Semisynthesis of Hyperphosphorylated Type I TGFβ Receptor: Addressing the Mechanism of Kinase Activation

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The pluripotent cytokine, TGFβ, transduces signals through a receptor composed of two transmembrane serine/threonine kinases,[1] the type I and type II TGFβ receptors (TβR-I and TβR-II, respectively). Upon ligand induced oligomerization, TβR-II hyperphosphorylates TβR-I, activating it to phosphorylate transcription factors of the Smad family. Most protein kinases are regulated by phosphorylation of the so-called activation segment, an internal loop which lies close to the catalytic center[2] (Figure 1). By contrast, the activating phosphorylation events in TβR-I occur distal to the active site in an N-terminal regulatory segment called the GS region (named for the highly conserved ^{185}TTSGSGSG^{192} sequence contained within[1]). Phosphorylation of four to five of the serines and threonines within the ^{185}TTSGSGSG^{192} sequence is thought to be required for full activation of TGFβ signaling.[3] However, the level to which hyperphosphorylation activates the kinase toward its natural substrate remains unknown, and the molecular mechanism of how this posttranslational event leads to kinase activation has yet to be resolved. The crystal structure of the cytoplasmic domain of TβR-I[4] shows that, in its unphosphorylated state, the GS region adopts an inhibitory conformation that maintains the protein kinase domain in a catalytically inactive configuration. Thus, phosphorylation within the GS region would presumably activate the kinase by disrupting inhibitory interactions (a de-repression model for kinase activation). Mutagenesis data, however, suggest that the situation may be more complex.[3,5] The phosphorylated GS region may also activate the receptor in a positive way, perhaps by adopting a new structure that complements kinase activity, or by recruiting substrate.

The biochemical and structural analysis of TβR-I in its distinct phosphorylation states is of great interest in understanding its mechanism of activation. We were unable to produce homogeneously phosphorylated samples of TβR-I using standard techniques,[6] and developed the semisynthetic strategy described here to address the problem. Our approach uses native chemical ligation[7] to join a synthetic phosphopeptide to a recombinant protein expressed in SF9 cells. We have applied this method to the production of tetraphosphorylated TβR-I. The hyperphosphorylated product displays enhanced kinase activity relative to both unphosphorylated TβR-I as well as truncated TβR-I missing the GS region, demonstrating that the phosphorylated GS region plays a positive role in kinase activation and arguing against a simple de-repression model.

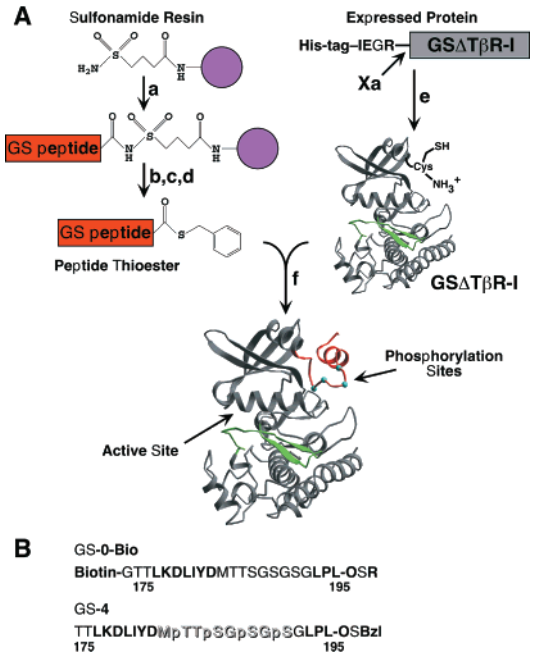

Figure 1. Semisynthesis of TβR-I fragments. (A) Strategy. The N-terminal α-thioester peptide is synthesized via Fmoc SPPS using an alkylsulfonamide resin. This peptide is ligated to the remainder of the TβR-I cytoplasmic domain (GSΔTβR-I), shown in gray ribbon (taken from the crystal structure of unphosphorylated TβR-I[4]). The synthetic peptide is shown in red, and the activation segment in green. The residues phosphorylated in this study are depicted as blue spheres in the model. Xa denotes the Factor Xa cleavage site. (a) Fmoc SPPS; (b) ICH_2CN, DIEA; (c) BzlSH, DIEA; (d) TFA, scavengers; (e) Factor Xa, pH 8.5; (f) 50 mM MESNA, pH 8.0, 4 °C. B) Peptides synthesized for this study. Residues that were double coupled in the GS-4 synthesis are highlighted. Numbering refers to the TβR-I amino acid sequence. R = $(CH_2)_2CONH_2$.

Two semisynthetic TβR-I targets were selected: (1) protein with an unphosphorylated GS region, to be used as a control, and (2) a tetraphosphorylated species in which Thr^{185}, Ser^{187}, Ser^{189}, and Ser^{191} were all phosphorylated (Figure 1). Residues 175−195, encompassing the first GS region helix and the loop containing all five of the phosphorylation sites, were synthesized as a peptide α-thioester. This peptide was then joined to the remainder of the TβR-I cytoplasmic domain (GSΔTβR-I, produced by recombinant methods[8]) by native chemical ligation under physiological conditions.[9]

The unphosphorylated peptide α-thioester, GS-0Bio, was prepared using N$^{\alpha}$ *tert*-butyloxycarbonyl (Boc) solid-phase peptide synthesis (SPPS) on a mercaptopropionamide support.[10] A biotin moiety and glycine spacer were included at the N-terminus of GS-0Bio to facilitate purification of the ligation product from unreacted GSΔTβR-I.[11] The tetraphosphorylated peptide GS-4 could not be synthesized using Boc SPPS because of the sensi-

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(8) GSΔTβR-I (residues 196−503, Leu^{196} mutated to Cys to enable ligation) was expressed in SF9 cells downstream of a polyhistidine tag. Following Ni^{2+} affinity purification, the tag was cleaved with the protease Factor Xa to expose Cys^{196}, and further purified using γ-phosphate conjugated ATP sepharose.
(9) The ligation site was chosen on the basis of analysis of the crystal structure of unphosphorylated TβR-I as well as inspection of the sequence conservation in that portion of the GS region.
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(11) The biotin moiety did not effect the kinase activity of the ligation product (data not shown).

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tivity of phosphoamino acid derivatives to the HF cleavage step.[12] We undertook an alternative strategy using N^{α} 9-fluorenylmethoxycarbonyl (Fmoc) SPPS and an alkanesulfonamide "safety-catch" resin. This approach has been developed recently to generate α-thioester peptides by Fmoc SPPS, and has proved particularly useful in cases where peptides with modified residues are required.[13,14] We anticipated that the close apposition of protected phosphate groups during the GS-4 synthesis would lead to slow acylation kinetics during this part of the chain assembly. Accordingly, residues from Met^{184} through $pSer^{191}$ were double coupled with extended coupling times (Figure 1), and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) was used as the activation agent in all phosphoamino acid couplings. The N-terminal Thr^{175} was incorporated as an N^{α} Boc derivative to prevent its amino functionality from attacking the sulfonamide linker during cleavage.[15] After chain assembly, the resin was N-alkylated using iodoacetonitrile, and the peptide cleaved using benzyl mercaptan in the presence of diisopropylethylamine (DIEA). Deprotection and subsequent HPLC purification afforded 6 mg of pure peptide from a 0.075 mmol scale synthesis, a final yield of 3%.[16]

Ligation reactions were performed in the presence of 50 mM mercaptoethanesulfonic acid (MESNA) as the thiol cofactor.[17] GSΔTβR-I displays a tendency to aggregate, and ligations were carried out at low protein concentration (0.05−0.1 mM) and at low temperature (4 °C) in order to slow this aggregation. Denaturants were not used, as their addition led to an irreversible loss in TβR-I kinase activity. Peptide concentrations were kept high, between 0.5 and 1 mM, to generate pseudo-first-order reaction kinetics. Unreacted peptide was recovered by gel filtration and could be recycled for multiple ligations. The GS-0Bio ligation reaction was efficient, proceeding to ∼70% completion after 10 h, as judged by denaturing polyacrylamide gel electrophoresis. The GS-4 ligation was significantly slower, only 60% complete after 24 h. After gel filtration, GS-0BioTβR-I was separated from unreacted GSΔTβR-I using monomeric avidin agarose. GS-4TβR-I was purified from GSΔTβR-I by anion exchange chromatography. Using these ligation and purification conditions, GS-0BioTβR-I and GS-4TβR-I can be routinely isolated in 10−20% overall yield (Figure 2A). These amounts are sufficient for detailed biochemical and biophysical studies.

To characterize the extent of TβR-I activation induced by hyperphosphorylation, we performed an in vitro kinase assay comparing GS-4TβR-I and GS-0BioTβR-I with each other and against GSΔTβR-I. A slightly larger recombinant fragment of TβR-I encompassing both the GS region and the kinase domain, termed TβR-Icyt and used in the previous crystallographic analysis,[4] was also included for comparison. The C-terminal domain of Smad2 was used as a substrate in this assay.[18] Under the conditions of this experiment, GS-4TβR-I displays more than a 40-fold increase in kinase activity relative to both GS-0BioTβR-I and GSΔTβR-I (Figure 2B). GS-0BioTβR-I does not demonstrate

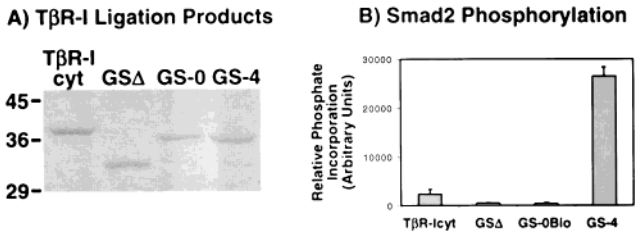

Figure 2. Characterization of ligation products. (A) SDS−PAGE gel showing the starting material and purified ligation products. Molecular weight markers are shown to the left. The identity of the ligation products was confirmed by mass spectrometry. GS-0BioTβR-I calculated: 37 695.2 Da, measured: 37 697.0 Da. GS-4TβR-I calculated: 37 731.9 Da, measured: 37 731 Da. (B) Relative kinase activity of the TβR-I fragments used in this study. Smad2 phosphorylation in three independent kinase assays has been combined and quantitated.

comparable activation relative to TβR-Icyt and GSΔTβR-I, indicating that the increase observed for GS-4TβR-I is dependent upon phosphorylation, and not a result of the ligation chemistry or of the Leu to Cys mutation. Furthermore, the observed increase in GS-4TβR-I kinase activity relative to GSΔTβR-I indicates that the phosphorylated GS region does indeed play an active role in the upregulated state of the kinase, and argues against a simple de-repression model. In this assay, a 3-fold difference in activity is observed between TβR-Icyt and both GSΔTβR-I and GS-0BioTβR-I. We cannot explain this difference at present, but do note that it does not affect our conclusions with regard to GS-4TβR-I, which is still activated by a factor of 10 relative to TβR-Icyt. A more detailed biochemical and structural analysis of TβR-I activation is currently in progress.

In conclusion, we have been able to effectively employ native chemical ligation to produce chemically defined samples of hyperphosphorylated TβR-I. Access to these molecules has allowed the level to which hyperphosphorylation activates the kinase to be quantified for the first time and, in addition, has provided evidence that the phosphorylated GS region may also activate the receptor in a positive way. To our knowledge, this work represents the first example in which a peptide has been ligated to a protein expressed in eukaryotic cells. Furthermore, the successful synthesis of the tetraphosphorylated GS-4 peptide on the alkanesulfonamide resin demonstrates the utility of the Fmoc approach for introducing multiple posttranslational modifications into α-thioester peptides. Native chemical ligation has been used previously to attach a phosphopeptide to the C-terminus of a protein.[19] Our work here demonstrates that phosphopeptides may be linked to the N-terminus and, by extension,[20] incorporated into the middle of a protein as well. We believe this strategy may be readily adapted to study posttranslational modifications in other systems.

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Supporting Information Available: Detailed descriptions of peptide syntheses, protein expression and purification, native ligation reactions, and kinase assays; analytical HPLC and mass spectra of GS-0Bio, GS-4, GS-0BioTβR-I, GS-4TβR-I, and GSΔTβR-I (PDF). This material is available free of charge at the website http://pubs.acs.org.

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(15) GS-4 was initially biotinylated at the N-terminus. Cleavage revealed a contaminating degradation product 144 Da less than the expected mass. Peptide mapping studies localized the modification to the N-terminus of the peptide. The contaminant did not bind avidin beads, and we concluded that the biotin had fragmented at some point during the activation/cleavage process.
(16) Mass analysis of side products and amino acid analysis of the resin after cleavage revealed incomplete deprotection of the phosphate groups and incomplete thiolysis of the peptide from the resin, respectively. This accounts for the modest yield of the GS-4 peptide.
(17) In a typical ligation reaction, 100 nmol of peptide were reacted with 10 nmol GSΔTβR-I in 100 μL of 100 mM Hepes pH 8.0, 200 mM NaCl, 50 mM MESNA at 4 °C for 12−24 h.
(18) In a typical kinase reaction, 10 pmol of TβR-I were mixed with 80 pmol of Smad substrate in 15 μL of kinase buffer containing trace amounts of [γ-^{32}P]ATP. Samples were subjected to gel electrophoresis and visualized by autoradiography.
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