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Point of VIEW

A Lock on Phosphotyrosine Signaling

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ABSTRACT Protein phosphorylation plays a major role in cell signaling and human disease, so understanding the effects of tyrosine phosphorylation on protein structure and function is an area of intense investigation. A new technique allows site-specific incorporation of a non-hydrolyzable phosphotyrosine analogue into recombinant proteins, providing a new strategy for research in this important area.

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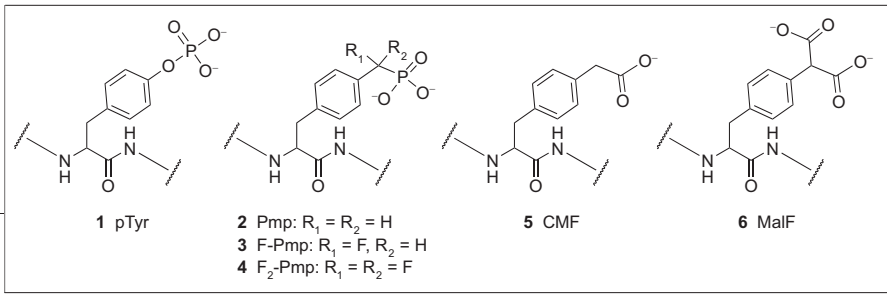
Since Hunter and Sefton reported that *v-src* was a protein tyrosine kinase (PTK) (*1*), researchers have been heavily invested in understanding the roles of PTKs in cancer and other diseases. There are ~10 clinically used drugs that block tyrosine kinases—including the kinases Abl, epidermal growth factor receptor (EGFR), c-Kit, Her2/neu, and vascular endothelial growth factor receptor—that are employed to treat chronic myeloid leukemia, breast cancer, lung cancer, and gastric stromal tumors, among other cancers (*2*). Phosphorylation can affect a protein's conformation, enzymatic activity, and binding interactions. In this issue, Xie *et al.* (*3*) report on the site-specific incorporation of a phosphotyrosine (pTyr, **1**) mimetic in recombinant proteins (Scheme 1). The mimetic is non-hydrolyzable and can be placed at any position in a protein. Consequently, the effects of any particular phosphorylation site on a protein's structure and function can be studied.

Phosphorylation sites and the effects of these post-translational modifications have been characterized for some protein targets. However, ~90 human PTKs (*4*) and >600 tyrosine phosphorylation sites exist, so much work is still needed (*5*). pTyr modifications are known to mediate cellular effects by three primary mechanisms: inducing binding to Src homology 2 (SH2) domains (*6*), conferring interactions with pTyr binding (PTB) domains (*7*, *8*), and negatively influencing intramolecular hydrogen bonds involving the tyrosine phenol side chain (*9*).

SH2 domains are found in non-receptor tyrosine kinases such as Src, Abl, and Jak; several protein tyrosine phosphatases (PTPs) like SHP-2; transcription factors such as the signal transducers and activators of transcription (STATs); and many adaptor proteins like Grb2. In the human genome, 109 SH2 domains have been identified (*10*), and many are believed to mediate sequence-specific protein–protein interactions that require pTyr. SH2–pTyr interactions can stimulate (*e.g.*, SHP-2) (*11*) or inhibit (*e.g.*, Src) enzymatic activity in an intramolecular fashion (*12*). In addition, some SH2–pTyr interactions serve in recruitment. For example, the SH2 domain of Grb2 is recruited to the cell membrane by binding to the C-terminal tail of activated receptor tyrosine kinase EGFR (*13*), a transmembrane protein. Many high-resolution crystal and NMR structures of SH2 domains bound to pTyr-containing peptides illustrate extensive interactions between the phosphate dianionic oxygens and a highly conserved SH2 arginine side chain (Figure 1, panel a) (*14*). Neighboring residues upstream and downstream of the pTyr typically make specific contacts with the SH2 domain, fine-tuning specificity. Thermodynamic studies reveal that the phosphorylation event results in 10,000-fold tighter binding to an SH2 domain compared with the unphosphorylated peptide (*15*).

PTB domains, which number ~50 in the human genome (*10*), also mediate pTyr recognition. Unlike SH2 domains, only a subset of PTB domains apparently requires pTyr modifications for high-affinity interaction (*7*, *8*). The best-characterized member of this class is Shc, which plays a major role in growth factor receptor signaling by inter-



Scheme 1. pTyr and some effective mimetics.

acting with growth factor receptors. Like canonical SH2 domains, PTB domains utilize one or more arginine residues for phosphate interaction (Figure 1, panel b) (*16*). Many of these domains are themselves phosphorylated and act as adapter proteins: they serve as a linker between a ligand and an SH2-containing protein.

Tyrosine phosphorylation of protein kinases within their activation loop is commonly a critical element that regulates their activity. For example, the insulin receptor kinase (IRK) is phosphorylated on multiple residues, including Tyr1162 in the activation loop (*17*). Prior to autophosphorylation, Tyr1162 forms a hydrogen bond with the catalytic base Asp1132 and maintains IRK in an inactive state (Figure 2) (*9, 18*). After insulin-induced phosphorylation, this hydrogen bond is disrupted, and IRK becomes a powerful catalyst.

Another key element of tyrosine kinase signaling pathways is the role of PTPs in their regulation. Scientists have demonstrated that intracellular tyrosine phosphorylation events can have a <4 min lifetime after the kinase is turned off (*19*). Thus, isolating and characterizing tyrosine-phosphorylated proteins can represent a significant challenge. This is particularly true for the not uncommon cases in which PTPs are themselves phosphorylated on tyrosines.

Typically, scientists assess the roles of pTyr modifications by making mutants with substitutions that abolish phosphorylation, but it is also desirable to study the effects of the presence of a phosphate in the protein. In the case of serine or threonine phosphorylation, mimicking phosphorylation by substitution with the acidic amino acids, aspartate and glutamate, has been possible. However, no natural amino acids are analogous to pTyr, so studies of the effects of these phosphorylation events have had to rely on more sophisticated chemical methods.

The development of pTyr analogues was first studied in the context of synthetic peptide experiments (*20*). As predicted by geometry and charge, 4-phosphonomethyl phenylalanine (Pmp, **2**), the mono- and difluorinated Pmp analogues (**3** and **4**), and malonylphenylalanine (**6**) have shown an impressive ability to mimic pTyr. Another analogue that has been used with some success is *para*-carboxymethyl phenylalanine (*p*CMF, **7**). This analogue is structurally very much like a glutamate mimic of phosphoserine, and *p*CMF-containing peptides bind to the SH2 domain of the PTK Lck (*21*). The potential of achieving site-specific incorporation of unnatural amino acids into proteins is a relatively new frontier in biochemistry. The semisynthetic method of expressed protein ligation is one alternative, but it is most effective when substitutions are near the protein termini (*22, 23*).

The site-specific incorporation of unnatural amino acids *via* nonsense suppression has been an exciting development in the field of protein engineering. In earlier studies, efforts from the groups of Hecht, Chamberlin, and Schultz showed it was possible to use *in vitro* translation methods to generate small quantities of recombinant proteins (reviewed in ref 24). However, a major technical advance was achieved when Schultz and co-workers first reported the successful incorporation of *O*-methyltyrosine into recombinant proteins generated in *Escherichia coli* (*25*). In this technique, a transfer RNA (tRNA) specific for a given codon (*i.e.*, the amber stop codon, TAT) and an aminoacyl-tRNA synthetase (responsible for charging the tRNA with the correct amino acid) are evolved *in vivo* under selective pressure to specifically incorporate a non-natural amino acid. They have reported success in *E. coli*, yeast (*26*), and mammalian (*27*) expression systems and extended amino acid selection to a broad range of unnatural residues. In the latest report from Xie *et al.* (*3*) on page 474, an aminoacyl-tRNA synthetase has been evolved to incorporate *p*CMF into recombinant proteins in *E. coli*. They demonstrated the utility of the method by showing that *p*CMF incorporation into a recombinant STAT1 transcription factor was sufficient to confer DNA binding properties. This is likely to have utility for a wide variety of signaling experiments where site-specific incorpora-

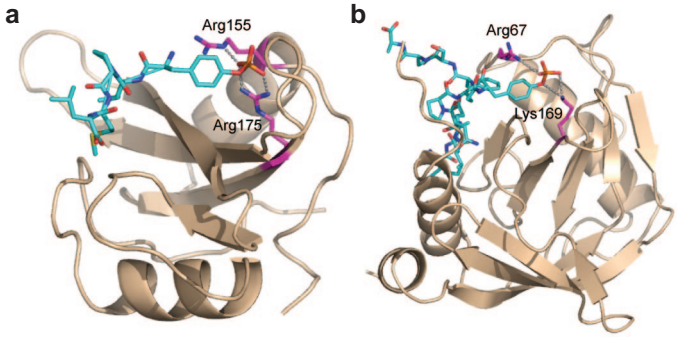


Figure 1. pTyr interaction domains. Cartoon representations of pTyr recognition domains (tan) bound to peptide (blue stick representation). Basic residues that interact with the phosphate group are shown as magenta sticks with gray dotted lines indicating the salt bridges. a) The SH2 domain of Src bound to a segment of platelet-derived growth factor receptor (Protein Data Bank (PDB) code 1SHA). b) The PTB domain of Shc bound to a segment of TrkA (PDB code 1SHC). PyMOL was used to create this figure (DeLano Scientific, Palo Alto, CA).

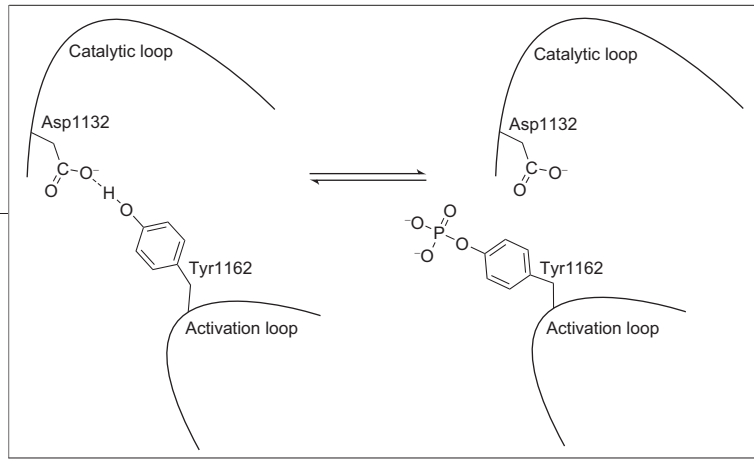


Figure 2. Effect of activation loop phosphorylation in tyrosine kinases. The autoinhibited form (left) of IRK has a hydrogen bond between Tyr1162 and the catalytic base, Asp1132. After phosphorylation (right), pTyr1162 can no longer form this hydrogen bond, and the activation loop moves out of the active site, clearing the way for a substrate to interact with Asp1132.

tion of a pTyr mimic in a recombinant protein can be used for functional analysis.

The authors note that it is not yet possible to incorporate higher fidelity phosphono-phenylalanine mimics such as Pmp because of the poor cell permeability of these amino acids. Because *p*CMF can be 500-fold less faithful at substituting for pTyr in SH2 domain binding (*21*), this is currently a limitation. One expects that masked phosphono-phenylalanine derivatives that can be deprotected inside cells should be able to overcome the cell penetration challenge. Acyloxymethylene-substituted phosphates have been very effective in this regard (*28*). Nonetheless, it is likely that for activation loop phosphorylation events, where precise docking interactions may not be crucial, *p*CMF replacements may be especially valuable. Accordingly, we predict that unnatural amino acid incorporation *via* nonsense suppression will prove to be an important tool in pTyr proteomics analysis in the years ahead.

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