CHAPTER SIX

Fluorescent Sensors of Protein Kinases: From Basics to Biomedical Applications

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Abstract

Protein kinases constitute a major class of enzymes underlying essentially all biological processes. These enzymes present similar structural folds, yet their mechanism of action and of regulation vary largely, as well as their substrate specificity and their subcellular localization. Classical approaches to study the function/activity of protein kinases rely on radioactive endpoint assays, which do not allow for characterization of their dynamic activity in their native environment. The development of fluorescent biosensors has provided a whole new avenue for studying protein kinase behavior and regulation in living cells in real time with high spatial and temporal resolution. Two major classes of biosensors have been developed: genetically encoded single-chain fluorescence resonance energy transfer biosensors and peptide/protein biosensors coupled to small synthetic fluorophores which are sensitive to changes in their environment.

In this review, we discuss the developments in fluorescent biosensor technology related to protein kinase sensing and the different strategies employed to monitor protein kinase activity, conformation, or relative abundance, as well as kinase regulation and subcellular dynamics in living cells. Moreover, we discuss their application in biomedical settings, for diagnostics and therapeutics, to image disease progression and monitor response to therapeutics, in drug discovery programs, for high-throughput screening assays, for postscreen characterization of drug candidates, and for clinical evaluation of novel drugs.

ABBREVIATIONS

AFP autofluorescent proteins
AMPK AMP-activated protein kinase
ATM Ataxia telangiectasia mutated kinase
BiFC bimolecular fluorescence complementation
BTF beta-turn-focused
CaMKII calcium/calmodulin-dependent protein kinase II
CDK cyclin-dependent kinase
CFP cyan fluorescent protein
CHEF chelation-enhanced fluorescence
CML chronic myelogenous leukemia
CPP cell-penetrating peptide
EGFP enhanced green fluorescent protein
EGFR epidermal growth factor receptor
DPA dipicolylamine
ELISA enzyme-linked immunosorbent assay
ERK extracellular signal-regulated kinase
FAK focal adhesion kinase
FHA domain forkhead-associated domain
FLIM fluorescence lifetime imaging
FRET fluorescence resonance energy transfer
GFP green fluorescent protein
GLUT Glutamate Transporter

HCS high content screening
HTS high-throughput screening
KAR kinase activity reporter
NBD nitrobenzofuran
NES nuclear export sequence
NIRF near-infrared fluorescence
NLS nuclear localization sequence
PAABD phosphoamino acid-binding domain
PBD Polo Box Domain
PKA cAMP-dependent protein kinase A
PKB protein kinase B also known as Akt
PKC protein kinase C
PKD protein kinase D
PTB phosphotyrosine-binding domain
PTK protein tyrosine kinase
RDF recognition-domain-focused
RFP red fluorescent protein
RGD Cyclic Arginine-Glycine-Aspartate
SAPK stress-activated protein kinases
SH2 domain Src-homology 2 domain
SH3 domain Src-homology 3 domain
Sox sulfonamide-oxine
YFP yellow fluorescent protein

1. INTRODUCTION

1.1. Protein kinases—Structure, function, and mechanism of action

The first report of a phosphorylated protein was the identification of phosphorylated vitellin (phosvitin) in 1906, followed by the detection of phosphorylated casein in 1933.[1,2] Yet the enzymatic process of protein phosphorylation itself was described only in 1954, with the identification of an enzyme from rat liver mitochondria, namely, glycogen phosphorylase, that could catalyze the transfer of phosphate from adenosine triphosphate (ATP) to protein substrates, together with a complete characterization of the conditions underlying this process, and reference made to emphasize the high turnover rate of phosphorus and the reactivity of phosphoproteins in tumors.[3] Today, we know that protein phosphorylation is the consequence of a fine balance between the activities of protein kinases that add phosphate groups and protein phosphatases that remove phosphate groups from protein substrates, and that these activities are responsible for phosphorylation of up to 30% of all cellular proteins.[4–7]

Protein kinases catalyze the transfer of a phosphate group from ATP onto an amino acid of a substrate protein with a free hydroxyl group, namely, serine, threonine, and tyrosine (Fig. 6.1A). As such, protein kinases are classically divided into the families of serine/threonine kinases, tyrosine kinases, and dual-specificity kinases.[8] Protein kinases are further divided into either receptor or nonreceptor kinases, depending on whether they are localized at the surface of cells and play a role in integrating extracellular cues to be fed into the cell. The human genome is estimated to harbor 514 genes encoding protein kinases, known as the kinome, which corresponds to 2% of all eukaryotic genes.[9] Protein kinases are central to the regulation of most cellular processes, throughout development and cell differentiation, metabolism, gene transcription, cell growth and division, DNA replication, protein degradation, apoptosis, and immune response.[10,11] They play a major role in

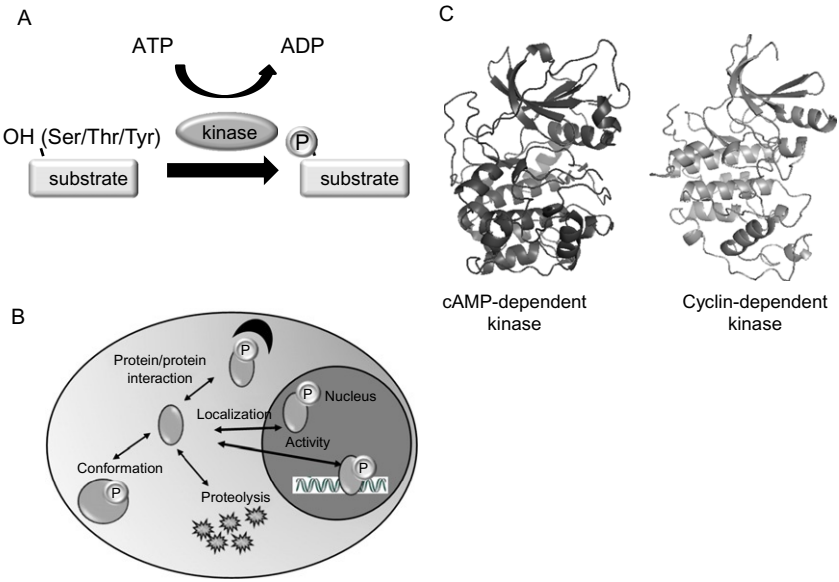

Figure 6.1 *Protein kinase: structure, function, and mechanisms of action*. (A) A protein kinase is an enzyme that catalyzes the transfer of a phosphate group from ATP to a substrate protein, a process known as phosphorylation. This reaction consists in the removal of a phosphate group from ATP, followed by its covalent attachment to amino acids with a free hydroxyl group, namely, serine, threonine, and tyrosine. (B) Phosphorylation usually results in a functional change of the target protein (substrate) by changing enzyme activity, conformation, subcellular localization, or association with partners. (C) Structural fold of two protein kinases—cAMP-dependent kinase (PDB structure 2CPK) and cyclin-dependent kinase 2 (PDB structure 1B38).

signal transduction pathways in healthy cells, and their activity is normally tightly controlled and finely regulated. Phosphorylation affects substrate proteins in a variety of ways, by affecting their activity, subcellular localization, or their stability through routing to the ubiquitin–proteasome pathway for degradation, by promoting a conformational change in the structure of the substrate, its association with cofactors, partner proteins, inhibitors, or gene promoters[12] (Fig. 6.1B).

Protein kinases present a very similar structural fold, yet a high degree of structural plasticity.[13–16] Indeed, the structural conformation of active kinases differs from that of inactive kinases, yet the catalytic domains of active kinases adopt similar structures (Fig. 6.1C). Despite this structural homology, the primary sequences and modes of regulation vary widely from one kinase to another. Their specificity toward substrate recognition is equally very fine-tuned, and many kinases use binding sites that are remote from the catalytic site to recruit substrates with higher affinity, thereby leading to greater catalytic efficiency and specificity.[17,18]

Protein kinase activity is finely regulated through a variety of mechanisms, including activating phosphorylation (cyclin-dependent kinases, CDKs) or inhibitory phosphorylation (Src); through dimerization or multimerization (insulin receptor, EGF receptor); through allosteric regulation, upon binding of activator or inhibitor proteins, cofactors, or small molecules; through control of accessibility to their substrates; or through autoinhibition (PKA/PKI).[16] Protein kinases are "molecular switches" which may adopt at least two conformations corresponding to an "ON" state and an "OFF" state. Activation is associated with a net conformational change of a dynamic segment, termed "the activation loop", thereby providing an accessible binding site for the substrate.[16,19]

Protein kinases have been the focus of many studies not only because of their central role in major signaling pathways and biological processes but also because of their implication in disease. Indeed, a large number of genetic alterations result in deregulated protein kinase activity, thereby prompting or contributing to the development of pathological conditions, including cardiovascular diseases, neurodegenerative and endocrinological disorders, immune deficiency and viral infection, psoriasis, cancer, and diabetes.[20–23] As such, protein kinases constitute disease biomarkers and pharmacological targets in a variety of human diseases, and several small-molecule inhibitors have been developed to interfere with their function, some of which have become therapeutics administered in the clinic.[21–28]

1.2. Strategies for probing and studying protein kinases *in vitro* and *in cellulo*

Despite the central role of protein kinases in physiological pathways, and their implication as disease biomarkers in pathological disorders, there are very few means of studying their activity in real time in a noninvasive fashion and in their natural environment. Most kinase activity studies have been traditionally performed using biochemical assays based on purified enzymes produced as recombinant proteins from insect or mammalian cells in culture. Commonly implemented approaches to study protein kinase activity rely essentially on the incorporation of radioactive phosphate into artificial substrates, or on antigenic approaches, which further depend on highly specific antibodies to recognize the phosphorylated form of the kinase substrate(s). These assays have been widely used in the laboratory and further adapted to high-throughput drug screening, but they are irreversible endpoint assays, which do not allow for real-time analysis of kinase activity, and further lack the physiological cellular environment. Cell-based methods that monitor kinase activity have been developed that rely on the incorporation of ^{32}P into cells; this approach requires cell lysis, substrate protein isolation, and purification to determine its relative degree of phosphorylation by measuring the amount of radioactivity incorporated. However, such cell-based assays are labor intensive, show poor sensitivity, and have the disadvantage of requiring high levels of radioactivity. Other cell-based assays for the study of kinase activity involve the use of radiolabeled phosphorylation-specific antibodies (i.e., antibodies that can discriminate between phosphorylated and nonphosphorylated proteins); the phosphorylated substrate can then be detected and quantified by immunoprecipitation, gel electrophoresis, or Western blotting after cell lysis. Although these assays generally require lower levels of radioactivity than ^{32}P-based methods, they are equally labor intensive, time consuming, and complex to automate. Nonradioactive cell-based methods have emerged that use an ELISA (enzyme-linked immunosorbent assay) approach to measure the activation of specific kinase signaling pathways. These kinase assays, which employ phosphorylation-specific antibodies, have been demonstrated to be suitable for high-throughput drug screening.[29] More recently, a nonradioactive method for probing kinase activity has been developed, which is based on the use of a fluorogenic peptide substrate (rhodamine 110, bis peptide amide) that is cleaved in its unphosphorylated form, thereby releasing free rhodamine 110; phosphorylation prevents cleavage, and the compound remains as a nonfluorescent peptide conjugate.[30] This approach has been successfully applied to

high-throughput screening (HTS) and does not require cell lysis. However, it does not allow for real-time studies, as the nonphosphorylated substrates are cleaved and degraded. Moreover, besides their disruptive character, these approaches merely provide a snapshot of a dynamic process and do not provide real insights into the dynamics and enzymatic kinetics of the target kinase in its native environment.

Faced with a real need for tools that enable qualitative and quantitative assessment of enzymatic activities in their native environment, biologists and chemists have designed a new generation of tools designated as "fluorescence-based reporters" or "fluorescent biosensors," which enable probing the biochemical function, activity, and dynamics of specific enzymes through sensitive changes in their fluorescent properties. Fluorescence detection is currently one of the most widely used approaches in biomolecular imaging because of its high intrinsic sensitivity and selectivity. Indeed, fluorescence lends itself to nondestructive imaging, thereby preserving the sample and the molecules of interest within. Moreover, fluorescence imaging provides a high degree of temporal and spatial resolution and allows for real-time tracking of a molecule in motion in a complex solution or environment.

As such, when fluorescent biosensors are designed to recognize a target with high specificity and to report on this event with high sensitivity, they constitute extremely useful tools for the detection of biomolecules, both *in vitro* and *in vivo*, and for monitoring dynamic molecular events, such as enzymatic activities, conformational changes, or protein/protein interactions.[31] High-resolution imaging and real-time measurements of fluorescent biosensors provide precious information on the spatial and temporal localization of a wide variety of intracellular targets and have proved a successful means of visualizing dynamic processes in living cells. Fluorescent biosensors that specifically probe protein kinases allow imaging the behavior of kinases in a dynamic and quantitative fashion, in space and in time, in cells, tissues, and whole organisms, thereby allowing tackling questions that could not be addressed previously. In addition, fluorescent reporters and biosensors can be exploited to develop high-throughput and high content screens for drug discovery purposes and for further assessing the efficacy and pharmacokinetics of lead compounds of potential therapeutic utility. Moreover, fluorescent biosensors offer promising perspectives for diagnostic strategies, for early detection of protein kinase dysregulation associated with the onset of disease, and for monitoring disease progression and response to therapeutics.

Two large classes of kinase biosensors have been developed: genetically encoded autofluorescent protein (AFP) biosensors and nongenetic fluorescent

peptide biosensors. The discovery of the green fluorescent protein (GFP) led to the development of genetically encoded reporters, which have been successfully applied to the study of protein kinase behavior in living cells with high spatial and temporal resolution (for review, see Refs. 32–36). In parallel, combined efforts in fluorescence chemistry and in chemical biology have led to the design of an entirely different family of biosensors, based on peptide, polypeptide, or polymeric scaffolds, onto which small synthetic probes with particularly attractive photophysical properties can be incorporated. These nongenetic biosensors have been applied to monitor protein kinase activities *in vitro* and in more complex biological samples, including cell extracts and living cells, with an equally successful outcome (for review, see Refs. 35–38). While biosensors from each of these classes exhibit specific advantages, they equally suffer from characteristic drawbacks and shortcomings, which will be discussed further below and are summarized in Table 6.1. Genetically encoded biosensors are user-friendly, allowing for easy manipulation and transfection into cells. However, most of these biosensors are based on changes in fluorescence resonance energy transfer (FRET) between two AFPs, which are often fairly weak compared to the fluorescence changes associated with nongenetic, environmentally sensitive biosensors. In contrast, whereas peptide and protein biosensors are easy to engineer and handle *in vitro*, it is difficult to introduce these into living cells and *in vivo*. However, they offer complete control over the concentrations used and the timing of their availability *in cellulo*, whereas genetically encoded biosensors are heterogeneously expressed both in terms of levels and timing.

2. GENETICALLY ENCODED REPORTERS OF PROTEIN KINASES

The discovery of the GFP and its engineering into a wide variety of AFPs prompted the development of GFP-based reporters and the design of genetically encoded biosensors, which were rapidly applied to the field of protein kinases.[32–36,39–45] Several groups of genetically encoded biosensors have been developed (for review, see Refs. 34,44): (i) single-chain biosensors, which bear a pair of FRET AFPs within the same molecule that are brought together and undergo energy transfer due to intramolecular conformational changes in response to the recognition event; (ii) two-chain biosensors, in which two AFPs lie on two different molecules capable of interacting and undergoing intermolecular FRET

Table 6.1 Pros and cons of genetically encoded versus fluorescent peptide biosensors

Genetically encoded fluorescent biosensors	**Fluorescent peptide/protein biosensors**
Features	Features
• Genetically encoded constructs based on two AFPs • Essentially FRET/FLIM	• Peptide or protein backbone onto which a synthetic fluorescent probe is introduced during or postsynthesis through chemical or enzymatic conjugation • Environmentally sensitive, chelation-enhanced, self-reporting
Pros	Pros
• Easy to engineer through molecular biology approaches • Easy to transfect or microinject into cultured cells	• Small size • Versatility in design and engineering • Easy and cheap to synthesize or express through recombinant protein engineering • Allow control over concentration and time of addition to target or cells • Allow technological improvements: introduction of quenchers, amino acid caging
Cons	Cons
• Large size—essentially due to the size of the autofluorescent proteins • Require ectopic expression • Lack of control over expression levels and timing • Heterogeneous expression in a population of cells • Difficult to obtain optimal expression for *in vivo* imaging	• Require means for efficient introduction into living cells and *in vivo* • Require choice of NIRF probes for *in vivo* applications

when the two chains are brought together for a protein/protein interaction; (iii) biosensors based on bimolecular fluorescence complementation (BiFC), in which two fragments of a split AFP are brought together as a consequence of the recognition event, thereby reconstituting the intact and fully fluorescent protein; (iv) single-chain biosensors which bear a single AFP whose spectral properties change in response to the recognition of a target by an encoded element other than the AFP; and (v) biosensors

constituted by the AFP itself, whose spectral properties respond directly to the target or analyte, as pH-dependent or redox-dependent AFP biosensors (Fig. 6.2).

The most widely developed genetically encoded biosensors developed to probe protein kinase activities are single-chain FRET biosensors that report on kinase activity through phosphorylation-induced changes in FRET between

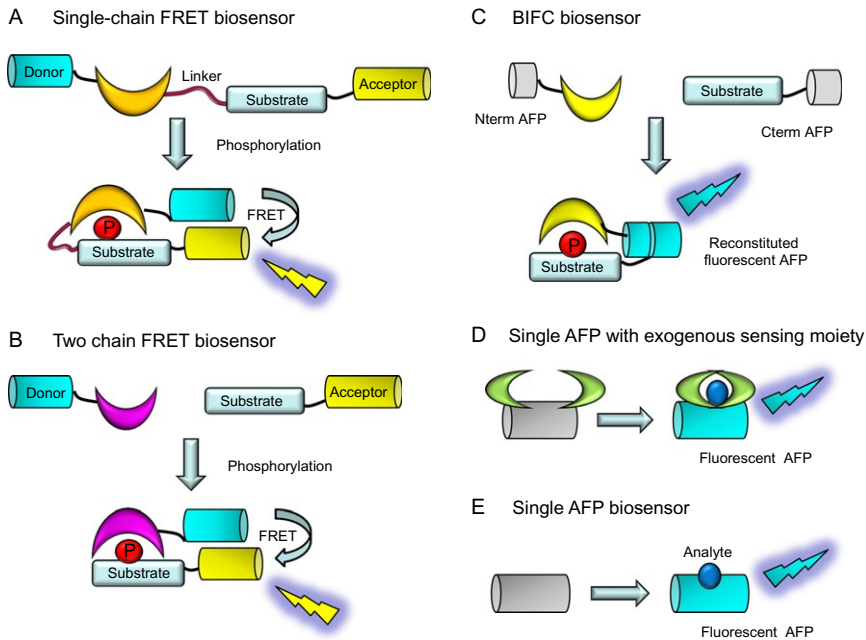

Figure 6.2 Genetically encoded biosensors. Genetically encoded biosensors of kinase activity are derived from at least one genetically encoded autofluorescent protein. (A) Single-chain biosensors bear a pair of AFPs, within the same molecule, which are brought together and undergo changes in fluorescence resonance energy transfer due to intramolecular conformational changes in response to the phosphorylation event. (B) Two-chain biosensors, in which two AFPs lie on two different molecules susceptible for interacting and undergoing intermolecular FRET when the two chains are brought together for a protein/protein interaction. (C) Biosensors based on bimolecular fluorescence complementation (BiFC), in which two fragments of a split AFP are brought together as a consequence of target recognition, or in this case phosphorylation, thereby reconstituting the intact and fully fluorescent protein. (D) Single-chain biosensors composed of a single AFP whose spectral properties change in response to the recognition of a target by an exogenous sensing element (other than the AFP). (E) Single AFP biosensors, whose spectral properties respond directly to the target or analyte, such as pH-dependent or redox-dependent AFP biosensors.

two AFPs due to an intramolecular conformational change. A wide variety of these genetically encoded biosensors have been developed (see Table 6.2 and Figs. 6.3–6.6), but the basic structure of these kinase activity reporters (KARs) is essentially the same: a kinase-specific substrate sequence bearing a consensus phosphorylation site connected to a matching phosphoamino acid-binding domain (PAABD) by a flexible linker and flanked by a pair of AFPs that can transfer fluorescence resonance energy between each other. Following phosphorylation of the substrate sequence by the kinase, the PAABD binds the phosphorylated sequence, thereby promoting an intramolecular conformational change which alters the distance and orientation of the AFPs.

Several factors have to be considered when aiming to design an optimally responsive and selective genetically encoded FRET biosensor. Moreover, the specificity and selectivity of a kinase/biosensor couple should always be characterized *in vitro* and *in cellulo*.

First, needless to say, the size and sequence of the substrate have to be tailored to the most suitable sequence to gain the highest level of selectivity. The choice of the substrate sequence is essential for selectivity and may either be identified through an *in silico* approach through database mining from knowledge-based libraries, or simply through rational design of a sequence derived from a known substrate protein of a given kinase. The specificity of a kinase for a protein substrate is generally dependent on other regions of the protein that "dock" onto the kinase to offer higher affinity/recognition. Therefore, several designs include a docking domain distinct from the substrate sequence, so as to increase specificity for the target kinase while also increasing the efficiency of phosphorylation of the substrate by the kinase. This is well exemplified by MAPK protein kinases (ERK, p38, and JNK), which have very similar phosphorylation sites, yet distinct docking sites that can be made use of to improve substrate targeting and selectivity.[81,82]

Second, the choice of the PAABD is critical for an efficient and sensitive biosensor. The sequence of the PAABD should present high affinity and efficient recognition of the phosphorylated substrate, as opposed to poor affinity for the unphosphorylated substrate, and should display a fully reversible behavior between both forms. Several modular phosphobinding domains (PBDs) have been well characterized, including Src-homology 2/3 (SH2 SH3) and phosphotyrosine-binding (PTB) domains, 14.3.3 proteins, forkhead-associated (FHA) domains, WW domains, WD40 domains, and the PBD of Plk1 (for reviews, see Refs. 83–89).

Table 6.2 Genetically encoded single-chain FRET biosensors of protein kinases

Protein kinase	Biosensor name	AFP FRET pairs	Author and year	Reference
Abl/EGFR	CrkII-based reporter	CFP/YFP	Ting *et al.* (2001)	46
c-Abl	Picchu	CFP/YFP	Kurokawa *et al.* (2001)	47
Bcr–Abl	Pickles	ECFP/ Venus	Mizutani *et al.* (2010)	48
Aurora B		CFP/YFP	Fuller *et al.* (2008)	49
AKT	AKTAR	Cerulean/ cpVenus	Gao and Zhang (2008)	50
AKT	AKTUS	CFP/YFP	Sasaki *et al.* (2003)	51
AKT	BKAR	ECFP/ citrine	Kunkel *et al.* (2005)	52
AMPK	AMPKAR	ECFP/ cpVenus	Tsou *et al.* (2011)	53
ATM	ATOMIC	CFP/YFP	Johnson *et al.* (2007)	54
CAMKII	Camui	CFP/YFP	Erickson *et al.* (2011)	55
CDK1/cyclin B activity		mCerulean/ Ype	Gavet and Pines (2010)	56
ERK	EKAR	EGFP/ mRFP1	Harvey *et al.* (2008)	57
ERK1	Erkus	CFP/YFP	Sato *et al.* (2007)	58
IR	Phocus	CFP/YFP	Sato *et al.* (2002)	59
JNK kinase	JNKAR	EGFP/ citrine	Fosbrink *et al.* (2010)	60
FAK kinase		ECFP/YPet	Seong *et al.* (2011)	61
Histone phosphorylation		CFP/YFP	Lin and Ting (2004)	62
MLCK	MLCK-FIP (Ca^{2+}/ calmodulin)	CFP/YFP	Chew *et al.* (2002)	63

Table 6.2 Genetically encoded single-chain FRET biosensors of protein kinases—cont'd

Protein kinase	Biosensor name	AFP FRET pairs	Author and year	Reference
PKA	ART	BGFP/RGFP	Nagai *et al.* (2000)	64
PKA	AKAR1	ECFP/YFP	Zhang *et al.* (2001)	65
PKA	AKAR2	ECFP/citrine	Zhang *et al.* (2005)	66
PKA	AKAR	EGFP/cpVenus	Allen and Zhang (2006)	67
PKA (sarcoplasmic reticulum)	SR-AKAR3	CFP/YFP	Liu *et al.* (2011)	68
PKC	CKAR	ECFP/citrine	Violin *et al.* (2003)	69
PKC-delta	deltaCKAR	CFP/YFP	Kajimoto *et al.* (2010)	70
PKC	KPC-1 (pleckstrin based)	GFP/EYFP	Schleifenbaum *et al.* (2004)	71
PKA and PKC	KPAC-1 (pleckstrin based)		Brumbaugh *et al.* (2006)	72
PKD	DKAR	CFP/YFP	Kunkel *et al.* (2007)	73
Plk1		CFP/YFP	Macůrek *et al.* (2008)	74
SAP3K		Venus/SECFP	Tomida *et al.* (2009)	75
Src		CFP/YFP	Wang *et al.* (2005)	76
c-Src	Srcus	CFP/YFP	Hitosugi *et al.* (2007)	77
SYK		ECFP/YPet	Xiang *et al.* (2011)	78
ZAP-70	ROZA	CFP/YFP	Randriamampita *et al.* (2008)	79

The linker between these domains is generally designed to be flexible (i.e., rich in glycine, alanine, and proline residues), and its length has proved to be a critical factor in affecting FRET efficiency.[90–92]

Last but not least, the choice of the AFP/FRET pair is essential. Ideally, AFPs that do not photobleach too rapidly, that have high quantum yield, and that are red-shifted should be chosen to maximize FRET efficiency and minimize phototoxicity.[93,94] Komatsu *et al.* recently proposed an optimized backbone for genetically encoded FRET biosensors bearing the cyan fluorescent protein (CFP) or Turquoise as donors, the yellow fluorescent protein (YFP) or YPet as an acceptor fluorophore, and a very long linker connecting the ligand to the sensor domain, which basically abolishes dimerization of the FRET pair, and showed that this backbone was ideal for both kinase and GTPase FRET biosensors reporting on ERK, PKA, S6K, RSK, Ras, and Rac1.[92]

In this section, we provide several examples to illustrate the different strategies that have been devised in designing genetically encoded FRET biosensors and describe how they have been employed to study the behavior of specific kinases in living cells, rather than attempt an exhaustive review of all the kinase biosensors that have been developed so far.

2.1. Phosphotyrosine kinase (PTK) biosensors

Ting *et al.* developed the first genetically encoded fluorescent reporters of tyrosine kinases to probe the activities of Src, Abl, and EGFR (epidermal growth factor receptor). These single-chain FRET biosensors consist of fusions of the CFP/YFP FRET pair, an SH2 phosphotyrosine-binding domain, and a substrate sequence, the phosphorylation of which on a critical tyrosine promotes binding to the SH2 domain, thereby inducing a conformational change that brings the two AFPs close enough to undergo FRET between the donor and the acceptor. While the EGFR and Src indicators were designed by combining an SH2 domain with an appropriate substrate, separated by a linker, the Abl reporter was directly engineered by introducing a domain derived from Crk protein, which includes an SH2 domain, two SH3 domains, and an intervening Abl phosphorylation site[46] (Fig. 6.3A and B). The Picchu sensor was constructed in a similar fashion to monitor specific phosphorylation by c-Abl, with a CFP/YFP FRET pair and an SH2–2 SH3 domain derived from the CrkII adaptor protein.[47] More recently, Mizutani *et al.* developed a sensitive and specific FRET biosensor, termed Pickles, for measuring the tyrosine kinase activity of Bcr–Abl, which

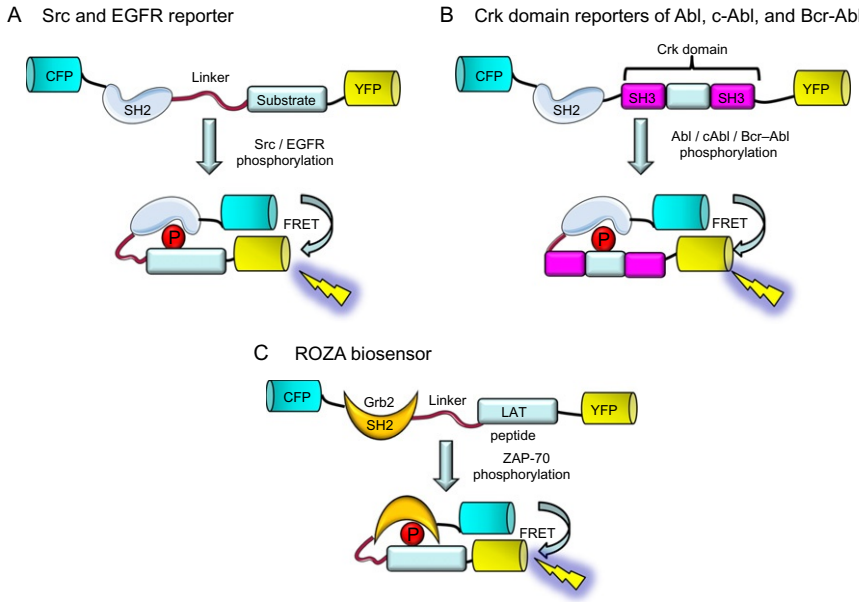

Figure 6.3 *Genetically encoded protein tyrosine kinase reporters*. Genetically encoded FRET biosensors of tyrosine kinases are composed of a substrate sequence and a matching phosphoaminoacid binding domain sandwiched between a FRET pair of GFP variants. (A) Fluorescent reporters of Src and EGFR (epidermal growth factor receptor), based on an SH2 phosphotyrosine-binding domain and a separate substrate sequence joined by a linker and flanked by the CFP/YFP couple.[46] (B) Structure of fluorescent reporters of Abl,[46] c-Abl (Picchu),[47] and Bcr–Abl (pickles),[48] derived from Crk adaptor proteins bearing an SH2 domain, two SH3 domains, and an intervening phosphorylation site. (C) ZAP-70 biosensor ROZA constituted of the SH2 domain of mouse Grb2 and a peptide substrate sequence derived from LAT sandwiched between the CFP–YFP pair.[79]

again incorporates one SH2 and two SH3 domains derived from CrkL, the most characteristic substrate of Bcr–Abl, sandwiched between Venus, a variant of YFP, and ECFP[48] (Fig. 6.3B). The high sensitivity of this biosensor allows the assessment of Bcr–Abl activity from patient cells to monitor the disease status and response to therapy, as well as the detection of the onset of drug-resistant cells within a heterogeneous population.[95,96]

Src is a nonreceptor tyrosine kinase that is activated by a variety of mechanisms and constitutes a major kinase in the signaling pathways involved in cancer and metastasis.[97] An Src-specifc biosensor based on an ECFP/YFP couple, an SH2 domain, and a substrate peptide derived from the c-Src substrate p130cas was developed to probe this kinase and visualize its dynamics

of activation following mechanical stimuli.[76] More recently, a different variant, Srcus, was engineered including a nuclear export signal, which was employed to study Src activation by steroids in the cytosol and at the plasma membrane.[77] Another tyrosine kinase of particular relevance to cancer is the spleen tyrosine kinase Syk.[98] A FRET-based biosensor was developed to image Syk tyrosine kinase activity in living cells based on an ECFP/YPet FRET couple, a substrate derived from VAV2 and an SH2 PAABD.[78] This biosensor allowed following and quantifying real-time activation of Syk, associated with an increase in the FRET signal, upon immunoreceptor activation and following stimulation by the platelet-derived growth factor.

In order to examine the dynamics of the ZAP-70 tyrosine kinase activity at the immunological synapse, Randriamampita *et al.* developed the ROZA biosensor (*R*eporter *O*f *Z*AP-70 *A*ctivity). This single-chain FRET biosensor bears an SH2 domain from mouse Grb2 and a peptide substrate sequence from mouse LAT sandwiched between the CFP–YFP pair (Fig. 6.3C). ROZA was applied to image ZAP-70-dependent phosphorylation in T-cell lines and primary human lymphocytes with subcellular resolution during the formation of an immunological synapse.[79]

2.2. Biosensors for protein kinase PKA, PKB, PKC, and PKD

PKA (cAMP-dependent protein kinase) constitutes an essential enzyme involved in major biological processes including gene expression, metabolism, cell growth, and cell proliferation.[99] Genetically encoded single-chain FRET biosensors of PKA, combining the consensus kinase substrate sequence, with a matching PAABD sandwiched between a FRET pair of GFP variants, were amongst the first kinase reporters developed to characterize their endogenous activity in living cells in a dynamic fashion. The first genetically encoded biosensor of PKA activity, termed ART (cAMP-responsive tracer), was composed of BGFP/RGFP, the kinase-inducible domain (KID) of the CREB transcription factor (cAMP-responsive element-binding protein), which undergoes a conformational change upon phosphorylation[64] (Fig. 6.4A). As opposed to most other FRET biosensors, phosphorylation of KID by PKA disrupts the FRET between the flanking AFPs. An entirely different biosensor called AKAR (cAMP-dependent KAR) was later generated by Zhang *et al.*[65] (Fig. 6.4B). AKAR1 was based on a 14-3-3 phosphorecognition domain but was later optimized and replaced by an FHA domain in AKAR2,[66] so as to improve the reversibility of the biosensor and therefore its response to cellular phosphatases, for

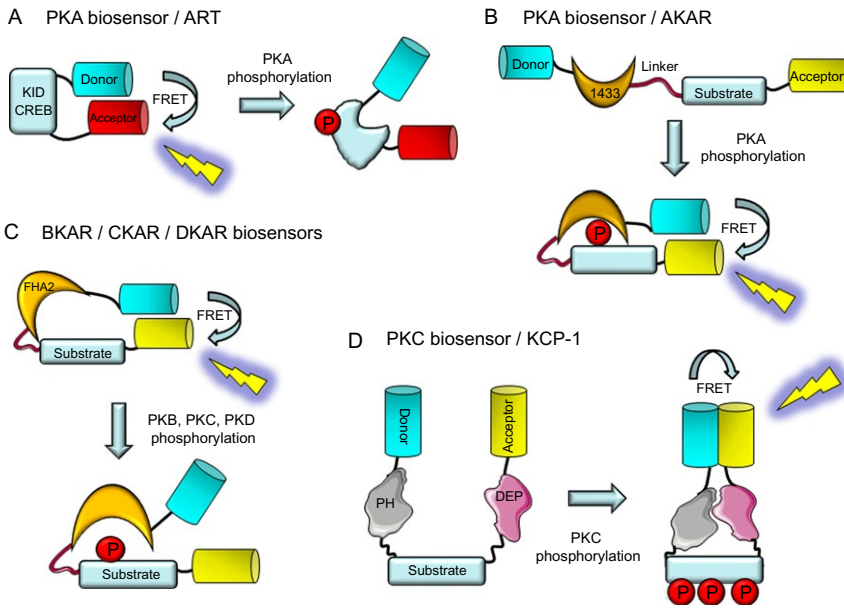

Figure 6.4 *Genetically encoded biosensors of PKA, PKB, PKC, and PKD.* (A) ART: genetically encoded biosensor of PKA activity, composed of two AFPs joined by the kinase-inducible domain (KID) of the CREB transcription factor (cAMP-responsive element-binding protein).[64] Phosphorylation of KID by PKA promotes a conformational change which promotes FRET between the flanking AFPs. (B) PKA biosensor AKAR1 based on a 1433 PAABD and a substrate peptide of PKA sandwiched between a CFP/YFP pair.[65,66] (C) BKAR, CKAR, and DKAR biosensors of PKB, PKC, and PKD kinases, respectively.[52,69,73] These reporters are constituted of a consensus phosphorylation sequence and the FHA2 domain of Rad53 sandwiched between an mCFP/mYFP pair that undergoes FRET in the unphosphorylated state; phosphorylation of the substrate sequence results in a conformational change that alters the FRET ratio. (D) PKC biosensor KCP-1 based on a GFP/EYFP FRET pair, and a domain derived from pleckstrin constituted by a PH domain and a DEP domain flanking a loop that bears several phosphorylation sites.[71] Upon phosphorylation of this intervening loop by PKC, the pleckstrin moiety undergoes a conformational change, which prompts the PH and the DEP domains to interact.

measuring the cellular dynamics of PKA activity.[67,100] More recently, a variant SR-AKAR3 was developed to monitor PKA activity in the sarcoplasmic reticulum in cardiomyocytes.[68]

PKC kinases form a family of 15 isoforms that contribute to well-defined signaling pathways.[101] The first genetically encoded PKC biosensor, CKAR, was designed to incorporate a consensus phosphorylation sequence

and the FHA2 domain of Rad53 sandwiched between the mCFP/mYFP pair, which undergo FRET in the unphosphorylated state, whereas phosphorylation of the substrate sequence results in a conformational change that alters the FRET ratio[69] (Fig. 6.4C). Targeting of this reporter to the plasma membrane where PKC is activated revealed an oscillatory phosphorylation in response to histamine, which is closely associated with a calcium oscillation. This original biosensor was not designed to discriminate between the different isoforms of PKC. However, more recent developments have led to reporters designed specifically to monitor PKCdelta activity.[70] A different strategy was employed by Schultz *et al.* To develop the KCP-1 biosensor, a PKC reporter that incorporates the GFP/EYFP FRET pair, together with a truncated form of pleckstrin that habors a PH domain (pleckstrin homology) and a DEP domain (Disheveled, Egl-10, pleckstrin) separated by a 14-amino acid loop kinase-specific substrate sequence bearing three phosphorylation sites was designed. Upon phosphorylation of this intervening loop by PKC, the pleckstrin moiety undergoes a conformational change, which prompts the PH and the DEP domains to interact. The major advantage of this reporter design is that it does not rely on interactions between the substrate and a distinct PAABD. This probe responds rapidly to PKC activation through phorbol ester stimulation or upon activation of physiologically relevant pathways[71] (Fig. 6.4D). The KPC-1 biosensor was further engineered to KCAP-1, which bears a PKA substrate sequence in the C-terminal loop just before the GFP moiety. This GFP/EYFP FRET biosensor consequently responds to PKC phosphorylation through an increase in FRET, as opposed to a decrease in FRET induced by PKA phosphorylation, thereby allowing monitoring PKA and PKC activities independently in living cells.[72]

PKB/Akt is a serine/threonine protein kinase that plays a key role in a wide variety of cellular processes, including glucose metabolism, apoptosis, cell proliferation, transcription, and cell migration, and integrates many oncogenic signals from the phosphatidylinositol 3-kinase pathway signaling pathway, thereby promoting cellular growth, survival, and proliferation together with decreased apoptosis. Akt is constitutively phosphorylated, activated, and recruited to the plasma membrane in a large variety of solid tumors and hematologic malignancies, and is known to be actively involved in tumor initiation and progression.[102,103] While several reporters based on bioluminescence have been developed, which will not be described here, several fluorescence-based reporters have also been engineered to monitor PKB/Akt signaling and study its dynamics in living cells.[50–52] The Aktus reporters are based on a CFP/YFP FRET pair, a protein kinase B (PKB)

substrate sequence derived from Bad, and a PAABD domain derived from 14-3-3n, similar to the structure of AKAR. Aktus was further fused to the Golgi-targeting and mitochondrial-targeting domains of eNos and Bad, respectively, so as to generate variants that would preferentially colocalize with these endogenous substrates and thereby optimize their chances of phosphorylation by PKB compared to the nontargeted sensor.[51] Another PKB sensor, BKAR, was developed with the same structural design as the CKAR reporter[69] and employed to image phosphorylation catalyzed by PKB in real time and to investigate differences in the dynamics of this activity in the nucleus, cytosol, and when targeted to the plasma membrane.[52] This study revealed differences in biosensor response associated with different subcellular compartments, inferring differences in PKB signal propagation and termination. Another PKB reporter, AktAR, was developed to examine dynamic Akt activity in membrane microdomains and was based on the CFP/Venus FRET couple, an FHA1 domain, and a FOXO1 substrate.[50] This biosensor revealed that Akt activities are differentially regulated in different membrane microdomains and present overall higher activity within lipid rafts.

A reporter of protein kinase D, DKAR, based on the design of BKAR and CKAR,[52,69] was applied to investigate the dynamics of this kinase, revealing its dependence on calcium through positive feedback regulation of diacylglycerol production.[73]

2.3. Genetically encoded ERK/MAPK biosensors

ERK1/2 (extracellular signal-regulated kinases) play central roles in growth, cell proliferation, and differentiation. As their name suggests, these kinases are activated following different stimuli, including mitogenic factors, hormones, and cytokines, and they are themselves involved in regulating the activity of a wide variety of substrates including transcription factors and antiproliferative genes during G0 and G1 phases.[104] The first genetically encoded FRET biosensor of ERK, Erkus, was designed by Sato *et al.* in order to study the spatiotemporal dynamics of protein phosphorylation by activated ERK. Erkus is a single-chain biosensor encoding the CFP and YFP AFPs that flank an FHA2 domain from Rad53p, a short linker, and a substrate domain derived from the EGFR T669 peptide, as well as a short docking motif, the D-domain, derived from p90 ribosomal S6 kinase[58,80] (Fig. 6.5A). While this biosensor allowed the visualization of the dynamics of ERK kinase in real time, additional variants bearing either a nuclear localization sequence (NLS) or a nuclear export sequence (NES)

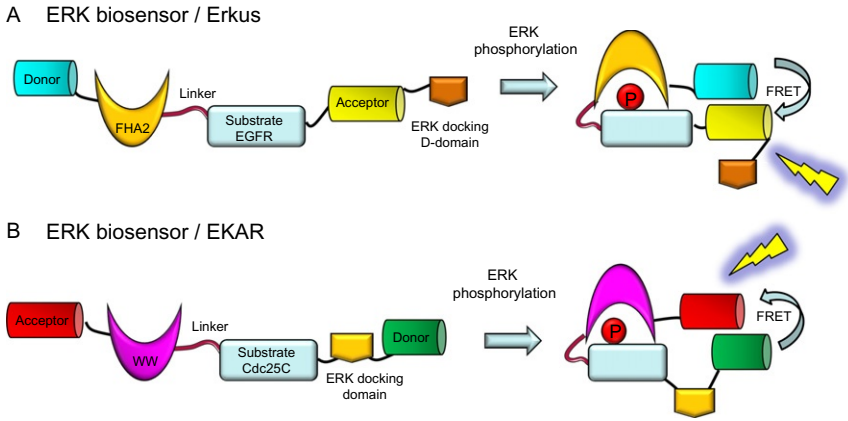

Figure 6.5 *Genetically encoded biosensors of ERK kinases*. (A) The first genetically encoded FRET biosensor of ERK, Erkus, is a single-chain biosensor encoding the CFP/YFP FRET pair flanking an FHA2 domain from Rad53p, a short linker and a substrate domain derived from EGFR T669 peptide, together with a short docking motif, the D-domain, derived from p90 ribosomal S6 kinase.[58,80] (B) EKAR comprises a WW domain and a substrate derived from Cdc25C, immediately flanked by an ERK-specific docking domain, sandwiched between an mRFP/EGFP FRET pair.[57]

further allowed the comparison of the cytosolic and nuclear activities of ERK in living cells. More recently, Harvey *et al.* developed a different biosensor, termed EKAR, which comprises a WW domain and a substrate derived from Cdc25C, immediately flanked by an ERK-specific docking domain and separated by a 72-glycine linker, sandwiched between an mRFP and an enhanced green fluorescent protein (EGFP)[57] (Fig. 6.5B). Expression of EKAR biosensor was observed to be essentially nuclear; therefore, a cytoplasmic version of EKAR was developed, EKARcyto, by appending an NES to the C-terminus of the biosensor. Moreover, EKAR bears an ERK-specific docking site, which differs from that of Erkus, is adjacent to the substrate, and appears to contribute to the superior FRET signal of EKAR over Erkus. EKAR was successfully applied to measure the spatiotemporal signaling dynamics of this kinase in neurons from intact brain tissue by fluorescence lifetime imaging.

2.4. Cell cycle kinases: ATM, Plk, Aurora, CDK/cyclin biosensors

Several genetically encoded biosensors have been developed to study protein kinases involved in cell cycle regulation, although there have been fewer developments in this field than in the field of protein tyrosine kinases or of

PKA/PKC signaling. Aurora and Plk kinases are mitotic kinases whose central function and subcellular dynamics have recently been uncovered, thanks to fluorescent reporters of their activity. A biosensor of Aurora B kinase was developed to study the dynamics of protein phosphorylation by this mitotic kinase during anaphase.[49] This biosensor encodes a CFP/YFP FRET pair of AFPs, an Aurora B substrate peptide, and an FHA2 domain and was further targeted at chromosomes (histone H2B fusion) or centromeres (CENP-B fusion) (Fig. 6.6A). Fluorescence imaging of Aurora B, thanks to this FRET sensor in mitotic cells, revealed that Aurora kinase activity organizes the targeted microtubules to generate a structure-based feedback loop and a spatial phosphorylation gradient of multiple substrates during anaphase that predicts the cellular cleavage site. Along the same lines, a genetically encoded FRET biosensor of the mitotic Plk1 kinase was developed to probe the activity of this kinase in human cells in a physiological context and upon

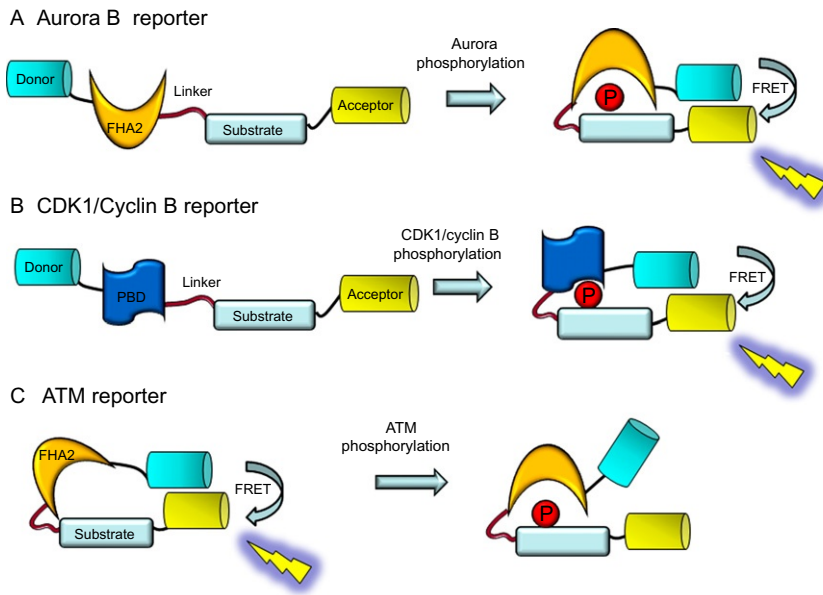

Figure 6.6 *Genetically encoded biosensors cell cycle kinases*. (A) Aurora B kinase reporter encodes a CFP/YFP FRET pair, an Aurora B substrate peptide, and an FHA2 domain.[49] (B) CDK1/cyclin B kinase reporter comprises an autophosphorylation site from cyclin B1 fused to the PBD of Plk1 and flanked by mCerulean and YPet.[56] (C) ATM reporter encodes a CFP/YFP FRET pair, an FHA phosphobinding domain, and an ATM substrate sequence, the phosphorylation of which promotes a conformational change of the biosensor which disrupts FRET between the AFPs.[54]

checkpoint recovery.[74] This study revealed that Plk1 activation occurs several hours before entry into mitosis, thanks to Aurora A-mediated phosphorylation of Plk1, and that Bora/Aurora-A-dependent phosphorylation is a prerequisite for Plk1 to promote mitotic entry after a checkpoint-dependent arrest.

CDKs constitute the molecular engines that drive cell cycle progression. In particular, CDK1/cyclin B is essential for driving cells through mitosis. However, functional studies of this kinase have remained limited to classical antigenic approaches following cell fixation or extraction. More recently, Gavet and Pines developed a FRET sensor to probe CDK1/cyclin B kinase activity in living cells based on an autophosphorylation site from cyclin B1 fused to the Polo box domain (PBD) of Plk1 and flanked by mCerulean and YPet[56] (Fig. 6.6B). Thanks to this probe, imaging of CDK1/cyclin B activity was performed with high temporal precision, showing that this kinase is inactive in G2 until just before nuclear envelope breakdown, contributing to initiate prophase.

ATM (Ataxia telangiectasia mutated kinase) is an intracellular protein kinase that coordinates the DNA damage signaling pathway upon recognition of DNA double strand breaks. Activation of ATM leads to phosphorylation of transducer and effector proteins that coordinate a cellular response including DNA repair, cell cycle arrest, or apoptosis. A genetically encoded CFP–YFP FRET reporter was developed to probe ATM kinase activity in living cells, based on an FHA PBD and an ATM substrate sequence, the phosphorylation of which leads to changes in FRET efficiency between CFP and YFP associated with a conformational change of the biosensor[54] (Fig. 6.6C). This reporter allowed monitoring the ATM activity in a specific and quantitative fashion, providing a measurable output in response to double strand breaks, while also providing information on the spatiotemporal dynamics of ATM activity in living cells.

2.5. Other kinase biosensors

To visualize signal transduction based on protein phosphorylation in living cells, Sato *et al.* developed genetically encoded fluorescent indicators, named "phocuses" (fluorescent indicator for protein *pho*sphorylation that can be *cus*tom-made) that report on phosphorylation by the insulin receptor[59] (Fig. 6.7A). These reporters encode a CFP/YFP pair, a substrate sequence for the insulin receptor, a flexible linker sequence, and a phosphorylation recognition domain derived from SH2, PTB, or WW domains, or single-chain antibodies that recognize the phosphorylated substrate sequence.

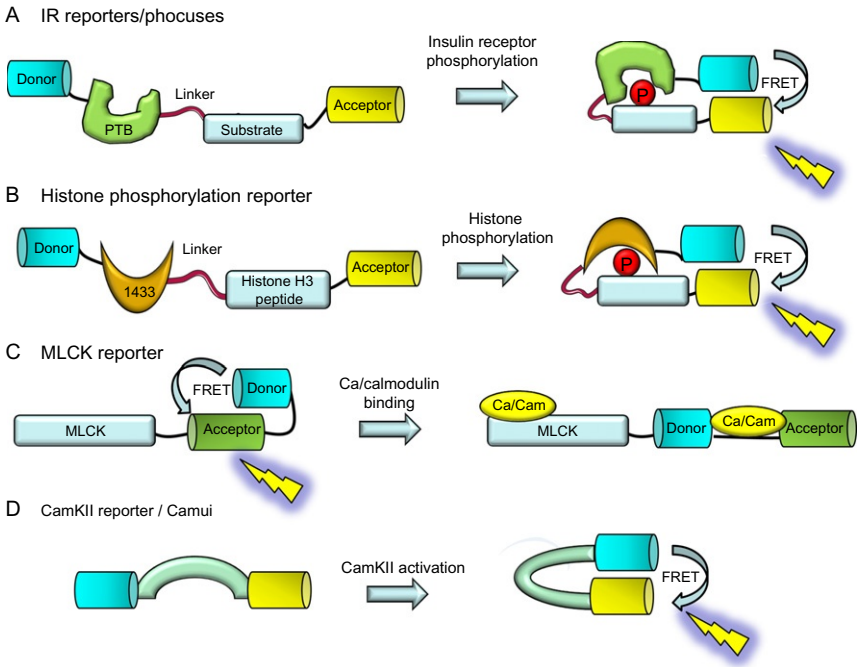

Figure 6.7 *Genetically encoded biosensors of other kinases*. (A) Phocuses are fluorescent indicators that report on phosphorylation by the insulin receptor based on CFP/YFP FRET pair, a substrate peptide for the insulin receptor, a flexible linker sequence, and a phosphorylation recognition domain.[59] (B) Histone phosphorylation reporter by appending a substrate sequence from histone H3 bearing a phosphorylatable serine residue to a phosphoserine-binding domain, derived from 14-3-3, flanked by YFP and CFP, respectively.[62] (C) MLCK-FIP is a reporter of MLCK activity associated with Ca^{2+}/calmodulin binding. This reporter is based on a calmodulin-binding domain derived from MLCK and a BFP/GFP couple that undergoes FRET in the absence of Ca^{2+} and calmodulin.[63] (D) Camui, a FRET biosensor of CaMK II, constituted of full-length CaMKII flanked by CFP and YFP, allows measurement of CaMKII activation associated with a conformational change.[55]

Upon phosphorylation, the intramolecular interaction between the substrate and the phosphorecognition domain promotes a conformational change which induces a consequent increase in FRET between CFP and YFP.

Lin and Ting engineered a histone phosphorylation reporter by appending a substrate sequence from histone H3 bearing a phosphorylatable serine residue to a phosphoserine-binding domain derived from 14-3-3 flanked by YFP and CFP, respectively[62] (Fig. 6.7B).

A biosensor of the Ca^{2+}/calmodulin-bound and active form of myosin light chain kinase (MLCK), MLCK-FIP, was developed by Chew *et al.*, based on a calmodulin-binding domain derived from MLCK and a BFP/GFP couple that undergoes FRET in the absence of Ca^{2+}/calmodulin and displays a decrease in FRET upon activation of MLCK through binding of Ca^{2+}/calmodulin (Fig. 6.7C). This biosensor allowed imaging the spatial and temporal pattern of MLCK activation, revealing that it is enriched at the spindle equator during late metaphase and maximally activated just before cleavage furrow constriction.[63]

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a key mediator of intracellular signaling in the heart. Erickson *et al.* developed Camui, a FRET biosensor of CaMKII, constituted of full-length CaMKII flanked at its N- and C-termini by CFP and YFP, respectively, which undergo robust FRET in the compactly folded autoinhibited state. Activation of the kinase results in a conformational change associated with a decrease in fluorescence transfer between CFP and YFP. This reporter was applied to study the activation of CamKII in living neurons and in cardiomyocytes[55] (Fig. 6.7D).

JNK kinase is involved in signal transduction of external stimuli such as stress and cytokines into functional responses that mediate apoptosis, proliferation, differentiation, and inflammation. Several genetically encoded fluorescent biosensors were engineered to detect endogenous JNK activity in living cells, based on an EGFP/citrine FRET pair; an FHA1, FHA2, or WW PAABD; a substrate domain derived from c-Jun or phosphoacceptor sites from JDP2 or ATF3; and docking domains from JDP2 or JIP1.[60] The JNKAR biosensor (for JNK activity reporter) specifically detects stress (ribotoxic and osmotic) and cytokine (TNF-α) induced JNK activity in living cells, in the cytoplasm, nucleus, mitochondria, and plasma membrane, associated with a phosphorylation-dependent increase in FRET between the two AFPs.

The stress-activated protein kinases (SAPKs) p38 and JNK are members of the mitogen-activated protein kinase family and are important determinants of cell fate when cells are exposed to environmental stresses such as UV and osmostress. SAPKs are activated by SAPK kinases (SAP2Ks), which are in turn activated by various SAP2K kinases (SAP3Ks). A genetically encoded FRET biosensor was developed to probe the dynamic behavior of SAP3K activity toward the MKK6 SAP2K, which was based on a Venus/SECFP pair, an FHA1 domain, and MKK6 substrate.[75] This biosensor allowed the study of the dynamic behavior of SAP3K activation in living cells, associated with stress stimuli such as epidermal growth factor and

osmostress on the plasma membrane, anisomycin and UV in the cytoplasm, and etoposide in the nucleus.

AMP-activated protein kinase (AMPK) is activated when the AMP/ATP ratio in cells is elevated as a result of energy stress. A fluorescent biosensor of AMPK activity, AMPKAR, was developed to study the function of this kinase upon cellular stress.[53] This biosensor is based on an ECFP/cpVenus FRET pair, an FHA1 domain, and a substrate peptide. AMPKAR exhibits enhanced FRET in response to phosphorylation, allowing probing of the spatiotemporal dynamics of AMPK activity in living cells, thereby revealing that its activation takes place in the cytosol in response to energy stress but occurs in both the cytosol and the nucleus in response to calcium elevation.

Focal adhesion kinase (FAK) is crucial for many cellular processes. To visualize FAK activity and activation at different membrane microdomains, a genetically encoded FRET biosensor was developed, which is based on an ECFP/YPet FRET pair, an SH2 domain derived from c-Src, and a substrate peptide derived from FAK. This biosensor was either targeted into or outside of detergent-resistant membrane regions at the plasma membrane. This study revealed that FAK is activated at membrane microdomains but that its activation mechanisms vary in response to different physiological stimuli.[61]

3. FLUORESCENT PEPTIDE/PROTEIN BIOSENSORS

Concerted efforts of chemists and biologists in designing fluorescent probes for biological applications have led to the development of a very different class of biosensors that do not rely on genetically encoded AFPs. Fluorescent peptide, polypeptide, or protein biosensors constitute attractive alternatives to genetically encoded biosensors in that they offer a high degree of versatility, yet also a high degree of control. This class of biosensors is engineered by exploiting peptide substrate sequences or protein domains that bind a specific analyte or interface, which serves as platforms for site-selective coupling of one or more fluorescent probe(s). The fluorescent probe may be coupled by different means to the peptide backbone—most often chemically, enzymatically, or through replacement of a fluorescent amino acid analog (for review, see Ref. 105). The design allows for freedom in the choice of the fluorescent probe, amongst a wide variety of wavelengths and synthetic probes,[106] and for its incorporation at virtually any position within the peptide.

Peptides and polypeptides offer several major advantages inherent to their nature. They can be readily produced through synthetic chemistry or recombinant protein engineering; are easy to handle, store, and characterize; and can further be modified with a wide variety of unnatural substituents, such as modified amino acids, to improve specificity and selectivity, as well as small synthetic fluorescent probes. Peptides are very small yet can serve as substrates, docking sequences, or complementary biomolecular recognition interfaces, thereby offering a wide array of possibilities and strong potential for development of fluorescent biosensors. Peptides also constitute scaffolds for a wide variety of technological improvements, including introduction of quenchers or caging of specific amino acids. Moreover, compared to antibodies, they are cheaper to produce, display low antigenicity, and are rapidly eliminated by the organism, thereby generating relatively low cytotoxicity. Peptide biosensors are readily applicable *in vitro* and in cell lysates. Their applicability in living cells and *in vivo*, however, remains challenging, requiring suitable methods to facilitate their intracellular delivery. Notwithstanding, major advances in the field of protein and peptide delivery over the past 20 years have provided efficient means of introducing this class of biomolecules into living cells and *in vivo* based on protein transduction domains and cell-penetrating peptides (CPPs).[107,108] Once the issue of delivery is solved, peptide biosensors offer a major advantage over genetically encoded biosensors, in that they allow for immediate and controlled use, compared to genetically encoded biosensors, which require long periods of time for their expression and/or maturation (Table 6.1).

Several strategies have been employed to design and engineer peptide biosensors of kinase activity, which are quite different from the strategies developed to generate genetically encoded KARs. In all cases, phosphorylation induces changes in the spectral properties of the fluorophore(s) incorporated into the peptide scaffold, which may involve a shift in its emission wavelength and/or an important change in its quantum yield. Fluorescent peptide biosensors can be broadly divided into three different groups, depending on the mechanism through which fluorescence reports on phosphorylation: environmentally sensitive biosensors, chelation-enhanced fluorescence through metal-ion binding, and biosensors involving quenching/unquenching strategies. We will describe the different categories of fluorescent peptide biosensors that have been developed to probe kinase activities, together with some representative examples to illustrate their mechanism of action and their applications (summarized in Table 6.3). Additional details

may be found in the original papers or in comprehensive reviews on this class of sensors.[35–38,134–136]

3.1. Environmentally sensitive kinase biosensors

Environment-sensitive fluorophores respond to changes in the polarity of their environment.[105] A good probe will be poorly fluorescent in a fairly polar aqueous solution but will become highly fluorescent in nonpolar solvents or upon docking into a hydrophobic protein pocket or membrane. Several solvatochromic dyes have been described in detail, and their features

Table 6.3 Fluorescent peptide and protein biosensors of protein kinases

Protein kinase	Biosensor features	Author and year	Reference
	Environmentally sensitive peptide biosensors		
PKC	NBD probe proximal to phosphorylation site	Yeh *et al.* (2002)	109
PKC	NBD probe proximal to phosphorylation site, light activatable	Veldhuyzen *et al.* (2003)	110
Src	Phosphorylation-driven protein–protein interaction, based on an SH2 domain	Wang and Lawrence (2005)	111
Src	Merobody: fibronection monobody conjugated to merocyanine dye	Gulyani *et al.* (2011)	112
CDKSENS	Reports on CDK/cyclin abundance/cyanine probe	Kurzawa *et al.* (2011)	113
CDKACT	Reports on CDK/cyclin activity/WW phosphobinding domain/cyanine probe	Van *et al.* (in preparation)	114
	Metal ion-induced and chelation-enhanced fluorescence		
PKCalpha	Ca^{2+} dependent	Chen *et al.* (2002)	115
PKA, PKC, Abl	Mg^{2+} dependent, Sox based	Shults *et al.* (2003), Shults and Imperiali (2003)	116,117

Continued

Table 6.3 Fluorescent peptide and protein biosensors of protein kinases—cont'd

Protein kinase	Biosensor features	Author and year	Reference
Akt, PKA, MK2	Mg^{2+} dependent, Sox based, cell lysates	Shults *et al.* (2006)	118
PKC, PKA, Akt1, MK2, CDK2, PIM2	Mg^{2+} dependent, Sox based, multiplexed assay in cell lysates	Shults *et al.* (2005)	119
PKC, Pim2, Akt1, MK2, PKA, Abl, Src, IRK	Mg^{2+} dependent, Sox based, recognition-domain focused chemosensors	Lukovic *et al.* (2008), Gonzalez-Vera *et al.* (2010)	120,121
ERK1, 2	Mg^{2+} dependent, Sox based, protein-based docking domain (Sox-PNT)	Lukovic *et al.* (2009)	122
p38 alpha	Mg^{2+} dependent, Sox based, protein-based docking domain (Sox-MEF2A)	Stains *et al.* (2011)	123
CDK9/cyclin T	Hybrid biosensor: WW domain of Pin1 and a Zn^{2+}–DPA complex	Anai *et al.* (2007)	124
Src and Abl	Lanthanide biosensors: carbostyril group sensitizer, Tb^{3+} or Eu^{3+} chelator	Tremblay *et al.* (2008)	125
	Quenching/unquenching strategies		
Src kinase peptide biosensor	Self-reporting biosensor: tyrosine quencher/pyrene group	Wang *et al.* (2006)	126
Src kinase peptide biosensor	Self-reporting biosensor: tyrosine quencher/Cascade Yellow, Cascade Blue, or Oregon Green	Wang *et al.* (2006)	127
Bcr–Abl and Lyn	Self-reporting biosensor: tyrosine quencher/Cascade Yellow and Oxazine	Wang *et al.* (2010)	128
PKA	Deep quench: pyrene/Rose Bengal/14-3-3 phosphoserine binding domain	Sharma *et al.* (2007)	129
PKA	Deep quench: pyrene/ Coumarin probe and Acid	Agnes *et al.* (2010)	130

Table 6.3 Fluorescent peptide and protein biosensors of protein kinases—cont'd

Protein kinase	Biosensor features	Author and year	Reference
	Green/14-3-3 phosphoserine binding domain		
PKA, PKC, p38, MAPKAP K2, Akt, Erk1, Src	Micelle/auto-assembly	Sun *et al.* (2005)	131
	Ratio metric strategies		
PKA	Single probe/wavelength shift/ phosphorylation-sensitive Cd (II)-cylcen aminocoumarin	Kikuchi *et al.* 2009	132
Src	Dual probe/merocyanine and mCerulean	Gulyani *et al.* (2011)	112
	Photoactivatable biosensors		
PKC	NBD probe proximal to phosphorylation site, caged serine	Veldhuyzen *et al.* (2003)	110
PKC beta	NBD probe proximal to phosphorylation site, caged serine	Dai *et al.* (2007)	133
Src	Self-reporting biosensor, caged tyrosine	Wang *et al.* (2006)	127

when coupled to peptide biosensors for biological applications have been readily characterized by several groups of chemists (see Ref. 105 and references therein). Although most of these probes possess excellent photophysical properties for *in vitro* applications, their excitation and emission wavelengths tend to be incompatible with *in vivo* applications. Notwithstanding, several groups of chemists are currently devoting their effort to synthesize environmentally sensitive red-shifted dyes with suitable properties for *in vivo* sensing applications.

Environmentally sensitive kinase biosensors constitute a unique class of sensors in that they rely on changes in the environment of the fluorescent probe to report on phosphorylation of the kinase biosensor. Different means have been employed to achieve this goal: phosphorylation itself, phosphorylation-induced conformational changes, and binding of the phosphorylation site by a

phosphorecognition-binding domain (for review, see Refs. 134–136). Environmentally sensitive kinase biosensors have been generated through incorporation of fluorescent probes on the phosphorylatable residue itself, immediately adjacent to the residue or proximal to it (i.e., 2–5 residues away). One of the first set of fluorescent peptide probes based on the propinquity effect was generated by labeling a PKC peptide substrate at its N-terminal serine hydroxyl moiety, thereby yielding a library of 417 compounds through incorporation of different fluorescent labels. Although many of the dyes used did not result in a significant increase in fluorescence of the peptide probe upon phosphorylation by PKC *in vitro*, an NBD (nitrobenzofuran)-labeled variant displayed a robust increase in fluorescence intensity (2.5-fold) (Fig. 6.8A). Moreover, this peptide probe was successfully applied to monitor PKC activity in cell lysates and further microinjected into living cells to visualize and monitor the spatiotemporal dynamics of PKC activity.[109] To further improve this biosensor, Lawrence and coworkers synthesized a light-activatable variant of the NBD–PKC probe through incorporation of a single photolytically sensitive cage on the phosphorylatable serine group, thereby offering a precise means of controlling biosensor availability in living cells through photoactivation.[110]

Environmentally sensitive dyes will also respond to changes in their local environment associated with protein–protein interactions. In the case of kinase sensors, this has been exploited by using phosphopeptide recognition domains, including 14-3-3, SH2, or WW domains,[83–89] that bind the phosphorylated peptide but not the unphosphorylated species. The fluorescent probe is consequently embedded in a hydrophobic environment, which modifies the local polarity and consequently affects its spectral properties. This strategy tends to enhance the fluorescence of environmentally sensitive probes more significantly than simply proximal phosphorylation, and further increases the selectivity of the biosensor, because the PAABD is more likely to bind the phosphorylated substrate than any other nonspecific protein found in cell extracts. This strategy was employed to generate a very sensitive probe for Src tyrosine kinase based on a fluorescent peptide labeled with dapoxyl, NBD, or Cascade Yellow two residues away from the phosphorylation site, thereby leading to a fivefold increase in fluorescence upon phosphorylation, thanks to interaction with an SH2 domain[111] (Fig. 6.8B). More recently, Gulyani *et al.* generated a new Src biosensor based on a fibronectin monobody scaffold, which was employed to quantify the dynamics of Src activity at the edge of living cells, in correlation with protrusion and retraction activities.[112] This merobody

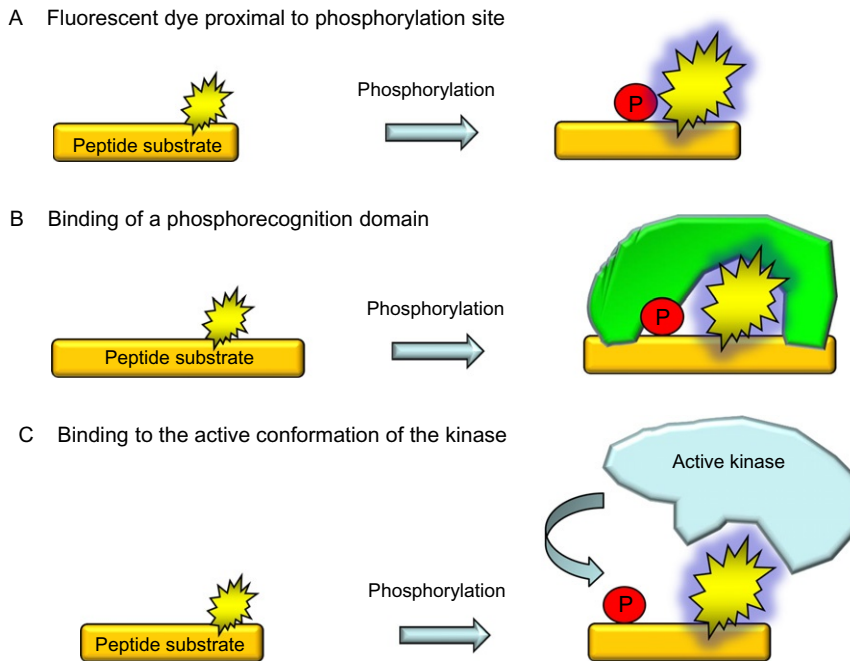

Figure 6.8 *Environmentally sensitive peptide-based kinase biosensors*. Environmentally sensitive peptide biosensors bear a fluorophore whose spectral properties are directly modified/affected by proximal phosphorylation (at ± 1–5 residues). Environmentally sensitive peptide biosensors of kinase activity have been developed with a fluorescent dye coupled directly at or proximal to the phosphorylation site whose fluorescence is enhanced (A) by phosphorylation itself,[109] (B) upon binding of a phosphopeptide recognition domain such as SH2 for phosphotyrosine,[111] and (C) upon binding of the kinase itself through an interface that does not affect its catalytic activity.[112]

biosensor was engineered by a HTS approach, by derivatizing a monobody that specifically recognizes the active conformation of Src kinases with an environmentally sensitive merocyanine dye, the fluorescence of which is enhanced upon target binding, yet without interfering with target binding or phosphorylation (Fig. 6.8C).

3.2. Metal-ion-induced, chelation-enhanced fluorescence

Metal-ion-induced, chelation-enhanced fluorescence (CHEF) relies on fluorophores that can coordinate metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, or lanthanides) and whose ability to chelate these ions is further aided by proximal phosphorylation of a peptide substrate. Upon chelation, the electronic structure of the fluorophore is modified, and consequently its spectral properties.[137]

3.2.1 Ca^{2+}-chelating biosensors

Lawrence and coworkers developed a set of PKC peptide probes through incorporation of a Ca^{2+}-sensitive fluorophore with a tetracarboxylate-chelating site previously developed by Tsien and collaborators.[138] The fluorophore was directly incorporated onto the N-terminal phosphorylation site of a consensus PKC peptide substrate, lying within only a couple of angstroms from the phosphorylation site. The unphosphorylated probe has poor affinity for Ca^{2+}, but phosphorylation by PKC creates a divalent metal-ion-binding site, and coordination of Ca^{2+} by the fluorophore induces a twist that affects its spectral properties, promoting a 3.6-fold increase in fluorescence[115] (Fig. 6.9A).

3.2.2 Mg^{2+}-chelating biosensors—Sox biosensors

Imperiali and collaborators developed a solvatochromic probe, the Sox (sulfonamide-oxine) dye, derived from 8-hydroxyquinoline, that chelates Mg^{2+} and undergoes fluorescent enhancement upon coordination of a phosphate group on a phosphopeptide.[116] The Sox dye is resistant to photobleaching, is small, and causes minimal perturbation of substrate affinities with the kinase. Based on the Sox amino acid, Imperiali and collaborators developed a "versatile kinase kinase activity scaffold" that incorporates a consensus phosphorylation site coupled to a β-turn sequence that preorganizes the binding site for Mg^{2+} and a Sox dye, together with a kinase recognition motif[117] (Fig. 6.9B). This β-turn-focused (BTF) design allows the phosphorylation site to be either N- or C-terminal of the kinase recognition motif. Mg^{2+} binding is weak in absence of phosphorylation (Kd 100–300 mM) and considerably increased upon phosphorylation (Kd 4–20 mM). Sox-based BTF biosensors were successfully developed for PKC, PKA, and Abl substrate peptides with a phosphorylatable serine, threonine, or tyrosine residue with consequent increases in fluorescence intensity between three- and fivefold.[117] Further, this class of biosensors was applied to develop a multiplex fluorescence-based assay of Akt, PKA, and MK2 kinase activity in cell lysates.[118,119] To further increase the specificity of recognition, recognition-domain-focused (RDF) chemosensors were engineered with extended binding sequences to maximize recognition of a variety of well-characterized Ser/Thr and Tyr kinases, including PKC, Pim2, Akt1, MK2, PKA, Abl, Src, and IRK[120] (Fig. 6.9C). These biosensors reported on phosphorylation with robust fluorescence enhancement and high sensitivity, and were further used in 96-well plate formats, indicating that they were amenable to HTS of kinase inhibitors.

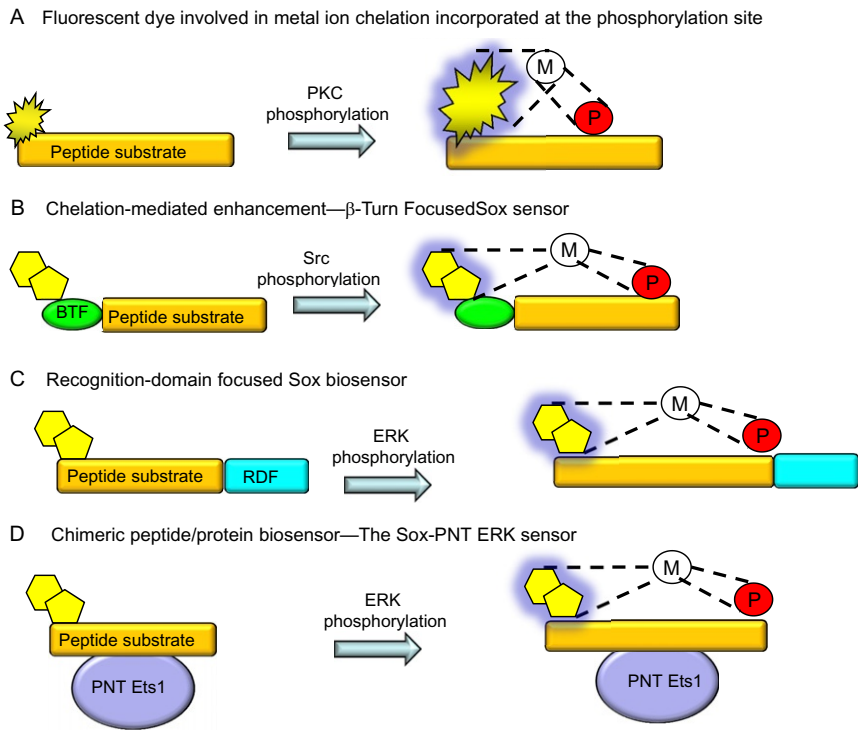

Figure 6.9 *Metal-ion-induced, chelation-enhanced fluorescence*. (A) Fluorescent dye involved in metal-ion chelation incorporated at the phosphorylation site, such as the Ca^{2+}-sensitive PKC probe.[115] (B) Chelation-mediated enhancement—β-turn-focused (BTF) Sox sensor.[117] (C) Recognition-domain-focused (RDF) Sox biosensor.[120] (D) Chimeric peptide/protein biosensor—the Sox-PNT ERK sensor.[122,123]

Moreover, a high-throughput approach was developed to generate Sox-based chemosensors with the highest possible selectivity using combinatorial libraries.[121] More recently, the RDF strategy has been extended to engineer Sox protein biosensors that incorporate a kinase-specific docking domain distal to the phosphorylation site, which enhances the specificity of recognition significantly more than an extended peptide substrate motif. For instance, a Sox-based ERK1/2 sensor (Sox-PNT) engineered through incorporation of the PNT domain of the Ets1 transcription factor into the Sox-ERK1/2 peptide substrate sequence was developed and readily applied to probe ERK1/2 kinase in cell lysates, displaying high selectivity against other kinases from the JNK, p38, and CDK families.[122] Likewise, a Sox-based p38α-selective sensor was

developed to probe p38α kinase in cell lysates in a selective fashion, by incorporating an MEF2A docking peptide and p38α phosphorylation site bearing a CSox amino acid two residues before the phosphorylation site into the same scaffold[123] (Fig. 6.9D).

Although this strategy is extremely powerful, it should be kept in mind that the spatial constraint and proximity required between the phosphorylation site and the position of the chelating fluorophore may be a limitation for recognition by certain kinases.

3.2.3 Zn^{2+}-chelating biosensors

Zn^{2+} chelators have been developed based on binuclear Zn(II)–dipicolylamine (DPA) complexes spanned by a fluorescent module (stilbene, anthracene, phenyl, or biphenyl) that is sensitive to the recognition of phosphate by the Zn^{2+} moiety[139] (Fig. 6.10A). Based on these Zn^{2+} chelators, a hybrid biosensor was designed for enhanced phosphopeptide recognition based on the phosphopeptide-binding WW domain of Pin1 coupled with the Zn^{2+} chemosensor[124] (Fig. 6.10B). This combinatorial strategy was successfully employed to monitor phosphorylation of a pSer-CTD peptide by CDK9/cyclin T1.

3.2.4 Lanthanide-chelating biosensors

Yet another class of environmentally sensitive probes worth mentioning report on lanthanide ions (Eu^{3+}, Tb^{3+}), and constitute extremely attractive tools to develop biosensors, given their photophysical features.[140] Lanthanide biosensors display poor affinity for lanthanides (such as Eu^{3+} or Tb^{3+}) in their unphosphorylated state and are essentially nonfluorescent, compared to their phosphorylated counterparts whose affinity for Eu^{3+} or Tb^{3+} increases significantly owing to the presence of the phosphate which coordinates with the metal[140] (Fig. 6.10C). Several kinase biosensors have been developed, including a kinase recognition domain for Src and Abl, a carbostyril group that acts as a luminescence sensitizer, and an iminodiacetate-chelating moiety that displayed up to 10-fold increase in signal upon phosphorylation[125] (Fig. 6.10D). The major limitations of this approach are that it requires the presence of free Tb^{3+} or Eu^{3+} as well as photosensitizing groups coupled to the peptide biosensor and that it cannot be applied *in vivo* because of the very short excitation wavelength (262 nm).

3.3. Biosensors involving quenching–unquenching strategies

3.3.1 Self-reporting biosensors

Aromatic amino acids, such as tyrosine and tryptophan, constitute good quenchers of organic dyes through a process that involves π/π stacking between the aromatic groups. In particular, tryptophan has been used in a countless number of assays to quench the fluorescence of probes.[141] A similar

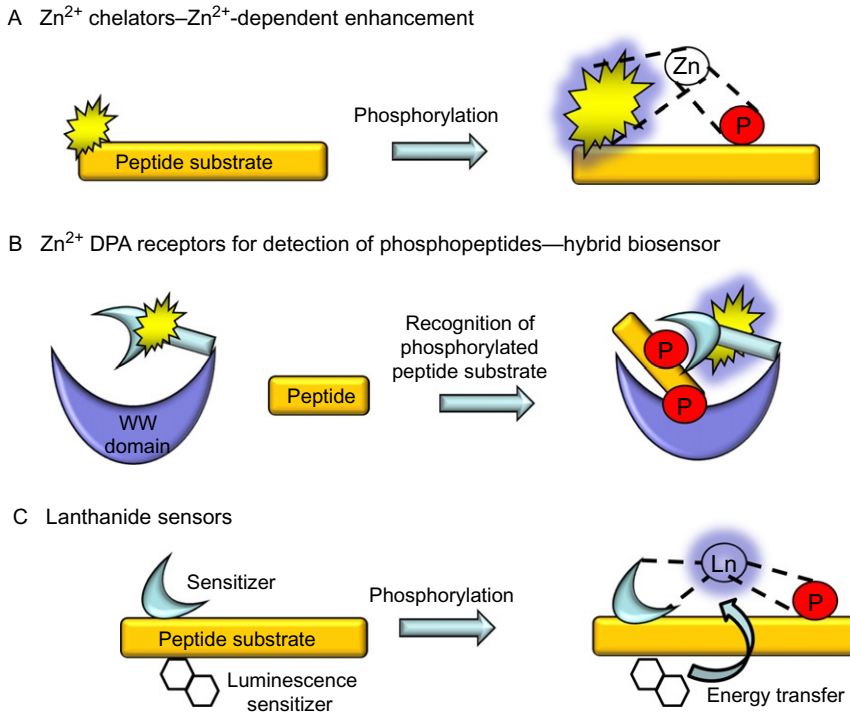

Figure 6.10 *Metal-ion-induced fluorescence: Zn^{2+} and lanthanides.* (A) Zn^{2+} chelators—Zn^{2+}-dependent enhancement of fluorescence.[139] (B) Zn^{2+}–DPA receptors for detection of phosphopeptides—hybrid biosensor between a phosphopeptide-binding domain (WW) and a fluorescent Zn^{2+}–DPA chemosensor that binds phosphate through cooperative recognition.[124] (C) Lanthanide sensors.[140] (D) Kinase biosensor including a recognition domain for Src and Abl, a carbostyril group that acts as a luminescence sensitizer, and an iminodiacetate-chelating moiety.[125]

strategy was employed to generate a fluorescent "self-reporting" peptide biosensor of protein tyrosine kinase Src, in which the phosphorylatable tyrosine directly quenches the fluorescence of a proximal dye (pyrene) until phosphorylation of tyrosine disrupts the stacking interaction, thereby releasing the fluorophore and promoting full enhancement of fluorescence[126] (Fig. 6.11A). Further variants of this biosensor were then successfully developed for applications *in cellulo*, through incorporation of Cascade Yellow, Cascade Blue, or Oregon Green.[127] More recently, two orthogonal biosensors of Lyn and Abl kinases bearing Cascade Yellow and Oxazine were developed and successfully applied to visualize these kinases in chronic myelogenous leukemia (CML) drug-resistant cell lines.[128]

3.3.2 The deep quench strategy

The strategy described above is not applicable to residues that are not aromatic. Therefore, for Ser/Thr kinase biosensors, Lawrence and collaborators developed the "deep quench" strategy, which involves shielding of the fluorophore by a quencher in solution, which will be displaced upon phosphorylation of the biosensor because of binding of a phosphorecognition domain to the phosphorylated serine or threonine, which will in turn promote

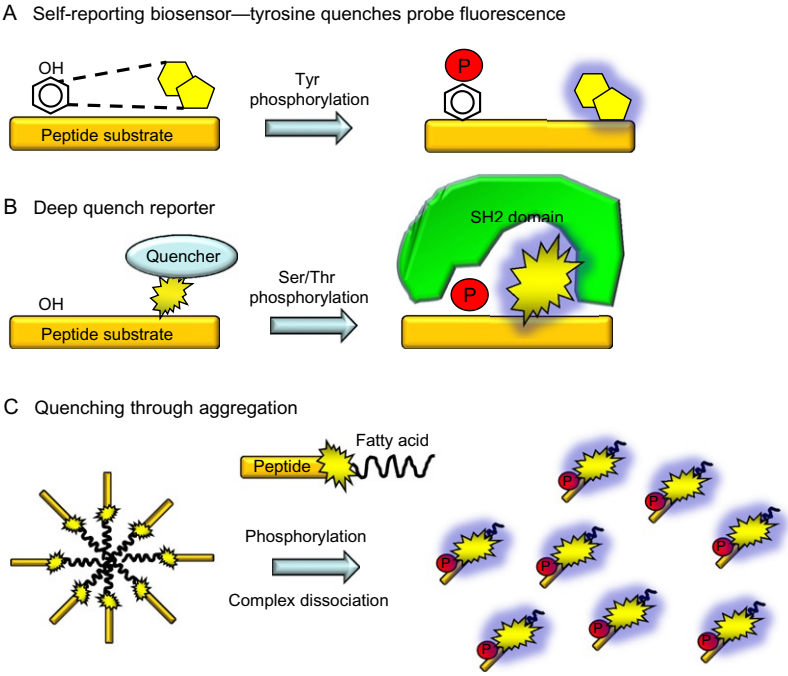

Figure 6.11 *Biosensors involving quenching–unquenching strategies.* (A) Self-reporting biosensor—the phosphorylatable tyrosine residue serves as a quencher that silences the fluorescence of a proximal fluorophore; phosphorylation disrupts this interaction, thereby leading to complete enhancement of fluorescence.[126–128] (B) Deep-quench reporter developed for Ser/Thr peptides—fluorescence of a probe is quenched by a compound in solution; phosphorylation promotes recruitment of a phosphoSer/Thr-binding domain (e.g., 14-3-3) which promotes enhancement of probe fluorescence.[129,130] (C) Quenching through aggregation—an amphiphilic peptide biosensor composed of a substrate domain with a phosphorylatable residue, labeled with fluorescein, and an N-terminal carbon tail that promotes auto-assembly or aggregation of the peptides. Peptide aggregation leads to fluorescence quenching within the micelle; phosphorylation disrupts the micelle structure, thereby releasing the individual probes whose fluorescence is enhanced.[131]

significant enhancement of the fluorescence of the environmentally sensitive probe (Fig. 6.11B). This strategy has proved extremely successful in generating a PKA biosensor bearing a pyrene probe, using Rose Bengal as a quencher and a 14-3-3pi as a phosphoserine-binding domain, which displays 64-fold increase in fluorescence,[129] and more recently, another PKA biosensor bearing a Coumarin probe and Acid Green as a quencher, which displays up to 150-fold increase in fluorescence, was used to monitor PKA activity in mitochondria.[130] Despite its apparent potency, the complexity of this approach makes it difficult to apply in living cells and *in vivo*.

3.3.3 Quenching through aggregation

A variant of these strategies was devised by Sun *et al.* based on an "auto-assembly" or "micelle" approach to quench fluorescence of peptide kinase biosensors for PKA, PKC, p38, MAPKAP K2, Akt, Erk1, and Src.[131] This involves the design of an amphiphilic peptide biosensor labeled with fluorescein, composed of a substrate domain with a phosphorylatable residue and of an N-terminal carbon tail that promotes auto-assembly or aggregation of the peptides, thereby quenching their fluorescence. Phosphorylation of the peptide biosensor disrupts the micelle structure, thereby leading to fluorescence (Fig. 6.11C).

3.4. Ratiometric strategies

3.4.1 Intramolecular ratiometric strategy—Single-probe biosensor

Two different ratiometric strategies have been developed. The first consists of a single probe that is sensitive to environmental changes that will affect its spectral properties sufficiently to allow for ratiometric quantification. A metal-ion, anion-sensitive ratiometric peptide kinase biosensor has been developed to probe PKA activity, consisting of a Cd(II)-cylcen appended aminocoumarin coupled to a substrate peptide for PKA. Upon phosphorylation by PKA, the metal-complex moiety binds to a phosphorylated residue, which in turn displaces the coumarin fluorophore, resulting in ratiometric change of the probe's excitation spectrum[132] (Fig. 6.12A). A different strategy is based on the incorporation of two fluorophores within the same biosensor, the first displaying sensitivity to target recognition and the second being inert. This concept was employed to generate the Src merobody biosensor, through incorporation of a merocyanine dye onto the fibronectin biosensor scaffold, as well as the mCerulean autofluorescent protein for ratio imaging *in vivo*[112] (Fig. 6.12B).

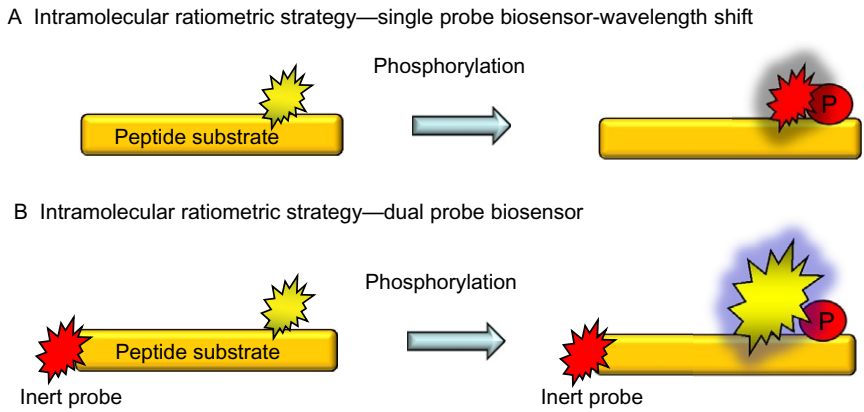

Figure 6.12 *Ratiometric strategies.* (A) Intramolecular ratiometric strategy—single-probe biosensor based on a coumarin/Cd^{2+}-cyclen probe that undergoes a wavelength shift upon phosphorylation (from 360 to 410 nm) due to chelation of the phosphate group by the Cd^{2+} center—anion sensor-based ratiometric probe.[132] (B) Intramolecular ratiometric strategy—based on incorporation of two different fluorescent probes on a single biosensor scaffold—one involved in the sensing process, the other serving as an internal control, such as the Src merobody biosensor based on a fibronectin monobody that is conjugated to a merocyanine dye and fused with a mCerulian which serves as an internal control.[112]

3.5. Optimizing peptide biosensors through quenching and caging strategies

3.5.1 Intramolecular quenching

One of the means of optimizing the signal-to-noise ratio in applications involving the use of peptide or protein kinase biosensors consists in silencing the basal fluorescence of the fluorescent probe, thanks to a quencher moiety conjugated within the same biosensor. This strategy implies that the quencher is in spatial proximity with the fluorescent probe and that the quenching mechanism will be reversed upon phosphorylation of the biosensor by the kinase. Different quencher strategies have been employed (Fig. 6.13). The simplest strategy involves incorporation of a synthetic quencher into the peptide backbone so that it quenches the fluorescent probe until phosphorylation of the biosensor allows the disruption of the interaction between the quencher and the fluorophore, much in the same way as tryptophan or tyrosine residues quench proximal fluorophores[126,141] (Fig. 6.13A). An alternative approach, which consists in intramolecular homoquenching between two fluorophores (through homo- or

heteroquenching) conjugated to the peptide biosensor, can be employed to reduce basal fluorescence of the biosensor, although this does not necessarily ensure that fluorescence will be completely quenched[142] (Fig. 6.13B).

3.5.2 Light activation

Another means of optimizing the signal-to-noise ratio and increasing the sensitivity of the response involves generating light-activatable probes that can be selectively activated in response to UV irradiation. Light-activatable probes are generated through molecular caging, by masking the main function with a photolabile group, which will be selectively released upon illumination. This provides spatial and temporal control over the activation of the probe, thereby allowing dampening or silencing any nonspecific fluorescence and reducing nonspecific noise attributable to basal fluorescence prior to activation[143] (Fig. 6.13C). The first caged fluorescent reporter of intracellular enzymatic activity was a light-activatable variant of the NBD–PKC probe, engineered through incorporation of a single photolytically sensitive cage on the phosphorylatable serine group of the peptide, which precluded

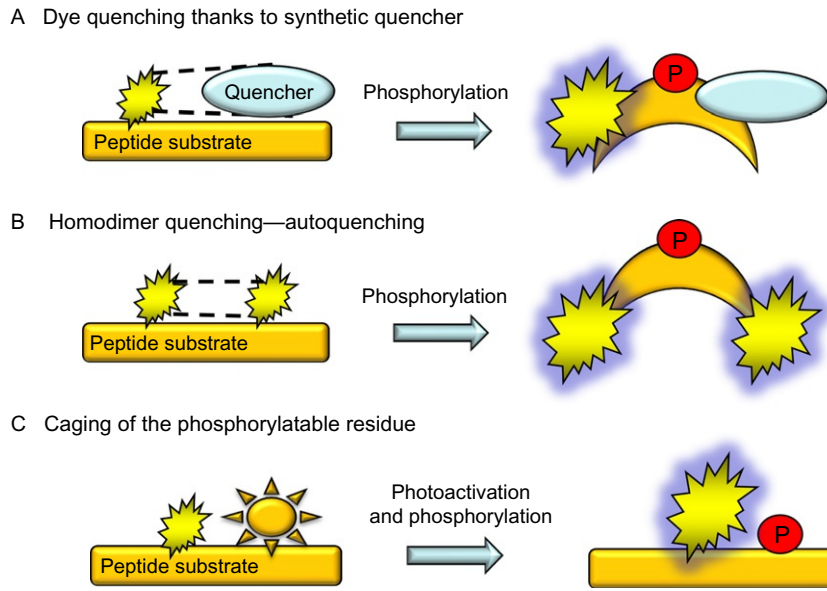

Figure 6.13 *Quenching and photoactivation strategies for peptide biosensors.* (A) Incorporation of a synthetic quencher into the peptide backbone. (B) Intramolecular homoquenching between two fluorophores conjugated to the peptide biosensor. (C) Selective photoactivation of caged compounds by UV irradiation.[110,127,133]

its phosphorylation by PKC.[110] This variant offers the advantage of controlling both the timing and the relative concentration of biosensor released upon illumination and was readily microinjected into HeLa cells to monitor intracellular PKC activity. Further, a caged biosensor of PKC was microinjected into PTK2 cells to follow its kinase activity at mitosis, with high temporal precision, thereby revealing that kinase activity is required for the progression from prophase into metaphase and that robust kinase activity of the PKCb isoform occurs just prior to nuclear envelope breakdown. This biosensor provides "visual snapshots of intracellular kinase activity" with high spatial and temporal control.[133] Similarly, a caged Src reporter was generated by modifying the phosphorylatable tyrosine of a self-reporting peptide biosensor with a photolabile nitrobenzyl moiety and microinjected into A549 cells expressing high levels of Src, allowing the control of the timing of kinase sensing.[127]

3.6. Peptide biosensors for cell cycle targets: CDKSENS and CDKACT

Enzymes involved in regulation of cell cycle progression are central for processes such as cell growth, DNA replication, and cell division, and therefore constitute pillars of cellular physiology. Many kinases involved in coordination of cell cycle progression have been well documented to undergo mutations that alter their function, aberrant expression, or hyperactivation in human disorders, and thereby constitute attractive pharmacological targets and biomarkers of disease.[144,145,27] However, despite the pharmacological importance of these kinases, there are barely any means for probing these kinases, which limits the development of diagnostic approaches to monitor alterations in their levels or activities associated with human cancer. The development of cell cycle reporters and biosensors to probe some of these kinases is now paving the way for developing strategies to detect anomalies in these kinases associated with cancer.[146]

3.6.1 CDKSENS and CDKACT—Fluorescent peptide biosensors for probing the relative abundance and activity of CDKs

Cyclin-dependent kinases (CDK/cyclins) are heterodimeric protein kinases that coordinate cell cycle progression through phosphorylation of well-defined enzymatic and structural targets.[147–149] CDK/cyclins are known to contribute to sustain aberrant cell proliferation in human cancers and constitute attractive pharmacological targets for the development of anticancer drugs. Unfortunately, there are no direct means of studying

the levels and activities of these kinases in their natural environment in living cells. From a fundamental perspective, this limits our understanding of the physiology of these enzymes in their natural environment. From a diagnostic perspective, reporters that provide information on alterations in CDK/cyclin levels and activity would provide the means of identifying cancer cells or tumors in which these kinases constitute relevant targets for therapeutic intervention.

We have developed two new families of peptide biosensors that report on the relative abundance and activity of CDK/cyclins, respectively, through changes in fluorescence of an environmentally sensitive probe.[113,114] CDKSENS is a biligand peptide composed of a CDK-binding pseudosubstrate sequence and a cyclin-binding sequence that allows docking of the biosensor onto the CDK/cyclin complex with high affinity and specificity. This peptide bears a fluorescent probe (cyanine dye) which undergoes significant enhancement upon recognition and docking onto CDK/cyclin complexes.[113] CDKACT is a modular peptide biosensor constituted of a peptide substrate, onto which a fluorescent probe is coupled proximal to the phosphorylation site, and a PBD that recognizes the phosphorylated peptide sequence, separated by a short proline/glycine-rich linker. This biosensor undergoes changes in fluorescence intensity upon phosphorylation by active CDK/cyclin complexes and is fully reversible, thereby allowing monitoring kinase activity in real time through changes in fluorescence intensity of an environmentally sensitive probe.[114] Both CDKSENS and CDKACT were validated using recombinant CDK/cyclin complexes as well as cell extracts derived from healthy fibroblasts and cancer cell lines expressing endogenous CDK/cyclins. The design of CDKSENS and CDKACT is depicted in Fig. 6.14.

CDKSENS and CDKACT constitute sensitive tools for reporting on CDK/cyclin levels and activity *in vitro* and in cell extracts. Furthermore, these peptide biosensors can be introduced into living cells, thanks to CPPs,[107,108] thereby allowing monitoring of the CDK/cyclin levels and activities in their natural environment through live-cell fluorescence imaging and ratiometric quantification. Indeed, CDKSENS and CDKACT allow the detection of subtle differences when tampering with a single CDK or cyclin (through siRNA downregulation or through ectopic expression) or following treatment with CDK/cyclin drugs. They further allow the detection of the differences between different healthy and cancer cell lines, thereby enabling identification of cells that express

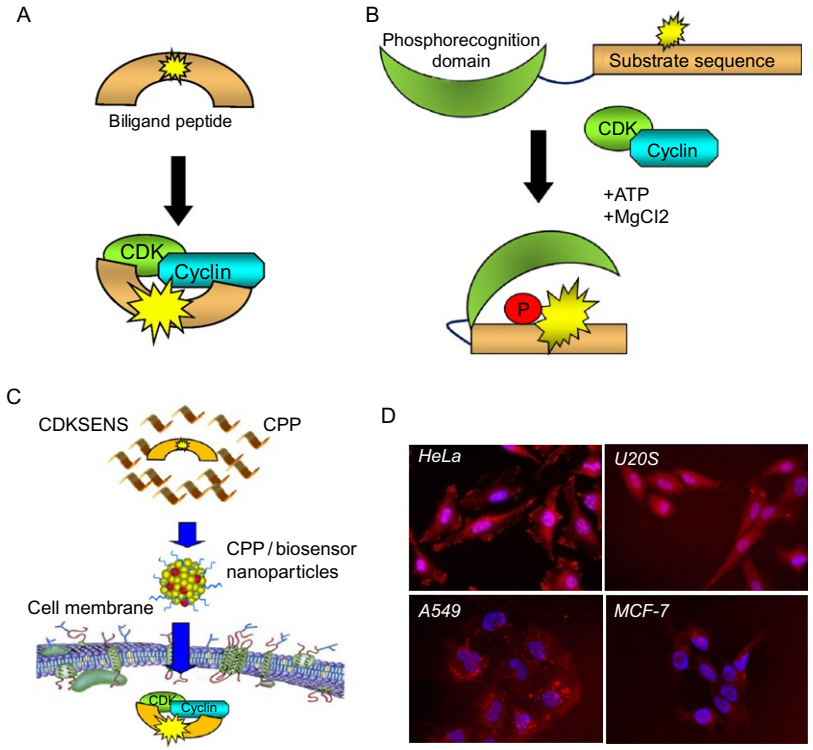

Figure 6.14 *CDKSENS and CDKACT biosensors*. (A) CDKSENS and (B) CDKACT biosensors are fluorescent peptide biosensors that allow probing the relative abundance and the kinase activity of cyclin-dependent kinases, respectively, *in vitro* and (C) in living cells following delivery mediated by cell-penetrating peptides. (D) A representative example of CDKSENS labeled with Cy3 internalized into different cancer cell types.

high levels or activities of CDK/cyclin kinases from cells that present decreased or defective assemblies.

CDKSENS provides information on CDK/cyclin complexes that is not conveyed by antibodies that recognize individual CDK or cyclin subunits. Likewise, CDKACT allows circumventing the requirement for antigenic or radioactive assays to report on CDK/cyclin kinase activity. As such, these technologies offer promising perspectives for the development of fluorescence-based cancer diagnostics, present strong potential for monitoring response to therapeutic strategies targeting CDKs or cyclins, and constitute attractive tools for drug discovery programs.

4. APPLICATIONS

Protein kinases are central to most biological signaling pathways and constitute disease biomarkers in many pathological conditions. As such, they have been the focus of intensive studies using different technologies, mostly based on antigenic and radioactive assays. The more recent development of fluorescent reporters and biosensors has provided the means of imaging the activity and dynamic behavior of protein kinases in real time in their natural environment, with high spatial and temporal resolution, without perturbing their native structure or function. Fluorescent biosensors provide a wealth of opportunities over other approaches used for fundamental studies of protein kinases and allow for countless practical applications in analytical chemistry and biotechnology, as well as in biomedical and drug discovery programs (Fig. 6.15).

4.1. Fundamental studies of protein kinase localization, function, and regulation

From a fundamental perspective, fluorescent biosensors have proved useful for studying the dynamics of subcellular localization, function, and regulation of individual kinases in real time. While these tools may be employed to study the kinetics of protein kinase activation *in vitro*, they may equally well be used to probe associated activities in cell extracts and further be applied to characterize the spatiotemporal dynamics and activities of these enzymes in living cells with high spatial and temporal resolution. Biosensor technology allows addressing those aspects of protein kinase biology that had not been characterized previously because of the lack of appropriate technologies. Indeed, fluorescent biosensors allow imaging the behavior of protein kinases within distinct subcellular compartments and, in response to stimuli, involving different cofactors, agonists, or coregulators, thereby contributing to unravel complex signaling cascades that lead to kinase activation in a physiological context and providing a comprehensive understanding of their overall function in a cellular context. Moreover, fluorescent biosensors are very useful for comparative studies of protein kinases in healthy and pathological conditions, highlighting molecular and cellular alterations associated with disease in a qualitative and quantitative fashion (for review, see Refs. 150–152).

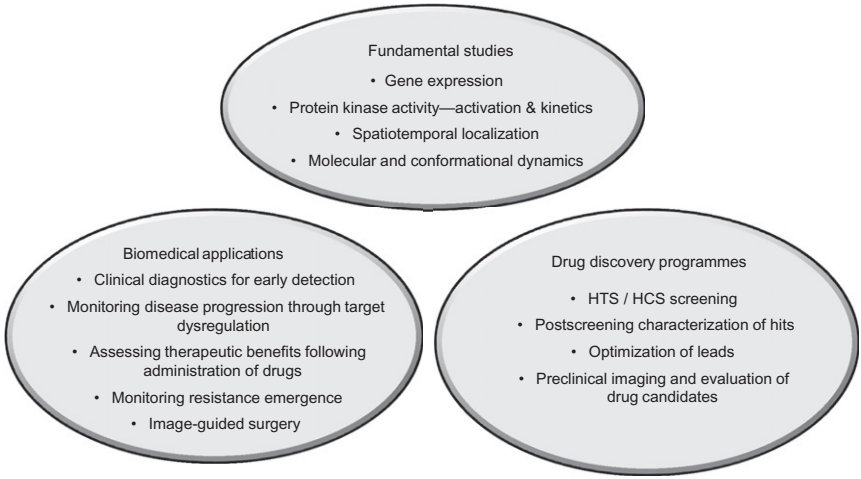

Figure 6.15 *Applications of fluorescent kinase biosensors*. Fluorescent biosensors can be applied to probe the function, regulation, and spatiotemporal dynamics of protein kinases in fundamental studies. Aside from their utility in fundamental research, they constitute potent tools for biomedical and drug discovery approaches. Biomedical applications include development of clinical diagnostics, through imaging disease biomarkers, monitoring disease progression, and response to therapeutics. Drug discovery approaches include high-throughput screening and postscreening evaluation of lead compounds and drug candidates.

4.2. Monitoring protein kinase biomarkers in clinical diagnostics

From a biomedical perspective, fluorescent biosensors constitute very attractive tools for probing differential or aberrant activation of protein kinases in a pathological context and have a number of practical applications in several areas of health and disease, including clinical diagnostics and monitoring response to therapeutics. For instance, imaging protein kinases that constitute disease biomarkers, thanks to biosensors whose fluorescence is affected by their activity, can provide a wealth of information on the functional status of these enzymes. In this respect, fluorescent biosensors provide a basis for the development of diagnostic strategies involving detection of protein kinase biomarkers whose activity is dysregulated in pathological disorders or involved in disease pathogenesis. Although there are still very few fluorescent biosensors that have actually been applied to detect protein kinases in a diagnostic perspective, a couple of noteworthy examples are currently employed for clinical diagnostics of dysregulated protein kinases in cancer.

The genetically encoded FRET biosensor developed by Mizutani *et al.* for detection of Bcr–Abl kinase activity allows the detection of cancerous

and drug-resistant cells and can further be applied for direct evaluation of kinase inhibitor efficacy.[48] This highly sensitive biosensor was therefore applied to assess Bcr–Abl activity from patient cells and further employed to establish a correlation with the disease status: a clinical diagnosis of Bcr–Abl kinase activity in CML. Moreover, this probe has been used to monitor response to therapy and to detect the onset of drug-resistant cells, thereby allowing foreseeing when and how to employ second-generation inhibitors or novel compounds to treat drug-resistant mutants.[95,96] Wang *et al.* developed fluorescent peptide biosensors that are enzymatically and photophysically distinct, allowing the simultaneous monitoring of Bcr–Abl and Lyn tyrosine kinase activity through multicolor imaging. In particular, this combination of orthogonal probes revealed significant differences in Lyn kinase activity, but not in Abl kinase activity, between CML cell lines that are sensitive to imatinib or which develop resistance to this drug.[128]

It can be expected that the potential of fluorescent biosensors will be harnessed for clinical diagnostics in the coming years, not only for early stage detection but also for monitoring kinase activities throughout disease progression and for assessing the benefits of therapeutic intervention, drug efficiency, and resistance, in particular, but not exclusively in the field of cancer.[152]

4.3. Drug discovery strategies—HTS/HCS assays and postscreening evaluation

Protein kinases constitute one of the major classes of therapeutic targets for drug discovery programs developed by academia and the pharmaceutical industry. In this respect, fluorescent biosensors constitute sensitive and selective tools that provide a means of monitoring protein kinase activity in a continuous fashion *in vitro* or in cell-based assays, in high-throughput and high content screening assays. Indeed, fluorescent biosensors are particularly well suited to screen large, complex libraries for inhibitors that affect enzymatic activity or function in high-throughput formats because of the high sensitivity of response to compounds that affect kinase activity. Moreover, as fluorescent reporters allow visualization of the activity of target enzymes in a continuous fashion, they allow establishing screens that follow the action of compounds on kinase activity over time, rather than at a static point. Fluorescent biosensors are further well suited to the qualitative and quantitative evaluation of kinase inhibitor efficacy in living cells at a postscreening stage, to gain insight into the pharmacokinetics and pharmacodynamics of hits and to determine the efficacy of novel leads and optimize derivative compounds for therapeutic applications. Finally, biosensor technology can be employed to

assess the potential of candidate drugs in preclinical evaluation trials and to monitor response over time as well as the emergence of resistance.

To this aim, several strategies have been developed successfully: FRET-based approaches and positional biosensors whose changes in subcellular localization reflect the activity or the inhibition of the target enzymes. The gold standard for high-throughput formats is an assay that can be easily miniaturized without losing signal intensity or signal-to-noise ratio and that does not suffer from off-target effects.[153]

Several HTS assays have been developed for screening small-molecule compounds from complex libraries, based on genetically encoded FRET biosensors.[154] Likewise, nongenetic, environmentally sensitive peptide biosensors are perfectly well suited to HTS assays and Sox biosensors have been readily applied to probe kinase activities in HTS formats using cell lysates.[118,119,122,123] As such, fluorescent biosensors constitute attractive tools for drug discovery and, in particular, for assays based on cellular and molecular imaging.[155–159] Fluorescence imaging technologies based on biosensors facilitate drug discovery in a complex physiological environment of a cell in a quantitative fashion. Fluorescence near-infrared and multispectral imaging modalities are also becoming increasingly popular in preclinical studies in animal models to image disease and drug targets, drug distribution, and therapeutic response in a noninvasive fashion, thereby contributing to accelerated preclinical drug development *in vivo*.

5. CONCLUSIONS AND PERSPECTIVES

The opportunities offered by fluorescent biosensors for fundamental research and biomedical applications are countless. Although a large number of challenges have been overcome, there are still many technological developments required to move toward further applications. Future directions include the development of biosensors for multiplexed detection of protein kinases; strategies for improving delivery, targeting, and activation of biosensors; and *in vivo* imaging and biomedical applications, fluorescence-based diagnostics, theragnostic approaches, and image-guided surgery.

The future of these nanotools looks very bright, as their versatility and potency allows for their application in a myriad of different applications. Fluorescent biosensors can be expected to contribute to early detection of disease, in particular, cancer, thereby paving the way for personalized diagnostics and theranostic applications, multiplexed sensing technologies, and *in vivo* applications.

5.1. Multicolor imaging—Multisensing, multiplex, or multiparameter imaging

Multicolor imaging approaches have already been well described for studying protein/protein interactions. However, multiplex analysis of kinase activities through multicolor imaging constitutes a challenging objective, as it implies using different sets of fluorescent probes, which are sufficiently distinct with respect to their spectral properties and which allow imaging the complex dynamic patterns of kinases from different signaling pathways. Multisensing, multiplex, or multiparameter imaging consists in devising means of probing several targets using different fluorescent probes or biosensors simultaneously by measuring different excitation and emission wavelengths. This strategy is extremely useful for fundamental research, as it potentially allows the following of several signaling pathways in parallel and in real time, to gain insight into their hierarchy of activation and study the dynamics of their interconnections or their independent behavior. Obviously, from a diagnostics perspective, the multisensing approach allows a more detailed readout of the biomarker status. Indeed, most pathologies are characterized by the concomitant activation of multiple kinases from different signaling pathways. Moreover, in drug discovery strategies, multisensing allows gaining a larger set of information concerning the specificity of pharmacological inhibitors to characterize the underlying differences in potency, off-target effects, and genetic backgrounds.

The main challenge, however, consists in combining different tools into one single experimental design and finding suitable means to acquire, discriminate, and quantify their signals. This proves extremely challenging from a technological perspective when using FRET-based biosensors, as it requires the use of spectrally distinct FRET pairs for multiparameter live-cell fluorescence imaging.[160,161] Notwithstanding, this strategy has been successfully developed for imaging of dual FRET-based caspase-3 biosensors[162] for simultaneous imaging of cAMP and cGMP in single cells, thanks to the sapphire/RFP (red fluorescent protein) and CFP/YFP set of FRET biosensors, respectively,[163] and for monitoring three calcium-dependent signaling events, thanks to a cytosolic CamKIIα-CFP/YFP biosensor, a membrane-bound PKC-CFP/YFP sensor, and a translocating annexin A4-mOrange/mCherry FRET biosensor.[161] Likewise, multiplex strategies have been developed using nongenetic biosensors coupled to synthetic probes with different spectral properties. For example, Lawrence and collaborators developed a set of orthogonal fluorescent peptide biosensors whose combination allows for simultaneous multicolor monitoring of

Bcr–Abl and Lyn tyrosine kinase activity in cell extracts.[128] The combination of probes that provide a readout of different enzymatic activities offers a means of assessing the differential behavior of distinct targets in a given signaling pathway. Moreover, from a therapeutic perspective, it can provide information on the efficacy of drug cocktails and dosing regimens prior to therapy and during therapy, depending on disease progression and emergence of resistance.

5.2. *In vivo* imaging of protein kinase activities

Despite the complexity of enzymatic reactions in living systems, major advances have been made in probe design and application for *in vivo* imaging.[164,165] Efforts have been made in developing fluorescent proteins for imaging in living cells and tissues,[94] and several synthetic near-infrared fluorescence (NIRF) probes have been developed by chemists for *in vivo* application.[166] Moreover, imaging technologies have improved significantly[167] and strategies have been designed for *in vivo* imaging through engineering of activatable or targeted probes.[168–173] Nevertheless, *in vivo* imaging of fluorescent biosensors remains extremely challenging for several reasons. With respect to genetically encoded biosensors, the challenge resides essentially in the heterogeneity and lack of control associated with ectopic expression. This said, recent developments are contributing to establish stable expression of FRET biosensors in cell lines and expression in transgenic mouse models.[174,175] With respect to nongenetic biosensors, the challenge lies in the choice of synthetic probes that are compatible with *in vivo* imaging and in delivering peptide or polypeptide scaffolds *in vivo* in an efficient fashion. As mentioned above, the development of NIRF probes which are appropriate for *in vivo* applications[166] solves part of the problem. Moreover, the development of nanoparticle strategies for delivery of peptides and proteins into cells[107,108] will be of considerable help in promoting the successful application of nongenetic biosensors *in vivo*.

5.3. Delivery, targeting, and activation strategies

A major bottleneck for *in vivo* applications concerns the delivery of fluorescent biosensors into cells, tissues, and organs. In practice, this involves engineering biosensor formulations with nanocarriers which are stable in bodily fluids and which can be delivered to their target passively, through prolonged circulation and enhanced permeability retention in the tumor, for instance, or actively, thanks to specific targeting sequences. One of the future priorities for biosensor application *in vivo* therefore concerns the

refinement of noninvasive strategies for the delivery of fluorescent biosensors into living organisms.

With respect to targeting strategies, there are two different levels that may have a major impact on the signal reported by the biosensor. The first consists in targeting a specific intracellular compartment, thanks to subcellular targeting sequences. Indeed, several kinase biosensors have been specifically targeted to the nucleus, the Golgi, the endoplasmic reticulum, the mitochondria, or the plasma membrane, revealing differences in kinase activities and dynamics, as well as differences attributable to differential localization of discrete subpopulations or specific kinase isoforms. Hence, subcellular targeting allows monitoring the differential activity associated with subcellular localization, whereas it may not be as clear-cut if the biosensor is simply distributed randomly within the cellular cytoplasm. The second level of targeting consists in targeting specific cell types, which involves a more complex approach, requiring conjugation or complexation of the biosensors with formulations that direct the biosensor to cell-surface receptors.

Aside from targeting strategies, "smart" strategies based on specific activation of probes are being developed, notably for imaging cancer and metastasis, and some of these have been successfully applied to image-guided surgery.[168,176] These activatable probes make use of properties inherent to cancer cells for specific penetration and/or activation within tumor vicinity. For instance, metalloproteases secreted in the vicinity of tumors cleave target sequences which normally prevent the probe from penetrating cells in a nonspecific fashion, thereby releasing a cell-penetrating sequence that allows for endosomal uptake of the probe.[169] Other systems are activated by the acidic pH that characterizes the tumor environment, such as pH-activatable fluorescent moieties coupled to cancer-targeting antibodies[172] and pH-activatable CPPs conjugated to a fluorescent probes (pHLIP technology).[173] Yet another strategy consists in silencing a fluorescent probe through molecular quenching until specific enzymes release the molecular cage. This strategy has been applied to generate a fluorescent probe activated by tumor-specific γ-glutamyl transferases (GGT),[177] which can be applied topically to probe fluorescence at local tumor sites and which allows for rapid visualization of surface lesions upon intraoperative detection of tumors and metastases.

Other examples include targeting of NIRF probes to specific cell-surface antigens and receptors, such as integrins and Glutamate Transporter receptors (GLUT), through conjugation with Cyclic Arginine-Glycine-Aspartate (RGD) and 2-deoxyglucose, respectively, to image tumors *in vivo*.[178,179]

5.4. Looking toward the future—Biomedical imaging for diagnostics

Visualizing the activities of protein kinases in disease such as cancer and metastasis can provide insights into its pathogenesis while also providing a means for early detection of target dysregulation. Fluorescent biosensors unquestionably constitute one of the most promising classes of tools for detection of biomarkers *in situ*, as they allow circumventing the biopsy and extraction required for traditional *ex vivo* detection of biomarkers using antigenic approaches.

While the perspective of developing diagnostics through molecular imaging may still seem far-fetched, advances in fluorescence imaging technologies, associated with ongoing developments in medical devices for whole-animal and patient imaging, are paving the way toward this possibility. Moreover, together with the development of novel synthetic probes and smart chemical biology approaches, new generations of molecular tracers and fluorescent biosensors are being proposed to report on disease biomarkers. The concomitant development of nanocarriers and nanoparticles that are suited to introduce these fluorescent probes *in vivo* is now providing new opportunities to "illuminate disease" through molecular imaging. Fluorescent imaging technology can easily be combined with endoscopy or tomographic optical imaging methods, thereby raising expectations for the application of fluorescent biosensors to detect target dysfunction or hyperactivation *in vivo* in diseased tissues, tumors, or metastatic cancer cells. Finally, this approach can potentially be applied to monitor response to therapeutics and thereby develop a theragnostic approach allowing the determination of the status of a given biomarker over time while administrating a drug and assessing the benefits of treatment.

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