The origins of protein phosphorylation

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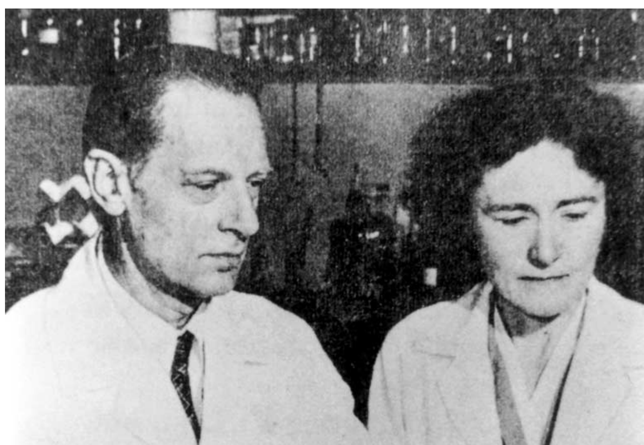
The reversible phosphorylation of proteins is central to the regulation of most aspects of cell function but, even after the first protein kinase was identified, the general significance of this discovery was slow to be appreciated. Here I review the discovery of protein phosphorylation and give a personal view of the key findings that have helped to shape the field as we know it today.

The days when protein phosphorylation was an abstruse backwater, best talked about between consenting adults in private, are over. My colleagues no longer cringe on hearing that "phosphorylase kinase phosphorylates phosphorylase" and their eyes no longer glaze over when a "'kinase kinase kinase" is mentioned. This is because protein phosphorylation has gradually become an integral part of all the systems they are studying themselves. Indeed it would be difficult to find anyone today who would disagree with the statement that "the reversible phosphorylation of proteins regulates nearly every aspect of cell life". Phosphorylation and dephosphorylation, catalysed by protein kinases and protein phosphatases, can modify the function of a protein in almost every conceivable way; for example by increasing or decreasing its biological activity, by stabilizing it or marking it for destruction, by facilitating or inhibiting movement between subcellular compartments, or by initiating or disrupting protein–protein interactions. The simplicity, flexibility and reversibility of phosphorylation, coupled with the ready availability of ATP as a phosphoryl donor, explains its selection as the most general regulatory device adopted by eukaryotic cells.

It is thought that perhaps 30% of the proteins encoded by the human genome contain covalently bound phosphate, and abnormal phosphorylation is now recognized as a cause or consequence of many human diseases. A number of naturally occurring toxins and tumour promoters exert their effects by targeting particular protein kinases and phosphatases. A topical example is the cyclic heptapeptide microcystin, which has just been listed as a "notifiable dangerous substrance", along with anthrax, in the Anti-terrorism, Crime and Security Act of 2001 recently approved by the British parliament. Microcystin, produced by toxic blue-green algae, is a potent hepatotoxin and liver carcinogen that inhibits members of one of the major families of protein phosphatases[1].

In view of these developments, it seems timely to reflect on the early days of research on protein phosphorylation. How was this phenomenon originally discovered as a control mechanism and why did it take so long before its general significance was appreciated?



Carl and Gerty Cori, the 1947 Nobel Laureates. Picture: Science Photo Library.

Regulating by phosphorylation

In the late 1930s Carl and Gerty Cori discovered that there were two forms of glycogen phosphorylase (called b and a), the enzyme that catalyses the rate-limiting step of glycogenolysis. Phosphorylase b was only active in the presence of 5′ AMP, whereas phosphorylase a was active in the absence of this nucleotide. They reasoned (incorrectly) that phosphorylase a must contain tightly bound 5′ AMP, and that the enzyme that converts phosphorylase a to phosphorylase b, discovered in 1943 (ref. 2), must catalyse the removal of 5′ AMP. The effect of 5′ AMP on phosphorylase b was the first example of allosteric activation, but, because this term would not be coined for another 20 years, they called the a-to-b converting enzyme 'prosthetic-group-removing' (or PR) enzyme[2]. But the Coris' never demonstrated that PR enzyme released 5′ AMP from phosphorylase a and, although they received a Nobel Prize in 1947 for "discovering the course of the catalytic conversion of glycogen", many years passed before the true nature of the reaction was discovered.

Protein kinase activity was first observed in 1954 when Gene Kennedy described a liver enzyme that catalysed the phosphorylation of casein[3]. Soon after, Fischer and Krebs[4,5], as well as Wosilait and Sutherland[6], found that the interconversion of phosphorylase b to phosphorylase a involved a phosphorylation/dephosphorylation mechanism. In particular, Fischer and Krebs[4,5] demonstrated that the b form could be converted to the a form in the presence of Mg-ATP and an enzyme they termed phosphorylase kinase[4,5]. Phosphorylase kinase was subsequently shown to catalyse the transfer of the γ-phosphoryl group of ATP to a specific serine residue on phosphorylase b [7]. The reconversion of phosphorylase a to phosphorylase b was therefore catalysed by a 'phosphate-releasing' (or PR!) enzyme, today called protein phosphatase 1 to reflect its much wider use in cell regulation[8].

In 1950, Earl Sutherland showed that glycogenolysis could be stimulated if liver slices were incubated with adrenalin or glucagon; he subsequently showed that the activity of phosphorylase a was increased under these conditions (reviewed in ref. 9). This was the first demonstration that a hormone could influence the activity of a specific enzyme, although the response was lost if the liver slices were homogenized. But, when the activation mechanism of phosphorylase was discovered, it became obvious that Mg-ATP would be necessary for

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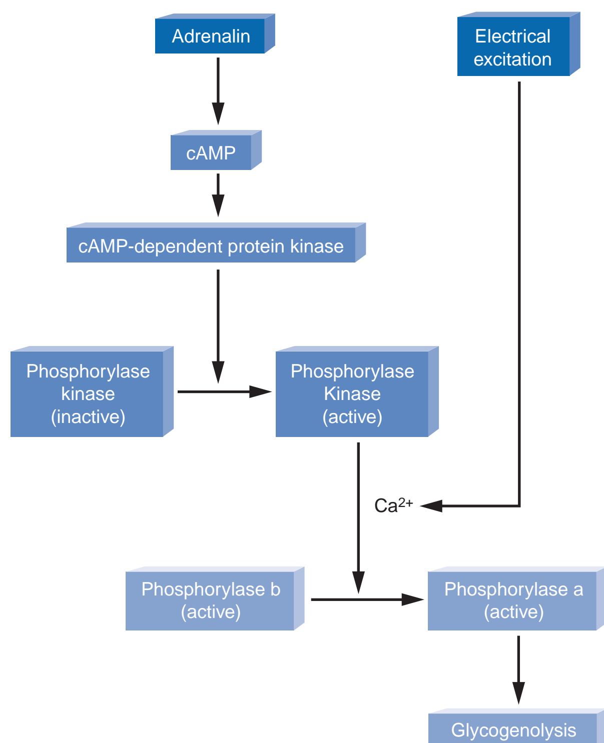

Figure 1 **The glycogenolytic cascade in mammalian skeletal muscle. Adrenalin stimulates the production of 3′ 5′ cyclic adenosine monophosphate (cAMP) leading the sequential activation of cAMP-dependent protein kinase and phosphorylase kinase. The latter converts glycogen phosphorylase from the inactive dephosphorylated b form to the active phosphorylated a form, stimulating glycogenolysis in advance of an increased energy demand. The activity of phosphorylase kinase also depends on calcium ions and is therefore also switched on during muscle contraction. This provides energy (via the breakdown of glycogen) to sustain muscle contraction.**

activation; addition of Mg-ATP did indeed restore the response to hormones. The reconstruction of a hormone response in a cell-free system was a major breakthrough that led to the discovery that adrenalin exerted its effects by generating a small, heat-stable factor later identified as 3′5′ cyclic adenosine monophosphate (or cyclic AMP). The remarkable story of how the first 'second messenger' was identified is beautifully described in the first chapter of *Cyclic AMP* (ref. 9) published in 1970, the year before Sutherland received a Nobel Prize.

It took much longer before other important missing pieces of the jigsaw were put in place. These included the discovery of cAMP-dependent protein kinase (PKA) and the finding that it activated phosphorylase kinase[10], the first example of a 'cascade' in which one protein kinase activates another. PKA was also found to inhibit glycogen synthase[11,12], the first example of enzyme inhibition by phosphorylation. Another crucial finding was that phosphorylase kinase activity also depends on another second messenger, namely calcium ions[13,14], and that calmodulin (one of major Ca^{2+} receptors of eukaryotic cells) was one of its subunits[15]. These findings explained how glycogenolysis and muscle contraction were synchronized (Fig. 1). But by the end of the 1960s, 15 years after phosphorylase kinase had been discovered, phosphorylation was still thought of as a rather specialized control mechanism largely confined to the regulation of one metabolic pathway (glycogen metabolism).

Phosphorylation develops

It was through the 1970s and early 1980s that the general significance of protein phosphorylation came to be appreciated. Lester Reed's discovery in 1969 that the mitochondrial pyruvate dehydrogenase complex was inactivated by phosphorylation[16] was one of the first hints that this control mechanism might operate in other metabolic pathways and organelles. This view was strengthened when PKA was shown to activate hormone-sensitive lipase[17] and to inhibit L-type pyruvate kinase[18]. These observations also helped to explain how adrenalin stimulates lipolysis in adipose tissue and how glucagon inhibits glycolysis in the liver. But the widespread distribution of PKA in animal tissues and other organisms suggested an even wider range of functions[19]. More substrates were identified, such as cardiac troponin I (ref. 20) and phospholamban[21], which explained how adrenalin regulates the rate and force of heart-muscle contractility. This extended the involvement of phosphorylation to proteins that are not enzymes, although the demonstration by Tom Langan in 1969 that PKA phosphorylates histone H1 at a specific serine residue[22] had already hinted at this possibility.

The first calmodulin-dependent protein kinases were identified in the late 1970s and included myosin light-chain kinase[23], phosphorylase kinase[15] and calmodulin-dependent protein kinases I and II in the brain[24]. The subsequent realization that calmodulin-dependent protein kinase II has multiple functions in Ca^{2+}-signalling akin to PKA (ref. 25), and especially the discovery that protein kinase C (ref. 26) is activated by the second messenger diacylglycerol, broadened the concept of second-messenger-dependent protein kinases.

Some of the major serine/threonine-specific protein phosphatases were classified during the late 1970s and early 1980s (ref. 8), and mechanisms by which they are regulated began to be identified. Prominent among these was the characterization in 1981 of the calmodulin-dependent protein phosphatase 2B (also termed calcineurin)[27], which 10 years later was shown to be the target for cyclosporin[28], the immunosuppressant drug that made organ transplants possible.

In 1975, PKA was shown to phosphorylate peptides in proteolytic digests of myelin basic protein[29], and this led to the realization that PKA phosphorylates serine residues in specific amino-acid sequence motifs[30,31]. These studies paved the way for the development of synthetic peptide substrates that have been a key technical advance in the study of protein phosphorylation.

In retrospect, the determination of the amino-acid sequence of the first protein kinase (PKA) in the early 1980s (ref. 32) was more significant than it seemed at the time (at least to me!), because it allowed geneticists to understand the functions of several regulatory genes that they had identified. In particular, cdc2, the cell-cycle control gene identified by Paul Nurse, was shown to be a protein kinase[33], a discovery recognized last year by a Nobel Prize.

The 1970s also furnished the first examples of proteins that are phosphorylated on two or more residues by two or more kinases, termed multisite phosphorylation[34], which we now know to be the norm rather than the exception. Ed Fischer and Ed Krebs have often said they were fortunate in studying glycogen phosphorylase, because

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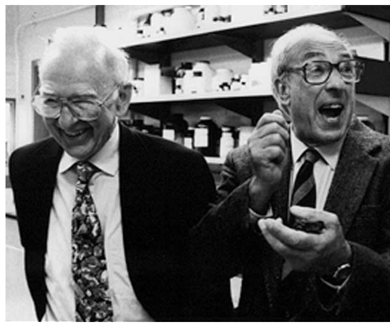
it is one of the few proteins phosphorylated at a single site by a single kinase. Had this enzyme been as complex as, say, glycogen synthase (which eventually turned out to be phosphorylated at nine serine residues by at least six different kinases[35]), it might have taken much longer to sort out what was going on.

The 1980s also led to the realization that protein phosphatases and kinases do not always find their substrates by simple diffusion, but are frequently directed to particular subcellular locations by 'targeting' proteins with which they interact. The glycogen-targeting subunit of protein phosphatase-1 (ref. 36) and proteins that interact with the RIIβ regulatory subunit of PKA (ref. 37), later called A-kinase anchoring proteins (AKAPs; reviewed in ref. 38) were the first examples of this important and widespread phenomenon.



Ed Krebs and Eddy Fischer in 1992, after hearing about their award of the Nobel Prize in Physiology and Medicine.

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Protein tyrosine kinases

The discovery by Ray Erikson that v-Src, the protein encoded by the transforming gene of Rous sarcoma virus, was a kinase[39] was a landmark event in the late 1970s. This led Tony Hunter to the surprising finding that v-Src phosphorylates tyrosine residues in proteins[40]. The epidermal growth factor (EGF) receptor was also shown to be a protein tyrosine kinase, switched on when EGF engages the receptor[41]. Similar findings were made for the insulin receptor a couple of years later[42].

The discovery that many growth-factor receptors were protein tyrosine kinases stimulated the search for their physiological substrates. But, surprisingly, it was the receptors themselves that seemed to be the most prominent cellular substrates, frequently becoming phosphorylated at multiple tyrosine residues. These puzzling observations were explained when it was shown that proteins containing the Src homology 2 (SH2) domain[43] are able to bind directly to phosphorylated growth-factor receptors because of their ability to recognize particular phosphotyrosine-containing sequences[44]. Receptor 'autophosphorylation' is therefore critical in inducing the binding sites for cytoplasmic targets with SH2 domains, which then stimulate 'downstream' pathways to mediate the effects of the signal.

The first protein tyrosine phosphatase (PTP1B) was purified in the late 1980s (ref. 45) and there was great excitement when it was found to be homologous to leukocyte common antigen CD45 (ref. 46), often found on the surface of haematopoietic cells. Before this, the function of CD45 was unknown. These discoveries generated enormous interest in this new family of enzymes and nearly 100 members were subsequently identified, including many receptor tyrosine phosphatases.

The late 1980s and early 1990s saw the cloning of the JAK kinases, a new family of protein tyrosine kinases. JAK is an acronym of 'just another kinase' (in recognition that, like many other kinases, it emerged from a PCR-based screen) or Janus kinase (in recognition of their two-kinase domain structures reminiscent of the two-faced Roman god Janus)[47]. These enzymes are activated at the plasma membrane after cytokines and interferons have been engaged by their receptors. After the JAK kinases are activated, their substrates, the 'signal tranducers and activators of transcription' (STATs; reviewed in ref. 48), are phosporylated; modified STATs then mediate transcription directly. The identification of a signalling pathway in which the substrate is phosphorylated at the plasma membrane and then migrates to the nucleus to regulate transcription without any other intervening steps was initially met with astonishment. But the persuasive genetic evidence validating these conclusions led to rapid acceptance by the scientific community.

Cascading protein kinases

Some have dubbed the 1990s as the decade of protein kinase cascades. It is surprising that, although the first protein kinase cascade was identified in 1968 (ref. 10), it took more than 20 years before further examples of this phenomenon were identified. An insulin-stimulated protein kinase that phosphorylated microtubule-associated protein-2 (MAP2) was identified in the late 1980s and termed MAP kinase[49] (its name was later changed to mitogen-activated protein kinase — still MAP kinase — to reflect its activation by many mitogens and growth factors in different cells). This enzyme was found to be activated by the phosphorylation of a threonine and a tyrosine residue[50], catalysed by a 'dual specificity' MAP kinase kinase[51,52], through a Ras-dependent signalling pathway[53]. This 'classical' MAP kinase cascade was worked out during the early 1990s through the efforts of a number of laboratories. This was followed by the dissection of many other MAP kinase cascades that are important in protecting cells against cellular stresses, cell-damaging agents and infection by pathogenic organisms.

A phosphatidylinositol (PtdIns) 3-kinase, or PI(3)K, activity associated with Src and the platelet-derived growth factor (PDGF) receptor was identified in the late 1980s (ref. 54). After the discovery of PtdIns(3,4,5)trisphosphate (PIP3) in neutrophils as an inositol phospholipid produced in response to fMet–Leu–Phe (ref. 55), it became clear that this compound was formed from PtdIns(4,5)bisphosphate by the action of class 1 PI(3)Ks (reviewed in ref. 56). The importance of PIP3 as a second messenger in insulin and growth-factor signalling emerged when relatively specific inhibitors of PI(3)K (refs 57,58) were found to block many of the effects of these signals. A PIP3-dependent protein kinase cascade that has a major function in mediating cellular responses triggered by insulin and growth factors was identified in 1995 (refs 59–61), culminating in the discovery of 3-phosphoinositide-dependent protein kinase in 1997 (ref. 62).

Future trends

Our understanding of protein phosphorylation has now reached the stage where its importance in almost every physiological event is recognized. A well-deserved Nobel Prize was awarded to Ed Fischer and Ed Krebs in 1992 for their pioneering studies in this area. But there are, undoubtedly, many surprises still in store, and our understanding of signal integration is still in its infancy. In particular, we now realize that regulatory circuits are 'wired up' in distinct ways in different cells. Therefore, although transformed cell lines have been invaluable in helping us to dissect particular signalling pathways, I would expect the emphasis of research (apart from cancer) to shift increasingly to the analysis of 'real' cells and tissues.

One of the major gaps in our knowledge concerns the identities of the key substrates of protein kinases and how their phosphorylation contributes to the changes in cell physiology evoked in response to particular signals. If a third of the 30,000 proteins encoded by the human genome contain covalently bound phosphate, an 'average' protein kinase (on the basis of the probable number of protein kinases) would be expected to phosphorylate about 20 different proteins *in vivo*, and an 'average' protein phosphatase would be expected to dephosphorylate 60 proteins. These numbers are conservative, in part because closely related protein kinase and protein

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phosphatase isoforms would be expected to have overlapping specificities, and many proteins are regulated by multisite phosphorylation. Moreover, although some protein kinases are dedicated to the phosphorylation of one, or just a few substrates (e.g. phosphorylase kinase and MAP kinase kinases), others must have several hundred substrates. Furthermore, the substrates of protein kinases are frequently cell-specific, explaining the distinctive effects of different signals in different tissues. The identification of the major substrates of each protein kinase and phosphatase is a massive undertaking, which is going to take at least several decades to solve. But it is at the heart of much that we want to know in the twenty-first century, including the molecular basis of embryogenesis and the functioning of the brain. More powerful methods are needed to identify the substrates of particular protein kinases and phosphatases, coupled with further exploitation of important methodological and technical advances, such as the development of phospho-specific antibodies, specific cell-permeant inhibitors of protein kinases and phosphatases, and cell lines that do not express a particular protein kinase or phosphatase.

In a review article I wrote for *Nature* 20 years ago[63], I used the term 'silent' to describe phosphorylation sites that did not seem to influence the activities of enzymes directly. Many such sites have been identified. Do silent phosphorylations have important physiological functions that we do not yet understand, or is there, as Ed Krebs has suggested, a considerable amount of noise in the system?

There was evidence many years ago that there are protein kinases that phosphorylate histidine and lysine residues[64]; it was also pointed out at that time that the acid lability of these phosphorylated residues make them difficult to study with conventional methods. Although protein kinases that phosphorylate histidine residues are now well established as integral components of sensing mechanisms in bacteria[65], it is still unclear whether the phosphorylation of residues other than serine, threonine and tyrosine is widespread or significant in mammalian cells. Are protein lysine kinases, protein arginine kinases[66], or even protein kinases that use phosphoryl donors other than ATP (e.g. phosphoenolpyruvate[67]), represented among the many proteins in the human genome for which a function has yet to be ascribed? Providing the answers to these and other intriguing questions will be a major challenge for many years to come.

Finally, I should mention that recent events have propelled the development of specific protein-kinase inhibitors for treating disease right to the top of the pharmaceutical agenda. There is going to be a huge push over the next 20 or 30 years to develop drugs that modulate the activities of specific protein kinases and phosphatases. But the remarkable surge of interest in this area over the past few years is another story, which I cover in another article[68]. □

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Acknowledgements
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