroblasts are detached from the culture dish c-Abl kinase activity is significantly reduced, when immunoprecipitates are assayed using RNA polymerase II CTD as a substrate.[13] Conversely, when detached cells are replated on fibronectin, c-Abl activity is increased.

Because there is an F-actin binding domain in c-Abl and because the actin cytoskeleton undergoes rearrangement when adherent cells are detached, we set out to test whether F-actin itself might regulate Abl kinase activity. We found that purified F-actin inhibits the kinase activity of purified Abl *in vitro* in a dose-dependent fashion.[14] A c-Abl mutant that lacks the C terminal F-actin binding domain is not inhibited by F-actin. Further analysis showed that the binding and inactivation of c-Abl by F-actin requires both the F-actin binding domain and the SH2 domain. Moreover, when various c-Abl mutants are stably expressed in Abl−/− MEFs, deletion of either the F-actin-binding domain or the SH2 domain is sufficient to prevent the inactivation of c-Abl upon detachment. We are not exactly sure how F-actin inhibits catalytic activity. No structure of full length c-Abl is available, and there are only individual structures of the catalytic domain and the SH2 and SH3 domains. In consequence, we cannot say how this very large protein might fold up to adopt an inhibited conformation in the presence of F-actin. There is evidence that the SH3 domain binds to the SH2 catalytic domain linker in a proline-dependent fashion and that this contributes to the inhibitory mechanism, and binding of the N terminal cap to the SH3 and SH2 domains is also important. Perhaps, F-actin binding to the C-terminal domain stabilizes the inhibited conformation through interaction with the SH2 domain. A possible model for F-actin regulation of c-Abl is shown in Figure 5A.

After obtaining evidence that c-Abl activity was inhibited by F-actin *in vitro*, we next wanted to try to show that this also happens in the cell. For this purpose, we did three types of experiment.[15] First, we showed that when fibroblasts are detached the amount of actin present in c-Abl immunoprecipitates increases with time, and that this parallels the loss in c-Abl kinase activity. In addition, using immunofluorescence staining we could show that there is significant colocalization of F-actin and Abl around the periphery of detached cells, whereas there is relatively little colocalization in attached cells. Finally, we obtained compelling evidence that F-actin actually does inhibit c-Abl activity *in vivo* by using latrunculin-A, an inhibitor of F-actin polymerization that acts by binding to actin monomers and preventing them from incorporating into growing chains. When detached cells are treated with the latrunculin-A, c-Abl kinase activity is restored, and at the same time there is a decrease in actin association.

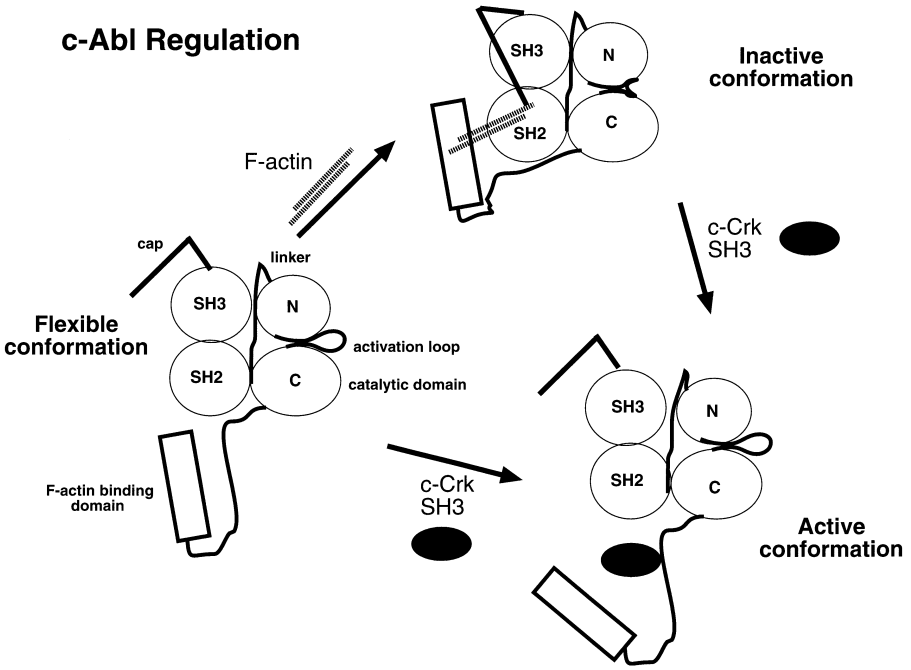

Fig. 5A c-Abl regulation by F-actin.

Moreover, latrunculin A treatment abolishes the colocalization of F-actin and c-Abl in detached cells. Thus, we conclude that F-actin is a negative regulator of c-Abl in the cell.

What about the converse possibility that c-Abl activity regulates the actin cytoskeleton? One piece of evidence in favor of this idea is that c-Abl−/− fibroblasts are delayed in spreading on fibronectin compared to c-Abl+/+ cells, and normal spreading is restored when an endogenous level of c-Abl is reexpressed.[15] When fibroblasts spread on fibronectin they send out filopodial surface microspikes that contain F-actin. Abl−/− MEFs send out fewer microspikes than cells expressing wild type c-Abl, whereas cells expressing the ΔF-actin c-Abl mutant, which is resistant to F-actin inhibition, show a more extensive array of F-actin-positive surface protrusions. Even more strikingly, expression of ΔF-actin c-Abl mutant induces the formation of F-actin positive surface filopodia in detached cells, whereas expression of wild type c-Abl does not. The STI571 Abl inhibitor blocks peripheral microspike formation in detached cells expressing ΔF-actin c-Abl proving that this phenotype is due to Abl kinase activity. Interestingly, when these cells are stained not only for F-actin, but also for P.Tyr, the very tips of the microspikes are strongly positive for P.Tyr, suggesting that Abl-dependent tyrosine phosphorylation occurs at the tips of these microspikes, and may be responsible for their formation. We are now trying to identify the protein(s) that are phosphorylated by c-Abl in order to promote the protrusion of filopodia.

Based on these experiments, we conclude that c-Abl can be negatively regulated *in vitro* and *in vivo* by F-actin. We also conclude that Abl kinase activity can promote F-actin assembly in fibroblasts, and that c-Abl/Arg activity is required for efficient cell spreading and F-actin assembly. To extend our studies we turned to the interaction of c-Abl and F-actin in neurons, where there is strong genetic evidence from mouse and *Drosophila* mutant phenotypes of the importance of these tyrosine kinases in regulating F-actin and neuronal function. In collaboration with David Litwack and Dennis O'Leary we examined E18 rat embryonic cortical neurons where after two days in culture long extended neurites send out F-actin containing side branches or filopodia.[15] In contrast when these cells are grown in the presence of STI571 to inhibit c-Abl and Arg kinase activity, the formation of these branches is almost completely blocked. Conversely, when ΔF-actin c-Abl is transiently expressed in E18 cortical neurons, the number and length of the branches is greatly increased compared to those in neurons exogenously expressing wild type c-Abl. Quantification shows that there are on average 2.7 one μm branches per 100 μm neurite length in c-Abl expressing cells. Whereas cells expressing ΔF-actin c-Abl have 8.0 branches. As an alternative approach we made use of c-Abl knockout mice to see whether we could establish genetically a requirement for c-Abl in neurite branching. Abl+/+ E17 cortical neurons had on average 7.0 branches per

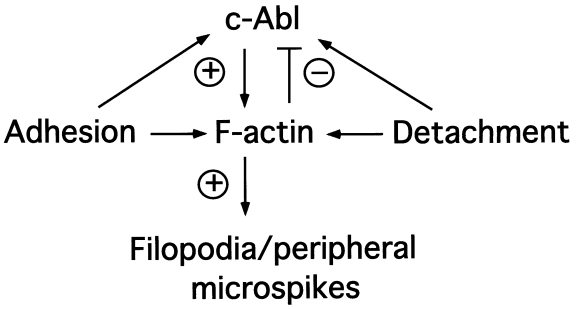

Fig. 5B Reciprocal regulation of Abl and F-actin.

100 μm, Abl+/− neurons had 5.3 branches, and Abl−/− neurons had 3.0 branches Clearly, there is a c-Abl gene dosage dependent increase in branching frequency, consistent with the data obtained with STI571.

Let me summarize the relationships we have discerned between F-actin and c-Abl. In fibroblasts, expression of activated forms of Abl results in an increase in peripheral active microspike formation; and conversely loss of Abl function decreases this. The same appears to hold true for neurite branching in cortical neurons, where inhibition of c-Abl/Arg or reduction of c-Abl; level results in reduced branching, and expression of activated forms of c-Abl stimulates branching. Surface microspike or filopodium formation is believed to be important for the exploratory behavior of cells as they migrate, and in the case of neurons these filopodia may make contact with neighboring axons, and be the precursors to synapses formed by dendritic spines. Overall, we conclude that there is bidirectional regulation of Abl and the F-actin cytoskeleton. Active Abl can stimulate an F-actin polymerization, whereas F-actin reduces Abl kinase activity and may act as a feedback mechanism to restrict the action of c-Abl in promoting F-actin assembly (Fig. 5B).

Epilogue

Let me conclude by saying that it is a rare privilege to have witnessed the events and the progress that have led from discovery of tyrosine phosphorylation over 20 years ago to the development of drugs that block the activity of oncogenic tyrosine kinases and are being used successfully to treat cancer and other human disease.

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