Communication

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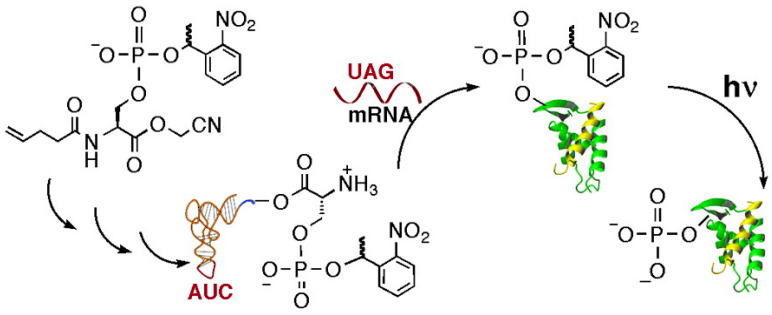
J. Am. Chem. Soc., **2005**, 127 (3), 846-847• DOI: 10.1021/ja043875c • Publication Date (Web): 30 December 2004



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Published on Web 12/30/2004

Caged Phosphoproteins

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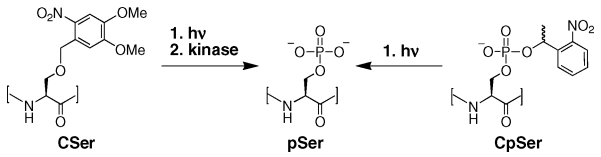
The phosphorylation of serine, threonine, and tyrosine residues in proteins is a central mechanism of cellular regulation.[1] Phosphorylation can profoundly modulate the role of the protein in a biological system, either by altering the activity of the isolated protein or by influencing interactions with other proteins. The phosphorylation state of a protein is dynamic; it is determined by the interplay of kinases, which append the phosphoryl group, and phosphatases (PPases), which remove it.[2] Currently available biological experiments, such as chemical inhibition, gene knockout, or point mutation studies, do not allow real-time studies of the effects of protein phosphorylation in complex biochemical signaling pathways.

Caged amino acids afford researchers spatial and temporal control over the effective phosphorylation state at a specific site in a target protein (Scheme 1).[3] In general, the "cage" comprises a photocleavable protecting group that masks the essential functionality. Photolysis of the protecting group generates a bioactive protein. Caged amino acids have been available for incorporation into full-length proteins for some time.[4] Caged phosphopeptides have recently become accessible as tools for studying cell signaling pathways both in vitro and in vivo.[5] These peptides have already proven useful for elucidating the role of a specific protein family in real-time studies of cell cycle regulation.[6] Due to the centrality of protein phosphorylation in cell biology, a general method for preparing caged phosphoproteins is highly desirable.

Scheme 1. Control of Phosphorylation Dynamics with Caged Residues

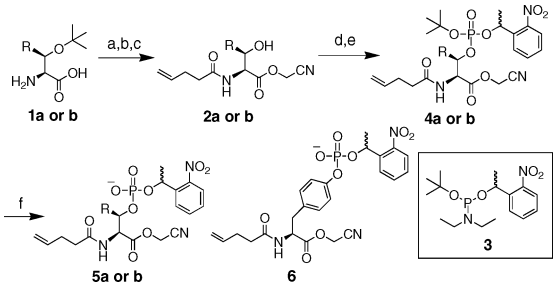


Herein is presented the chemical and biological synthesis of caged phosphoproteins using the in vitro nonsense suppression methodology.[7,8] Although Hecht has used this method for the incorporation of phosphonoserine and phosphotyrosine into firefly luciferase,[9,10] this is the first report of caged phosphoamino acids (CpAAs) in proteins via in vitro translation. Caged phosphoproteins will allow the real-time study of kinase targets with phosphorylated residues in any position of a native protein sequence.

In these studies, phosphoamino acid analogues with a *single o*-nitrophenylethyl-caging moiety were strategically targeted (Scheme 1). The cage renders the phosphoprotein stable to phosphatases in biological systems and affords temporal control over the release of the target phosphoprotein. Use of a single cage avoids anticipated poor expression of bulkier, bis-caged analogues. For this purpose, a new phosphitylation reagent (**3**; Scheme 2) was developed to enable the synthesis of the singly caged phosphate. Transient, acid-labile *t*-butyl protection enables manipulation of the serine and threonine amino acid derivatives and avoids β-elimination reactions that would result during the basic deprotection of groups such as the common cyanoethyl moiety. The phosphoramidite (**3**) was synthesized from hexaethyl phosphorus triamide in a one-pot procedure. Each of the target amino acids (Ser, Thr, and Tyr) was synthesized as the corresponding N^{α}-4-pentenoylphospho(1-nitrophenylethyl)cyanomethyl ester (**5a**, **5b**, and **6**; Scheme 2) for coupling to the dinucleotide, dCA.[7,8] The tyrosine derivative **6** was synthesized using a slightly modified version of the CpSer/CpThr route.[7]

When coupling the CpAAs to dCA, tetrabutylammonium acetate (NBu_4OAc) salt beyond the typical 2.4 equiv was required; we believe that the additional salt aided in solubilizing the negatively charged amino acids in the presence of the negatively charged dCA. It was found that 250 mM NBu_4OAc optimally increased coupling efficiency with minimal β-elimination side reactions. Next, the aminoacylated dCAs were ligated to truncated tRNAs[11] using a slightly modified literature procedure.[7,8] This gave full-length, aminoacylated tRNAs ($tRNA_{CUA}$−CpAAs), designed to deliver the CpAA to a position specified by an amber (UAG) codon.

Scheme 2. Syntheses of Amino Acid Derivatives[a]



[a] (a) 4-Pentenoic anhydride, THF/water; (b) chloroacetonitrile, DBU; (c) TFA; (d) 4,5-dicyanoimidazole, **3**, THF; (e) *t*BuOOH, CH_2Cl_2; (f) TFA/TIS, CH_2Cl_2. **1a**, **2a**, **4a**, **5a**: R = H, **1b**, **2b**, **4b**, **5b**, R = Me.

With the $tRNA_{CUA}$−CpAAs in hand, the suppression efficiency of these bulky and negatively charged residues was tested in a system known to accept natural and non-natural amino acids using nonsense suppression: position 122 of the nicotinic acetylcholine receptor (nAChR) α-subunit.[12] The mRNA for nAChR α with an amber stop codon in position 122 was introduced into a rabbit reticulocyte lysate translation system, and the results were monitored by Western blot analysis (Figure 1). Uncaged, phosphorylated amino acids (pAA) appear to incorporate, but with relatively low efficiency (Figure 1, lanes 4, 7, and 10), consistent with earlier findings from Hecht.[9,10] However, adding a single caging group (CpAA) does

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10.1021/ja043875c CCC: $30.25

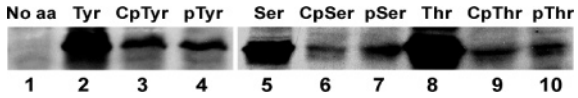

Figure 1. Test suppression in nAChR α subunit: A122TAG mRNA with $tRNA_{CUA}$ charged with amino acids listed above lanes. "No aa" refers to full-length, but uncharged, tRNA.

not further diminish suppression efficiency (Figure 1, lanes 3, 6, and 9). The inefficient suppression may be attributable to low affinity binding of the aminoacyl tRNAs by elongation factors (i.e., EF-1A). Uhlenbeck has shown that elongation factor Tu (EF-Tu) binds tRNAs bearing negatively charged amino acids poorly,[13] and that glutaminyl tRNAs (from which our $tRNA_{CUA}$ is derived) have a low inherent EF-Tu affinity.[14] It should be noted that masking this charge by doubly caging the phosphate moiety resulted in far lower incorporation efficiency for tyrosine derivatives,[7] and use of bis-caged phosphoserine and threonine was intractable due to β-elimination side reactions.

Next, the suppression ability of the $tRNA_{CUA}$−CpAAs was tested in a biologically significant system relating to phosphoregulated signaling with a centrally targeted residue. The vasodilator-stimulated phosphoprotein, VASP, is involved in cell migration processes. Specifically, phosphorylation of serine 153 has been associated with cell leading edge protrusion and forward cell movement.[15] VASP with CpSer153 would thus be a valuable tool for determining the precise role of this phosphoserine in the complex process of cell migration. Figure 2A depicts the translation of the wild-type (WT) VASP in rabbit reticulocyte lysate (lane A1). The VASP appears as two bands due to a gel shift caused by phosphorylation at serine 153; the shift is not caused by the increase in molecular weight, but rather by a conformational change and altered SDS binding capacity of VASP that results in decreased mobility in the SDS−PAGE analysis.[16]

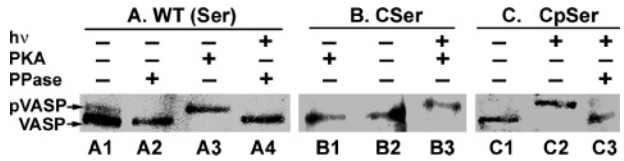

Figure 2. Suppression at VASP position 153 with CSer or CpSer. Lanes (left to right): (A) 1, WT translation; 2, WT with λ-PPase; 3, WT with PKA; 4, WT with λ-PPase, then UV-irradiated; (B) 1, CSer with PKA; 2, CSer; 3, CSer subjected to UV, then PKA; (C) 1, CpSer; 2, CpSer subjected to UV; 3, CpSer subjected to UV, then λ-PPase.

To ascertain that the proteins produced by in vitro translation behave as expected, several experiments were performed. Phosphorylation of the wild-type protein with protein kinase A (PKA) caused all of the protein to migrate as the phospho-species (Figure 2, lane A3), and dephosphorylation by λ-phosphatase (λ-PPase) caused all of the protein to migrate as the unphosphorylated species (lane A2). Next, mRNA for VASP−S153TAG was suppressed with caged serine (CSer, protected with the 6-nitroveratryl group), and the behavior of the protein product was examined (Figure 2B). With the caging group on the serine hydroxyl group, the full-length protein migrated as the unphosphorylated species in the presence or absence of PKA (lanes B1 and B2, respectively). Upon uncaging with 350 nm light, the serine hydroxyl group was liberated, and subsequent phosphorylation by PKA caused the protein to migrate as the phosphorylated species (lane B3). Next, the suppression of VASP−S153TAG mRNA with caged phosphoserine was evaluated (Figure 2C). The full-length protein with the caged phosphoserine migrated as the unphosphorylated species (lane C1). The uncaged protein migrated with a shift characteristic of that for the phosphorylated species (lane C2). Finally, upon uncaging and subsequent reaction with λ-PPase, the protein migrated as the unphosphorylated species (lane C3). As a negative control, wild-type VASP (λ-PPase-treated) was irradiated under the uncaging conditions to show that this did not alter the gel mobility of the wild-type protein (lane A4).

In conclusion, we present a synthesis of tRNA aminoacylated with caged phosphoserine, threonine, and tyrosine. Several suppression reactions were performed in order to evaluate the ability of the native ribosomal machinery to incorporate these large and charged residues into full-length proteins. Additionally, the protein products were tested for nativelike behavior in biochemical studies. Access to such caged phosphoproteins will enable studies of a large number of kinase targets in real-time using both in vitro and in vivo[4] translation systems.

Acknowledgment. The work has been supported by the Cell Migration Consortium Grant GM64346 to B.I., NS-34407 to D.A.D, and the HFSP Foundation for M.E.V. The authors thank Professor F. Gertler for the VASP gene and antibody.

Supporting Information Available: Full experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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