Minireview

Multisite and Hierarchal Protein Phosphorylation*

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Phosphorylation of proteins at Ser, Thr, and Tyr residues is one of the most frequent forms of posttranslational modification in eukaryotic cells and is linked to the control of a multitude of cellular functions (1, 2). The historical prototype for proteins controlled by phosphorylation is mammalian glycogen phosphorylase, an enzyme activated by modification of a single Ser per subunit by a single protein kinase (3). As the number of examples of phosphorylated proteins has escalated in recent years, it has become apparent that the majority of phosphoproteins contain multiple sites. This review seeks to survey the phenomenon of multisite phosphorylation and to evaluate its significance.

Which Proteins Are Multiply Phosphorylated?

In assessing any specific role of multiple, as opposed to single site, phosphorylation, an immediate question is whether the proteins so modified fall into any particular classes. Almost certainly, only a fraction of multiply phosphorylated proteins have yet been identified; and efforts to survey multiply phosphorylated proteins, as in Fig. 1, inevitably involve a restricted database. In compiling Fig. 1, the guiding principle was to identify proteins for which the location of phosphorylations was either known precisely or at least localized to some specific domain of the protein. The best response to the question posed at the outset of this paragraph is that the occurrence of multiple phosphorylation appears no more restricted than the occurrence of phosphorylation in general.

The survey of Fig. 1 does reveal some interesting features. First, the number of phosphorylation sites observed in proteins varies from 1 to over 100 (Fig. 1) and no special numerology emerges. The only distinction that can perhaps be made is between proteins that contain relatively few sites and those that are heavily phosphorylated. The egg yolk protein phosvitin, neurofilaments, and the COOH-terminal tails of the large subunit of eukaryotic RNA polymerases are good examples of the latter phenomenon. Second, Ser(P), Thr(P), and Tyr(P) can be found in the same protein. Third, multiple phosphorylation sites tend not to be randomly distributed and are usually concentrated in relatively short segments of the polypeptide chain. Often, these phosphorylated regions are located at the extreme NH2 or COOH termini of proteins. Since phosphorylation requires interaction with protein kinase(s), regions of phosphorylation will be defined in part by their accessibility, such as on the surface of a globular protein. In addition, their location must also be related to function. In this regard, it is often relevant that related proteins in different species or tissues (or subunits of the same protein) contain similar subunits or the other. The α-subunit contains an ~100-residue insertion that harbors some seven sites while the β-subunit has an NH2-terminal extension that contains two sites. In this regard, it is often relevant that related proteins in different species or tissues (or subunits of the same protein) contain similar subunits. However, the greatest divergence is seen precisely in these regions.

The yeast versions lack entirely the NH2-terminal regulatory domain, and the other major differences are in the region of COOH-terminal phosphorylation sites. Two messages for acetyl-CoA carboxylase have been detected that differ as to the presence or absence of sequences encoding an 8-amino acid segment just upstream of an important phosphorylation site (8). The insertion disrupts the ability of CAMP-dependent protein kinase to phosphorylate this site (Ser-1200 in the shorter message).

How Are Proteins Multiply Phosphorylated?

Any discussion of mechanism leads to consideration of the specificity of protein kinases. These enzymes range from being highly specific, designed to phosphorylate even a single substrate, to having very broad substrate specificity. The historical prototype for proteins modified in regions of sequence repeats. Examples of proteins modified in regions of sequence repeats include the following: the largest subunit of eukaryotic RNA polymerase II large subunit which, depending on species, can have as many as 52 repeats of a heptameric sequence unit (13, 14). Microtubule-associated protein 2 has been reported to contain more than 30 phosphates per polypeptide (15) and is a substrate in vitro for numerous protein kinases (see Ref. 16). Identification of these sites is incomplete, but the multiplicity of sites in this case is not related to any sequence repeat but rather to the presence of numerous Ser and Thr residues in a generally polar protein. Many other proteins (Fig. 1) contain more moderate numbers of sites whose modification can be explained by the independent action of one or more protein kinases and do not involve global sequence features like sequence repeats.

A mechanism unique to multiply phosphorylated proteins is when the introduction of phosphate groups influences the subsequent phosphorylation reaction(s) (Fig. 2). The first example worked out in molecular terms was that of the phosphorylation

* Work from my laboratory was supported by National Institutes of Health Grants DK27221 and DK42676.

1 For this article, multisite phosphorylation is defined as the occurrence of non-identical phosphorylation sites in a protein complex, usually but not necessarily in a single polypeptide. Phosphorylation of the same site in an oligomer composed of identical subunits is not considered multisite.

2 Space limitations prohibit a totally comprehensive citation of all the information contained in Fig. 1. Refs. 4 and 5 contain listings of many of the individual phosphorylation sites.

α and β regulatory subunits of phosphorylase kinase (6). These polypeptides have significant overall sequence homology, but 9 out of the 10 phosphorylation sites are in sequences specific to one subunit or the other. The α-subunit contains an ~100-residue insertion that harbors some seven sites while the β-subunit has an NH2-terminal extension that contains two sites. In this regard, it is often relevant that related proteins in different species or tissues often differ most at their termini. Glycogen synthase is an enzyme with its phosphorylation sites localized to the termini of the polypeptide (7). In comparing enzymes, the greatest divergence is seen precisely in these regions. The yeast versions lack entirely the NH2-terminal regulatory domain, and the other major differences are in the region of COOH-terminal phosphorylation sites. Two messages for acetyl-CoA carboxylase have been detected that differ as to the presence or absence of sequences encoding an 8-amino acid segment just upstream of an important phosphorylation site (8). The insertion disrupts the ability of CAMP-dependent protein kinase to phosphorylate this site (Ser-1200 in the shorter message).
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> **FIG. 1. Some multiply phosphorylated proteins.** In compiling this figure, it became apparent that anything close to a comprehensive listing was impossible, and there are many omissions. Multiple entries of related proteins were curtailed unless a specific point was to be made. For example, many receptors of the β-adrenergic/rhodopsin family, more than 30 members characterized by seven membrane-spanning segments, are likely to have multiply phosphorylated cytosolic tails. Some phosphorylations not yet proven unequivocally to occur in vivo are also included. Phosphorylation sites are indicated by a vertical line, above for Ser/Thr or below for Tyr. Where no number is shown, a single site is implied.

**FIG. 2. Mechanisms of multisite phosphorylation.** Some phosphorylations involve independent recognition of multiple sites in the substrate by one or more protein kinases (left). Interdependent phosphorylations occur when one phosphorylation event influences another (right). In these hierarchal schemes, one can distinguish primary phosphorylations which affect the course of subsequent secondary phosphorylations. Usually, different protein kinases are involved (upper right), but it is formally possible for a single protein kinase to act in both a primary and secondary way (lower right), as may be exemplified by casein kinase II (see text). The shadings represent different recognition determinants in the substrate.

of glycogen synthase by glycogen synthase kinase-3 (GSK-3)\(^3\) and casein kinase II (7). Totally dephosphorylated glycogen synthase is not a substrate for GSK-3. Upon introduction of phosphate at one specific site by casein kinase II, the protein becomes a substrate for GSK-3, which sequentially modifies 4 Ser residues. The results are explained if GSK-3 recognizes sites in the motif -S-X-X-X-S(P)- (17). In this example, another feature of the reaction is that GSK-3 introduces multiple phosphates, due to the presence of adjacent repeats of the -S(P)-X-X-S- motif. As one phosphate is introduced, a new GSK-3 site is generated. At the time of writing, seven examples of hierarchal phosphorylation involving more than a dozen GSK-3 sites have been recorded (Table I). In three instances the primary protein kinase is cAMP-dependent protein kinase and in three cases casein kinase I. The important feature for recognition by GSK-3 is the presence of a phosphate in an appropriate site and not the kinase that introduced it. Another enzyme that can act as a secondary protein kinase in the sense above is casein kinase I which has a particular selectivity for sites in the motif -S(P)-X-X-S- (10). Again, sites in glycogen synthase provide the best examples to date. The Golgi

\(^3\)The abbreviation used is: GSK-3, glycogen synthase kinase-3.
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"Hierarchal," as explained further in the text, refers to multiple phosphorylations in which the introduction of one phosphate group influences the introduction of subsequent ones.

| Primary protein kinase | Secondary protein kinase | Ref. | Substrate |
|------------------------|--------------------------|------|-----------|
| Synergistic            |                          |      |           |
| CK II                  | GSK-3                    | 7    | Glycogen synthase |
| CK II                  | GSK-3                    | 18   | cAMP-dependent protein kinase RI subunit |
| CK II                  | GSK-3                    | 19   | Inhibitor-2 of type 1 phosphatase |
| cAMP PK                | GSK-3                    | 20, 21 | G-subunit of type 1 phosphatase |
| cAMP PK                | GSK-3                    | 22   | ATP-citrate lyase |
| cAMP PK                | GSK-3                    | 23   | CREB |
| ?                     | GSK-3                    | 24   | N-CAM |
| cAMP PK’               | CK I                     | 25   | Glycogen synthase |
| CK I                   | cAMP PK 26              |      | DARPP-32 |
| Antagonistic           |                          |      |           |
| AMP PK                 | cAMP PK 27              |      | Hormone-sensitive lipase |
| cAMP PK AMP PK 27     | Hormone-sensitive lipase |
| cAMP PK AMP PK 28     | Acetyl-CoA carboxylase |
| Casein kinase I        | S(P)-X-X-S              |      |           |
| Casein kinase II       | S-X-E/S(P)              |      |           |
| Mammary gland casein kinase | S-X-E(S)           |      |           |
| GSK-3                  | S-X-X-S(P)              |      |           |
| jARK                   | E-X-S                   |      |           |

Table I

Hierarchal phosphorylation systems

The exact role of the phosphate in hierarchal phosphorylation schemes has not been defined. One possibility is that the phosphoserine itself is involved in recognition contacts at the active site of the secondary kinase. This idea fits with the fact that most enzymes so far identified as acting in a secondary manner are acidotropic, their recognition motifs characterized by acidic residue(s) close to the modified amino acid (Table II). In the case of GSK-3, it should be noted that -S-X-X-S(P)- is the shortest unit recognition sequence and in two substrates, phosphatase inhibitor 2 and cAMP-dependent protein kinase RI subunit, the phosphates are more distant in the linear sequence. One can speculate that the folding of the protein might bring the target and the recognition serine phosphate into similar juxtaposition as in -S-X-X-S(P)- unit. Two multiply phosphorylated GSK-3 substrates, c-Jun and c-Myb, do not fit the recognition criteria noted above and are reported to be phosphorylated without prior phosphorylation (57).

A second possibility is that the introduction of a phosphate group changes the conformation of the substrate into one that is recognized by a secondary protein kinase without the protein group itself participating in kinase-substrate contacts. One potential example is the enhanced phosphorylation of Thr-34 in DARPP32 by camp-dependent protein kinase once Ser-45 and/or Ser-102 has been phosphorylated by casein kinase II (26). The cAMP-dependent protein kinase typically recognizes basic motifs such as -R-R-X-S-, and a phosphate group would be expected to participate directly in recognition. Hierarchal phosphorylation mediated by conformational changes in the substrate would not require the secondary kinase to be acidotropic.

Why Are Proteins Multiply Phosphorylated?

There are two related but distinct perspectives on the possible role of multisite phosphorylation. One is at the level of the structural changes elicited by the introduction of phosphate groups and the subsequent effects on protein function. Multiple phosphorylations could correlate with the generation of a variety of protein forms, in which one or more properties are altered. Different phosphorylations could thus be linked to distinct protein functions or graded effects on a single function. Alternatively, multiple phosphates might be necessary to cause one critical conformational change. The other perspective is at the level of the regulation of the different phosphorylations. Obviously, if the actions of more than one kinase influence the functional status of a target protein, more complex regulation could be exerted relative to the actions of a single kinase. Examples below are selected to illustrate some of the features that may be exclusive to multiple phosphorylations.

Hormones regulate the phosphorylation of glycogen synthase, and the multisite phosphorylation is in part linked to the occurrence of the hierarchal mechanism described above (7). An important feature is that the primary phosphorylations, such as mediated by casein kinase II and cAMP-dependent protein kinase, at best have moderate effects on activity; effective inactivation requires the occurrence of the secondary phosphorylations catalyzed by GSK-3 or casein kinase I. Thus, some phosphorylations function to alter activity whereas others influence kinase recognition. Not all the details linking hormone action to the control of phosphorylation have been worked out, but an important aspect of glycogen synthase control is that, mechanistically, the down- or more phosphate modifications do not occur totally independently and a smaller number of multiply phosphorylated units can be defined.

Acetyl-CoA carboxylase is another metabolic enzyme whose multiple phosphorylation is regulated by hormones (28, 38). In this protein, there is evidence that different phosphorylation sites have different influences on the kinetic properties of the enzyme.

Most of the known independent phosphorylations involve a positive role for the initial phosphorylation though a priori there is no reason why phosphorylation might not impair the action of a second kinase. For example, hormone-sensitive lipase (27) has two phosphorylation sites, Ser-563 (site 1) and Ser-565 (site 2). Phosphorylation of Ser-563 by cAMP-dependent protein kinase activates the enzyme whereas Ser-565 modification is without effect. However, phosphorylation of the two sites is mutually exclusive so that modification of Ser-565 could control activity indirectly by reducing phosphorylation at Ser-563. A similar situation holds for acetyl-CoA carboxylase. Phosphorylation of Ser-77 and Ser-1200 by cAMP-dependent protein kinase prevents phosphorylation of Ser-79 by the AMF-dependent protein kinase (28).
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Summary
Multisite phosphorylation is a prevalent form of protein modification whose full implications are just beginning to be understood. Multiple protein phosphorylations expand the repertoire of structural changes that can be elicited in proteins and permit more intricate regulatory circuits to operate.

Acknowledgments—Special thanks go to Peter Kennelly and Edwin Krebs, and to Kew Kemp and Richard Pearson for allowing me access to their reviews and compilations of phosphorylation sites prior to publication. Robert Swift of Eli Lilly provided invaluable help with the figures.

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allosteric activator citrate. It has also been reported that phosphorylation of the enzyme by casein kinase II at site 6 (Ser-29), itself without effect on activity, may influence dephosphorylation of other sites that do control activity (39).

The tyrosine protein kinase pp60 src provides an example of a protein in which different tyrosine phosphorylations can potentially modulate protein function (40). The autophosphorylation site, Tyr-416, may activate the kinase, whereas Tyr-527 is an inactivating site that lies, interestingly, in precisely the region of the molecule missing in the retroviral transforming gene product pp60 v-src. The viral protein therefore lacks the negative control, and the unconstrained activity of pp60 src is thought to be linked to transformation by Rous sarcoma virus. pp60 src is also phosphorylated at several Ser and Thr residues close to the NH2 terminus, including sites for CAM-dependent protein kinase and protein kinase C that may also be linked to activation of the kinase. The activity of pp60 src may thus depend on the phosphorylation of multiple sites.

The -adrenergic receptor undergoes phosphorylation in two sets of functionally distinguishable sites (41), modified by CAM-dependent protein kinase and the -adrenergic receptor kinase, respectively. Receptor activation promotes its own phosphorylation by CAM-dependent protein kinase whose full implications are just beginning to be understood. Multiple protein phosphorylations expand the repertoire of structural changes that can be elicited in proteins and permit more intricate regulatory circuits to operate.