Regulation of Cellular and Molecular Functions by Protein Phosphorylation

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Ghanshyam Swarup is a scientist at the Centre for Cellular and Molecular Biology, Hyderabad. He has been working on various aspects of protein phosphorylation and on the molecular biology and cellular functions of enzymes involved in this process, namely protein tyrosine phosphatases and protein tyrosine kinases.

Modification of proteins by phosphorylation is the major general mechanism by which many cellular functions in eukaryotic cells such as cell division, malignant transformation, differentiation, signal transduction etc. are controlled by external physiological stimuli. At the molecular level protein phosphorylation-dephosphorylation can alter various properties of the substrate molecules such as enzymatic activity, sub-cellular location, ligand binding, interaction with other proteins, DNA binding and some other functional properties. The changes in molecular properties of proteins brought about by protein phosphorylation play a critical role in regulating various cellular functions.

Introduction

Many proteins in eukaryotic cells after synthesis undergo modifications of the side chains of amino acids. Over one hundred such modifications are known to occur on various amino acids. Phosphorylation of proteins is one of the post-translational modifications, which is reversible and plays a critical role in the regulation of many cellular functions. Phosphorylation of proteins is carried out by a group of enzymes known as protein kinases which transfer terminal phosphate of ATP (sometimes GTP) to the hydroxyl group of amino acids – serine, threonine and tyrosine. Phosphate group on these three hydroxy amino acids is acid stable. In addition to these three, phosphorylation of histidine, lysine and arginine is also known to occur which is acid labile. This article is mainly restricted to the phosphorylation-dephosphorylation at Ser, Thr and Tyr.

[1] ΔG° is the energy that is released upon hydrolysis.

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The protein bound phosphate is a high-energy linkage. The free energy of hydrolysis [1] of protein bound tyrosine phosphate

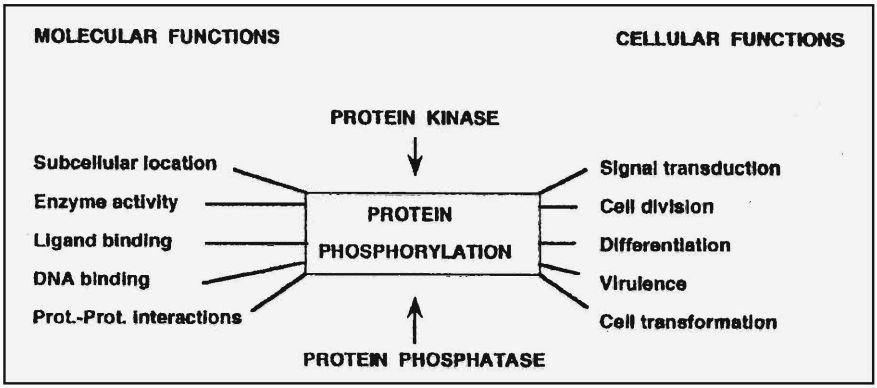

Figure 1. Protein kinases and phosphatases regulate various molecular properties of substrate proteins, which play a critical role in mediating cellular functions.

has been reported to be –9.48 Kcal (assuming an approximate ΔG° of –10 Kcal for hydrolysis of ATP). Free tyrosine phosphate (or serine, threonine phosphate) is not an energy rich linkage. Hydrolysis or formation of such a high-energy protein phosphate bond could conceivably bring about a conformational change in the protein resulting in an altered functional state of the molecule.

Phosphorylation of proteins at serine and threonine has been known for a long time but phosphotyrosine was discovered in proteins in 1979. This was partly due to the fact that tyrosine phosphate represents less than 1% of total acid stable protein phosphorylation (90–95% on Ser and 5–10% on Thr) in eukaryotic cells. However the number of protein tyrosine kinases is not small and is perhaps equal to those that phosphorylate on Ser, Thr. It has been estimated that in human cells about 1% (or 1000 out of an estimated number of 100,000 genes) of the genes code for protein kinases and perhaps 1000 genes code for protein phosphatases[2]. In budding yeast, *S. cerevisiae*, for which the complete nucleotide sequence is known, there are 113 protein kinase genes, which correspond to about 2% of the total genes. Such a large number of genes devoted to protein phosphorylation is not surprising considering the central and critical role played by this process in controlling various cellular processes.

[2] Protein phosphates are enzymes that remove the phosphate group from proteins.

All protein kinases have a conserved catalytic domain of about 250 amino acids; in addition there are various non-catalytic sequences at *C*-terminal and *N*-terminal ends which have various functions. For example, receptor type protein

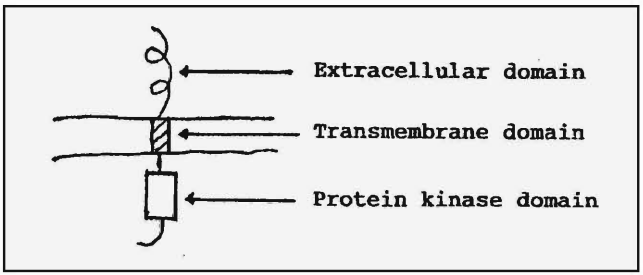

Figure 2. Structure of a typical receptor tyrosine kinase such as epidermal growth factor receptor.

kinases have an extracellular domain for ligand binding, a short transmembrane domain and a *C*-terminal cytoplasmic catalytic domain (*Figure 2*). The catalytic domain consists of an ATP binding site and a site for binding of protein substrate.

Protein phosphorylation by its nature is a regulatory modification reaction. Therefore, it is necessary that the activity of protein kinases in turn must be regulated. This is in fact the case. The activity of protein kinases is regulated by small molecules such as cyclic AMP, cyclic GMP, calcium ion, diacylglycerol etc. Hormones and growth factors such as epidermal growth factor regulate some protein kinases: platelet derived growth factor, insulin etc. The activity of many protein kinases is regulated by phosphorylation by other protein kinases e.g. mitogen activated protein kinases, cdc2 kinase (which regulates cell division cycle) and related cdc kinases.

Although protein phosphorylation catalysed by protein kinases is thermodynamically reversible, the reverse reaction i.e. dephosphorylation of proteins is carried out *in vivo* by another group of enzymes known as protein phosphatases. The activity of protein phosphatases is also regulated and these enzymes play an important role in regulating the phosphorylation state of substrate proteins in an active manner.

Regulation of Enzyme Activity by Phosphorylation

Since phosphate linkage in proteins is a high-energy bond, formation of this bond can provide energy for local or global changes in conformation of the protein. This conformational change driven by the formation of phosphate-protein linkage

would lead to changes in functional properties such as enzyme activity. Phosphorylation (or dephosphorylation) regulates activity of many enzymes at one or more Ser, Thr or Tyr residues in response to extracellular signals such as hormones or growth factors. Phosphorylation can activate or inhibit enzyme activity. In its simplest form phosphorylation at a single Ser residue of glycogen phosphorylase by a protein kinase (known as phosphorylase kinase) increases enzyme activity. Dephosphorylation by a protein phosphatase (known as phosphorylase phosphatase) decreases enzyme activity. This was the first example of phosphorylation dependent regulation of activity of an enzyme. For this discovery in mid fifties by Edwin G Krebs and Edmond H Fisher, these scientists were awarded the Nobel Prize in 1992. Phosphorylation also regulates the activity of phosphorylase kinase and glycogen synthase, the enzymes involved in regulating glycogen metabolism. The activity of glycogen synthase decreases upon phosphorylation. Thus the key regulatory enzymes in glycolysis are regulated by protein phosphorylation. Some other enzymes whose activity is regulated by phosphorylation are pyruvate kinase, pyruvate dehydrogenase, phosphofructokinase-2, hydroxymethylglutaryl co-enzyme A reductase, tyrosine hydroxylase, triacylglycerol lipase etc.

Phosphorylation can activate or inhibit enzyme activity.

A protein tyrosine kinase TK-1 isolated from rat spleen phosphorylates itself on Tyr residues and gets activated (*Table 1*). The activated TK-1 can be inactivated by dephosphorylation or by purified tyrosine phosphatase. Subsequently the dephosphorylated inactive TK-1 could be rephosphorylated and activated after inhibiting the phosphatase by an inhibitor sodium orthovanadate. Typical data from such an experiment are shown in *Table 1*.

Regulation of the activity of many enzymes by phosphorylation is much more complex. Rous Sarcoma virus carries a gene coding for a protein kinase name *v-Src* (for viral Src). A homologous cellular protein is present in vertebrates and chicken termed as *c-Src* (for cellular Src). The protein kinase activity of *v*-Src protein is responsible for cell transformation by this virus.

Table 1. Regulation of kinase activity of a protein tyrosine kinase TK-1 by autophosphorylation and dephosphorylation.

Experiment	Kinase activity
Unphosphorylated TK-1	11
Phosphorylated TK-1	100
Phosphorylated TK-1 + phosphatase	13
Rephosphorylated TK-1	102

The *c*-Src plays a role in cellular signalling. The Src family of protein kinases such as Hck, Src etc. are regulated by phosphorylation at two distinct Tyr residues. Phosphorylation at one of the residues (Tyr 527 in *c*-Src) leads to a decrease in the enzyme activity, whereas phosphorylation at Tyr 416 increases enzyme activity. Dephosphorylation at these residues has the reverse effect. Thus at least two distinct protein kinases and two distinct protein phosphatases are involved in the regulating activity of the *c*-Src kinase.

How does phosphorylation of an enzyme influence enzyme activity? In case of *c*-Src kinase extensive work has shown that phosphorylation at Tyr 527 inhibits enzyme activity due to intramolecular interaction of phosphorylated Tyr 527 with its SH2 domain resulting in a closed conformation. SH2 domain or Src homology 2 domain is a protein module of about 100 amino acids located adjacent to the catalytic domain in Src family kinases. A conformational change has also been observed in the form of *c*-Src kinase phosphorylated at Tyr 416, which activates the enzyme to some extent.

Some of the enzymes are activated by phosphorylation at two distinct sites. MAP kinase or mitogen activated protein kinase is a Ser, Thr specific protein kinase which is activated in response to various extracellular stimuli such as mitogen or growth factor. It requires phosphorylation at a Thr and a Tyr residue for activation. Dephosphorylation at one of these sites is sufficient to inactivate the enzyme. The function of MAP kinase is to regulate the activity of substrate proteins in the nucleus.

Phosphorylation dependent activation or inhibition of enzyme activity in some cases behaves like a molecular switch. Many tyrosine specific protein kinases phosphorylate themselves. In some cases autophosphorylation results in an increase in the activity of the enzyme. Autophosphorylation dependent activation of a tyrosine kinase in response to a stimulus would behave like a molecular switch. In addition, the cells use phosphorylation cascades. MAP kinase is phosphorylated by MAP kinase kinase, which in turn is phosphorylated by a protein kinase known as Raf which in turn is activated by an activator generated in response to an extracellular signal. This kinase cascade offers several advantages; it converts graded inputs into switch-like outputs. In addition, this cascade results in amplification of the signal. But the main advantage of this kinase cascade is the large increase in sensitivity of the response to a given stimulus. One of the ways of increasing sensitivity in the cell is by an enzyme showing positive cooperativity. Positively cooperative enzymes are multimeric and show cooperative binding of substrate molecules resulting in more than expected increase in enzyme activity with increase in substrate or ligand concentration. The sensitivity of MAP kinase corresponds to that of an enzyme showing a high degree of positive cooperativity with Hill coefficient[3] of 4 or 5.

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Regulation of Sub-cellular Location of Proteins by Phosphorylation

Phosphorylation dependent changes in sub-cellular location of a protein are a relatively recent development. Some transcription regulatory factors such as STAT proteins (signal transducers and activators of transcription) are present in the inactive state in the cytosol. In response to an extracellular signal a tyrosine kinase is activated in the cytosol which phosphorylates STAT proteins. Upon phosphorylation at tyrosine these proteins dimerise and migrate to the nucleus where these proteins bind to regulatory sequences in genes and activate or inhibit transcription. Another transcription factor which migrates to the nucleus upon growth factor stimulated phosphorylation is

[3] Hill coefficient is a measure of cooperativity; Hill coefficient of 1 implies no positive cooperativity).

The main advantage of kinase cascade is the large increase in sensitivity of the response to a given stimulus.

c-Fos protein. *c*-Fos protein is the cellular homologue of a viral protein *v*-Fos which is responsible for cell transformation by the virus. This protein plays a role in the control of transcription. In addition to transcription factors, certain protein kinases, known as ERKs (extracellular signal regulated kinases) upon phosphorylation at Thr and Tyr migrate to the nucleus. The phosphorylation of ERKs is carried out by a set of dual specificity kinases, which in turn are also regulated by phosphorylation; the initial trigger for these phosphorylations is an extracellular signal such as a growth factor. Thus phosphorylation dependent activation and migration to the nucleus of ERKs is a very important link in the signal transduction pathway.

Regulation of Protein-Protein Interactions by Phosphorylation

Phosphorylation state of a protein can affect its interaction with other proteins. One of the best-studied examples is the interaction of SH2 domains of proteins with short peptide sequences containing phosphotyrosine. SH2 domains were identified in Src family of protein kinases and are present in many other proteins involved in signal transduction pathways. Activation of growth factor receptors such as EGF (epidermal growth factor) receptor or PDGF (platelet derived growth factor) receptor upon binding of the growth factor results in autophosphorylation at many tyrosine residues. These phosphorylated tyrosines

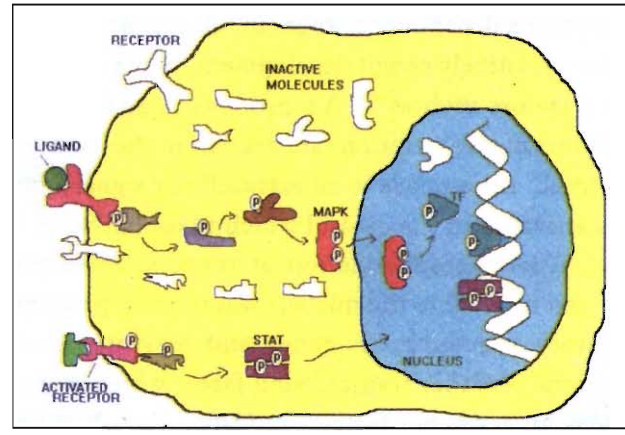

Figure 3. In response to extracellular or internal signals, phosphorylation-dephosphorylation dependent changes in enzyme activity, subcellular location, protein-protein interactions etc. are affected.

provide high affinity binding sites for various signalling proteins through their SH2 domains such as *c*-Src kinase, phosphatidyl inositol 3 kinase, adaptor protein Grb2 etc. Studies with short peptide sequences have shown that the dissociation constant for such interactions is in the range of few nM. Removal of phosphate from phosphorylated tyrosine reduces the affinity for SH2 domain by over 1000-fold, essentially abolishing this high affinity interaction.

Phosphorylation dependent protein-protein interactions not only bring two molecules together but can also alter the sub-cellular location of the SH2 domain protein, for example, from cytosol to plasma membrane. In addition, binding of the signalling proteins to phosphotyrosines in the receptor molecules also results in the functional activation of the signalling proteins.

Regulation of Transcription Factor Function by Phosphorylation

Most of the eukaryotic transcription regulatory proteins, which bind to specific sequences of DNA, are phosphorylated at Ser, Thr or Tyr. Phosphorylation can regulate DNA binding, transcriptional activation, protein stability, protein-protein interaction and sub-cellular location of transcription factors. One of the well-studied examples is CREB (cyclic AMP response element binding) protein which binds to a specific DNA sequence TGACGTCA and regulates transcription in response to phosphorylation by cyclic AMP dependent protein kinase. Phosphorylation of CREB by cyclic AMP dependent kinase at a particular Ser (Ser 133) *in vitro* and *in vivo* leads to an increase in transcription by several fold. Ca^{2+} activated protein kinases can also phosphorylate CREB protein and activate transcription. The activator protein AP-1 consists of two components, *c*-Fos and *c*-Jun, which combine to form a heterodimer. These two proteins are phosphorylated at several Ser, Thr residues. In the *C*-terminal domain of *c*-Fos there are three Ser residues, phosphorylation of which inhibits transcription. Interestingly the oncogenic form of Fos protein (*v*-Fos) lacks this part of the

Suggested Reading

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Protein phosphorylation is the most widely used and perhaps the most important mechanism for regulation of cellular functions in eukaryotic cells.

protein; mutagenesis studies have shown that the lack of phosphorylation domain is responsible for constitutive activation of v-Fos protein and also towards its oncogenic properties.

Regulation of Protein Degradation by Phosphorylation

Some proteins involved in cell cycle progression are synthesized when they are needed and degraded subsequently. How is this timely degradation regulated? One of the ways a cell marks a protein for degradation is by phosphorylation at a particular site. For example cyclin E, a regulatory protein which activates a protein kinase involved in the regulation of cell division cycle and which is synthesized when the cells enter a division cycle is phosphorylated at a specific threonine residue; this phosphorylation serves the purpose of marking the protein for degradation by the proteolytic enzymes using a complex pathway.

Thus phosphorylation of proteins regulates a variety of cellular functions. In fact survival of mammalian cells is dependent on signals known as survival factors which activate protein kinases. The examples listed in this article provide an idea about the range of cellular functions, which are controlled by phosphorylation. Translation, transcription, DNA replication, control of certain ion channels, neuronal functions etc. are also regulated by phosphorylation. The entry of cells into mitosis in all eukaryotic cells is governed by the activity of a protein kinase known as cdc2, originally discovered in yeast as a cell division cycle mutant. Activation of cdc2 kinase upon dephosphorylation by a protein phosphatase known as cdc25 leads the cells into mitosis. Thus protein phosphorylation is the most widely used and perhaps the most important mechanism for regulation of cellular functions in eukaryotic cells.

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1947: *Edwin H Land is reported to have invented a camera that develops its own film, in about 60 seconds, without the need for a darkroom. The Polaroid instant camera is marketed a year later (the color version appears in 1963).*

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