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Phosphotyrosine – a new protein modification

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Abstract

Tyrosine protein kinases carry out a newly described form of protein modification – phosphorylation of tyrosine residues. The transforming proteins of certain tumor viruses are tyrosine protein kinases and unscheduled phosphorylation of tyrosine in cellular proteins appears to be crucial for malignant transformation by these viruses. A primary effect of two cellular growth factors is to activate intracellular tyrosine protein kinases.

A new type of protein modification which appears to be involved in a number of crucial metabolic processes has been recognized only in the past 2 years. Phosphotyrosine had not been detected as a constituent of proteins until the discovery that the amino acid phosphorylated by certain viral transforming proteins was tyrosine^{1–3}. Previously, the only acid stable phosphoamino acids known in proteins were phosphoserine and phosphothreonine. The failure to observe phosphotyrosine stemmed in part from the dearth of this modified amino acid in normal cells. Phosphotyrosine accounts for only about 0.05% of the total acid-stable phosphate in protein in animal cells⁴. Nevertheless, despite the scarcity of phosphotyrosine, evidence is accumulating that tyrosine-specific protein kinases may be an important class of regulatory enzymes.

Tyrosine phosphorylation and viral transformation

Tyrosine phosphorylation was initially observed in conjunction with the transforming proteins of tumour viruses. As a result, the best characterized tyrosine-specific protein kinases are those associated with viral transforming proteins. With one exception the viruses in question are retroviruses. Retroviruses possess an RNA genome but replicate by way of a DNA intermediate which becomes stably integrated into the DNA of the infected cell. Some retroviruses are highly oncogenic and cause acute disease with a short latent period. A common feature of this type of retrovirus is that their genomes are chimeric. Both termini are derived from the genome of a non-defective weakly oncogenic retrovirus; However, the central part of the genome originated from cellular genes, presumably by a recombination event. The best documented example of a virus of this type is Rous sarcoma virus (RSV). The *src* gene of RSV is very closely related to a gene which is found in all normal vertebrate cells. Although it has only been formally proved in a few cases, the expression of the cellular information in these viral genomes seems to be required for transformation by these viruses and, as would be anticipated, virus-specific proteins encoded by the acquired cellular genes have been identified in every virally transformed cell of this sort. The cellular genes homologous to the sequences acquired by these viruses are expressed as proteins in normal cells of the appropriate type, although usually at much lower levels than their viral

counterparts in transformed cells. The available evidence suggests that the viral transforming proteins display the same basic function as the products of the normal cellular genes.

The idea that protein phosphorylation might be a mechanism of viral transformation stems from the observations of Collett and Erikson⁵ and Levinson *et al.*⁶. They found that p60^{src}, the 60,000 dalton transforming protein of RSV, was tightly associated with a protein kinase activity. Subsequently, it was shown that this protein kinase had the novel specificity of phosphorylating tyrosine³. Initially, it was not clear whether the tyrosine protein kinase activity associated with p60^{src} was an intrinsic activity or due to a bound cellular enzyme. Considerable evidence has now been accumulated showing that p60^{src} has the inherent ability to transfer phosphate from ATP to tyrosine in protein. Perhaps the most persuasive observation is that p60^{src} synthesized in *Escherichia coli* from recombinant DNA molecules containing the RSV *src* gene has tyrosine-specific protein kinase activity^{7,8}. Not only does p60^{src} function as a protein kinase *in vitro*, but there is a strong indication that it acts this way in the intact cell. Cells transformed by RSV contain about ten times as much phosphotyrosine in protein as uninfected cells⁴. Indeed it seems likely that phosphorylation of proteins on tyrosine is crucial for malignant transformation by RSV.

The transforming proteins of several other retroviruses have been found to have similar tyrosine protein kinase activities, although it is by no means a universal feature of retroviruses that cause acute disease. To date ten viruses are known whose transforming proteins have this property. In every case the virally transformed cells have a characteristically increased amount of phosphotyrosine. It is presumed, but not proven, that this reflects the presence of viral transforming proteins like p60^{src} with intrinsic tyrosine protein kinase activities. This type of virus can be further subdivided according to the nature of the acquired cellular information. By cross hybridization of nucleic acid probes specific for the cellular sequences and by comparison of the cell-derived portions of the transforming proteins, these viruses can be placed into five distinct groups representing five separate cellular genes. In the case of RSV, the homologous cellular gene has been shown to express a protein very similar to the viral transforming gene product and this protein also has tyrosine-specific protein kinase activity³. The products of the cellular genes homologous to the other classes of virus have not been studied in such detail, but there is reason to believe that all five cellular genes will turn out to code for tyrosine protein kinases. Although some of these cellular genes are expressed only in specialized cell types, it seems that eukaryotic cells have the genetic potential for at least five such enzymes. Presumably the phosphotyrosine seen in normal cells results from the expression of cellular tyrosine protein kinase genes.

Substrates for viral tyrosine protein kinases

Mostly because of the relevance to viral transformation a considerable effort has been put into identifying intracellular substrates of tyrosine protein kinases. The major difficulty is to pinpoint such proteins when only a small percentage of phosphate in protein is phosphotyrosine. Several methods however, have been developed to recognize proteins that contain phosphotyrosine. One technique makes use of the marked alkali-stability of the ester linkage of phosphotyrosine⁹. Two-dimensional gel analysis of ³²P-labeled cellular proteins

can resolve several hundred phosphoproteins, most of which contain phosphoserine and phosphothreonine. Treatment of such gels with strong alkali greatly simplifies the pattern of phosphoproteins. Some, but by no means all, of the alkali-resistant phosphoproteins are found to contain phosphotyrosine. Substrates for the viral tyrosine protein kinases should be proteins containing increased amounts of phosphotyrosine in transformed cells. By comparing the phosphoprotein patterns from normal and transformed cells, half a dozen phosphotyrosine-containing proteins with this characteristic have been identified⁹. The most prominent of these, a protein of 36,000 daltons¹⁰, has been purified and shown to be a substrate for p60^{src} *in vitro*¹¹. All the phosphoproteins identified by this means are cytoplasmic and have been found to be modified forms of relatively abundant cellular proteins, comprising 0.1–0.5% of total cellular protein. However, the functions of these proteins remain obscure.

An alternative approach is to look for phosphotyrosine in specific proteins which are known to be involved in processes affected by viral transformation, such as growth control or nutrient transport. The drastic disruption of cytoskeletal architecture in transformed cells suggests that one or more cytoskeletal proteins might be a target for the viral tyrosine protein kinases. One cytoskeletal protein, vinculin, has been found to show increased amounts of phosphotyrosine in RSV-transformed cells¹². Vinculin has been localized to the adhesion plaques of cells. These are sites at which the cell makes direct contact with its substratum. Inside, the cell bundles of actin-containing microfilaments terminate at these structures. Vinculin appears to be interposed between the ends of the filaments and the inner surface of the plasma membrane and therefore may act as a linker providing internal anchorage for the cytoskeleton. Possibly tyrosine phosphorylation of vinculin decreases its effectiveness as a linker and this contributes to the characteristic rounded shape of RSV transformed cells. Since vinculin from normal cells also contains phosphotyrosine, tyrosine phosphorylation may be part of the cellular machinery for changing shape.

Other ways of detecting phosphotyrosine-containing proteins are being developed. One promising avenue is the generation of antibodies to phosphotyrosine or analogues of

phosphotyrosine¹³. Using this approach a phosphotyrosine-containing protein of 110,000 daltons has been detected in virally transformed cells.

Tyrosine phosphorylation and control of cell growth

Increased phosphorylation of tyrosine is not limited to virally transformed cells. Two other situations have recently been described, both involving polypeptide growth factors. Epidermal growth factor (EGF) binds to specific cell surface receptors and, as a consequence, stimulates cells to divide. One of the most rapid responses to the binding of EGF is the activation of an intracellular tyrosine protein kinase^{14, 15}. The induced protein kinase phosphorylates a number of cellular proteins including the receptor itself¹⁴. One of these is the same 36,000 dalton protein which is phosphorylated in response to viral transformation¹⁵. The identity of the EGF-induced tyrosine protein kinase is not known. Since the kinase is tightly associated with the receptor, one possibility is that it is an intrinsic function of the receptor. In any case, this kinase appears to be distinct from the viral tyrosine protein kinases. The second instance is platelet derived growth factor (PDGF), the major growth factor in serum. Although PDGF is recognized by a surface receptor distinct from that for EGF, binding of PDGF is also accompanied by an increase in intracellular tyrosine protein kinase activity¹⁶. In neither case has it been shown that the phosphorylation of cellular proteins is necessary for subsequent mitogenic events. Furthermore, other growth factors, such as fibroblast growth factor and insulin, do not appear to lead to increased phosphorylation of tyrosine. Nevertheless, it is an appealing idea that one pathway of growth control involves tyrosine phosphorylation, particularly in view of the fact that viral transformation leads to unrestricted growth. Viruses encoding tyrosine protein kinases could stimulate growth by usurping a cellular growth control pathway which uses tyrosine phosphorylation.

General properties of tyrosine protein kinases

Although little is known about the detailed enzymic properties of tyrosine protein kinases, some generalizations can be made about these enzymes. All those described so far are cytoplasmic proteins. Many of them appear to be affiliated with the inner surface of the plasma membrane and thus are in a position to transduce external signals into the cell. All of the viral tyrosine protein kinases themselves have at least one site of tyrosine phosphorylation and there are indications that phosphorylation at such sites may increase enzymic activity. It is therefore possible to envision regulatory cascades of tyrosine protein kinases. Most of the viral transforming proteins are also phosphorylated at serine residues and thus may in turn be regulated by serine protein kinases. While the tyrosine protein kinases display rigid amino acid acceptor specificity for tyrosine, the basis on which they select their substrates is not clear. For the serine and threonine protein kinases, the primary sequences around the target residues play some role. The amino acid sequences surrounding several phosphotyrosine residues have been determined¹⁷. One common, but not universal feature, is the presence of a cluster of acidic residues to the NH₂-terminal side of the phosphorylated tyrosine. However, factors other than primary sequence are almost certainly involved. For instance, the fact that the protein specificities of tyrosine protein kinases *in vitro* are less exacting than those in intact cells, suggests that the subcellular location of the

enzymes and their substrates may be crucial. It is also evident that there are intracellular substrates, such as the 36,000 dalton protein, which are common to several of the tyrosine protein kinases.

Tyrosine phosphorylation is reversible. Phosphotyrosine-specific phosphatases have been described although they are not extensively characterized¹⁸. In addition, intracellular turnover of phosphate on tyrosine can readily be demonstrated⁴. The turnover rate seems to be rather rapid, with an average half-life of less than an hour. This is ideal for a responsive regulatory system.

In addition to the tyrosine protein kinases themselves, about ten cellular proteins containing phosphotyrosine have been identified in different systems. Probably these represent only the most abundant of such proteins, and many other phosphotyrosine-containing proteins await discovery. With the exception of vinculin and the EGF receptor very little is known about the phosphoproteins which contain phosphotyrosine. From the point of view of understanding the mechanisms of viral transformation and growth control, it is obviously important to determine the functions of these various proteins and the effect, if any, of phosphorylation of tyrosine residues on their function. This approach may also provide insights into what other cellular processes are controlled by tyrosine phosphorylation.

Evolutionary significance of multiple tyrosine protein kinases

The universality of tyrosine phosphorylation as a protein modification remains to be determined. The existence of phosphotyrosine in *E. coli* has been reported, but no individual protein with this modification has been isolated. Further up the evolutionary tree, free phosphotyrosine is found in *Drosophila* larvae, but so far phosphotyrosine has not been reported in *Drosophila* proteins. Nevertheless, because cellular genes homologous to at least two of the viral genes coding for tyrosine protein kinases have been detected in *Drosophila*, phosphotyrosine is likely to be found in *Drosophila* proteins. The impressive degree of conservation of the cellular homologues of such viral transforming genes implies that tyrosine phosphorylation may be a centrally important metabolic function. In this context, the evolution of tyrosine protein kinases is of interest. So far five tyrosine protein kinase genes have been positively identified in higher eukaryotes and there could be several more. Although the five viral tyrosine protein kinase genes are distinct from one another, indications are emerging that they may be closely related and therefore possibly derived from a single ancestral gene. Why should cells need so many similar enzymes? Possibly the tyrosine kinase genes are expressed and regulated independently during development or differentiation. The multiplicity of tyrosine protein kinases could then be a result of the evolution of complex multicellular organisms with their increasing problems of intercellular communication.

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