TRANSDUCTION MECHANISMS IN CELLULAR SIGNALING

EDITED BY
EDWARD A. DENNIS AND RALPH A. BRADSHAW

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Preface

Since cell signaling is a major area of biomedical/biological research and continues to advance at a very rapid pace, scientists at all levels, including researchers, teachers, and advanced students, need to stay current with the latest findings, yet maintain a solid foundation and knowledge of the important developments that underpin the field. Carefully selected articles from the 2nd edition of the *Handbook of Cell Signaling* offer the reader numerous, up-to-date views of intracellular signal processing, including membrane receptors, signal transduction mechanisms, the modulation of gene expression/translation, and cellular/organotypic signal responses in both normal and disease states. In addition to material focusing on recent advances, hallmark papers from historical to cutting-edge publications are cited. These references, included in each article, allow the reader a quick navigation route to the major papers in virtually all areas of cell signaling to further enhance his/her expertise.

The Cell Signaling Collection consists of four independent volumes that focus on *Functioning of Transmembrane Receptors in Cell Signaling, Transduction Mechanisms in Cellular Signaling, Regulation of Organelle and Cell Compartment Signaling,* and *Intercellular Signaling in Development and Disease*. They can be used alone, in various combinations or as a set. In each case, an overview article, adapted from our introductory chapter for the Handbook, has been included. These articles, as they appear in each volume, are deliberately overlapping and provide both historical perspectives and brief summaries of the material in the volume in which they are found. These summary sections are not exhaustively referenced since the material to which they refer is.

The individual volumes should appeal to a wide array of researchers interested in the structural biology, biochemistry, molecular biology, pharmacology, and pathophysiology of cellular effectors. This is the ideal go-to books for individuals at every level looking for a quick reference on key aspects of cell signaling or a means for initiating a more in-depth search. Written by authoritative experts in the field, these papers were chosen by the editors as the most important articles for making the Cell Signaling Collection an easy-to-use reference and teaching tool. It should be noted that these volumes focus mainly on higher organisms, a compromise engendered by space limitations.

We wish to thank our Editorial Advisory Committee consisting of the editors of the Handbook of Cell Signaling, 2nd edition, including Marilyn Farquhar, Tony Hunter, Michael Karin, Murray Korc, Suresh Subramani, Brad Thompson, and Jim Wells, for their advice and consultation on the composition of these volumes. Most importantly, we gratefully acknowledge all of the individual authors of the articles taken from the Handbook of Cell Signaling, who are the 'experts' upon which the credibility of this more focused book rests.

Ralph A. Bradshaw, San Francisco, California
Edward A. Dennis, La Jolla, California
January, 2011

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Section A

Overview

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Chapter 1

Intracellular Signaling*

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Cell signaling, which is also often referred to as signal transduction or, in more specialized cases, transmembrane signaling, is the process by which cells communicate with their environment and respond temporally to external cues that they sense there. All cells have the capacity to achieve this to some degree, albeit with a wide variation in purpose, mechanism, and response. At the same time, there is a remarkable degree of similarity over quite a range of species, particularly in the eukaryotic kingdom, and comparative physiology has been a useful tool in the development of this field. The central importance of this general phenomenon (sensing of external stimuli by cells) has been appreciated for a long time, but it has truly become a dominant part of cell and molecular biology research in the past three decades, in part because a description of the dynamic responses of cells to external stimuli is, in essence, a description of the life process itself. This approach lies at the core of the developing fields of proteomics and metabolomics, and its importance to human and animal health is already plainly evident.

ORIGINS OF CELL SIGNALING RESEARCH

Although cells from polycellular organisms derive substantial information from interactions with other cells and extracellular structural components, it was humoral components that first were appreciated to be intercellular messengers. This idea was certainly inherent in the 'internal secretions' initially described by Claude Bernard in 1855 and thereafter, as it became understood that ductless glands, such as the spleen, thyroid, and adrenals, secreted material into the bloodstream. However, Bernard did not directly identify hormones as such. This was left to Bayliss and Starling and their description of secretin in 1902 [1].

Recognizing that it was likely representative of a larger group of chemical messengers, the term *hormone* was introduced by Starling in a Croonian Lecture presented in 1905. The word, derived from the Greek word meaning 'to excite or arouse,' was apparently proposed by a colleague, W. B. Hardy, and was adopted, even though it did not particularly connote the messenger role but rather emphasized the positive effects exerted on target organs via cell signaling (see Wright [2] for a general description of these events). The realization that these substances could also produce inhibitory effects, gave rise to a second designation, 'chalones,' introduced by Schaefer in 1913 [3], for the inhibitory elements of these glandular secretions. The word autocoid was similarly coined for the group as a whole (hormones and chalones). Although the designation chalone has occasionally been applied to some growth factors with respect to certain of their activities (e.g., transforming growth factor), autocoid has essentially disappeared. Thus, if the description of secretin and the introduction of the term hormone are taken to mark the beginnings of molecular endocrinology and the eventual development of cell signaling, then we have passed the hundredth anniversary of this field.

The origins of endocrinology, as the study of the glands that elaborate hormones and the effect of these entities on target cells, naturally gave rise to a definition of hormones as substances produced in one tissue type that traveled systemically to another tissue type to exert a characteristic response. Of course, initially these responses were couched in organ and whole animal responses, although they increasingly were defined in terms of metabolic and

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other chemical changes at the cellular level. The early days of endocrinology were marked by many important discoveries, such as the discovery of insulin [4], to name one, that solidified the definition, and a well-established list of hormones, composed primarily of three chemical classes (polypeptides, steroids, and amino acid derivatives), was eventually developed. Of course, it was appreciated even early on that the responses in the different targets were not the same, particularly with respect to time. For example, adrenalin was known to act very rapidly, while growth hormone required a much longer time frame to exert its full range of effects. However, in the absence of any molecular details of mechanism, the emphasis remained on the distinct nature of the cells of origin versus those responding and on the systemic nature of transport, and this remained the case well into the 1970s. An important shift in endocrinological thinking had its seeds well before that, however, even though it took about 25 years for these 'new' ideas that greatly expanded endocrinology to be enunciated clearly.

Although the discovery of polypeptide growth factors as a new group of biological regulators is generally associated with nerve growth factor (NGF), it can certainly be argued that other members of this broad category were known before NGF. However, NGF was the source of the designation *growth factor* and has been, in many important respects, a Rosetta stone for establishing principles that are now known to underpin much of signal transduction. Thus, its role as the progenitor of the field and the entity that keyed the expansion of endocrinology, and with it the field of cell signaling, is quite appropriate. The discovery of NGF is well documented [5] and how this led directly to identification of epidermal growth factor (EGF) [6], another regulator that has been equally important in providing novel insights into cellular endocrinology, signal transduction and, more recently, molecular oncology. However, it was not till the sequences of NGF and EGF were determined [7, 8] that the molecular phase of growth factor research began in earnest. Of particular importance was the postulate that NGF and insulin were evolutionarily related entities [9], which suggested a similar molecular action (which, indeed, turned out to be remarkably clairvoyant), and was the first indication that the identified growth factors, which at that time were quite limited in number, were like hormones. This hypothesis led quickly to the identification of receptors for NGF on target neurons, using the tracer binding technology of the time (see Raffioni *et al.* [10] for a summary of these contributions), which further confirmed their hormonal status. Over the next several years, similar observations were recorded for a number of other growth factors, which in turn, led to the redefinition of endocrine mechanisms to include paracrine, autocrine, and juxtacrine interactions [11]. These studies were followed by first isolation and molecular characterization using various biophysical methods and then cloning of their cDNAs, initially for the insulin and EGFR receptors [12–14] and then many others. Ultimately, the powerful techniques of molecular biology were applied to all aspects of cell signaling and are largely responsible for the detailed depictions we have today. They have allowed the broad understanding of the myriad of mechanisms and responses employed by cells to assess changes in their environment and to coordinate their functions to be compatible with the other parts of the organism of which they are a part.

INTRACELLULAR SIGNALING MECHANISMS

At the same time that the growth factor field was undergoing rapid development, major advances were also occurring in studies on hormonal mechanisms. In particular, Sutherland and colleagues [15] were redefining hormones as messengers and their ability to produce second messengers. This was, of course, based primarily on the identification of cyclic AMP (cAMP) and its production by a number of classical hormones. However, it also became clear that not all hormones produced this second messenger nor was it stimulated by any of the growth factors known at that time. This enigma remained unresolved for quite a long time until tyrosine kinases were identified [16, 17] and it was shown, first with the EGF receptor [18], that these modifications were responsible for initiating signal transduction for many of those hormones and growth factors that did not stimulate the production of cAMP.

Aided by the tools of molecular biology, it was a fairly rapid transition to the cloning of most of the receptors for hormones and growth factors and the subsequent development of the main classes of signaling mechanisms. These data allowed the six major classes of cell surface receptors for hormones and growth factors to be defined, which included, in addition to the receptor tyrosine kinases (RTKs) described previously, the G-protein coupled receptors (GPCRs) (including the receptors that produce cAMP) that constitute the largest class of cell surface receptors; the cytokine receptors, which recruit the soluble JAK tyrosine kinases and directly activate the STAT family of transcription factors; serine/threonine kinase receptors of the TGFβ superfamily; the tumor necrosis factor (TNF) receptors that activate nuclear factor kappa B (NFκB) via TRAF molecules, among other pathways; and the guanylyl cyclase receptors. Structural biology has not maintained the same pace, and there are still both ligands and receptors for which we do not have three-dimensional information.

In parallel with the development of our understanding of ligand/receptor organization at the plasma membrane, a variety of experimental approaches have also revealed the general mechanisms of transmembrane signal transduction in terms of the major intracellular events that are induced by these various receptor classes. There are three

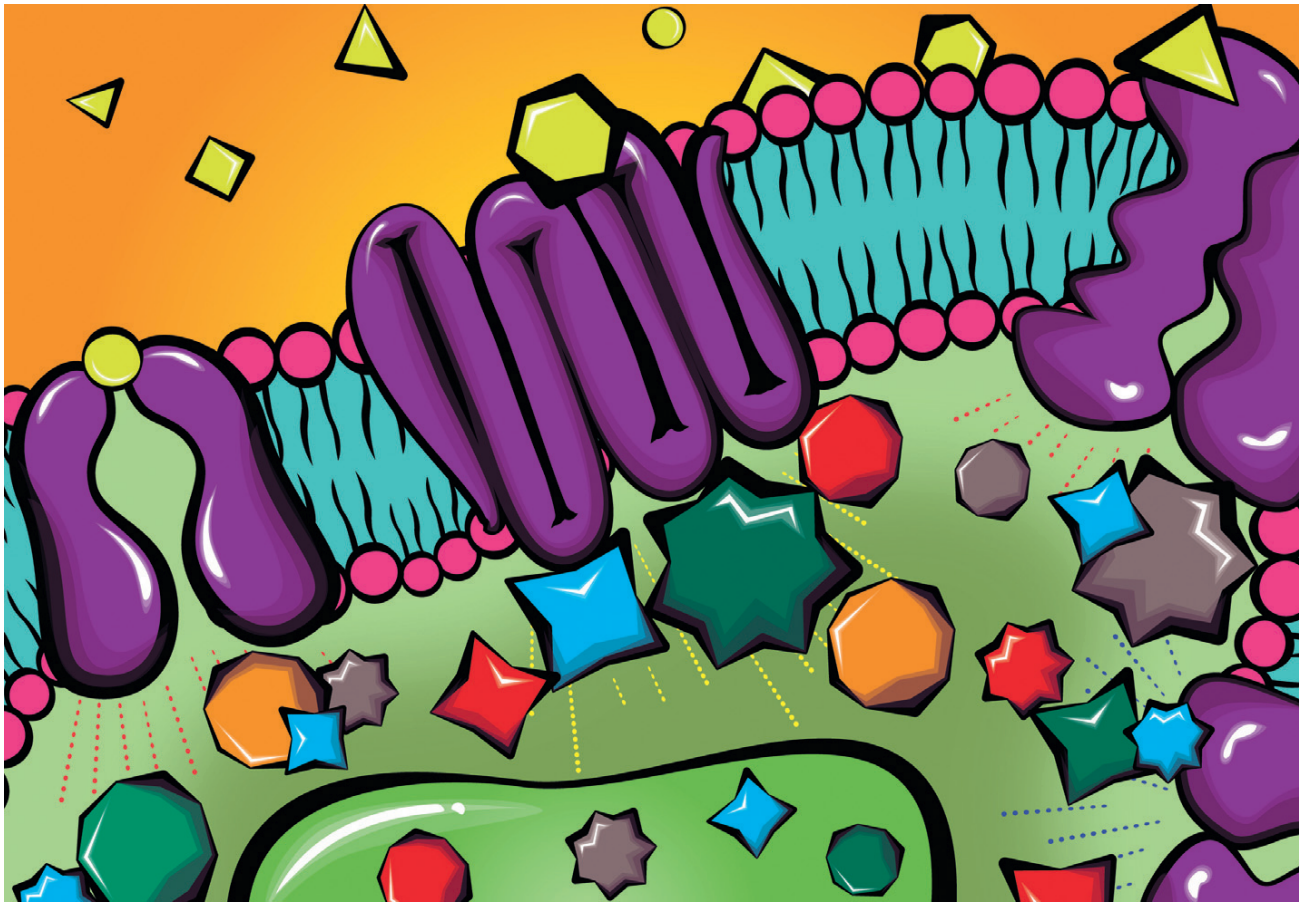

FIGURE 1.1 Intracellular events following receptor activation include the activation of kinase/phosphatase cascades, phospholipases liberating a variety of lipid mediators, cyclic nucleotide production and their downstream events, and numerous G-protein triggered pathways. The immediate signaling pathways often amplify their signals by a series of posttranslational events that in turn release various messengers, often lipids or ions, which over time ultimately result in the modulation of transcriptional events in the nucleus.

principal means by which intracellular signals are propagated: protein posttranslational modifications (PTMs), lipid messengers, and ion fluxes (see Figure 1.1). There are also additional moieties that play significant roles, such as cyclic nucleotides, but their effects are generally manifested in downstream PTMs. There is considerable interplay between the three, particularly in the more complex pathways.

By far the most significant of the PTMs is phosphorylation of serine, threonine, and tyrosine residues (although phosphorylation of several other residues is also known albeit that these modifications are usually found in lower organisms). As already noted, the RTK and cytokine receptors initiate their responses with tyrosine phosphorylation, and there are more than 30 additional nonreceptor tyrosine kinases that also have significant roles in many signaling responses that can be activated by these and other types of receptors. However, the vast bulk of protein phosphorylation occurs downstream from the receptors and is mainly on serine and threonine residues in a ratio of about 20:1. These are produced by a myriad of protein kinases that are activated themselves through PTMs or through the production of lipid messengers (see below). Indeed, there are over 500 protein kinases in the human genome with more than 100 phosphatases, which emphasizes the investment that has been made in this modification by higher eukaryotes [19]. Therefore, it is perhaps not surprising that through the agency of substantive technological advances in proteomic analyses, mainly in the area of mass spectrometry and its quantitative applications, it has become clear that the level of this PTM, both in terms of type and amount, is significantly greater than originally envisioned [20]. It could not have been readily anticipated from the pioneering studies of Krebs and Fischer in the 1960s [21] when they observed the regulation of muscle phosphorylase activity by protein phosphorylation that this modification would occur essentially universally in cells and that hundreds, if not thousands, of enzyme activities and protein–protein interactions would be regulated by it. Nonetheless, thousands of phosphorylation events have indeed been detected in cellular paradigms that have been appropriately stimulated by one or another growth factor (see, e.g., Olsen

et al. [22]) and in no case has the complete set of modifications been identified. Clearly, the new challenges are to determine which of these modifications are physiologically meaningful and which kinases (or another type of modifying enzyme) are responsible for which alterations. Further findings using proteomic methodology have demonstrated that other PTMs are also important, if not as widespread. O-GlyNAcylation (also on serine and threonine residues) [23] and N^{ε}-acetylation of lysine residues [24] are examples of modifications that are receiving increasing attention.

As intracellular signaling was being unraveled, it became increasingly clear that receptor activation and subsequent activations through PTM additions were inducing more than just enzyme activations. Rather, many modifications were providing new, specific sites for forming protein complexes. These were appropriately designated as 'docking sites,' and it introduced the concept of both adaptors and scaffolds, with activated enzymes being called 'effectors.' Adaptors, such as Grb or Shc proteins, and the larger, multisite scaffolds, such as the insulin receptor substrate (IRS), recognize newly formed sites through specific motifs and as the process is repeated, successively build up multicomponent signaling structures [25]. There has now emerged a significant number of binding motifs, recognizing, in addition to PTMs, phospholipids and proline-rich peptide segments to name a few, that are quite widely scattered through the substantial repertoire of signaling molecules and that are activated by different types of receptors in a variety of cell types.

The elucidation of cell signaling mechanisms and the variety of molecules that are employed in these myriad of processes is particularly well exemplified by the lipid messengers. With the exception of steroid hormones, lipids have long been thought to function mainly in energy metabolism and membrane structure. Experimental work for the last two decades has revealed a broad recognition that membrane phospholipids provide many of the important cell signaling molecules via phospholipases and lipid kinases. Key is the role of phospholipase C of which there are four subtypes that are activated by various receptor systems to hydrolyze phosphatidylinositol bisphosphate (PIP_2) to release diglyceride that activates protein kinase C (PKC) and inositol triphosphate (IP_3), which mobilizes intracellular Ca^{2+} central to so many regulatory processes. The phosphorylation of PIP_2 at the 3-position to produce PIP_3 promotes vesicular trafficking and other cellular processes. Phospholipase D releases phosphatidic acid, and phospholipase A2 provides arachidonic acid, which is converted into prostaglandins, leukotrienes, lipoxins, and various P450 products; these ligands in turn bind to unique families of receptors as does platelet activating factor (PAF). The more recent recognition, in the last decade, of the importance of sphingolipids and ceramide in signaling and the discoveries of the unique lysophosphatidic acid and sphingosine phosphate families of receptors have sparked the search for other new lipid messengers and their receptors. The newly emerging field of lipidomics (see www.lipidmaps.org) holds the promise of expanding our ability to interrogate in greater detail the specificity of agonists and receptors and their effects on lipid signaling events [26–28].

The extent and complexity of GPCRs, in terms of both the ligands that bind them and the effectors they in turn activate, is unparalleled in the other signaling systems. The receptors of this family, with their seven transmembrane segments, function by linking to heteromeric G protein complexes composed of three subunits: α, β, and γ. The α-subunit binds GTP and the receptor-G-protein complex functions as a guanine nucleotide exchange factor (GEF). The ligand induces the G-protein to split into two components – α-GTP and $\beta\gamma$ – both of which are active in the further propagation of the signal. When the GTP is hydrolyzed to GDP, it recycles back to the GTP form so it is ready to be reutilized. This type of biochemical 'switch' is widely encountered in biological systems ranging from translation to vesicle transport and is also utilized (as Ras) in the major pathway leading to ERK activation by RTKs. GPCRs are utilized as sensors of peptide/protein hormones, neurotransmitters, amino acids, lipids, and various physiological processes such as light, taste, and smell. The adenylyl cyclases are a major effector for the GPCR signals and are affected by both the α-GTP and $\beta\gamma$ subunits. However, they also activate some of the nonreceptor tyrosine kinases, PI-3-K and the β-type of PLC among others.

cAMP, the product of adenylyl cyclase activation, was of course the discovery of Sutherland and colleagues [15], and it exerts much of its effects by the activation of PKA. This is one of the most important mediators in signal transduction pathways. It is composed of two regulatory and two catalytic subunits and is activated when cAMP binds to the regulatory subunits, causing dissociation of the heterotetramer and the concomitant activation of the catalytic subunits. In addition to its multiple cellular roles, it has been an important model for understanding the structure–function relationships of the protein kinase superfamily.

FOCUS AND SCOPE OF THIS VOLUME

The chapters of this volume have been selected from a larger collection [29] and have been organized to emphasize receptor organization and transduction mechanisms functioning in cell signaling. They have been contributed by recognized experts and they are authoritative to the extent that size limitations allow. It is our intention that this survey will be useful in teaching, particularly in introductory courses, and to more seasoned investigators new to this area.

It is not possible to develop any of the areas covered in this volume in great detail, and expansion of any topic is left to the reader. The references in each chapter provide an

excellent starting point, and greater coverage can also be found in the parent work [29]. It is important to realize that this volume does not cover other aspects of cell signaling such as the structure and role of cell surface receptors in signaling activities, transcriptional activation and responses in other organelles, and organ-level manifestations, including disease correlates. These can be found in other volumes in this series [30–32].

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Section B

Phosphorylation/ Dephosphorylation

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Part 1

Kinases

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Chapter 2

Eukaryotic Kinomes: Genomics and Evolution of Protein Kinases

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INTRODUCTION

Ever since the discovery 50 years ago that reversible phosphorylation regulates the activity of glycogen phosphorylase [1], there has been intense interest in the role of protein phosphorylation in regulating protein function. With the advent of DNA cloning and sequencing in the mid-1970s it rapidly became apparent that a large family of eukaryotic protein kinases exists, and the burgeoning numbers of protein kinases led to the speculation that a vertebrate genome might encode as many as 1001 protein kinases [2]. Since then, the importance of protein phosphorylation as a regulatory mechanism has continued to grow, and recent phosphoproteomic analyses suggest that the majority of intracellular proteins can be phosphorylated at one or more sites under an appropriate condition. Protein phosphorylation not only regulates enzymatic activity through inducing conformational changes or through direct steric effects, but also modulates the function of structural proteins through conformational and charge effects. In addition, a major function of protein-linked phosphates is to provide docking sites for other proteins, thus promoting inducible protein–protein association [3].

The catalytic domains of eukaryotic serine/threonine- and tyrosine-specific protein kinases are related in sequence, and belong to the eukaryotic protein kinase (ePK) superfamily, which in turn is a subset of PKL (protein-kinase like) kinases that share a common fold and catalytic mechanism [4]. A few structurally unrelated proteins also have reported protein kinase activity, and a wide variety of protein families can phosphorylate non-protein substrates [5]. Non-ePK protein kinases are termed aPKs, or atypical protein kinases. In addition to Ser, Thr, and Tyr, several other amino acids in proteins can be phosphorylated, including Lys, Arg, and His. The provenance of the responsible protein kinases remains unclear, although NDPK-B has recently been reported to be a *bona fide* mammalian histidine kinase [6]. The prokaryotic two-component protein kinases, commonly known as "histidine" kinases, form yet another distinct family. These autophosphorylate on histidine, and then transfer the phosphate to an aspartate on a substrate protein. These kinases are also found in plants and protists, but are absent from animals, apart from the unusual mitochondrial PDHK family members, which phosphorylate Ser/Thr.

The ~270 amino acid ePK catalytic domain is characterized by a series of conserved sequence motifs, which define 11 subdomains, and serve as key catalytic elements of the kinase domain [7, 8]. These motifs in combination with the overall catalytic domain sequence can be used to identify other protein kinases through pairwise and HMM profile sequence searches. aPKs can be found using similar approaches. Using this strategy, we have surveyed a series of sequenced eukaryotic genomes to define the protein kinase complement (kinome) of each organism [9–16] and used this as a basis to explore the evolution and global functions of all protein kinases.

THE HUMAN KINOME

In our original survey completed in 2002 [11], we predicted that the human genome has 518 protein kinase genes (2.3 percent of all ~22,500 genes) (Figure 2.1). Of these, 478 encode ePKs, with the others divided between 9 small aPK families, which include the PIKK (PI3 kinase-like kinase), the PDHK (pyruvate dehydrogenase kinase) and alpha kinase (E2F kinase) families. There are 90 tyrosine kinase genes (16 percent of all protein kinases). The complexity of the kinome is further increased by alternative splicing of

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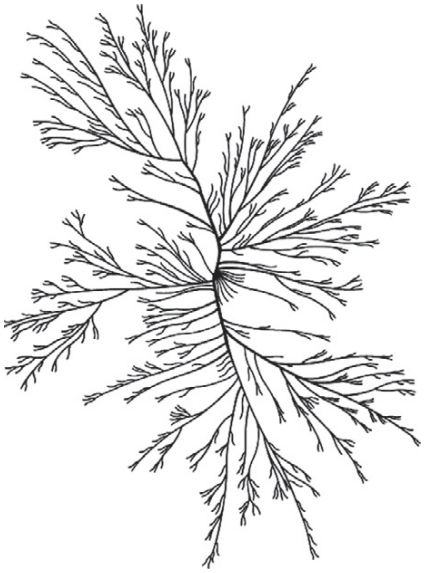

FIGURE 2.1 The human kinome.
A stylized phylogenetic tree represents the 492 ePK domains in the human genome, shaded by group classification. For further details, see http://kinase.com/human/kinome/. Reproduced by permission of *Science* magazine and *Cell Signaling Technologies*.

over half of all kinases [17], which in many cases is known to modulate function, as well as by the existence of regulatory subunits and differential targeting within the cell through association with scaffolding proteins.

Since the 2002 catalog, we have added 7 Ser/Thr kinases (6 four-jointed kinases and a second copy of PITSLRE/CDC2L2), lost 2 Tyr kinases (twinfilin/A6 family), and added NDPK-B (and 8 homologs) as a *bona fide* histidine kinase, to give a total of 532. The list may grow further, as humans have members of other classes of PKLs that are usually thought of as small-molecule kinases but have members known to phosphorylate proteins (e.g., ACAD10/11 from the CAK family [4]) Other ATPases or structurally distinct proteins may also emerge to have kinase activity (as seen in the MinD family of bacterial tyrosine kinases [18]). A few kinases currently in the catalog, such as SgK424 and PRKY are dubious, and may be relegated to pseudogenes.

The major control functions of protein kinases are reflected in their involvement in disease. Thirty-five percent of the kinome (175 genes) has been directly implicated in human disease, through mutation, mis-expression, or copy number changes [19]; 121 protein kinases are implicated in cancer, including 51 of the 90 tyrosine kinases. Many more protein kinases are weakly implicated and are emerging from genome-scale studies, including recent efforts to re-sequence the entire kinome in a wide variety of human cancers to pinpoint driver mutations involved in carcinogenesis. For instance, 164 kinases were mapped to common amplicons [11], and many more such data are now emerging.

Protein kinase catalytic function is often dependent on additional domains in the protein, which regulate activity, localize, and recruit regulatory proteins/second messengers and substrates. About half the protein kinases are predicted to have additional domains, many of which are implicated in signaling. Of the tyrosine kinases, 25 have P.Tyr binding SH2 domains that play a cardinal role in establishing tyrosine-phosphorylation based signaling networks. In contrast, perhaps surprisingly, only one serine kinase contains a P.Ser/Thr binding domain (an FHA domain in CHK2). In addition, 46 protein kinases have domains that interact with other proteins (e.g., SH3); 55 tyrosine and serine kinases have lipid interaction domains (e.g., PH); 38 have domains linked to small GTPase signaling; and 28 serine kinases have domains linked to calcium signaling. Generally, most members of a protein kinase family have the same set of ancillary domains, but there are some exceptions, and alternative splicing is often used to generate distinct domain combinations from a single gene. A complete listing of additional domains found in human protein kinases is given at http://kinase.com/.

The kinase catalytic domain itself often has ancillary functions. In fact, close to 10 percent of all kinase domains are predicted to have lost enzymatic function, but are retained for non-catalytic reasons. Of the 492 human ePK catalytic domains, 48 are predicted to be inactive, based on loss of key catalytic residues (Lys72/Asp166/Asp184 in PKA) and review of experimental data [20]. These "pseudokinase domains" may serve as docking platforms or scaffolds (e.g., ErbB3 and ILK), structural elements (receptor guanylyl cyclase kinase homology domains), and/or regulatory domains, which might bind and sense ATP levels [21]. Alternatively, they can act as regulators of protein kinases, mimicking mechanisms used by active protein kinases. Most human pseudokinase domains are conserved in all vertebrates, and several are even more ancient: CCK4 is inactive in all metazoans, and the inactive second kinase domain of GCN2 is found in almost all eukaryotes, suggesting that these domains play vital biological roles [11].

There were predicted to be 106 kinase pseudogenes in the human genome. These have sequence similarity to protein kinases, but have stop codons or frameshifts within their sequence, and in many cases (75) lack introns, indicating that they are retrotransposed copies of expressed kinases. For reasons that are unclear, some protein kinase families have a very high ratio of pseudogenes to functional genes (e.g., MARK 28:4). The mouse genome has a similar count of 97 kinase pseudogenes. None of these are orthologous to human, and the families that have high pseudogene counts are distinct from human, implying that no cryptic function remains for most kinase pseudogenes. On the other hand, retrotransposition appears to be the origin of several recently-derived functional kinases, such as the primate-specific TAF1L, CK1α2, and PKACγ genes [14]. Pseudogenes are rare in most invertebrate kinomes, although *C. elegans* has 24 kinase pseudogenes, mostly in recently expanded families [22].