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Phosphorylation and activation of a transducible recombinant form of human HSP20 in *Escherichia coli*

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Abstract

Protein-based cellular therapeutics have been limited by getting molecules into cells and the fact that many proteins require post-translational modifications for activation. Protein transduction domains (PTDs), including that from the HIV TAT protein (TAT), are small arginine rich peptides that carry molecules across the cell membrane. We have shown that the heat shock-related protein, HSP20 is a downstream-mediator of cyclic nucleotide-dependent relaxation of vascular smooth muscle and is activated by phosphorylation. In this study, we co-expressed in *Escherichia coli* the cDNAs encoding the catalytic subunit of protein kinase G and a TAT-HSP20 fusion protein composed of the TAT PTD (-YGRKKRRQRRR-) fused to the N-terminus of human HSP20. Immunoblot and HPLC-ESI-MS/MS analysis of the purified TAT-HSP20 demonstrated that it was phosphorylated at serine 40 (equivalent to serine 16 in wild-type human HSP20). This phosphorylated TAT-HSP20 was physiologically active in intact smooth muscles in that it inhibited 5-hydroxytryptamine-induced contractions by 57% ± 4.5. The recombinant phosphorylated protein also led to changes in actin cytoskeletal morphology in 3T3 cells. These results delineate strategies for the expression and activation of therapeutic molecules for intracellular protein based therapeutics.
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Smooth muscle relaxation can be induced by a variety of endogenous and pharmacologic agents such as nitric oxide (NO) through cyclic nucleotide signaling cascades. NO released from stimulated endothelial cells or by NO donors such as sodium nitroprusside (SNP), diffuses across cell membranes activating guanylyl cyclase, increasing intracellular cGMP concentrations, and activating cGMP-dependent protein kinase [1]. An alternate pathway for smooth muscle relaxation involves activation of adenylyl cyclase (by agonists such as forskolin), increasing intracellular cAMP concentrations, and activation of cAMP-dependent protein kinase [2].

One downstream target of both cyclic nucleotide signaling cascades is the heat shock-related protein 20, HSP20. HSP20[1] is highly and constitutively expressed in skeletal, cardiac, and smooth muscle and increases in the phosphorylation of HSP20 are associated with cyclic nucleotide-dependent relaxation of vascular smooth muscle [3,4]. Like other members of the heat shock protein family, HSP20 requires phosphorylation at a serine residue (S16) to become functionally active [5,6]. HSP20 is a specific substrate protein of PKA and PKG and accumulating evidence suggests HSP20 has several physiological and

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[1] *Abbreviations used:* cPKG, catalytic subunit of cGMP dependent protein kinase; HSP20, heat shock related protein 20 kD; PTD, protein transduction domain.

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biochemical roles including controlling muscle tone, regulating cell motility, modulating actin filament dynamics and protecting against intimal hyperplasia and ischemia/reperfusion injury [7–14]. Although the molecular mechanisms of action for HSP20 have not been determined fully, it is an actin-associated protein and we have shown that HSP20 peptide treatment leads to loss of actin stress fibers and disruption of focal adhesion complexes [9–11]. Phosphorylated HSP20 contains a 14-3-3 binding motif that may mediate changes in the actin cytoskeleton. The actin depolymerizing protein, cofilin, has also been shown to bind to 14-3-3. Treatment with phosphopeptide analogs of HSP20 leads to a dissociation and activation of cofilin resulting in depolymerization of the actin cytoskeleton [10]. Collectively, these data suggest HSP20 may modulate cytokinetic processes via alterations in actin filament dynamics.

Recently, we used a direct approach to establish the role of phosphoHSP20 in smooth muscle relaxation. We transduced phosphoproteins and phosphopeptides into tissues using protein transduction domains (PTDs) and in doing so prevented smooth muscle contraction [12,13]. The use of PTDs as tools for intracellular delivery is a relatively new approach that is receiving increased attention as new methods for its implementation are developed and evidence for its efficacy is established. PTDs, first discovered independently by the Green and Frankel laboratories, are positively charged amino acid sequences facilitating the entry of proteins into cells through macropinocytotic mechanisms [15–17]. Our laboratory has successfully implemented the use of these sequences to transduce different proteins and peptides into numerous tissues and cells [8–14].

One limitation of previous approaches was the requirement for *in vitro* phosphorylation of expressed, purified protein prior to physiological testing. In view of the potential shortcomings of this method for large-scale preparations, an alternate phosphorylation scheme was undertaken. The bacterial co-expression of the catalytic subunit of cGMP-dependent protein kinase (cPKG) and a fusion protein composed of a transduction domain (TAT) and the PKG substrate, HSP20 (TAT-HSP20) was performed to determine if biologically-active recombinant phospho-proteins could be produced. The results delineate possible strategies for the production of other proteins through co-expression of the enzyme responsible for post-translational modification.

Materials and methods

cDNA cloning and co-expression

A 1600 bp cDNA encoding the type I catalytic subunit of cGMP-dependent protein kinase from *Bos taurus* (encoded residues 334–670) was liberated from pBKS+ cPKG (gift from Dr. Thomas Lincoln, University of South Alabama) by NcoI-XhoI digestion and cloned into a NcoI-XhoI-digested shuttle vector pET29a yielding pET29a PKG. pACYC Duet PKG was constructed by ligating the BglII-XhoI insert of pET29a PKG and the backbone of BglII-XhoI-digested pACYC Duet (Stratagene). Construction of pET14b TAT-HSP20 has been described previously [13].

pACYC Duet PKG and pET14b TAT-HSP20 were co-transformed into the *Escherichia coli* expression strain BL21(DE3) and a single colony resistant to 50 mg/L ampicillin and 30 mg/L chloramphenicol used to inoculate 400 mL of Luria Broth (LB) seed culture and grown overnight on a platform shaker (220 rpm at 37 °C). The following morning, 4 L of LB was inoculated with the seed culture and the culture was induced with 2 mM isopropyl-β-D-thiopyranogalactoside (IPTG—Research Organics, Cleveland, OH) at an OD of 0.5. Induced cells were grown overnight (14 h) and the next morning harvested from media by centrifugation at 6000*g* for 15 min at 4 °C.

TAT-HSP20 purification and refolding

The purification scheme used to purify phosphorylated TAT-HSP20 is shown in Table 1. Frozen cells were thawed and thoroughly resuspended in 100 mL 1X TNE buffer (50 mM NaCl, 1 mM EDTA, and 500 mM Tris–HCl, pH 8.0, 10 nM Caliculin A, 50 μl Sigma Phosphatase Inhibitor Cocktail II, 50 μl Sigma Protease Inhibitor Cocktail and 200 μg deoxyribonuclease) and incubated at 4 °C for 30 min. After sonication on ice, the inclusion bodies containing recombinant protein were harvested by centrifugation (19,000*g*, 10 min). Metal chelation affinity chromatography was used to purify recombinant TAT-pHSP20. Briefly, inclusion bodies were first resuspended in binding buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 50 mM imidazole, pH 7.4, 8 M urea). The sample was then added to Ni^{2+}-charged Chelating Sepharose Fast Flow (Pharmacia Biotech, Peapack, NJ) and incubated for 30 min at room temperature. The resin was then loaded in a water-chilled XK-26 column and washed extensively with binding buffer on an AKTÄ fast-performance liquid chromatography system (Pharmacia Biotech). Protein was refolded by using an overnight linear gradient of urea from 8 to 0 M and eluted with binding buffer containing 500 mM imidazole. The eluate was concentrated and the imidazole removed by repeatedly reducing eluate volume 50% using a stirred ultrafiltration cell (8200, Millipore, Bedford, MA) with a 10,000-nominal

Table 1
Summary of purification scheme used to purify phosphorylated TAT-HSP20

Step 1	Inclusion body isolation
Step 2	Inclusion body solubilization in buffer containing 8 M urea
Step 3	Immobilized metal affinity chromatography
Step 4	Overnight resin wash using 8 M to 0 M urea gradient
Step 5	Elution using imidazole
Step 6	Imidazole removal via stepwise dialysis against physiological saline solution