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SCIENCE DIRECT
Protein Expression and Purification 48 (2006) 151–159
Protein Expression & Purification
www.elsevier.com/locate/yprep

Refolding, purification, and characterization of human and murine RegIII proteins expressed in *Escherichia coli*

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Received 7 December 2005, and in revised form 21 January 2006
Available online 13 February 2006

Abstract

The regenerating (Reg) family comprises an extensive, diversified group of proteins with homology to C-type lectins. Several members of this family are highly expressed in the gastrointestinal tract under normal conditions, and often show increased expression in inflammatory bowel disease. However, little is known about Reg protein function, and the carbohydrate ligands for these proteins are poorly characterized. We report here the first expression and purification of Reg proteins using a bacterial system. Mouse RegIIIγ and its human counterpart, HIP/PAP, were expressed in *Escherichia coli*, resulting in the accumulation of aggregated recombinant protein. Both proteins were renatured by arginine-assisted procedures and were further purified using cation-exchange chromatography. The identities of the purified proteins were confirmed by SDS–PAGE, N-terminal sequencing, and MALDI-TOF mass spectrometry. Size exclusion chromatography revealed that both proteins exist as monomers, and circular dichroism showed that their secondary structures exhibit a predominance of β-strands which is typical of C-type lectins. Finally, both RegIIIγ and human HIP/PAP bind to mannan but not to monomeric mannose, giving initial insights into their carbohydrate ligands. These studies thus provide an essential foundation for further analyses of human and mouse RegIII protein function.

Keywords: C-type lectins; Regenerating protein family; Carbohydrate binding; Mucosal injury; Inflammatory bowel disease

C-type lectins are proteins that contain carbohydrate recognition domains (CRDs)[1] and bind selectively to specific carbohydrate structures, often in a Ca^{2+}-dependent manner. They mediate a variety of functions including cellular adhesion, clearance of circulating proteins, and recognition of microbe-associated molecular patterns (reviewed in [1]). The *Reg* gene family encodes an extensive group of secreted proteins that contain conserved sequence motifs found in all C-type lectin CRDs. The family is so named because the first member to be identified was cloned from a cDNA library derived from regenerating pancreatic islets [2]. Subsequently, several members of this multigene family have been identified in mice and humans, and are grouped according to homology into four subfamilies: RegI, RegII, RegIII, and RegIV. Despite their similarities to well-characterized C-type lectins, the members of the Reg family have poorly defined functions and their carbohydrate ligands have not been clearly identified.

Members of the RegIII family are constitutively expressed at high levels in mouse and human gastrointestinal tissues. RegIIIα, β, and γ are expressed in mouse small intestine [3], while human hepatocarcinoma–intestine–pancreas/pancreatitis associated protein (HIP/PAP) is made in human small intestine. RegIIIβ and γ expression levels increase dramatically in response to bacterial colonization and other inflammatory stimuli in mice [4–6]. In addition, HIP/PAP expression is upregulated in the mucosal tissues

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[1] *Abbreviations used:* CRD, carbohydrate recognition domain; HIP/PAP, hepatocarcinoma–intestine–pancreas/pancreatitis associated protein; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; IB, inclusion body; MALDI-TOF, matrix assisted laser desorption/ionization time of flight; CD, circular dichroism; DC, detergent compatible.

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doi:10.1016/j.pep.2006.01.014

of patients with inflammatory bowel disease [5,7]. Despite these insights into the forces regulating RegIII protein expression, almost nothing is known about the biological functions of RegIII proteins or their role in disease.

An abundant source of purified recombinant mouse and human RegIII proteins is needed to delineate the role of RegIII proteins in intestinal biology and human disease. Human HIP/PAP has been purified previously from the milk of transgenic mice engineered to express the protein in mammary gland [8], and as a glutathione *S*-transferase (GST) fusion protein in an *Escherichia coli* expression system [9]. Although the transgenic approach yielded quantities of protein sufficient for crystallographic analysis [10], this method is technically challenging, time-consuming, and expensive. The recombinant fusion protein procedure produced only microgram quantities of the GST-tagged protein [9]. We therefore wished to develop a system for the rapid expression and purification of recombinant RegIII proteins that is simple, high yield, and readily adaptable to other Reg family members.

In this report, we describe a new method for high level expression of mouse RegIIIγ and HIP/PAP using a bacterial expression system. Initial problems with low HIP/PAP expression were solved by introducing silent mutations into the 5′ end of the gene that were designed to relax local mRNA secondary structure. We present details of a procedure for the refolding and purification of RegIIIγ and HIP/PAP from bacterial inclusion bodies. This simple protocol yields milligram quantities of both proteins, and is the first example of high level Reg protein purification from a bacterial expression system. Finally, we show initial evidence suggesting that both RegIIIγ and HIP/PAP bind polymeric but not monomeric mannose.

Materials and methods

Vectors, strains, and supplies

The expression vector pET3a was from Novagen. *E. coli* BL21-CodonPlus (DE3)-RIL and *E. coli* BL21-CodonPlus (DE3)-RILP competent cells were from Stratagene. Oligonucleotides and restriction enzymes were supplied by Invitrogen. Other DNA modifying enzymes and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Roche Molecular Biochemicals. Luria Broth was purchased from VWR. Sephacryl S-100 high resolution gel filtration medium and size exclusion chromatography standards were from GE Healthcare. All other chemicals and reagents were from Sigma.

Construction of the mouse RegIIIγ expression vector

A 474 bp amplicon was generated by RT-PCR from mouse small intestinal RNA using the specific primers 5′-ATTGCGAGGCATATGGAAGTTGCCAAGAAAGATGCCCCAT-3′ (forward primer) and 5′- CTATGGGGATCCCTAGGCCTTGAATTTGCAGACATAGGGT-3′ (reverse primer). The forward primer contained an *Nde*I restriction site (underlined) for cloning into pET3a. The reverse primer incorporated the native stop codon followed by an engineered *Bam*HI site (underlined). The resulting amplicon contained a methionine start codon in place of the signal sequence and thus encoded the mature secreted protein. PCR products and vector were digested with *Nde*I and *Bam*HI, gel-purified, and ligated. The recombinant plasmid (pET3a-RegIIIγ) was sequenced to confirm the absence of mutations, and was transformed into *E. coli* BL21-CodonPlus (DE3)-RIL for protein expression.

Construction of HIP/PAP expression strains

A 474 bp amplicon was generated by RT-PCR from human small intestinal RNA (Ambion) using the specific primers 5′-ATTGCGAGGCATATGGAAGAACCCCAGAGAGGAACTGC-3′ (forward primer) and 5′-CTATGGTGATCACTAGTCAGTGAACTTGCAGACATAGGGTAA-3′ (reverse primer). The forward primer contained an *Nde*I restriction site (underlined) for cloning into pET3a. The reverse primer incorporated the native stop codon followed by an engineered *Bcl*I site (underlined). The resulting amplicon lacked the HIP/PAP signal sequence and thus encoded the mature secreted protein [8]. The PCR product was digested with *Nde*I and *Bcl*I, ligated into *Nde*I/*Bam*HI-digested pET3a, and the resulting plasmid (pET3a-HIP/PAP) sequenced to confirm the absence of mutations.

A second expression construct (pET3a-HIP/PAP*mut*) was generated with silent mutations engineered into the 5′ end of the HIP/PAP coding sequence. Mutations were introduced by redesigning the forward primer that was used to generate the wild-type HIP/PAP construct: 5′- ATTGCGAGGCATATGGAAGAACCACAAAGAGAAACT GC-3′ (mutant bases are underlined; also see Fig. 1). A 474 bp amplicon was generated by PCR with this mutant primer and the *HIP/PAP*-specific reverse primer above, using pET3a-HIP/PAP as template. The amplicon was cloned into pET3a as described for pET3a-HIP/PAP. The resulting plasmid was sequenced to confirm incorporation of the silent mutations and the absence of additional mutations. Both pET3a-HIP/PAP and pET3a-HIP/PAP*mut* were transformed into *E. coli* BL21-CodonPlus (DE3)-RILP for protein expression.

Expression and purification of RegIIIγ

Escherichia coli BL21-CodonPlus (DE3)-RIL harboring pET3a-RegIIIγ were grown at 37 °C in 500 ml of LB medium supplemented with 0.1 mg/ml ampicillin to an absorbance of 0.6–1.0 (mid-log phase) at 600 nm. Protein expression was induced by the addition of 0.4 mM IPTG, and the culture was incubated for another 3 h at 37 °C with good aeration. Cells were collected by centrifugation at 6500*g* for 15 min at 4 °C, and the pellet resuspended in 1/20 culture volume (25 ml) of Inclusion Body (IB) Wash Buffer (20 mM Tris–HCl, 10 mM EDTA, 1% Triton X-100, pH 7.5). The cells were divided into