screening the HUMAN KINOME

expectations met or promises pending?

Protein kinases are important targets in drug discovery programmes aimed at treating many devastating diseases, including cancer, autoimmune disorders, diabetes and neurological disorders. Most 'classical' drug discovery efforts employ rational drug design methods based upon structural information to identify compounds targeting the enzyme catalytic domain. Novel information on kinase biology is opening up other approaches in the design of selective inhibitors which may provide more subtle modulation of these drug discovery targets. The identification of such modulators requires adoption of a new generation of HTS techniques. These will allow measurement of conformational changes in kinases, as well as protein-protein interactions via assessment of functional responses such as cellular translocation. These assay techniques, together with the understanding that numerous 'orphan' kinases may provide targets for therapeutics, suggest that a new era of kinase therapies is rapidly emerging.

By Dr Richard M. Eglen

Protein kinases are a large and diverse enzyme family that catalyse the transfer of γ phosphate groups from adenosine triphosphate (ATP) to hydroxyl groups on serine, threonine (ser, thr kinases; STKs) or tyrosine residues (tyrosine kinase; TKs)[1-4]. There are more than 500 genes encoding protein kinases[5], of which at least 30% are phosphorylated themselves by other kinases, often arranging in co-ordinated and cascading cellular pathways[6]. Protein phosphorylation, therefore, acts as a critical 'switching' mechanism in many cellular responses. Conversely, disregulation of cellular phosphorylation is now implicated in a large number of diseases[7].

Historically, much attention of the pathophysiological role of kinases has centred upon the TK family. More than 100 genes encode TKs in the human genome. A large proportion of TKs are intracellular, responding indirectly to extracellular stimuli. However, a subfamily acts as cell-surface receptors, activation of which catalyses phosphorylation of many cellular factors, including transcription factors, ultimately modulating gene expression. Ligand-dependent activation also catalyses autophosphorylation of these receptor tyrosine kinases (RTKs) resulting in sustained receptor activation. This constitutive activity appears to be critical in mediating cell proliferation with its disregulation being implicated in numerous cancers. Therefore, abnormalities in kinase catalytic activity, either due to altered expression levels or mutations in the kinase structure, are implicated in several disease pathologies. To this point, more than 250 protein kinase genes are known to map to human disease loci[8], with several cancers being induced by kinase mutations, such

as chronic myelogenous leukaemia (Bcr-Abl tyrosine kinase), chronic myelomonocytic leukaemia (TEL-PDGF receptor kinase), papillary renal cancer (c-Met receptor kinase) and non-Hodgkin's lymphoma (anaplastic lymphoma kinase)[9].

Due to their diverse physiology, and the large number of genetic mutations causing subtle but key changes in structure and function, it is unsurprising that kinases are implicated in the etiology of several diseases. Since several protein kinases have elevated activity in disease, most drug discovery efforts have been focused on developing selective inhibitors directed towards either a single kinase or a range of kinases. Their implicit role in cell cycle regulation and proliferation has resulted in intensive efforts being made to identify kinase inhibitors to treat cancer. However, kinase targeted drugs could also be useful in treating other disorders, including diabetes. Particular interest has focused on identifying inhibitors of receptor TKs, such as epidermal growth factor receptor (EGFR) and the non-receptor kinases, including cyclin dependent kinase (CDK), glycogen synthase kinase (GSK), c-jun-N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK) and protein kinase C (PKC).

The central role of TKs in disease has driven the biopharmaceutical industry to develop inhibitors of these enzymes as therapeutics[10-13]. Gleevec™ (imatinib, Novartis Pharmaceuticals), a potent inhibitor of the constitutively active Bcr-Abl fusion protein, was an early TK inhibitor approved for treatment of chronic myelogenous leukaemia and gastrointestinal stromal tumours[10-12]. Gleevec was followed by the development of EGFR inhibitors, Tarceva™ (erlotinib, Genentech Inc) and Iressa™ (gefitinib, AstraZeneca Pharmaceuticals), with the latter also receiving approval for treatment of non-small cell lung carcinoma[10-12]. In addition, there are late stage clinical programmes aimed at developing kinase inhibitors to treat other cancers, including inhibitors of growth factor receptors VEGFR and PDGFR (PTK787/vatalanib, Novartis; SU-11248/sunitinib, Pfizer Pharmaceuticals) to treat colorectal and gastrointestinal stromal tumours, inhibitors of MEK and RAF (CI-1044, Pfizer; BAY43-9006, Onyx) to treat renal cancer and solid tumours, CDK inhibitors (Flavopiridol, Sanofi-Aventis Pharmaceuticals) to treat head and neck cancers, and mTOR inhibitors (CCI-779) to treat renal and breast cancer[15]. Interest has also been increasing in the development of kinase inhibitors to treat other diseases. Eli Lilly Pharmaceuticals has applied for approval to use the protein kinase c (PKC-β) inhibitor, Ruboxistaurin, to treat diabetic retinopathy. The company has a second PKC inhibitor, Safingol, in late stage trials to treat atopic dermatitis. Furthermore, Schering AG has developed Fasudil, a protein kinase inhibitor in late stage trials for treatment of heart failure and has recently launched this same drug for treating cerebral ischaemia.

In summary, kinases are an important target class in drug development. This is due not only to their etiology in disease, but also that they are considered to be highly 'druggable', as they can also be purified in large amounts. To date the crystal structures of more than 40 different kinases have been published[15]. In parallel, significant advances in high throughput screening (HTS) technology development, both radioisotopic and non-radioisotopic, have accelerated identification of new structures of selective, high affinity inhibitors. These advances collectively have driven the development of large scale kinase profiling studies wherein compounds are characterised explicitly for multitarget inhibition.

Structure-function of kinases: new implications for drug discovery

Structural analysis of many different kinases reveal that the ATP binding pocket is similar[16]. Consequently, several compounds targeting the ATP binding domain act as non-selective inhibitors. These are known as type I inhibitors of which erlotinib and ZD-6474 are examples. However, some compounds, denoted as Type II inhibitors, inhibit kinase catalytic activity by recognising the 'inactive' conformation. In this state, the activation loop sterically hinders substrate binding (known as the DFG-out conformation). Examples of type II inhibitors are AZD-1152HQPA and MLN-518. Gleevec also binds to the closed, inactive state of a kinase, namely Bcr-Abl kinase[10,17]. In contrast, PD 173955 binds to the open conformation of the activation loop of Bcr-Abl kinase[17]. However, unlike Gleevec, PD 173955 is a potent inhibitor of src kinases, and is not selective for Bcr-Abl kinase. This is presumably due to the high degree of structural similarity in open kinase conformations. Consequently, inhibitors recognising enzymes in the open conformation are more likely to inhibit multiple kinases than those that bind to inactive kinases.

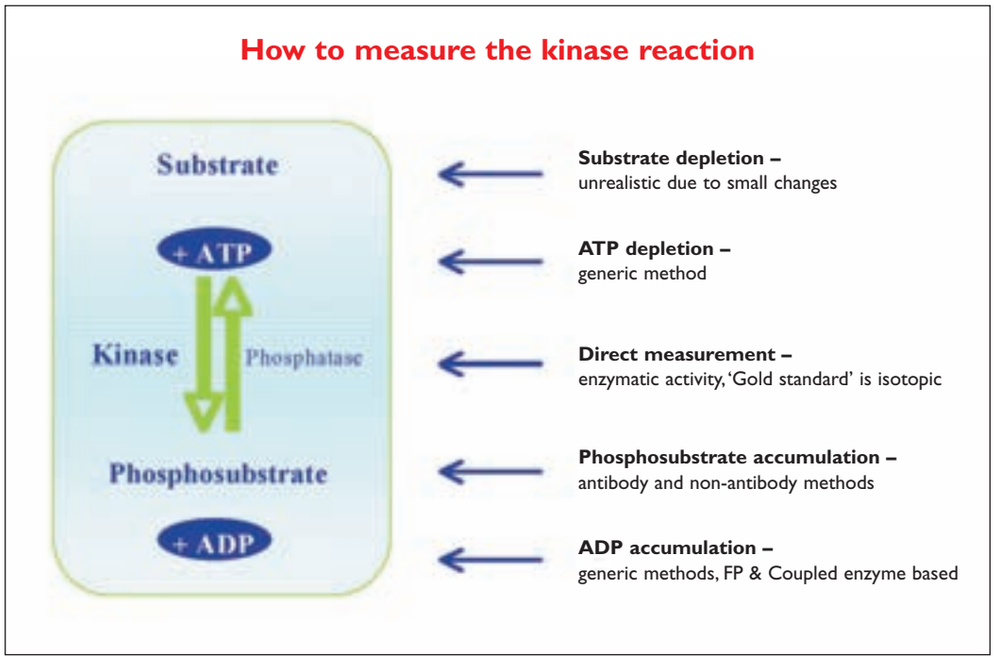
Although there are significant advantages of developing inhibitors of the inactive form of protein kinases, such as enhanced specificity and a lack of the necessity to compete with high millimolar ATP concentrations, there are also some

disadvantages. In the case of Bcr-Abl kinase, for example, tumour cell drug resistance develops from mutations in the Gleevec-binding site[18,19]. While such mutations do not affect kinase activity, they can attenuate the ability of the enzyme to interact with Gleevec. In contrast, these mutations do not affect the ability of PD 173955 to interact with and inhibit the kinase activity, further supporting that they selectively influence the Gleevec-binding site in an inactive conformation.

Clearly inhibitors of inactive kinases hold promise as novel therapeutic strategy approaches. However, screening technologies designed to identify such compounds are lacking, partly due to the fact that almost all assays are explicitly 'activity-based' thereby precluding detection of compounds that bind to the inactive kinase configuration. More pragmatically, assays that do not exploit the catalytic properties of a kinase to generate assay signal, theoretically require relatively large amounts of protein for direct competition binding studies. A further point is that the majority of studies on kinase structure focus on the ATP binding pocket. This has limited utility in aiding structure-based design of compounds recognising the inactive conformation. Taken together, assay technologies designed to measure kinase conformational changes, as they switch from inactive to active states, would be highly useful for inhibitor screening. However, such techniques are still at a nascent stage of development, even when considering the emerging label-free techniques, which to date have had little impact on the kinase screening area.

In consequence, the majority of TK inhibitors under development are directed towards the ATP binding pocket. Since domain structures in the ATP recognition site are highly conserved, it was originally suggested that compounds designed to block this site would be non-selective and therefore, promiscuously inhibit many kinases[20,21]. However, several therapeutically effective compounds have been identified all of which bind to the ATP recognition site, yet which exhibit minimal side-effects. This is derived from the fact that

Figure 1
Summary of assays methods for measuring the kinase reaction



there are numerous non-conserved, unique amino acid residues in the ATP binding pocket that do not participate in phosphate transfer (including a glycine-rich loop, hinge regions and a hydrophobic pocket also exists – all of which possess non-conserved amino acid residues). Consequently, some kinase inhibitors do not directly interact with the conserved phosphate transfer region; rather, they are directed at the hydrophobic pocket, as exemplified by several selective p38 inhibitors, such as BIRB-796, R-1487 and VX-745[22,23].

All protein kinases catalyse the transfer of the γ phosphate groups from adenosine triphosphate (ATP) to hydroxyl groups on serine, threonine (ser/thr kinases; STKs) or tyrosine residues (tyrosine kinase; TKs) in either peptide or protein substrates. Many assays have been developed to measure kinase activity, including substrate depletion (most frequently by assessing decreases in the ATP concentrations) or by product accumulation (generally by measuring increases in ADP or phosphosubstrate concentrations). To date, the most direct measure of enzyme activation is by measuring the radioisotopic incorporation of 33P into the reaction products.

Protein kinase assays for drug discovery

Structural analysis of kinases has been extensively used in the rational design of inhibitors. Indeed, drug design based on crystallographic information is widely used in kinase drug discovery. However, once compound scaffolds are generated a large series of analogues are then tested for potency and efficacy against the target kinase, as well as for mode of action. Given the size and diversity of the kinome, many counter screens against a large number of kinases are frequently employed to establish a profile of specificity.

In HTS, a range of technologies have now been developed to measure the kinase activity of small molecule or natural product libraries, as well as libraries generated from the structural analysis of kinase catalytic domains. HTS assays are also used to detect kinase inhibitors that are not readily identified by structural analysis. For example, HTS assays can be used to identify compounds that inhibit kinase activity by binding to regions in the catalytic domain distinct from the ATP binding site. Such assays may reveal allosteric interaction with the target kinase, as well as those that affect kinase function by abrogating cellular translocation. Now emerging are assays that can directly measure ligand binding or its consequences such as RTK dimerisation.

A growing area of kinase drug discovery is the high throughput profiling of putative inhibitors across the kinome and is beginning to replace the linear process of designing a compound to selectively inhibit a single kinase target[24]. Profiling, particularly when conducted using assay technologies that can facilitate a common assay protocol against a large portion of the human kinome, not

only allows assessment of any 'off-target' action of the novel inhibitor, but also facilitates the generation of an inhibitor with 'multitarget' activities. Indeed, many kinase inhibitors appear to exert their therapeutic action via inhibition of a discrete range of enzymes – as has been seen with a whole series of quinazoline based compounds, including Tarceva. This was opportunistically exploited with many first generation kinase inhibitors, such as Gleevec. An emerging issue, however, is that in many cases our understanding of the disease is incomplete. Consequently, it may be unclear as to the optimal inhibitor profile that is desirable in a novel kinase therapeutic[24].

The basic principles of most such HTS assays involve measuring the amount of substrate that undergoes phosphorylation by a kinase or the conversion of ATP to ADP as a measure of activity (**Figure 1**). These are discussed in detail below:

Biochemical kinase assays

Radioisotopic readouts

Measurement of kinase turnover via the catalytic incorporation of 32P into the substrate is widely used for assessing kinase activity, particularly in compound profiling. In this technique, phosphorylated substrate is separated from free 32P using gel electrophoresis or filtration[25-27]. A modification of this approach is the scintillation proximity assay (SPA, GE Healthcare), a pseudo-homogeneous assay in which the biotinylated substrate is labelled with 33P due to the kinase reaction. The 33P-labelled biotinylated substrate is captured on a streptavidin-coated SPA bead, and the light that is emitted from the bead is then detected either by a scintillation counter or CCD imager[28,29]. Alternatively, FlashPlate® (PerkinElmer) is used to capture the kinase substrate on a microtiter plate surface with either streptavidin (to capture a biotinylated substrate) or nickel chelate (to capture a His-tagged substrate). Incorporation of 33P into the solid phase substrate on the plate brings it in close proximity to a scintillant coating the plate, allowing the resulting signal to be measured. These two assays are relatively simple to use and can be used to measure activity of many different kinases in either crude preparations or purified, recombinant forms. The main advantage is that Michaelis-Menten kinetics can be assessed, since a range of ATP and substrate concentrations can be studied. Furthermore, since these assays do not require selective antibodies recognising the substrate, they can be readily modified to characterise the substrate profile of a specific kinase or kinase mutation (see below)[30]. However, the major disadvantage of these assays is the use of radioactivity, which generally requires washing steps to remove unbound isotopes from the substrate, thereby increasing waste disposal costs.

Non-radioisotopic readouts

With the development of numerous antibodies that distinguish phosphorylated from non-phosphorylated substrates, a wide variety of non-radiometric HTS approaches have been developed for kinase screening[31,32]. Dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA®, PerkinElmer) is a standard fluorometric assay that measures protein kinase activity using anti-tyrosine or anti-serine/threonine phosphoantibodies[33]. This assay employs a lanthanide-labelled antibody for detection of substrates using labels with a long decay time, as well as a wide Stokes' shift, both of which result in a large signal-to-noise ratio. In this assay, a biotinylated substrate is first incubated with the kinase, followed by the transfer of the phosphorylated substrate to streptavidin-coated microplates. Once the phosphorylated substrate binds to the streptavidin in the plate, it is incubated with a europium-labelled antibody directed against the phosphorylated substrate. Time-resolved fluorometry of the enhanced fluorescence of the lanthanide, which is dissociated by a proprietary enhancement solution, is then carried out to increase sensitivity.

Recently, time resolved fluorescence energy transfer (TR-FRET) has gained broader acceptance than DELFIA for kinase screening[31], principally due to the lack of wash steps in the protocol[33-36]. In this assay, the kinase substrate with acceptor moiety is first incubated with the kinase. The phosphorylated substrate is then detected with a specific anti-phospho-peptide antibody labelled with a europium chelate (Eu) in LANCE® and LANCE *Ultra* (PerkinElmer) assays, which serves as a donor[37]. The binding of the Eu-labelled antibody to the phosphorylated peptide substrate with an acceptor moiety (U*Light*™ in the LANCE *Ultra* assay) causes the donor and acceptor dyes to come in close proximity, and result in energy transfer and light emission upon excitation, which is detected as the assay response. Analogous TR-FRET assays to measure protein kinase activity are now commercially available including the Z'-LYTETM and LanthaScreen™ (Invitrogen) and HTRF® (Cisbio) assays[38,39].

The AlphaScreen® assay (PerkinElmer), which utilises a bead-based proximity approach, also employs donor and acceptor beads for signal generation[40,41]. Here, a biotinylated or GST-tagged

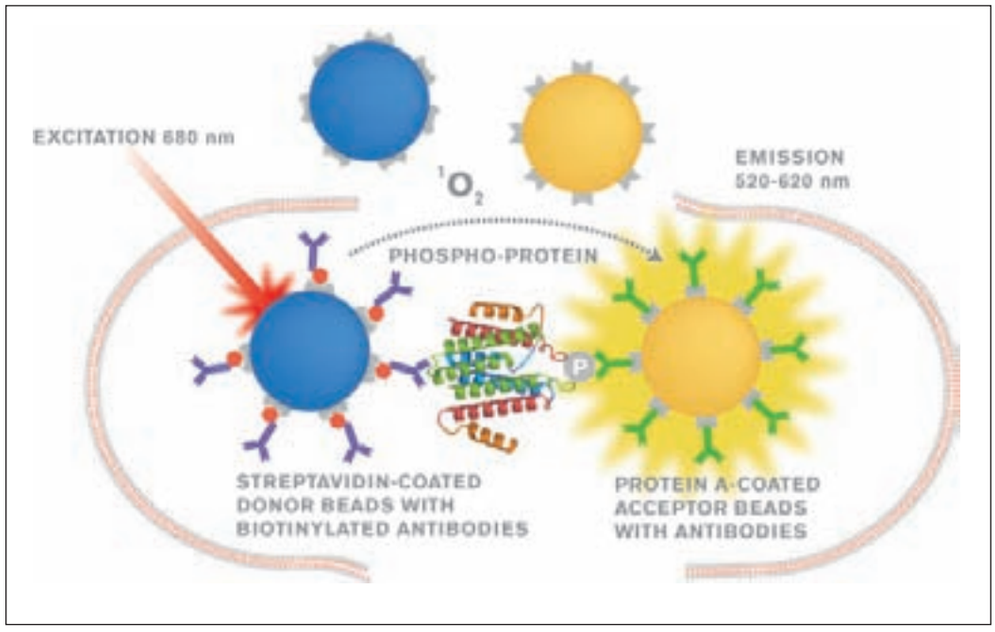


Figure 2
AlphaScreen SureFire Cell Based Kinase Assay Principle: The first antibody is biotinylated and captured by streptavidin-coated Donor bead which in turn, captures the endogenous substrate present in the cell lysate. The second antibody is captured by the Protein A-coated Acceptor bead and only recognises the phosphorylated protein substrate under investigation. The two beads are only brought into close proximity in the presence of the phosphoprotein. This assay format allows the development of extremely sensitive assay to be developed at kinases endogenously expressed in the cell

substrate is first phosphorylated by the kinase, followed by binding of the phosphorylated substrate to the donor beads via either streptavidin or glutathione. The acceptor bead that is tagged with either anti-tyrosine or anti-serine/threonine antibodies then binds to the phosphorylated substrate, bringing donor and acceptor beads in close proximity to generate a signal[41]. A major advantage of this approach, in marked contrast to TR-FRET techniques, is that it is also adaptable to the use of proteins as substrates, as opposed to short peptides. One extension of the technology is the PhosphoSensor assay (PerkinElmer) in which the acceptor beads are coupled to a Lewis metal chelate instead of an antibody[43,44]. The Lewis metal chelate binds to free, unincorporated phosphate as well as to phosphate that is incorporated into the substrate. Signal generation occurs when the metal chelate binds to phosphorylated substrate in proximity to the donor. The immobilised metal assay for phosphochemical (IMAPTM) TR-FRET (Molecular Devices; MDS Analytical Technologies) employs an analogous approach to detect kinase phosphorylation again without use of antibodies[45].

Several other assay technologies are also available for measuring levels of peptide substrate phosphorylation due to kinase activity. These include the Antibody Beacon™ tyrosine kinase fluorescence quenching assay (Invitrogen), the IQ™ kinase assay (Pierce; ThermoFisher Scientific)[36], the TruLight™ kinase assay (EMD Chemicals), and the HitHunter™ β galactosidase enzyme fragment complementation assay (DiscoveRx)[46,47]. These products use a competitive binding assay with an anti-phosphopeptide antibody to measure the formation of newly phosphorylated substrate as a result of kinase catalytic activity.

Measurement of ATP depletion

Kinase activity converts ATP into ADP via incorporation of the terminal phosphate group into a substrate. Consequently, either depletion of ATP or depletion of ADP also serves as a measure of kinases activity[48-50]. The Kinase-Glo® luminescent kinase assay (Promega Corporation) and the easylite-Kinase™ assay (PerkinElmer) reflect depletion of ATP using a luciferase-based technique. Although both assays are homogenous and do not require substrate-specific antibodies, they measure ATP depletion against the background concentration of ATP required for optimal kinase activity. Thus, high turnover enzymes produce relatively large changes in ATP, while weakly active kinases produce low depletion levels. Many kinases analysed by this approach need to be purified, since the technique does not distinguish ATP depletion induced by the kinase from one resulting from contamination with ATPases. Consequently, novel assays favour the detection of ADP as a measure of kinases activity. For example, the ADP Quest™ assay (DiscoveRx) measures ADP levels via a coupled enzyme reaction[48]. TR-FRET (Cisbio) and Transcreener™ fluorescent polarisation (FP) assays

(BellBrook Labs) have also been designed to measure ADP generation. The TR-FRET assay (Cisbio) currently employs a terbium-conjugated monoclonal antibody.

Cell-based kinase assays

While biochemical assays are the standard for primary HTS to identify kinase inhibitors, cell-based assays are critical in validating the leads from HTS. This is partly because the physiological function of kinases is dependent on numerous factors, such as cellular location, and interaction with adaptor and regulatory proteins. Importantly, cell-based assays can also determine whether compounds identified in initial screening efforts are cell permeable and if potential cellular toxicity is apparent. In the past, measurement of kinase activity involved loading cells with 32P-labelled ATP and analysing kinase activation by incorporation of 32P into cellular substrates. This approach provided information on the entire gamut of potential phosphorylation targets for a kinase. A non-radiometric modification of this approach is widely used today as a proteomic assay to identify kinase targets. Thus, as opposed labelling ATP pools with 32P-ATP, phosphorylation is accessed by MALDI-TOF mass spectroscopy and specificity determined by silencing kinase expression using RNAi. However, such approaches are used primarily for research and are not easily adapted for compound screening.

Competition immunoassays can also be employed to assess protein phosphorylation in response to cellular kinase activation. ELISA assays are widely used to distinguish phosphorylated proteins from those that are non-phosphorylated, but are limited in their use in HTS due to the multiple wash and separation steps required. An analogous homogeneous phase format uses AlphaScreen SureFire® technology (TGR Biosciences Ltd, PerkinElmer). This is a bead-based proximity assay for detecting phosphoproteins[51] (**Figure 2**) and quantitates the levels of phosphosubstrate in the crude cell lysate. It is easily adapted to simple microtiter based assay protocols, providing a cell-based HTS assay platform to screen for putative inhibitors.

Cellular FRET is an alternative approach for measuring kinase-induced activity in cells[52]. Cellular FRET has been used successfully with the C-kinase activity reporter (CKAR) of PKC, consisting of a peptide substrate of the kinase fused to cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and an FHA2 phosphopeptide-binding module, which is subsequently expressed in the fusion protein in target cells. A FRET signal is detected when the substrate is non-phosphorylated, while the FRET response is lost when PKC phosphorylates CKAR. The assay can be further modified so that CKAR is targeted to different cellular locations, such as the plasma membrane, cytosol, mitochondria, Golgi and nucleus, using sub-cellular targeting sequences. This technology has been useful, for example, to characterise compounds that can discriminate distinct functions of PKC[53].

Cellular imaging to measure kinase activity

Besides measuring protein phosphorylation, kinase activity can be detected using other techniques. As described above, interaction of kinases with other cellular proteins is critical for their activity and illustrates the complex pathways by which they regulate cellular function. Cell-based assays that can measure protein-protein interactions provide a way to detect the activation of different protein kinases and also yield assay formats that can be used to identify drugs that modulate activity without necessarily directly interacting with the catalytic domain of kinases. For example, if a compound inhibits the interaction of NF-κB essential modifier (NEMO) with IκB kinase, it could prevent kinase activation but not the basal activity. Such allosteric regulators may provide a number of therapeutic advantages over more classical drugs that directly affect the catalytic domain of kinases. Several technologies employing either FRET or bioluminescence resonance energy transfer (BRET) as assay readouts have been developed for assessing protein-protein interactions to provide other measures of protein kinase activity.

Cellular FRET has been used to study protein kinase dynamics notably the interactions of the MAPKs, ERK1/2 kinase and MEK kinase in intact cells. An assay was reported[54] to study intermolecular changes in ERK1/2 conformation by placing FRET probes (yellow and cyan fluorescent proteins) at the ends of ERK1/2. The FRET signal is minimal when ERK2 is in an open conformation, but a conformational change is induced when ERK1/2 binds to MEK and gets activated as a result of this interaction, causing an increase in FRET signal. Thus, this simple assay can be employed as a cell-based assay to identify inhibitors of the activation of ERK1/2 by MEK. Presumably this technology could be extended to study molecular interactions of other protein kinases and assess the effect of external stimulation of their activation and translocation, and can be further developed into high content screening assays for a range of different protein kinases.

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FRET assays configured for HTS protocols have also been used to measure protein-protein interactions using a protein fragment complementation assays (PCA, Odyssey Thera)[55]. This technology employs fragments of two protein reporters recombinantly fused to interacting proteins. The fragments themselves are inactive, but when target proteins to which they are fused associate they form an active biosensor that can be measured via a fluorescent signal. This technology has been extended for use with other reporters including β-lactamase, *Renilla* luciferase, green fluorescent protein (GFP), and yellow fluorescent protein (YFP) to detect intracellular protein-protein interactions[56].

As indicated above, kinases translocate between different cellular compartments upon activation, a process that is necessary for many kinases since their substrates are in a different cellular location than where they are activated. Monitoring the movement of kinases provides a means to detect different functions of kinases and also yields approaches to discover novel drugs capable of blocking their cellular functions, notably translocation. Imaging technologies, such as those employing confocal microscopy, have been available for several years to measure cellular movement of proteins[57]. While confocal microscopy is widely employed for research purposes, novel technologies have been developed to allow this approach for HTS using instrumentation, including high throughput confocal instruments such as the Opera™ (PerkinElmer), which can be used to detect protein translocation within individual cells[58].

Conclusion

The knowledge gained on the biology of kinases provides novel insights into developing small molecule kinase regulators that are more specific, and potentially more subtle, in their action than inhibitors that are presently available. Since these compounds can be used to block unique sites of catalytic domains to put kinases in inactive conformations, they are more selective than those currently available. Similarly, approaches to developing drugs that block the substrate binding domain of kinases could provide a high degree of selectivity and assure that mutations that develop in the kinase would not hinder drug action. Screening technologies are also becoming available to target allosteric regulatory sites of kinases to either inhibit or activate them. Such sites may confer a high degree of specificity given that the structures of the allosteric sites are unique for each kinase. While growth factor and cytokine receptors are important for normal physiological control of cells and are implicated in numerous diseases, the identification of small molecule regulators of these protein kinases has been limited to date. Antibodies have been developed to sequester circulating growth factors and inhibit growth factor receptor binding; however, there are many drawbacks of using protein therapeutics, including cost, immune reactivity and toxicity.

Taken together, the kinome has emerged as one of the most important targets in drug discovery. Based on emerging discoveries in the field, one would predict that the kinome will continue to play an important role in developing kinase therapies well into the future. DDW

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