Published in final edited form as:
Methods Enzymol. 2009 ; 462: 1–24. doi:10.1016/S0076-6879(09)62001-2.

Protein Phosphorylation by Semisynthesis: From Paper to Practice

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Abstract

Deconvolution of specific phosphorylation events can be complicated by the reversibility of modification. Protein semisynthesis with phosphonate analogues offers an attractive approach to functional analysis of signaling pathways. In this technique, N- and C-terminal synthetic peptides containing nonhydrolyzable phosphonates at target residues can be ligated to recombinant proteins of interest. The resultant semisynthetic proteins contain site specific, stoichiometric phosphonate modifications and are completely resistant to phosphatases. Control of stoichiometry, specificity, and reversibility allows for complex signaling systems to be broken down into individual events and discretely examined. This chapter outlines the general methods and considerations for designing and carrying out phosphoprotein semisynthetic projects.

1. Overview of Protein Phosphorylation

Since the discovery of cell surface receptors and second messengers, there has been intensive study of the mechanisms of cell signal transduction. Information flow from the plasma membrane to the nucleus and then back again involves an array of enzymes, adaptor proteins, lipids, and other small molecules whose structural interactions govern cell growth, differentiation, and movement. Among the many cell-signaling pathway regulatory mechanisms, reversible protein phosphorylation stands out as of critical importance. Protein kinases and phosphatases are members of large superfamilies in the human genome, and a large number of these enzymes have been implicated as drug targets for diseases including cancer, diabetes, immune disorders, inflammatory conditions, and cardiovascular conditions (Blume-Jensen and Hunter, 2001; Cohen, 2002; Hunter, 2000). Protein phosphorylation catalyzed by the eukaryotic protein kinase superfamily members are typically Ser/Thr or Tyr selective, and there are approximately 400 protein Ser/Thr kinase (PSK) and 100 protein Tyr kinase (PTKs) human genes (Manning *et al.*, 2002). The protein tyrosine phosphatase (PTP) superfamily has about 100 members, whereas the protein Ser/Thr phosphatase (PSP) family numbers about 15 (Alonso *et al.*, 2004). It has been estimated that 25% of cellular proteins undergo phosphorylation and are thus substrates of one or more kinase and phosphatase (Cohen, 2000). Upon phosphorylation, protein structural interactions can be affected in several ways. In many cases, the addition of a phosphate to a protein side chain can inhibit or promote intra- or intermolecular protein-protein interactions. A number of well-characterized domains have been implicated in selectively binding to pSer/pThr and pTyr, respectively (see Table 1.1). The first of these discovered was the Src homology 2 (SH2) domain, which binds pTyr motifs in proteins. There are about 100 SH2 domains found in human genes (Yaffe, 2002). In addition, phosphotyrosine-binding (PTB) domains also bind pTyr-containing sequences. A number of proteins or protein domains that have been identified as maintaining pSer/pThr binding include 14-3-3 adaptor proteins, Polo-Box domains, WW domains, BRCA1 C-terminal (BRCT) domains, and forkhead-associated (FHA) domains (Yaffe and Elia, 2001). Unraveling the complexity of protein

phosphorylation functionality is daunting. In general, the identification of specific kinase-substrate interactions and cellular phosphorylation actions for individual cases remains a major challenge. A variety of novel approaches have been developed in recent years to attempt to analyze protein kinases, phosphatases, and the function of individual phosphate modifications (Johnson and Hunter, 2005; Shogren-Knaack *et al*., 2001; Qiao *et al*., 2006; Williams and Cole, 2001; Zhang, 2005). In this review, we discuss the steps required to successfully use protein semisynthesis as a tool to investigate protein phosphorylation, and we outline the practical uses of these semisynthetic proteins.

2. Investigating Protein Phosphorylation with Phosphomimetics

Researchers have been searching for ways to investigate protein phosphorylation since the discovery that this modification can reversibly alter the activity of an enzyme through the combined action of kinases and phosphatases (Fischer and Krebs, 1955; Krebs and Fischer, 1955). Unfortunately, it is this reversibility that presents a challenging problem in *in vivo* studies of protein phosphorylation and attempts to elucidate complex signaling cascades. Although various techniques have been used to address this problem, all have limitations. These techniques are discussed subsequently.

2.1. Thiophosphate substitution as a phosphomimetic

One way to deal with the reversibility of phosphorylation is to enzymatically phosphorylate the protein of interest using ATPγS and catalytic amounts of kinase (Cole *et al.*, 1994; Eckstein, 1983). This resulting thiophosphate linkage is more stable to phosphatases and therefore of greater potential utility in *in vivo* assays. However, with this approach, it is difficult to control the site specificity and stoichiometry of the modification (Tailor *et al.*, 1997), and thiophosphates are not fully phosphatase resistant (Cho *et al*., 1993).

2.2. Using amino acid substitution as a phosphomimetic

A genetic approach to deal with reversibility of phosphorylation has been to mutate the phosphorylated residue to either a Glu or an Asp in hopes that the addition of a negative charge will mimic the phosphorylated residue (Fig. 1.1B). This approach was taken by Thorsness and Koshland (1987) in the early years of site-directed mutagenesis; they showed that Asp was a good mimic for pSer in isocitrate dehydrogenase. In that case, the mutation led to the predicted complete inactivation of the enzyme. Since this early report, there have been numerous examples of this approach being both successful (Hao *et al.*, 1996; Potter and Hunter, 1998) and unsuccessful (Huang and Erikson, 1994; Zheng *et al.*, 2003). Phosphates and carboxylates obviously differ in number of oxygen atoms available for hydrogen bonding, geometry, numbers of negative charges at neutral pH, and size. Moreover, there is no natural isostere for pTyr (Zisch *et al.*, 2000).

2.3. Incorporation of alternative genetically encoded phosphomimetics

More recently, the Schultz group described a general method to expand the number of genetically encoded amino acids in *Escherichia coli* by using unique tRNA/aminoacyl-tRNA synthetase pairs (Wang *et al.*, 2001). To date, more than 30 unnatural amino acids have been incorporated into proteins in *E. coli*, yeast, and mammalian cells (Xie and Schultz, 2005). One such amino acid, *p*-carboxymethyl-phenylalanine (Cmp), acts as a pTyr mimetic in the same way that Glu and Asp act as pThr/pSer mimetics (Fig. 1.1B). Although this methodology expands the number of genetically available phosphomimetics to include a pTyr mimetic, it has not yet been used for phosphate-containing amino acids, presumably because of their limited ability to traverse the cell membrane or wall.

3. Phosphonate Analogues and Protein Semisynthesis

Nonhydrolyzable phosphonate analogues offer a useful tool for investigating phosphorylated proteins *in vivo*, as proteins bearing these mimetics are completely resistant to phosphatase activity (Fig. 1.1C) (Berkowitz *et al.*, 1996; Chen *et al.*, 1995; Desmarais *et al.*, 1999; Zheng *et al.*, 2003, 2005). Therefore, by incorporating phosphonate analogues into proteins at positions that are normally phosphorylated, it is possible to identify novel interacting proteins by pull-down assays (e.g., phosphatases and phosphopeptide binding domains) and to evaluate protein stability and cellular localization by microinjection assays (Lu *et al.*, 2001; Schwarzer *et al.*, 2006; Zheng *et al.*, 2003, 2005). However, to incorporate the phosphonate analogues into proteins, the proteins must be amenable to semisynthetic technology. In these procedures, N- and C-terminal peptides (up to 50 amino acids in length) containing the phosphonate substitution(s) can be synthetically prepared using a standard Fmoc solid-phase peptide synthesis (SPPS) strategy and ligated to a recombinant portion of the protein of interest (Dawson *et al.*, 1994; Erlanson *et al.*, 1996; Evans *et al.*, 1998; Muir *et al.*, 1998). In this manner, homogenous, stoichiometrically labeled phosphoproteins can be generated. The marriage of phosphonate analogue technology and protein semisynthetic technology makes it possible to determine which protein domains and enzymes can interact with phosphorylated proteins in live cells and aids in the elucidation of complex signal transduction cascades.

The development of native chemical ligation (NCL) revolutionized the possibility of linking large unprotected peptide fragments in a chemoselective and mild fashion (Dawson *et al.*, 1994). In this methodology, ^{1}Cys-containing peptides can be efficiently ligated to $^{\alpha}$thioester peptides by initial transthioesterification, followed by rearrangement to a stable peptide bond. Extension of NCL for use in protein semisynthesis requires the generation of ^{1}Cys groups or $^{\alpha}$thioester functionality in recombinant proteins. N-terminal Cys production is most readily achieved using selective proteases (Erlanson *et al.*, 1996; He *et al.*, 2003; Tolbert *et al.*, 2005; Zheng *et al.*, 2003), whereas $^{\alpha}$thioesters can be generated using modified inteins (Chong *et al.*, 1997; Evans *et al.*, 1998). Expressed protein ligation (EPL) has been used to describe the technique of protein semisynthesis in which a ^{1}Cys-containing peptide is ligated by NCL to a recombinant protein fragment containing a C-terminal thioester (Evans *et al.*, 1998; Muir *et al.*, 1998). In EPL, the recombinant protein thioester fragment is typically generated via a C-terminal intein-chitin binding domain (CBD) fusion protein, whereby the intein catalyzes an *N*- to *S*-acyl shift at the junction of the fusion protein. In the presence of a thiol [e.g., sodium 2-mercaptoethanesulfonate (MESNA), thiophenol, or mercaptophenylacetic acid], this intermediate is trapped as an $^{\alpha}$thioester and intercepted with a ^{1}Cys synthetic peptide. The newly formed thioester-linked species spontaneously rearranges to yield a native peptide bond at neutral pH. As a result, a semisynthetic protein is generated that contains a chemoselectively ligated synthetic peptide at the C-terminus (Fig. 1.2) (Evans *et al.*, 1998; Muir *et al.*, 1998).

4. Methods

This section aims to guide investigators in designing and carrying out a phosphoprotein semisynthetic project. In doing so, we refer to specific published examples that have been successful in our laboratory.

4.1. Choosing a protein for semisynthesis

Many but not all proteins are attractive candidates for protein semisynthesis. At least three major factors should be considered. First, the target residue (or residues) for modification should be within approximately 50 residues of either the N-terminus or the C-terminus. This reflects a limitation of protein semisynthesis in that there is no easy method to incorporate

unnatural amino acids into the central regions of proteins, and SPPS becomes increasingly difficult for very long peptides. Second, in choosing the candidate protein, it is more favorable to focus on C-terminal modifications because recombinant protein $^{\alpha}$thioesters for semisynthesis are easily made by inteins, whereas investigating N-terminal modifications requires the synthesis of a peptide $^{\alpha}$thioester, which can be difficult when using Fmoc SPPS. Third, protein expression and solubility should be considered. It is easiest to work with a protein that is soluble and expresses well in *E. coli*; however, many proteins have poor solubility when expressed recombinantly in prokaryotic systems, and semisynthesis can be achieved only with insect cell expression techniques. It can be difficult to predict how a protein fragment of interest will behave when it is truncated for semisynthesis and expressed as either a C-terminal intein-CBD fusion protein or N-terminal fusion protein for semisynthesis. Breaking into the middle of an independently folded domain is often problematic and may result in poor solubility. However, this does not mean that the semisynthetic project is not feasible. As long as the protein can be refolded to its native state, the project is likely to succeed (e.g., K^+ channel, LMW-PTP) (Schwarzer *et al.*, 2006; Valiyaveetil *et al.*, 2002). Dealing with soluble versus insoluble proteins for semisynthesis is discussed further in later sections.

4.2. Choosing the peptide ligation site

When ligating a synthetic peptide to a recombinant protein by NCL, it is necessary to have a Cys residue at the ligation site. Depending on whether C-terminal or N-terminal semisynthesis is used, the Cys residue will need to be included in either the synthetic peptide or the recombinant protein, respectively. Ideally, a native Cys residue will be present in close proximity, interior to the target amino acid for modification. Often, this is not the case, and investigators must select where to insert the ligation site. This is somewhat more complex in N-terminal semisynthesis, as many of the protocols for synthesizing peptide $^{\alpha}$thioesters by Fmoc SPPS can result in epimerization at the alpha carbon of the thioester. In such cases, we prefer to use a Gly residue for the $^{\alpha}$thioester so that epimerization is not a problem. There are several factors that influence selection of the ligation junction. First is the secondary structure of the protein. If a crystal structure of the protein is available, potential ligation sites can be examined closely to inspect whether truncation of the protein is likely to disrupt the secondary or tertiary structure. It is preferable to place the ligation site in a nondisruptive (i.e., flexible) region. Second, if a native Cys residue is not available for ligation, then conservative mutation of a residue to Cys is desirable. We recommend replacement by Ser first, Ala second, and other residues if necessary. Third, the identity of the residue N-terminal to the Cys at the ligation site can influence efficiency of the NCL reaction. Hackeng *et al.* (1999) have published a detailed analysis describing the kinetics of the NCL reaction on a model peptide in which every amino acid was substituted immediately N-terminal to the Cys residue at the ligation site. The authors found that the NCL reaction rates depended on the identity of the residue. The time it took for the reactions to be completed were as follows: HGC (4 h) < FMYAW (9 h) < NSDQEKR (24 h) < LTVIP (48 h). We use these data as guidelines for selecting ligation junctions. Additional information on this topic can be found in the Impact-CN manual available online from New England Biolabs. Fourth, in choosing a site for N-terminal ligation, one has to consider that it is convenient to have a Gly residue adjacent to the $^{\alpha}$thioester on the synthetic peptide. Ideally, the protein would contain a native Gly-Cys, Gly-Ser, or Gly-Ala sequence interior to the amino acid targeted for modification. If no such sequence exists, one can consider insertion of a Gly-Cys sequence or make nonconservative mutations to yield the Gly-Cys sequence. Amino acid insertion should generally be avoided, but it can be necessary, as described for arylalkylamine-*N*-acetyltransferase (AANAT) (Zheng *et al.*, 2003). Finally, after the ligation site has been chosen, a full-length protein

harboring the mutation(s) should be purified and compared to wild-type protein to make sure that no deleterious effects arise from the mutation(s).

4.3. Design of recombinant constructs for semisynthesis

Once the candidate protein and ligation site have been selected and the mutant protein has been validated as behaving identical to the wild-type protein, the semisynthetic project can proceed. The truncated candidate protein must be subcloned into an appropriate vector for ligation. For C-terminal EPL, our lab generally subclones the C-terminally truncated candidate protein into both commercially available Impact-CN vectors pTYB2 or pTXB1 (New England Biolabs) which contain the *Saccharomyces cerevisiae* VMA1 intein and *Mycobacterium xenopi GyrA* intein, respectively. The VMA1 intein is larger than the GyrA intein and cannot be refolded, which can be limiting (Valiyaveetil *et al.*, 2002). However, because it is impossible to predict which fusion protein will yield good expression, soluble protein, and ligation efficiency, it is advisable to start with both vectors. After the subcloning is complete, expression levels should be checked on a small scale to determine whether the fusion protein is soluble or insoluble. The vector(s) that yield soluble protein are preferred; however, if both vectors yield insoluble fusion protein, then only the GyrA fusion can be carried forward.

For N-terminal ligation, a cleavable fusion partner for the N-terminus of the protein must be chosen. It is important to realize that cleavage of the N-terminal tag must yield a ^{1}Cys recombinant protein fragment for the NCL reaction. Proteases typically used for this task are factor Xa (Erlanson *et al.*, 1996; Zheng *et al.*, 2003), TEV protease (Tolbert *et al.*, 2005), and SUMO protease. Alternatively, one may rely on bacterial methionine aminopeptidase to expose an N-terminal Cys after translation; however, it is impossible to predict to what extent this will occur *in vivo* (He *et al.*, 2003). The GST–factor Xa vectors (pGEX) and factor Xa protease are commercially available from GE Healthcare Lifesciences (formerly Amersham Biosciences), a His_6 tag and the TEV protease recognition sequence (ENLYFQ/G) can be introduced easily by PCR and His_6 tagged TEV protease can be produced recombinantly, and the Champion pET-SUMO expression system is commercially available from Invitrogen. Although TEV and SUMO proteases are more specific than factor Xa, one should recognize that cleavage with any protease can lead to the formation of unwanted degradation products. After the desired N-terminally truncated candidate protein is subcloned into the appropriate vector, expression levels should be checked on a small scale to determine whether the fusion protein is soluble or insoluble. Larger-scale protein purification is then necessary to work out conditions for specific proteolytic cleavage to yield the ^{1}Cys recombinant protein fragment and its subsequent purification for use in N-terminal NCL.

4.4. Peptide synthesis

Once it can be established that the recombinant protein fragment is accessible for semisynthesis, peptide synthesis can be pursued. The use of either native phosphorylated amino acids or nonhydrolyzable mimics depends on the project's goals. For example, there is little benefit to using a phosphomimetic, which is a greater experimental investment, if the semisynthetic protein will not be exposed to phosphatases in subsequent experiments (Huse *et al*., 2001; Muir *et al*., 1998; Ottessen *et al*., 2004). With the exception of the pTyr mimetic phosphonomethylene phenylalanine (Pmp, Advance ChemTech), we are unaware of a commercial source for nonhydrolyzable phosphonates for use in Fmoc SPPS; therefore, these unnatural amino acids must be synthesized as needed. In general, the preferred phosphonates are phosphono-difluoromethylene alanine (Pfa), a pSer/pThr mimetic, and phosphono-difluoromethylene phenylalanine (F_2Pmp), a pTyr mimetic (Fig. 1.1C). Although the corresponding CH_2 phosphonates are a reasonable option and have been used

successfully by our group, the presence of the halogens act to lower the second pK_a of the phosphonate to be similar to that observed for the natural phosphorylated amino acid. A discussion of the synthetic schemes for these analogues is beyond the scope of this manuscript; however, we have had success with the procedures of Berkowitz *et al.* (1996) (Pfa) and Guo *et al.* (2002) (F_2Pmp).

When embarking on a new Fmoc SPPS project, our lab follows standard protocols. For incorporation of phosphonates, which are typically limiting, we use a single coupling reaction with 1.2 eq of Fmoc amino acid for 4 to 10 h, followed by N-terminal capping with acetic anhydride and normal peptide extension. N-terminal Cys peptide acids for EPL are typically assembled on the Wang resin, but when synthesizing $^{\alpha}$thioesters for N-terminal NCL, the weak acid labile 2-Chlorotrityl (2-Cl-Trt) resin can be used. Although there are now several methods for synthesizing $^{\alpha}$thioesters by Fmoc SPPS (Botti *et al.*, 2004; Gross *et al.*, 2005), our lab has had success using the method described by Futaki *et al.* (1997). The thioester peptides/protein fragments need to be stored at low pH (<5) and temperature to prevent hydrolysis.

4.5. C-terminal semisynthesis (EPL) on a soluble protein

C-terminal EPL on a soluble protein is probably the simplest of the semi-synthetic methods. As a starting point for C-terminal EPL, three HEPES-based buffers are typically used in our lab:

1. Cell lysis buffer: 25 m*M* HEPES (pH 7 to 7.5), 150 m*M* NaCl, 1 m*M* $MgSO_4$, 5% glycerol, 5% ethylene glycol (protease inhibitors as needed)
2. Chitin column buffer: 50 m*M* HEPES (pH 7 to 7.5), 250 m*M* NaCl, 1 m*M* EDTA, 0.1% triton X-100 (protease inhibitors as needed)
3. Ligation buffer: 50 m*M* HEPES (pH 7 to 7.5), 250 m*M* NaCl, 1 m*M* EDTA (protease inhibitors as needed)

To perform NCL, one of several thiols can be selected. The major options include MESNA, thiophenol, and mercaptophenylacetic acid (MPAA), and we usually compare the efficiency of each for a given case (Johnson and Kent, 2006). These thiols will intercept the recombinant protein $^{\alpha}$thioester to yield a more reactive $^{\alpha}$thioester for C-terminal EPL. Ligations in the presence of MESNA can be somewhat slower than in the thiophenol or MPAA reactions (Ayers *et al.*, 1999; Johnson and Kent, 2006). Thiophenol can be problematic because of its low solubility in aqueous buffers (generally 0.5 to 2% v/v is used) and consequently its presence may have deleterious effects on protein stability and solubility. However, MPAA offers a compromise between MESNA and thiophenol, as it is quite soluble and forms a very reactive $^{\alpha}$thioester (Johnson and Kent, 2006). Because MESNA can induce unexpected enzymatic properties (Zhang *et al.*, 2003), it is advisable to rule out unwanted effects of the selected thiol on a protein of interest prior to modification.

For a protein that expresses well in *E. coli* (i.e., ≥5 mg/L of culture), C-terminal EPL is generally conducted by starting with the cell paste from 1 L of culture. Cells are resuspended in ice-cold lysis buffer, lysed via double pass on a French press (16,000–18,000 psi), and clarified by centrifugation at 25,000 × *g* for 15 min (4°C). The clarified soluble protein is then double loaded onto a pre-equilibrated 5-mL chitin column (chitin available from New England Biolabs) at approximately 1 mL/min (4°C). The column is washed with 20 vol of chitin column buffer to remove impurities, followed by 10 vol of ligation buffer to remove detergent (4°C). At this point, the column is allowed to warm up to room temperature for 30 min. For EPL initiation, two solutions are needed:

1. 5 mL ligation buffer + 200 m*M* thiol

2. 5 mL ligation buffer + 200 m*M* thiol + 1 to 2 m*M* ^{1}Cys-peptide for ligation.

Because NCL is a bimolecular reaction, the rate of ligation should generally be proportional to the amount of peptide used. The solutions for initiation are prepared at room temperature and the pH must be readjusted to 7.0 to 7.5 on addition of thiol and synthetic peptide. For longer ligation times, the solutions should be purged with N_2 to prevent the oxidation of thiol. To initiate EPL, 5 mL of solution A (thiol solution) is quickly passed through the column and the flow-through discarded. This is followed immediately by 5 mL of solution B (peptide solution), but the flow-through is collected and loaded back onto the top of the column, both ends of the column are sealed, and the column is purged with N_2 and incubated at room temperature for the duration of the ligation reaction. Adding agitation (e.g., rotation in three dimensions) and increasing the temperature may improve or reduce ligation efficiency and depends on the protein of interest.

Typically, C-terminal EPL takes between 24 h and 96 h to reach greater than 80% completion depending on the thiol used. Reaction progress can be monitored via SDS-PAGE by pipetting some solution off the top of the column and running a gel. One can generally distinguish the bands corresponding to the ligated and unligated semisynthetic protein (this is easy when the mass difference is ≥1000 Da). Although MALDI-TOF MS is not strictly quantitative, it can be used to monitor reaction progress; however, the protein must be desalted before analysis. Desalting is usually achieved by C_4 zip-tip purification, but sometimes the high concentrations of thiol or salt cannot be removed, thus rendering MALDI-TOF MS analysis impossible at this stage.

On completion of C-terminal EPL, the protein is eluted from the column with ligation buffer (no thiol), and protein-containing fractions are pooled and dialyzed into storage buffer overnight to remove thiol. The semisynthetic protein is then concentrated and subjected to size exclusion chromatography to remove excess ^{1}Cys peptide. A 1-L preparation of a protein that expresses well can often yield 3 to 5 mg of semisynthetic protein. If expression is poor, the initial amount of culture can be scaled up for the desired production.

4.6. C-terminal EPL on an insoluble protein

Conducting protein semisynthesis on an insoluble protein requires a similar approach but should be undertaken only if the target protein can easily be refolded. As mentioned earlier, investigators are limited to using the smaller GyrA intein because it can be denatured and refolded to a functional state (Schwarzer *et al.*, 2006; Valiyaveetil *et al.*, 2002). In our experience, the GyrA intein is functional at up to 3 *M* urea. It should be noted that the CBD has lower affinity for chitin under these denaturing conditions. Therefore, it is beneficial to determine the optimum urea concentration to maintain fusion protein solubility, intein functionality, and chitin affinity.

Typically, the insoluble protein from washed inclusion bodies is resuspended in 8 *M* urea buffer and then the GyrA intein is refolded by dialysis down to ≤3 *M* urea for intein mediated protein $^{\alpha}$thioester formation. The refolded soluble fusion protein is then loaded onto pre-equilibrated chitin beads (when affinity permits) and extensively washed or used as is. The $^{\alpha}$thioester protein is generated by treating the intein fusion protein with 200 m*M* thiol (MESNA, thiophenol, or MPAA) in the absence of ^{1}Cys peptide. If the $^{\alpha}$thioester protein precipitates after cleavage from the intein fusion protein, it can be solubilized with a mixture of 50% trifluoroethanol (TFE) and 0.1% trifluoroacetic acid (TFA) in H_2O and dried on a lyophilizer. This lyophilized protein mixture will contain the desired protein $^{\alpha}$thioester and may contain the uncleaved fusion protein as well as the intein-CBD that results from cleavage. This mixture can be used directly for ligation to ^{1}Cys peptide by simply mixing 1 to 10 eq of synthetic ^{1}Cys peptide with recombinant protein $^{\alpha}$thioester in the presence of 200

m*M* thiol (MESNA, thiophenol, or MPAA) in pH 7.0 to 7.5 buffer. Additives such as sodium dodecylsulfate (SDS) can also be used to completely solubilize the mixture of proteins. It should be noted that if SDS is used, it must be removed after ligation. For this purpose, we have used Extracti-Gel D Detergent Removing Gel (Pierce). Optimizing the level of agitation and temperature of the ligation reaction may result in better yields of semisynthetic protein. Upon completion of peptide ligation, the semisynthetic protein can either be refolded and purified by a subsequent chromatography step (i.e., affinity, ion exchange, or size-exclusion chromatography) or kept in the denatured state and purified by RP-HPLC on a C4 column followed by refolding (Schwarzer *et al.*, 2006).

4.7. N-terminal semisynthesis

As has been discussed, in this complementary semisynthetic approach, the $^{\alpha}$thioester for ligation is generated synthetically and the ^{1}Cys protein for ligation is generated by proteolytic cleavage of a recombinant protein. Ligation is afforded by mixing 1 to 10 eq of synthetic peptide $^{\alpha}$thioester with recombinant ^{1}Cys protein in the presence of 200 m*M* thiol (MESNA, thiophenol, or MPAA) in pH 7.0 to 7.5 buffer (Zheng *et al.*, 2003). Typically this is done in a small volume so that, on completion of the ligation reaction, the mixture can be directly subjected to size-exclusion chromatography to remove excess synthetic peptide $^{\alpha}$thioester and small-molecule thiol. Again, this procedure can be carried out on an insoluble protein provided that the ^{1}Cys protein can be generated under denaturing conditions and the target protein can easily be refolded.

4.8. Purification of the semisynthetic protein

If the ligation reaction does not go to ≥95% completion, the semisynthetic protein must be further purified to remove the unligated protein and prevent its interference with biological evaluation. This separation may be problematic if there is not a great enough difference in charge, size, or affinity for a ligand on generation of the semisynthetic protein. In some cases, a biotin tag can be included on the synthetic peptide during Fmoc SPPS as a handle for purification, but this approach should be used with caution because it introduces another variable into the evaluation of the semisynthetic protein. With any semisynthetic project, the mass of the final purified semisynthetic protein should be confirmed by MALDI-TOF MS.

5. Practical Uses of Semisynthetic Phosphoproteins

5.1. Kinetic analysis of phosphonylated enzymes

The combination of protein semisynthesis, phosphonate analogue technology, and kinetic analysis yields a powerful tool that can be used to dissect complex signaling cascades. The combined approaches enable the investigator to determine the effect of stoichiometric phosphorylation on enzyme activity, which often provides unique insights. The methods proved especially valuable in analyzing the protein tyrosine phosphatases (PTPs) SHP-1, SHP-2, and low molecular weight (LMW) PTP.

Both SHP-1 and SHP-2 are cytosolic PTPs that share a common domain structure that includes two SH2 domains followed by the PTP domain and a C-terminal tail. SHP-1 plays a role as a negative regulator of cell signaling in cells of hematopoietic lineage (Zhang *et al.*, 2000), whereas SHP-2 is critical in the early development of many species, including mammals (Feng, 1999). The C-terminal tail of each PTP contains two sites of tyrosine phosphorylation that have been proposed to be a possible regulatory mechanism (Bennett *et al.*, 1994; Feng *et al.*, 1993; Vogel *et al.*, 1993). This hypothesis could not be tested in the absence of phosphonate analogues because of the inherent tendency of the PTPases to autodephosphorylate. EPL was used to incorporate nonhydrolyzable phosphonates at these positions, thus allowing for the effect of phosphorylation of each position to be determined.

With respect to SHP-1, phosphonylation with F_2Pmp at Tyr^{536} and Tyr^{564} led to 8-fold and 1.6-fold stimulation of PTPase activity, respectively. These findings formed the basis for a structural model in which $pTyr^{536}$ and $pTyr^{564}$ engage the SH2 domains intramolecularly to relieve basal inhibition (Zhang *et al.*, 2003). The findings with SHP-2 (Tyr^{542} and Tyr^{580}) were similar to those with SHP-1, thus allowing a similar model for regulation to be proposed (Lu *et al.*, 2001, 2003). Semisynthesis with SHP-2 was extended to include *bis*-phosphonylated enzyme, where it was shown that the Pmp groups have close to additive effects on PTPase activity, thereby suggesting dual occupancy of the SH2 domains. Furthermore, catalytically inactive forms of phosphorylated SHP-2 were generated for use in a systematic analysis of intermolecular autodephosphorylation. In these studies, autodephosphorylation was shown to be both dependent on the surrounding sequence and competitive with the intramolecular binding to the SH2 domains (Lu *et al.*, 2003).

LMW-PTP is a nonclassical PTPase involved in regulating growth factor responses and in reorganizing the cytoskeleton (Chiarugi *et al.*, 1995; Nimnual *et al.*, 2003; Raugei *et al.*, 2002). A potential mechanism for regulating LMW-PTPase is tyrosine phosphorylation at positions 131 and 132, which are close to the active site. Investigating the roles of these residues has been difficult because of the intrinsic tendency of the enzyme to autodephosphorylate. Previous groups addressed this problem by using ATPγS-mediated phosphorylation of LMW-PTP; however, this approach can be problematic, as described earlier. Nevertheless, these teams reported stimulation of PTPase activity on tail phosphorylation (Bucciantini *et al.*, 1999; Tailor *et al.*, 1997). Our lab revisited this problem using a combination of protein semisynthesis and phosphonate analogue technology to produce site-specific, stoichiometrically phosphonylated LMW-PTP for detailed kinetic analysis (Schwarzer *et al.*, 2006). With physiological phosphopeptide substrates derived from the PDGF receptor and p190RhoGap, it was shown that tail phosphorylation led to a decrease in PTPase activity and that the effect of the *bis*-phosphonylation was almost additive. This represents, to our knowledge, the first example of a PTPase being inhibited by tyrosine phosphorylation. In this manner, phosphorylation of LMW-PTP would act to remove a negative influence on PDGF receptor stimulation.

5.2. Microinjection of phosphonylated enzymes

Microinjection provides the investigator with a way to introduce semisynthetic proteins into a cell. This is achieved by applying direct pressure to the cell membrane with a glass Femtotip. The advantages of this technique are its versatility (almost anything can be injected into the cell with no limit on size or charge), temporal control, and the ability to target delivery to either the cytoplasm or the nucleus. With microinjection, one can rapidly increase the level of a modified kinase or phosphatase and follow its downstream effects via reporter construct. Protein levels and stability can be directly quantitated by immunofluorescence, and cellular localization can be mapped with confocal microscopy. The main disadvantages of microinjection are that it is time consuming, requires concentrated proteins (>1 mg/mL) and fixed cells, and is not amenable to high-throughput analysis. Other disadvantages of the technique include the small sample size (only about 100 cells can be microinjected per time point) and the expertise and apparatus needed for the experiments.

To perform microinjection experiments, one needs an inverted microscope, either a phase contrast scope (for flat specimens like those in tissue cell culture) or differential-interference contrast (DIC) scope (for rounded cells and nuclear injection). In addition, both a micromanipulator (to control where the needle is) and a microinjector (or air regulator to control the pressure of the fluid into the needle) are required. The needles used for microinjection (Femtotips) and the microloaders used to fill them are available from Eppendorf.

Before beginning microinjection experiments, an appropriate cell line must be selected. This can depend on the protein or signal pathway being studied and on the robustness of the cell line. Effects observed in some cell lines may be exaggerated or not observed at all in other cell lines. Cells should be plated (seeded) at least 12 h prior to microinjection, though 24 h is optimal. Most cell types are very sensitive to plasma membrane damage during the early hours after seeding, and microinjection works best at 70 to 80% confluency. Some cell lines are very sensitive to pH change that occurs while a dish is outside the CO_2 incubator. For this reason, cells cannot be microinjected for longer than 15 min. To circumvent this problem, bicarbonate-free media (Leibovitz L-15 medium) or a microscope fitted with a CO_2 incubator can be used.

Proper sample preparation can be a critical aspect of the microinjection experiments. Typical protein concentrations used are 1 to 5 mg/mL. Although this can be varied, samples that are too concentrated are likely to clog the needle tip, and samples that are too dilute may be difficult to detect by immunofluorescence. The sample needs to be diluted in a physiological buffer. An example of an appropriate sample buffer is 20 m*M* Tris pH 7.5, 20 m*M* NaCl, 1 m*M* $MgCl_2$, 150 m*M* KCl (if the protein is sensitive to oxidation, 1 to 5 m*M* 2-mercaptoethanol can be included). Each sample needs to be clarified via high-speed centrifugation prior to loading into the Femtotip to remove any particulate matter that can clog the tip (Ridley, 1995).

Adherent cells should be microinjected with the tip at 30 to 40° angle, so the tip penetrates the cell by moving in a vertical z-axis movement. The volume injected into cells depends on the pressure and is usually femtoliters to nanoliters. Typically pressures of 5 to 12 kPa are used and injection times are between 0.2 s and 1 s. Ideally injection volume is 10% of the cell volume (Minaschek *et al.*, 1989). Injections can be performed manually, in which the operator controls the movement of the tip and length of injection. Alternatively, one can use automatic injection. In this case, the Z-line (or vertical movement of the needle into the cell) and length of injection are fixed. This provides more consistency in the volumes being injected into the cells, but it can be troublesome if the cells are not flat or the dish is not level. A tip diameter of ≈0.3 μm is optimal for microinjection of mammalian cells. Increases in tip diameter result in increases in delivery rate, which can cause more cell damage. Decreasing the tip diameter can result in frequent clogging of the tip and loss of sample (once a tip is clogged, it can no longer be used and sample cannot be recovered). For nuclear injection, a smaller shoulder angle on the tip is necessary to limit the delivery of protein into the cytoplasm above the nucleus.

During the course of a microinjection, one needs to work quickly and keep track of the number of cells that were injected (typically 50 to 100 per time point). To practice the microinjection technique, FITC-labeled dextran (available from Sigma at 5 mg/mL) can be used. This provides a quick way to determine whether one's microinjection technique is successful. Because dextran is an inert polymer of high molecular weight, it will remain in the cell compartment into which it was injected. If the microscope is fitted with a fluorescent lamp and appropriate filter, the cells can be visualized immediately following the injection. One should not attempt to microinject a cell more than once. If it is desired that two or more proteins are injected into the same cell, they should be mixed together at the desired ratio before sample preparation and then injected together (Lamb *et al.*, 1996).

Immediately after injections are complete, the dishes with the cells should be returned to the incubator. Following suitable incubation times, the cells can be fixed in 4% formaldehyde/phosphate buffered saline (PBS) solution. After washing with 10 m*M* glycine in PBS, the cells are permeabilized with 0.2% Triton-X100. At this point, the cells can be immunostained with the primary antibody followed by a fluorophore-conjugated secondary

antibody. Blocking with 10 mg/mL BSA or other blocking agents is recommended to prevent nonspecific binding of the antibodies. Dilutions for the antibodies will most likely need to be optimized. If the signal is poor, it may be necessary to increase the protein concentration in the injected sample. The stained cells then can be analyzed and quantitated by fluorescence microscopy. Our lab has used microinjection to investigate the roles of phosphorylation on the cellular stability of AANAT and LMW-PTP, the cellular localization of SHP-2 and LMW-PTP, and the downstream effects of SHP-2 (Lu *et al.*, 2001; Schwarzer *et al.*, 2006; Zheng *et al.*, 2003, 2005).

The penultimate enzyme in the melatonin biosynthetic pathway is AANAT (Klein *et al.*, 1997). The diurnal rise and fall of melatonin is governed by the rhythmic phosphorylation of this enzyme at two sites (Thr^{31} and Ser^{205}) (Ganguly *et al.*, 2005). Microinjection experiments were key in elucidating the role of phosphorylation in regulation of AANAT activity. The results of studies in which AANAT modified with Pma at Thr^{31} was injected into cells provided the first direct evidence that Thr^{31} phosphorylation controls AANAT cellular stability (Zheng *et al.*, 2003). Semisynthetic AANAT-Pma^{31} injected into CHO cells and measured by immunofluorescent staining had 3-fold greater signal than that of unmodified AANAT when assessed at 1 h after cellular microinjection. After 4 h, AANAT-Pma^{31} continued to show substantial fluorescent signal, whereas the signal from the unmodified enzyme was no longer present. This finding confirmed that phosphorylation at Thr^{31} protects against proteolytic degradation. Note that the corresponding Glu replacement was unable to mimic this effect, consistent with our understanding of 14-3-3/phosphopeptide interaction (Zheng *et al*., 2003). Phosphorylation at Ser^{205} was also investigated and yielded the same result; phosphorylation of the residue was important for cellular stability of AANAT (Zheng *et al.*, 2005). The findings, coupled with earlier studies that identified 14-3-3 proteins as ligands for phosphorylated AANAT, helped confirm the 14-3-3/phosphorylation-dependent model for AANAT regulation (Ganguly *et al.*, 2001). In contrast, similar studies conducted with LMW-PTP indicated that phosphorylation did not play a role in regulating the cellular stability of the enzyme (Schwarzer *et al.*, 2006).

Phosphorylation may play a role in changing the cellular localization of a protein. In these cases, microinjection followed by confocal microscopy can be used to follow protein movement. With respect to the two proteins that our lab has investigated via this technique (SHP-2 and LMW-PTP), there has been no change in cellular localization on protein modification (Lu *et al.*, 2001; Schwarzer *et al.*, 2006). By contrast, a change in cellular localization on phosphorylation was observed with the semisynthetically prepared Smad2-MH protein (Hahn and Muir, 2004). Here, protein semisynthesis was used to introduce two photocaged (discussed later) phosphoserines at the C-terminus of the protein. The caged Smad2 was bound to SARA (Smad anchor for receptor activation) in the cytosol, but on uncaging by brief irradiation, it localized to the nucleus and promoted gene transcription (Hahn and Muir, 2004).

Microinjection can be combined with reporter plasmid technology to provide a functional assay for the injected protein by monitoring downstream effects. This methodology was useful in elucidating the role of SHP-2 phosphorylation *in vivo*. Co-injection of phosphonylated SHP-2 and the reporter construct 5xSRE-CAT, which allows the investigator to demonstrate the ability of the microinjected protein to transcriptionally activate the serum response element (SRE), indicated that single phosphorylation at Tyr^{542} was sufficient to activate the MAP kinase pathway (Lu *et al.*, 2001). This result was previously unattainable because of the ability of SHP-2 to autodephosphorylate.

5.3. Pull-down assays using phosphonylated enzymes as bait

Phosphorylation on proteins can directly lead to the formation of multimolecular signaling complexes through specific interactions between phosphoprotein binding domains and phosphorylated proteins. Enzymes bearing the nonhydrolyzable phosphonate modification can be used as bait to pull-down novel interacting proteins from cell lysates because of their stability to phosphatases. In addition, pull-down assays can be used to demonstrate specific binding between two purified proteins.

As was previously discussed, the phosphorylation of Thr^{31} and Ser^{205} of AANAT leads to its cellular stabilization. Through the use of pull-down studies, it could be demonstrated that the phosphorylation of these residues leads to the recruitment of 14-3-3, which in turn protects AANAT from degradation *in vivo* (Zheng *et al.*, 2005). Similar pull-down assays were performed with semisynthetic SHP-2 and demonstrated that phosphorylation at Tyr^{542} was sufficient to enable binding to Grb2, an SH2 domain containing adaptor protein (Lu *et al.*, 2001).

6. Future of Protein Semisynthesis in Signaling

Where does protein phosphorylation by semisynthesis go from here? We have seen in the previous examples that incorporation of phosphonates into native proteins and their subsequent analysis in activity assays, micro-injection assays, and pull-down assays gives the investigator access to data that is otherwise not available because of the reversible nature of the modification. In this manner, it is possible to deconvolute the specific roles of phosphorylation in complex systems (e.g., regulation of activity, stability, cellular localization, and protein-protein interactions) and piece together signaling cascades one phosphorylation event at a time.

Currently, our lab is expanding on the notion of phosphorylation by protein semisynthesis to include ATP linkage via a thiophosphate at sites of protein phosphorylation (Parang *et al*., 2001). In this manner, a high-affinity kinase ligand can be generated. This approach has recently been used to convert Src into a high affinity Csk ligand. Src-ATP was a fairly potent inhibitor of the Csk kinase activity, with a K_i of 100 n*M*, and could be used as bait to selectively pull-down recombinant Csk from a 1% spiked mammalian cell lysate (Shen and Cole, 2003). The studies outline a general strategy for identifying unknown kinases that might be responsible for the phosphorylation of a specific target protein and add another tool to be used in the dissection of signaling cascades. The utility of ATP linkage is not limited to identifying kinases. The high-affinity protein-ATP conjugates could be used to facilitate X-ray crystal structure determination of the kinase with its physiological substrate by stabilizing the complex.

Nonhydrolyzable phosphomimetics such as Pfa and Pmp are not the only phosphorylated amino acid substitutions that can be incorporated into proteins through semisynthesis. Photolabile caged analogues of phosphorylated residues allowing temporal and spatial control provide further tools for uncovering the regulation of signaling pathways (Rothman *et al*., 2005). Caged amino acids can be introduced into the cell in an inert form by microinjection and then switched on with a short burst of light in a spatially well-defined fashion (Lawrence, 2005). A caged thiophospho-Thr^{197} variant of PKA was prepared by semisynthesis and on uncaging of this protein 85 to 90% of activity was restored (Zou *et al.*, 2002). As previously discussed, Smad2 could be prepared with two photocaged phosphoserines at the C-terminus and on irradiation became activated (Hahn and Muir, 2004).

The advantages of protein semisynthesis are its versatility with respect to the protein of interest, the type of modification to be investigated, and the position of the modified amino acid within the protein. Therefore, the combination of this technology with unnatural amino acids and traditional experimental approaches yields a level of experimental control that was previously elusive. Such control of stoichiometry, specificity, and reversibility allows complex signaling systems to be broken down to individual events and discretely examined. Relatively unexplored is the use of semisynthesis to investigate other reversible posttranslational modifications including glycosylation, acetylation, methylation, and ubiquitination (He *et al*., 2003; Shogren-Knaak *et al.*, 2006; Thompson *et al.*, 2004). In the future, protein semisynthesis in combination with new technologies is likely to play an increasingly important role in the elucidation of mechanisms in complex biological systems.

Acknowledgments

We are grateful for the insightful comments of many of our colleagues, most of whose names are noted in the references. We thank the National Institutes of Health for financial support.

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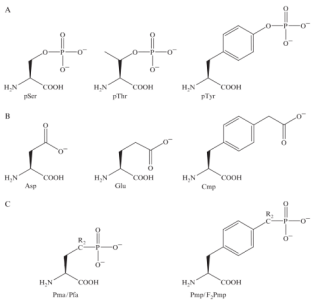

Figure 1.1.
Phosphorylated amino acids and their mimetics. (A) Naturally occurring phosphorylated amino acids, (B) genetically encoded phosphomimetics, and (C) nonhydrolyzable phosphonates, where R = H or F.

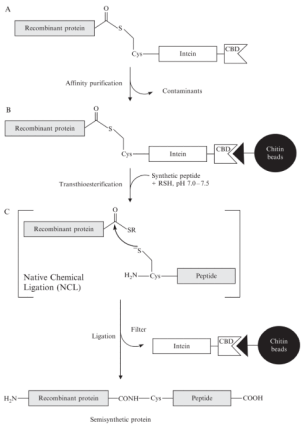

Figure 1.2.

Expressed protein ligation. (A) Recombinant protein for EPL is expressed as a C-terminal intein-CBD fusion protein and subjected to affinity purification on chitin beads. The modified inteins used for semisynthesis catalyze an *N*- to *S*-acyl shift resulting in the formation of a thioester-linked fusion protein. (B) The protein thioester is trapped by small-molecule thiol (RSH = sodium 2-mercaptoethansulfonate, thiophenol, or mercaptophenylacetic acid) as a more reactive $^{\alpha}$thioester for semisynthesis. (C) In the native chemical ligation reaction, the $^{\alpha}$thioester intermediate is intercepted by a ^{1}Cys synthetic peptide and the newly formed thioester-linked species spontaneously rearranges to yield a native peptide bond at pH 7.0–7.5. The semisynthetic protein is eluted from the column and now contains a chemoselectively ligated synthetic peptide at the C-terminus.

Table 1.1

Phosphoprotein binding domains

Ligand	Domains
Phospho-tyrosine	Src homology 2 domain (SH2)
	Phosphotyrosine binding domain (PTB)
Phospho-serine/threonine	14-3-3 protein
	WW domain
	Forkhead-associated domain (FHA)
	BRCA1 C-terminal (BRCT) domain
	Polo-box domain (PBD)