Structure-Function Analysis of Mouse Purβ II

CONFORMATION ALTERING MUTATIONS DISRUPT SINGLE-STRANDED DNA AND PROTEIN INTERACTIONS CRUCIAL TO SMOOTH MUSCLE α-ACTIN GENE REPRESSION

Received for publication, August 9, 2007, and in revised form, September 28, 2007. Published, JBC Papers in Press, September 28, 2007, DOI 10.1074/jbc.M706617200

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Previous studies from our laboratories have implicated two members of the Pur family of single-stranded DNA/RNA-binding proteins, Purα and Purβ, in transcriptional repression of the smooth muscle α-actin gene in vascular cell types. Although Purα and Purβ share substantial sequence homology and nucleic acid binding properties, genomic promoter and cis-element occupancy studies reported herein suggest that Purβ is the dominant factor in gene regulation. To dissect the molecular basis of Purβ repressor activity, site-directed mutagenesis was used to map amino acids critical to the physical and functional interaction of Purβ with the smooth muscle α-actin promoter. Of all the various acidic, basic, and aromatic residues studied, mutation of positionally conserved arginines in the class I or class II repeat modules significantly attenuated Purβ repressor activity in transfected vascular smooth muscle cells and fibroblasts. DNA binding and protein–protein interaction assays were conducted with purified recombinant Purβ and selected mutants to reveal the physical basis for loss-of-function. Mutants R57E, R57E/R96E, and R57A/R96A each exhibited reduced single-stranded DNA binding affinity for an essential promoter element and diminished interaction with corepressor YB-1/MSY1. Structural analyses of the R57A/R96A and R57E/R96E double mutants in comparison to the wild-type Purβ homodimer revealed aberrant self-association into higher order oligomeric complexes, which correlated with decreased α-helical content and defective DNA and protein binding in vitro. These findings point to a previously unrecognized structural role for certain core arginine residues in forming a conformationally stable Purβ protein capable of physical interactions necessary for smooth muscle α-actin gene repression.

Inflammation-induced activation of medial vascular smooth muscle cells (VSMCs) from a quiescent contractile state to a more migratory proliferative phenotype has been traditionally viewed as a key mechanistic step in early stage atherosclerosis (1). The importance of this process is highlighted by the fact that the degree of ensuing lipoprotein and inflammatory cell retention and fibrous cap formation may dictate the overall vulnerability of atherosclerotic plaques to rupture. However, accumulating evidence suggests that resident fibroblasts of the vascular wall adventitia as well as circulating progenitor cells may also contribute to neointimal hyperplasia in certain animal models of arterial disease and injury. Loss of conversion to a myofibroblast phenotype (5, 6). Notwithstanding the diversity of cell types implicated in vascular remodeling, the biochemical hallmark of a differentiated VSMC or myofibroblast is expression of smooth muscle α-actin (SMαA), a cytoskeletal protein that plays an essential role in facilitating vascular contractility and wound healing (7–12). As such, the gene encoding SMαA is a relevant target for elucidating mechanisms that contribute to phenotypic modulation of cell types involved in vascular development, disease, and repair.

Over the past fifteen years, work from many different investigators has led to a consensus view that SMαA gene regulatory mechanisms are highly cell type-dependent and, in the case of transcriptional control, typified by combinatorial interactions among multiple cis-elements, corresponding DNA-binding proteins, and associated tissue-restricted cofactors (13, 14). Among the various regulatory proteins identified to date, several single-stranded DNA (ssDNA)-binding proteins known as Purα and Purβ have been implicated in SMαA promoter repression in VSMCs and myofibroblasts by virtue of their strand-specific interaction with certain sequence elements.

4 The abbreviations used are: VSMCs, vascular smooth muscle cells; SMαA, smooth muscle α-actin; MEFs, mouse embryonic fibroblasts; SEC, size exclusion chromatography; CD, circular dichroism; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; β-gal, β-galactosidase; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; OB-fold, oligonucleotide/oligosaccharide-binding fold; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ChiP, chromatin immunoprecipitation.

1 This study was funded in part by Grant HL054281 (to R. J. K.) from the National Heart, Lung, and Blood Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5 and Table S1.

3 Supported by NHLBI, National Institutes of Health Institutional Training Grant T32 HL007594.

4 Supported by an American Heart Association Predoctoral Fellowship 0515620T.

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present in the 5′-flanking region of the gene (15–18). Each known Pur-interacting element in the SMαA promoter (−195 to −164 and −59 to −28) has a high degree of purine-pyrimidine asymmetry and a core motif that, in double-stranded configuration, serves as a recognition site for an essential activator. Mechanistically, Purα and Purβ appear to repress transcription by virtue of inhibitory protein-DNA and protein-protein interactions, which serve to impair efficient SMαA enhancer elaboration by basal and inducible activators including TEF-1, SRF, Sp1/3, and R-Smad2/3/4. Importantly, physical and functional interaction with YB-1/MSY1, which preferentially binds to the pyrimidine-rich strand complementary to Purα/β-binding element spanning −195 to −164 (15), seems to be necessary for augmentation of Purβ repressor activity and for ensuring site-specific ssDNA binding by Purβ (19).

Purα and Purβ are members of an evolutionarily-conserved family of ssDNA and RNA-binding proteins whose signature biochemical feature is specific and high affinity interaction with single-stranded nucleic acid sequences rich in purine nucleotides (20–22). All known Pur paralogs expressed in vertebrates (Purα, Purβ, and Purγ) exhibit a substantial degree of homology marked by an ordered array of highly conserved class I (basic aromatic) and class II (acidic leucine-rich) repeats in the central region of each protein (22). Together, these repeats comprise the ssDNA/RNA-binding domain of both Purα and Purβ (19, 23, 24). Despite the fact that Purα and Purβ are ~70% identical at the amino acid level, striking differences exist in the spacing of certain class I and II repeat modules and in the amino acid composition of sequence elements near N and C termini of each protein (25). In this regard, previous work from our group has indicated that non-conserved regions outside the minimal DNA-binding domain are necessary for high affinity binding of Purβ to both MSY1 (26) and ssDNA (19) as well as for elaboration of ectopic repressor activity in cultured VSMCs (19). Currantly, a recent study by Wortman et al. (27) identified point mutations in the first class I repeat or first class II repeat which specifically eliminates ssDNA binding by truncated versions of recombinant mouse and human Purα. However, how such mutations would affect DNA binding and transcriptional activity of full-length Purα is presently unknown.

In addition to repressing the expression of genes encoding contractile proteins expressed in the vascular wall, heart, and skeletal muscle (16–19, 28–30), Pur proteins have also been implicated in gene-specific transcriptional activation (20, 22), mRNA translation (26, 28, 31), and RNA transport (32, 33). The founding member of this family, Purα, has also been suggested to play a role in control of cell cycle progression (34–38) and in cell proliferation necessary for normal brain and myeloid cell development (39). Given the diversity of functions attributed to different members of the Pur family, molecular analysis of genetically engineered point mutants may be helpful in clarifying the mechanisms by which Pur paralogs modulate gene expression and cell phenotype. In the present study, we set out to test how mutation of certain conserved and non-conserved amino acid residues would change the physical and functional properties of Purβ in relation to SMαA promoter repression. This line of investigation has led to the novel finding that specific arginine residues present in the class I and class II repeats are essential to formation of a structurally stable protein, which can bind to ssDNA, interact with corepressor partner YB-1/MSY1, and inhibit SMαA gene transcription.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids Encoding Mouse Purβ Point Mutants—Mammalian expression vectors encoding full-length N-terminal His6 epitope-tagged Purβ with selected point mutations were created with the QuickChange® XL site-directed mutagenesis system (Stratagene) using template plasmid pCI-NHIS-Purβ (16). Primer sequences along with detailed methods describing mutant plasmid synthesis, propagation, and quality control are provided in the online data supplement. Large-scale purification of sequence-verified mutant expression plasmids was achieved by double cationic gradient centrifugation of DNA extracted from 1-liter Escherichia coli cultures. Supercoiled mutant Purβ expression plasmids were quantified by optical density measurement at 260 nm. Plasmid stocks used for transient transfection were subjected to analytical agarose gel electrophoresis to confirm both the absence of genomic DNA and the correct restriction fragment pattern after double digestion with BamHI and KpnI (Roche Applied Science). Selected mutant Purβ cDNA fragments released from plasmid by BamHI and KpnI digestion were subcloned into the bacterial expression plasmid pQE30 (Qiagen) and resequenced.

Recombinant NHIS-Purβ Purification and Structural Characterization—Expression, purification, and quantification of His6-tagged point mutants were performed as previously described for wild-type NHIS-Purβ except that isopropyl-1-thio-β-D-galactopyranoside induction was done at room temperature rather than 37 °C (18). After enrichment on HIS-Select™ Nickel Affinity Gel (Sigma), an additional chromatography step on Sephacryl® S200HR resin (Sigma) equilibrated in 50 mM sodium phosphate pH 7.5, 0.2 mM NaCl, 0.5 mM EDTA, and 2 mM dithiothreitol was included to remove low molecular weight fragments (40). Analytical size exclusion chromatography (SEC) was performed on selected preparations with the use of a HiPrep™ (16/60) Sephacryl™ S200HR column (GE Healthcare) coupled to a BioCAD® 700E work station (PerSeptive Biosystems) at a flow rate of 0.5 ml/min. The column was calibrated with a mixture of molecular weight standards (Sigma), which included blue dextran (2000 kDa), BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and DNP-aspartate. Wild-type or mutant NHIS-Purβ was loaded at a concentration of ~30 μM (0.6 ml) and eluted in buffer consisting of 50 mM sodium phosphate, pH 7.5, 0.5 mM EDTA, 10 mM β-mercaptoethanol with 0.2 mM NaCl or 1 mM NaCl. Protein elution was monitored by absorbance at 280 nm. Selected SEC-fractioned NHIS-Purβ preparations were analyzed by circular dichroism (CD) spectroscopy on an Aviv model 215 CD spectrometer fitted with a thermal equilibration chamber (UMDNJ Circular Dichroism Facility). Spectra were obtained from 260 to 200 nm at 0.5 nm intervals. Data were collected at protein concentrations of 0.2 mg/ml in buffer consisting of 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, and temperatures
ranging from 20 to 70 °C at 5 °C intervals. Smoothing and baseline correction of the data were performed as described (41).

**Cell Culture, Transient Transfection, Reporter Gene, and Pur Point Mutant Expression Assays—**AKR-2B mouse embryonic fibroblasts (MEFs) and A7r5 rat aortic VSMCs were cultured and transiently transfected with the use of GenePORTER® (Gene Therapy Systems, Inc.) or jetPEI™ (Qiagen) reagents as previously described (18, 42). Subconfluent cells were transfected with 3 μg of mouse SMαA promoter:chloramphenicol acetyltransferase (CAT) reporter plasmid plus 1 μg of pCI expression plasmid encoding wild-type or mutated His-tagged Purβ. As a control, cells were transfected with reporter plasmid plus 1 μg of empty pCI plasmid. After 48 h culture in growth medium, cells were washed three times with ice-cold phosphate-buffered saline and then extracted for 30 min with 1× CAT lysis buffer (Roche Applied Science) supplemented with 1 mM phenylmethylsulfonylfluoride and 1 μg/ml each of leupeptin, pepstatin, and aprotinin. Extracts were cleared of cellular debris by centrifugation at 14,000 rpm for 10 min at 4 °C and subjected to various biochemical assays. Total protein was determined by bichinchoninic acid assay (Sigma) with the use of BSA as a standard. CAT reporter protein was measured by enzyme-linked immunosorbent assay (ELISA) (Roche Applied Science). Expression of His-tagged Purβ was assessed by either immunocytochemistry (see supplemental methods) or by immunoblotting of cell extracts as previously described using a primary antibody directed against the N-terminal His6 epitope (19). Blots were reprobed with a monoclonal antibody against GAPDH (clone 6C5, Research Diagnostics, Inc.) to verify equivalent protein loading. Transfections were typically performed in triplicate and repeated two to three times. Data sets were subjected to analysis of variance. Differences between groups were further analyzed by Student’s t test with the alpha level set at 0.05 and Bonferroni adjustment for multiple comparisons.

**DNA Binding Assays—**Quantitative colorimetric assays in microtiter well format were used to assess the binding of cell extract-derived or recombinant Pur proteins to selected DNA probes as previously described (18, 19). Nucleoprotein binding buffer consisted of 20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA with 0.2% w/v BSA (Sigma, ELISA-qualified) and 0.05% v/v Tween 20 plus 5 μg/ml poly(dI-dC) (Roche Applied Science). In assays using cell extract-derived protein, 1.5 mM MgCl2 was substituted for EDTA in the binding buffer. In the assay design where synthetic 3′-biotinylated oligonucleotides (Sigma-Genosys) were immobilized on StreptaWells (Roche Applied Science), solid-phase Pur nucleoprotein complexes formed after incubation of DNA-coated wells with nuclear extract (42) or purified protein were detected by discontinuous ELISA. This involved incubating washed wells for 1 h with 1.0 μg/ml primary antibody directed against the C terminus of Purβ or Purα (15) followed by a secondary incubation with a 1:2000 dilution of goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Santa Cruz Biotechnology, Inc.) then tertiary incubation with HRP substrate solution (19). In the reverse orientation with purified Purβ immobilized on microtiter wells (Costar® 96-well Easywash™ high binding EIA/RIA plate, Corning, Inc.) at 50 nM, binding of biotinylated ssDNA probe in the presence or absence of fluid phase competitor protein was detected by incubating washed wells with a 1:2000 dilution of ExtrAvidin® peroxidase conjugate (Sigma) followed by secondary incubation with HRP substrate solution (18). In both assay formats, wells were washed three times with HEPES-buffered saline containing 0.05% v/v Tween 20 between each reagent application. To assess the association of Pur proteins with genomic DNA in living cells, chromatin immunoprecipitation (ChIP) assays were conducted by adapting established procedures (29, 43) to AKR-2B cells and the SMαA gene. A detailed methodological description of the assay including culture conditions, formaldehyde fixation, cell lysis, chromatin fragmentation, immunoprecipitation with Pur or MSY1 polyclonal antibodies, reverse cross-linking of isolated nucleoprotein complexes, and PCR amplification of SMαA promoter sequence is provided in the online data supplement.

**Protein-Protein Interaction Assay—**The binding of Purβ mutants to selected proteins was evaluated by ELISA (18, 42). To validate comparison of the binding capacity of wild-type and mutant Purβ proteins immobilized in the solid-phase, control assays were initially conducted to ensure that the coating efficiency of each mutant was similar to wild-type Purβ over a range of coating concentrations. Based on these results, wells were coated with wild-type or mutant Purβ at a saturating concentration of 100 nM in HEPES-buffered saline. Nuclear extracts from AKR-2B MEFs were prepared as previously described (42) and used as a source of Purβ interaction partners at 200 μg/ml in binding buffer consisting of 20 mM HEPES pH 7.5, 150 mM NaCl with 0.2% w/v BSA and 0.05% v/v Tween 20. Primary detecting antibodies against Purα (A291–313) or MSY1 (M242–267) (15) were applied at a concentration of 1.0 μg/ml followed by secondary HRP-conjugated goat anti-rabbit IgG at a 1:2000 dilution. Wells were washed three times between each reagent application. In all microplate-based assays, colorimetric signal was generated by application of HRP substrate solution (ABTS single reagent system, Chemicon). After 5–15 min, reactions were quenched by the adding an equal volume of 1% SDS. End point absorbance values at 405 nm were measured with a Vmax™ microplate reader (Molecular Devices). The binding of selected Purβ mutants to recombinant MSY1 was similarly assessed by ELISA in which purified MSY1 was immobilized in the solid phase (15).

**RESULTS**

**Biochemical Evidence for Purβ Dominance in SMαA Promoter and cis-Element Association in Vivo—**Results of recent gene knockdown and overexpression studies indicated that the repressor activity of Purβ exceeded that of Purα in both fibroblasts and VSMCs despite the fact that the recombinant proteins displayed nearly identical ssDNA binding properties in vitro (18). To reconcile these findings, we evaluated the binding of native cell extract-derived Purα and Purβ to biotinylated ssDNA probes immobilized on streptavidin-coated microtiter wells using an ELISA detection system. As shown in Fig. 1A, the apparent affinity of MEF or VSMC-derived Purβ for the purine-rich strand of the SMαA Pur/Pyr element (PE32-bF, −1.2 nM) was similar to that which was recently reported for the purified recombinant protein (18). Absence of detectable Purβ...
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**FIGURE 1.** Strand-specific binding by cell extract-derived Purβ to SMαA cis-elements. A, biotinylated oligonucleotides corresponding to the purine-rich forward strand or pyrimidine-rich reverse strand of the PE32 element were immobilized on streptavidin-coated microtiter wells at concentrations from 40 to 0.625 nM. After overnight incubation at 4 °C with 250 μg/ml nuclear protein from AKR-2B MEFs or C57BL/6 VSMCs, wells were washed and solid-phase Purβ nucleoprotein complexes were detected by ELISA. Data points were fit to an equation for a rectangular hyperbola to estimate the concentration of ssDNA where Purβ binding is half-saturated (EC50). B, microtiter wells coated with 10 nM of the indicated ssDNA or dsDNA probes were incubated with 250 μg/ml AKR-2B nuclear protein at 4 °C for 18 h. Wells were washed and solid-phase nucleoprotein complexes were detected by ELISA with the use of primary polyclonal antibodies against mouse Purα or Purβ. Single- or double-stranded probes correspond to putative Purβ recognition elements in the 5′-flanking region of the mouse SMαA gene; PE32 (−195 to −164), THR (−176 to −145), and SPUR32 (−59 to −28). F, forward strand; R, reverse strand; b, biotin; ds, double-stranded.

binding to the complementary pyrimidine-rich strand demonstrated the specificity of the purine-rich strand interaction and reliability of this experimental approach. To compare the relative ssDNA binding capacity of endogenous Purα to Purβ, we next assessed binding to different cis-elements derived from the SMαA promoter in either single- or double-stranded configurations. To ensure that the assay would be sensitive to differences in binding affinity and/or relative Pur expression levels, probes were immobilized at a fixed concentration of 10 nM. As shown in Fig. 1B, while nuclear Purα and Purβ from AKR-2B MEFs exhibited similar ssDNA binding specificity, the relative amount of Purβ bound consistently exceeded that of Purα for the PE32 (−195 to −164), SPUR32 (−59 to −28), and THR32 (−176 to −145) elements. Because the antibodies used for detection of Purα and Purβ were previously shown to possess identical antigen binding affinity (15), these results are likely reflecting true differences in ssDNA binding capacity of these proteins in a cellular milieu. To further test this interpretation, we performed ChIP assays to assess the proportional occupancy of the genomic SMαA promoter by Pur repressors in cultured AKR-2B MEFs (Fig. 2). Consistent with results of the cell-free ELISA, the intensity of the Purβ signal exceeded that of Purα or corepressor MSY1 under culture conditions in which the SMαA gene was rendered transcriptionally silent (44). While qualitative, these results point to Purβ as a dominant factor in gene repression and offer further justification for a detailed analysis of this protein at the level of individual amino acids.

**Rationale for Purβ Mutagenesis—**Specific amino acids in mouse Purβ were selected for mutation with the goal of determining their relative importance in Purβ structure-function vis-à-vis the SMαA promoter (Fig. 3). Analysis of the primary sequence of Purβ together with computer-based prediction of potential secondary structural elements present in the protein (45) suggested some weak similarity with ssDNA-binding proteins possessing an oligonucleotide/oligosaccharide-binding fold (OB fold). Two pairs of aspartic acid and phenylalanine residues with spatial arrangement ($D\alpha\alpha\alpha\alpha F$) found in certain proteins with OB folds (46) were selected for mutation to alanine. These residues (D61A, F70A, D247A) reside in the first and third basic aromatic class I repeats, which mark the boundary of the minimal ssDNA/RNA-binding domain of Purβ (19). A second set of mutants was generated to assess the role of selected positively charged residues in the same repeat modules. Point mutations within the first class I or class II repeats of Purβ were designed based on findings of Wortman et al. (27) who reported that $R\rightarrow E$ mutation of certain residues in Purα was sufficient to eliminate ssDNA binding capacity in the context of a truncated “mini” Purα protein lacking its C-terminal region. Because we had previously found that absence of the comparable C-terminal domain (amino acids 264−324) in Purβ
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Dramatically reduced its repressor activity and ssDNA binding affinity (19), we chose to evaluate the functional consequence of arginine mutations in the class I (R57E, R57A, R243E, R243A) and class II (R96E, R96A) repeats in the context of the full-length protein. Complementary DNAs (cDNAs) encoding both R → E and R → A point mutants were constructed to assess the functional significance of reversing versus neutralizing the charge of the amino acid side chain at these positions. Because of the presence of a unique non-conserved cysteine at position 250 together with a conserved cysteine at position 285, single and double mutants (C250A, C285A, C250A/C285A) were also generated to evaluate the potential role of these two cysteine residues in Purβ structure-function.

Repressor Activity of Purβ Mutants in VSMCs and MEFs—To evaluate the effect of the above listed point mutations on the transcriptional repressor activity of Purβ, transient cotransfec-
Calculated IC_{50} nearly identical to the wild type protein. On the other hand, mutants R57E and R57E/R96E, which were deficient in repressor activity in transfection assays, did not compete for binding of PE32-bF to wild-type Pur/\textsubscript{H9252}. Intriguingly, mutant R243A, which also appeared to lack repressor activity, demonstrated ssDNA binding affinity similar to the wild-type protein.

Biochemical Characterization of Pur/\textsubscript{H9252} Point Mutants: Protein-Protein Interaction—Because Pur/\textsubscript{H9252} is known to physically and functionally interact with corepressors Pur/\textsubscript{H9251} and YB-1/MSY1, we next tested whether the mutants displayed any change in their ability to bind to these proteins. To do so, we employed an ELISA in which each mutant was assessed relative to wild-type Pur/\textsubscript{H9252} for its ability to capture Pur/\textsubscript{H9251} and MSY1 from a nuclear extract of AKR-2B MEFs (Fig. 7). As a control, Pur/\textsubscript{H9252} mutants were first evaluated for coating efficiency on microtiter wells using antibodies directed against either the N-terminal His tag or C terminus of Pur/\textsubscript{H9252}. Similar to previous findings made when comparing the coating efficiency of Pur/\textsubscript{H9251} to Pur/\textsubscript{H9252} (18), no significant differences were observed. To compare the protein binding properties of wild-type Pur/\textsubscript{H9252} to selected mutants in a qualitative fashion, a saturating coating concentration of 100 nM was chosen. In keeping with the results of ssDNA binding studies, the C250A/C285A double mutant demonstrated binding capacity comparable to the wild-type protein. Conversely, arginine mutants showed reduced binding to Pur/\textsubscript{H9251} and MSY1 with R57E/R96E exhibiting the greatest deficiency followed by R57E, and to a lesser extent, R243A.

In an effort to validate these observations in a more quantitative fashion, we next evaluated the binding of fluid phase Pur/\textsubscript{H9252}, R243A, and the R57E/R96E double mutant to purified recombinant MSY1 by ELISA. In these experiments, MSY1 was immobilized at a coating concentration (50 nM) previously shown to support specific and saturable binding of nuclear Pur/\textsubscript{H9252} (15). BSA-blocked wells served as a negative control for nonspecific binding and a C-terminal-specific Pur/\textsubscript{H9252} antibody (B302–324) was used to detect Pur/\textsubscript{H9252}-MSY1 complex formation. As shown in Fig. 8, the R57E/R96E mutant displayed dramatically reduced binding affinity for MSY1 relative to wild-type Pur/\textsubscript{H9252} (\sim 10-fold difference). On the other hand, analysis of the R243A mutant revealed a potential difference in stoichiometry but no significant change in apparent binding affinity for MSY1. These results underscored the importance of Arg\textsuperscript{57} and Arg\textsuperscript{96} residues in Pur/\textsubscript{H9252} structure-function and implied a physical role beyond mere ssDNA recognition and binding.

Biochemical Characterization of Pur/\textsubscript{H9252} Point Mutants: Self-association and Conformational Stability—Whereas the preceding biochemical analyses of Pur/\textsubscript{H9252} mutants support a role for residues Arg\textsuperscript{57} and Arg\textsuperscript{96} in ssDNA binding and protein-protein interaction, given the tendency of Pur proteins to selfassociate, we felt it important to determine whether these muta-
tions also affect the quaternary structure of the protein (40). As shown in Fig. 9A and B, calibrated SEC analysis of R57E/R96E and R57A/R96A double mutants relative to wild-type Pur/H9252 revealed striking differences in the elution profiles consistent with enhanced monovalent-salt insensitive oligomerization by the mutant proteins. The relative proportion of R57E/R96E versus R57A/R96A in oligomeric versus homodimeric states suggested that replacement of arginine with glutamate had a more destabilizing influence on Pur/H9252 structure, and perhaps local protein folding, than the corresponding alanine substitutions. This supposition was independently corroborated by results of CD spectroscopy. As shown in Fig. 9C, the CD spectrum of wild-type NiHis-Purβ showed negative-ellipticity bands at 208 and 222 nm, which are indicative of a protein with α-helical content. The dimeric fraction of R57A/R96A demonstrated a similar CD profile at 20 °C. In contrast, the spectrum of the SEC-fractionated R57E/R96E oligomer showed a marked decrease in negative-ellipticity at 208 and 222 nm, suggesting a loss in α-helical content, a feature which likely contributed to aberrant folding and aggregation. In support of the argument for structural instability imposed by mutation of the Arg57 and Arg96 residues, evaluation of the thermal unfolding of Pur/H9252 by CD spectroscopy revealed a 10 °C decrease in the melting temperature of the dimeric R57A/R96A mutant compared with the wild-type Purβ bound to MSY1. Normalized values were then fit to a four parameter logistic equation to calculate an apparent EC50 for each protein.
PAGE revealed single homogenous bands for the various NHis-Purβ preparations separated by SEC (supplemental Fig. S4). Moreover, functional analysis of the soluble SEC-fractionated oligomeric and dimeric mutants confirmed that loss of ssDNA- and MSY1 binding affinity correlated closely with oligomerization status (Fig. 10).

DISCUSSION

In view of the fundamental role played by SMαA in modulating the phenotypic properties of VSMCs, myofibroblasts, and cardiomyocytes (7–9, 48–50), an understanding of the regulatory mechanisms governing SMαA gene expression in these cell types is essential to devising treatment strategies aimed at limiting destructive cardiovascular remodeling and fibrosis. Previous work from our laboratories has suggested that functional interplay among transcriptional activators, repressors, and their respective cis-element binding sites in the 5′-flanking region of the gene accounts for the plasticity of SMαA expression in cultured aortic VSMCs, embryonic fibroblasts, and growth factor-induced myofibroblasts. Among the transcription factors associated with SMαA gene regulation in these cellular contexts, two members of the highly conserved Pur family of ssDNA/RNA-binding proteins have been identified as key players in promoter repression by virtue of both site-specific ssDNA-binding and/or protein-protein interactions (15–18).

Prior biochemical and promoter mutagenesis studies indicated that Purα and Purβ each bind specifically and with high affinity to the purine-rich strands of the PE and SPUR elements in a manner, which may inhibit TEF-1 or Sp1/3-mediated activation (16–18). In the case of the PE element, physical and functional interaction of Purβ with YB-1/MSY1, which preferentially binds to the opposing pyrimidine-rich
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FIGURE 10. Differential ssDNA and MSY1 binding by homodimeric versus oligomeric Purβ. A, biotinylated oligonucleotide corresponding to the purine-rich forward strand of the PE32 element was immobilized on streptavidin-coated microtiter wells at concentrations from 5.0-0.078 nM. A fixed amount of recombinant wild-type or mutant Purβ protein (0.5 nM) fractionated by SEC was added to each well. After an overnight incubation at 4 °C, wells were washed and solid phase Purβ nucleoprotein complexes were detected by ELISA. B, microtiter wells coated with 400-6.25 nM wild-type Purβ (WT) or size-fractionated point mutants R57/E956A or R57/E96A were incubated with 200 μg/ml nuclear protein extracted from AKR-2B MEFs overnight at 4 °C. Wells were washed, and solid phase MSY1-Purβ complexes were detected by ELISA with a primary MSY1 antibody. A and B, raw absorbance values (A) were corrected for nonspecific antibody binding by subtracting background signal generated in control wells incubated with buffer only (A0).

strand, appears to be required for complete core MCAT enhancer encryption. In this regard, DNA structure studies have shown that certain nucleotides both within and surrounding the genomic PE element exhibit increased hypersensitivity to modification by chloroacetaldehyde or potassium permanganate in response to SMA promoter activation by TGFβ 1 in cultured AKR-2B fibroblasts (44). Based on these and other findings, we proposed a model whereby simultaneous binding by Purα, Purβ, and YB-1/MSY1 was envisioned to facilitate disruption of PE strand base-pairing and consequent MCAT enhancer repression (16). Although the purported ability of Purα and YB-1 to promote helix destabilization/strand separation in vitro would seem to support this model (27, 51–53), recent gain-of-function and loss-of-function studies have pointed to Purβ as the critical factor in SMA promoter repression (18). Because Purα and Purβ are apparently capable of interacting with ssDNA under certain conditions as either homo- or heteromers (15), it is possible that such complexes have different transcriptional activities. Moreover, despite the fact that purified recombinant Purα and Purβ exhibit similar binding affinity and specificity for PE and SPUR sequences in vitro, nucleoprotein interaction studies reported here suggest that cis-element/promoter occupancy by Purβ exceeds that of Purα in AKR-2B MEFs (Figs. 1 and 2), a feature which may help explain why Purβ was found to be the principal repressor of SMαA transcription in earlier gene knockdown studies (18).

In terms of Purβ structure-function, initial deletion mutagenesis studies indicated that this protein is modularly organized with a central core ssDNA/RNA-binding domain and two putative regulatory regions at the N and C termini, respectively (Fig. 3). The polyglycine stretch at the N terminus of Purβ appears to be important for the expression and overall stability of the protein in cells and may play a role in augmenting ssDNA binding affinity in concert with certain C-terminal residues (19). Moreover, the C-terminal region spanning amino acids 264-324, which is essential for the SMαA repressor activity of the ectopically expressed protein in VSMCs (19), has also been implicated in the binding of Purβ to corepressors Purα and MSY1 (26). Such interactions are presumably important for stabilizing a multi-protein repressor complex in the ssDNA-forming region of the SMαA promoter (18, 44). An important regulatory role for the C-terminal domain of Purα in DNA binding has also been espoused (27) although structural differences within this region suggest that Purα and Purβ are unlikely to be functionally identical (25). This contention is supported by the observation that Purα is a much weaker repressor of the SMαA promoter relative to Purβ when ectopically expressed in VSMCs (19). However, both proteins demonstrate a similar level of gene repressor activity when overexpressed in fibroblasts further pointing to the significance of cell type in gauging the transcriptional properties of Pur proteins in vivo (17–19).

The defining structural feature of the Pur family of gene regulatory proteins is the minimal ssDNA/RNA-binding domain formed by the highly conserved class I and class II repeat sequences (20–22). Curiously, while Purβ requires all five repeats (amino acids 37–263) to bind to purine-rich ssDNA (19), the third basic aromatic motif near the C terminus is evidently dispensable for ssDNA binding by Purβ (23, 24). Although these deletion mutation experiments were critical to identifying the core elements responsible for nucleic acid-binding by Purα and Purβ, relatively few studies have addressed how individual amino acids within or outside the conserved class I and class II modules contribute to the ssDNA binding specificity, protein–protein interaction capacity, and/or transcriptional regulatory activity of these proteins. Building on our own work (19) and that of Wortman et al. (27), we engineered cDNA sequences encoding full-length Purβ mutants in which specific basic, acidic, aromatic, or sulfhydryl-containing residues, positionally-conserved with Purα or unique to Purβ, were changed to either glutamate or alanine. Selected single or double point mutants were then expressed in cultured A7r5 VSMCs or AKR-2B MEFs and evaluated for their ability to repress the activity of the full-length SMαA promoter (Figs. 4 and 5) or a
minimal composite enhancer construct (supplemental Fig. S2) in relation to wild-type Purβ. Results were striking in that the mutations at positions Arg57, Arg96, and Arg243 either singly or in combination yielded a protein which was defective in repressor activity in both cell types. Conversely, mutation of aspartic acid and phenylalanine residues at putative OB-fold positions or the two cysteine residues in the C-terminal region did not demonstrably alter Purβ repressor activity. The latter finding is consistent with the notion that oxidation of the two lone cysteine residues located in the third class I repeat and psycho motif (Fig. 3) to form a disulfide bond is not a critical structural and/or functional requirement inside a cell where reducing conditions are likely to predominate. Results of biochemical assays conducted with the purified C250A/C285A double mutant (Figs. 6 and 7) and wild-type Purβ acetylated with iodoacetamide (supplemental Fig. S5) support this contention as do prior studies suggesting that in vitro oxidation of Purβ is deleterious to protein function (40).

While the results of our transfection studies do not formally eliminate the possibility that Purβ may assume a tertiary conformation similar to other SSBs or form an intrachain disulfide bond under certain physiological conditions, the preponderance of experimental data pointed us in the direction of the arginine mutations as having that most profound effect on Purβ structural-function. As the terminal guanidinium group on the side chain of arginine is capable of interacting via hydrogen bonding with acceptor atoms on guanine bases or via electrostatic contact with the phosphate oxygens of the DNA backbone, we predicted that these mutations would impair ssDNA binding in a manner similar to that which was previously reported for Purα (27). This hypothesis was seemingly validated for the repressor-defective R57E, R57E/R96E, and R57A/R96A mutants, which demonstrated reduced ssDNA binding affinity relative to wild-type Purβ (Figs. 6 and 10). The biochemical findings with the R243A mutant were quite different and implied a deficiency unrelated to ssDNA binding. This prompted us to evaluate the protein-protein interaction capacity of recombinant mutants in vitro as well as their apparent subcellular distribution in transiently transfected cells. R243A was unique in that this mutant retained significant Purα and YB-1/MSY1 binding capacity but exhibited comparatively weak nuclear expression in early passage A7r5 cells (supplemental Fig. S1). Hence, reduced stability and/or altered intracellular trafficking may explain why this mutant did not repress the SMoA promoter. On the other hand, mutants R57E, R57E/R96E, and R57A/R96A each showed robust nuclear expression but were also clearly defective in binding to Purα and YB-1/MSY1 in vitro (Figs. 7, 8, and 10). The latter result was both novel and thought-provoking because the paradigm established for the Purα protein only envisioned a role for these positionally conserved residues in local interactions with ssDNA (27). This conundrum compelled us to ascertain whether mutagenesis imposed a more extensive alteration in Purβ structure that could account for the multiple functional deficiencies conferred by changing two arginine residues.

A key observation that implied a molecular level defect in mutants R57A/R96A and R57E/R96E was made on the basis of calibrated SEC of the purified proteins. As we recently reported, recombinant mouse Purβ exists as an asymmetric homodimer in solution at protein concentrations in the micromolar range and exhibits a characteristic elution profile when analyzed by SEC (40). In contrast, mutants R57A/R96A and R57E/R95E exhibited salt-insensitive elution profiles consistent with anomalous oligomerization into higher order quaternary states (Fig. 9). Ensuing CD and biochemical analyses of the SEC-fractionated species suggested that the tendency of the mutants to aggregate was likely due to alteration of protein secondary structure leading to conformational instability and ultimately loss-of-function (Figs. 9 and 10). The extent of this instability was further validated by comparing the thermal unfolding of wild-type Purβ to both double mutants. Despite the fact that wild-type Purβ and the R57A/R96A mutant show similar CD spectra at 20 °C, the proteins exhibit distinctly different melting temperatures reinforcing the idea that structural instability is the probable cause of the functional deficits seen in transfection and nucleoprotein binding assays.

In conclusion, our findings establish a role for certain positionally conserved arginine residues in ensuring the structural and functional fidelity of Purβ. Future studies will be directed toward testing whether the same rules apply to Purα and how manipulation of these and other residues in the minimal ssDNA/RNA-binding domain affects folding, self-association, and nucleoprotein interaction. A better understanding of the structural features that account for the conformational stability of each protein will undoubtedly aid in resolving apparent discrepancies in the physical and functional properties attributed to Purα and Purβ. Such knowledge will also be necessary to facilitate the rational design of peptide- and/or nucleic acid-based inhibitors, which will permit a more detailed evaluation of the efficacy of Pur proteins as pharmacological targets in cardiac, skeletal, and vascular cell types.

Acknowledgments—We thank students Adam Lothrop, Nicholas Weir, and Jalanta Amblo for their assistance in performing ChIP and SEC experiments during research rotations in the Kelm laboratory. We also wish to extend special thanks to Dr. Norma J. Greenfield (UMDNJ Circular Dichroism Facility) for performing the CD analyses and helpful discussions regarding data interpretation. We are grateful to Dr. Doug Taatjes and Marilyn Wadsworth (UVM Microscopy Imaging Center) for providing technical training and advice on confocal microscopy, data acquisition, and image analysis.

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