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N-glycosylation influences the latency and catalytic properties of mammalian purple acid phosphatase

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Abstract

Purple acid phosphatase (PAP), also known as tartrate-resistant acid phosphatase or uteroferrin, contains two potential consensus N-glycosylation sites at Asn_{97} and Asn_{128}. In this study, endogenous rat bone PAP was found to possess similar *N*-glycan structures as rat recombinant PAP heterologously expressed in baculovirus-infected Sf9 insect cells. PAP from Sf9 cells was shown to contain two N-linked oligosaccharides, whereas PAP expressed by mammalian CHO-K1 cells was less extensively glycosylated. The extent of N-glycosylation affected the catalytic properties of the enzyme, as N97Q and N128Q mutants, containing a single oligosaccharide chain, exhibited a lower substrate affinity and catalytic activity compared to those of the fully glycosylated PAP in the native, monomeric state. The differences in substrate affinity and catalytic activity were abolished and partially restored, respectively, by proteolytic cleavage in the loop domain, indicating that the extent of N-glycosylation influences the interaction of the repressive loop domain with catalytically important residues.

Keywords: Tartrate-resistant acid phosphatase; Purple acid phosphatase; Uteroferrin; Acp5; Osteoclasts; Macrophages; N-glycosylation; Sf9 cells; CHO-K1 cells; Bone

Purple acid phosphatases (PAPs)[1] are acidic metallohydrolases that contain a binuclear Fe^{3+} M^{2+} center in their active site, where M is Fe or Zn [1,2]. In mammals, these enzymes are also referred to as tartrate-resistant acid phosphatases (TRAPs), uteroferrin or type 5 acid phosphatases. In rodents, PAP is abundantly expressed in bone-resorbing osteoclasts, dendritic cells, macrophages and neurons [3–5], and participates in bone remodeling, immune regulation, and lysosomal catabolism [6–9]. PAPs are glycoproteins with molecular weights between 35,000 and 37,000 Da with carbohydrate contents constituting 5–10% of molecular mass [10,11]. The enzyme is activated by limited proteolytic cleavage in a repressive loop domain leading to a two-subunit structure with the N-terminal glycosylated 23 kDa part joined to the C-terminal 16 kDa fragment [12–15]. Examination of PAP amino acid sequences reveals two potential consensus N-glycosylation sites at residues N97 and N128, conserved among all members of mammalian PAPs [16]. In uteroferrin purified from the allantoic fluid of pregnant pigs, carbohydrate analysis indicated a single unphosphorylated N-linked $Man_{5\text{-}6}GlcNAc_2$ high-mannose-type oligosaccharide [17,18], which is consistent with the crystal structure of uteroferrin which revealed only one carbohydrate chain linked to N97 [19]. In contrast, newly synthesized uteroferrin from endometrial explants contained two N-linked oligosaccharides [11]. The PAP isolated from rat bone and

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[1] *Abbreviations used:* PAPs, purple acid phosphatases; TRAPs, tartrate-resistant acid phosphatases; IPTG, isopropylthio-β-galactoside; PMSF, phenylmethylsulfonylfluoride; *p*NPP, *p*-nitrophenylphosphate; GNA, *Galanthus nivalis* agglutin; SNA, *Sambucus nigra* agglutinin; MAA, *Maackia amurensis* agglutinin.

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in secreted recombinant rat PAP expressed in insect cells was shown to contain both EndoH-sensitive and resistant N-linked oligosaccharides [20], and the crystal structures of recombinant rat PAP revealed a single carbohydrate chain linked to N97 [21,22]. Heterogeneity with regard to oligosaccharide substructure or extent of oligosaccharide substitution was also suggested for recombinant PAP expressed in CHO-K1 cells [23]. In bovine spleen PAP, carbohydrate analysis was consistent with the presence of two non-sialylated oligosaccharide chains per molecule [12]. Thus, different PAPs contain either a single oligosaccharide or become substituted at both sites. In this study, the possible functional consequences of differential glycosylation were addressed by site-directed mutagenesis to resolve whether individual N-linked oligosaccharides influence the catalytic properties of the PAP enzyme.

Materials and methods

Materials

Restriction enzymes, DNA purification system, mutation-specific primers, and baculovirus expression system were purchased from Life Technologies (Carlsbad, CA, USA).

Expression and purification of recombinant purple acid phosphatase in Sf9 insect cell construction of baculovirus expression vectors

Wild-type (wt) or mutant rat PAP cDNAs were cloned into baculovirus expression vector pFASTBAC1 using the *Eco*RI site in the donor plasmid. The correct orientation was determined by *Pst*I cleavage and further confirmed by DNA sequencing. The pFASTBAC1 donor vectors containing wt or mutant PAPs were transformed into DH10Bac cells for homologous recombination with bacmid. Recombinant bacmids were selected on Luria agar plates containing antibiotics (50 μg/ml kanamycin, 7 μg/ml gentamycin, 10 μg/ml tetracycline), 100 μg/ml Bluo-gel (halogenated indolyl-β-galactoside), and 40 μg/ml isopropylthio-β-galactoside (IPTG) and confirmed by PCR. Recombinant baculoviruses containing wt or mutant PAPs were produced in *Spodoptera frugiperda* (Sf9) insect cells according to the manufacturer's instruction.

Site-directed mutagenesis

The full-length 1.4 kb rat tartrate-resistant acid phosphatase cDNA [16] was cloned into pCMV5. Site-directed mutagenesis was performed using 5′–3′ MORPH site-specific plasmid DNA mutagenesis kit (5 Prime → 3 Prime,. Boulder, CO, USA). Primers used for each specific mutation were as follows: N97Q, Primer: 5′-GATCACCTTGGC**CAA**GTCTCGGCACAAATT G-3′; N128Q, 5′-CAAAGTTCCGCGGTCG**CAA**AT CACCGTGGC-3′; N97Q-N128Q, 5′-GATCACCTTG GC**CAA**GTCTCGGCACAAATTG-3′, and 5′-CAAA GTTCCGCGGTCG**CAA**ATCACCGTGGC-3′. The underlined bases indicate changes compared to the wild-type (wt) sequence. The primers employed for mutagenesis were phosphorylated by T4 kinase according to the manufacturer's instruction. The rPAP cDNA mutants were verified by DNA sequence analyses (Cybergene AB, Huddinge, Sweden).

Cell culture and transfection

Spodoptera frugiperda (Sf9) cells were cultured in SF900 II SFM medium and transfected with purified bacmids using the transfection reagent Cellfectin (Life Technologies). After transfection, the cells were kept and incubated at 27 °C for 3 days. The medium, which constitutes the scale up-1 virus stock, was harvested and stored at 4 °C. This scale up-1 virus stock was used to infect a 25-cm^2 flask seeded with 1×10^6 cells and culture was incubated at 27 °C for 5 days. The supernatant constitutes the scale up-2. The recombinant virus stock scale up-2 was amplified once more by infection of 100 mL Sf9 cells seeded at a density of 1×10^6 cells/mL with 3 mL scale up-2, and after 5 days, high-titer virus stock (10^8 pfu/ml) was harvested. The high-titer stock virus was used in protein production.

Protein expression and purification

The Sf9 cells were infected at a density of 1×10^6 cells/ml with recombinant baculovirus containing either wild-type or mutant rat PAP cDNA using a MOI of 0.1. This low MOI was chosen to minimize coexpression of proteolytically cleaved PAP. The production of wild-type and mutant PAP proteins was done in 1 L shaking flasks. After 5 days at 27 °C, cells were removed by centrifugation at 5000*g* for 30 min and proteinase inhibitors E64 (10 μg/ml), Pefabloc (1 mg/ml, Roche, Germany), and EDTA (5 mM) were added to the supernatants. For protein purification, an ÄKTA Purifier system (Amersham Bioscience, Uppsala, Sweden) was used. The protein samples were filtered through a 0.8 μm filter and thereafter loaded onto a cation exchange column (HiLoad 16/10, Amersham Bioscience, Uppsala, Sweden), equilibrated with 0.1 M NaAc buffer, pH 6.5. The column was washed with six column volumes (CV) equilibration buffer before starting a linear gradient from 0.1 M NaAc, pH 6.5–0.5 M NaAc, pH 6.5. Proteinase inhibitors were added to the fractions and the fractions containing TRAP activity were pooled followed by addition of solid ammonium sulfate to a final concentration of 1.1 M. After filtration through 0.8 μm filters, the samples were