Purα and Purβ are structurally related single-stranded DNA/RNA-binding proteins implicated in the control of cell growth and differentiation. The goal of this study was to determine whether Purα and Purβ function in a redundant, distinct, or collaborative manner to suppress smooth muscle α-actin gene expression in cell types relevant to wound repair and vascular remodeling. DNA interference-mediated loss-of-function analyses revealed that, although Purβ was the dominant repressor, the combined action of endogenous Purα and Purβ was necessary to fully repress the full-length smooth muscle α-actin promoter in cultured fibroblasts but to a lesser extent in vascular smooth muscle cells. The activity of a minimal core enhancer containing a truncated 5′ Pur repressor binding site was unaffected by knockdown of Purα and/or Purβ in fibroblasts. Conversely, gain-of-function studies indicated that Purα or Purβ could each independently repress core smooth muscle α-actin enhancer activity albeit in a cell type-dependent fashion. Biochemical analyses indicated that purified recombinant Purα and Purβ were essentially identical in terms of their binding affinity and specificity for GGN repeat-containing strands of several cis-elements comprising the core enhancer. However, Purα and Purβ exhibited more distinctive protein interaction profiles when evaluated for binding to enhancer-associated transcription factors in extracts from fibroblasts and vascular smooth muscle cells. These findings support the hypothesis that Purα and Purβ repress smooth muscle α-actin gene transcription by means of DNA strand-selective cis-element binding and cell type-dependent protein-protein interactions.

Phenotypic reprogramming of adventitial fibroblasts and medial vascular smooth muscle cells (VSMCs) plays a critical role in arterial remodeling associated with de novo atherosclerosis, restenosis after percutaneous coronary intervention, transplant arteriosclerosis, and vein-graft disease (1–5). Although the notion of phenotypic modulation of vascular cell types has existed for several decades, the molecular mechanisms leading to pathologic changes in cell migration, proliferation, or apoptosis are largely unknown. Among the well characterized markers of VSMC differentiation status, smooth muscle α-actin (SMAα) is unique in that its physiological role is not entirely restricted to regulation of vascular contractility (6). Indeed, experimental evidence indicates that reduced levels of SMAα may alter the tissue-invasive and motile properties of certain fibroblastic cell types in vivo (7, 8) and may be a contributing factor in elevating the risk of plaque rupture in vulnerable fibroatheroma in vivo (9, 10). On the other hand, enhanced SMAα expression is crucial to myofibroblast differentiation and physiological wound repair (11, 12) and, together with other contractile proteins such as smooth muscle myosin, may limit neointimal hyperplasia during vascular remodeling induced by mechanical injury or proatherogenic stimuli (13–15). Thus, identification of factors that modulate SMAα expression in myofibroblasts and VSMCs could provide useful targets for therapies designed to either stabilize, or perhaps favorably alter, the phenotypic composition of vascular lesions.

Among the multitude of transcription factors implicated in SMAα gene regulation (16, 17), a group of functionally novel single-stranded DNA (ssDNA)-binding proteins known as Purα, Purβ, and MSY1 have been reported to restrain muscle-CAT (MCAT) enhancer-dependent SMAα gene transcription in both fibroblasts and VSMCs (18). These repressor proteins were originally identified based on their ability to interact with opposing strands of a highly conserved, asymmetric polypurine-polypyrimidine (Pur/Pyr) tract located in the 5′ region of the mouse SMAα gene (19). In its double-stranded configuration, the Pur/Pyr sequence contains a consensus MCAT enhancer motif, which serves as a recognition site for transcription enhancer factor 1 (TEF-1) (18). Importantly, computer modeling of the extended Pur/Pyr tract coupled with ssDNA-selective chemical modification of genomic DNA in cultured fibroblasts indicated that this region has the propensity to assume a partially unpaired configuration in response to transforming growth factor β1 (TGFβ1) signaling (20). These findings coupled with results of in vitro DNA binding and cell-based SMAα promoter mutagenesis studies, led to the development of two alternative models for cryptic MCAT enhancer regulation. The salient feature of both models involves ssDNA-specific interactions by endogenous Purα, Purβ, and MSY1, which either mask TEF-1 from other components of the transcription machinery or alter cis-element structure in such a way as to preclude TEF-1 binding to double-stranded DNA (dsDNA) (18). Although speculative, the latter scenario is plausible because several independent studies have demonstrated that Pur and Y-box proteins are...
individually capable of promoting DNA strand separation in vitro (21–24). However, based on more recent work, it is now clear that Purα and Purβ have additional targets and mechanistic roles to play in suppressing smooth and cardiac muscle-specific gene transcription depending upon the cell type studied, Pur expression levels, and/or TGFβ1 signaling (25–27).

There are four known Pur family members in mice and men, Purα, Purβ, and two isoforms of Purγ (28–30). Although a comprehensive analysis of the tissue expression of Pur paralogs at the protein level has not yet been reported, evidence from RNA transcript analysis suggests that Purα and Purβ are ubiquitously expressed while Purγ exhibits a more restricted distribution (30). All Pur family members exhibit a substantial degree of homology particularly in domains that confer nucleic acid binding capacity (25, 31, 32). By virtue of their ability to interact with high affinity and specificity with single-stranded nucleic acid sequences rich in purine nucleotides of the form (GGN)ₙ, Pur proteins appear to have evolved to regulate cellular processes essential to genetic metabolism, including DNA replication and transcription, RNA transport and localization, and miRNA translation (33). The founding member of this family, Purα, has also been implicated in regulation of cell cycling and proliferation in vitro (34–38) and in brain and myeloid development in vivo (39). Despite their structural relatedness, it is unknown at present whether Purα and Purβ are functionally redundant or distinct. Hence, the objective of this study was to perform a rigorous comparative evaluation of mouse Purα and Purβ with the use of gain-of-function and loss-of-function strategies in SMαA-expressing cell types. In combination with results of biochemical assays conducted with purified recombinant proteins, our findings provide insight into the physical and functional interactions that account for the ability of Purα and Purβ to repress SMαA gene expression in a cell-type-specific manner.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transient Transfection, and Reporter Gene Assay—**Aortic segments from C57BL/6 mice were obtained following protocols approved by the University of Vermont Institutional Animal Care and Use Committee. VSMCs were isolated by cell outgrowth from aortic tissue explants and characterized as previously described (40, 41). Primary VSMCs were cultivated in a 90% air/10% CO₂ incubator at 37 °C in growth medium consisting of Dulbecco’s modified Eagle’s medium, 1 mM insulin-transferrin-selenium supplement (Invitrogen), and 20% v/v heat-inactivated fetal bovine serum (FBS, HyClone). For transient transfection studies, primary VSMCs were seeded in six-well plates at a density of 10,000 cells/well and transfected with the use of jetPEITM reagent at a ratio of 2 µl/µg of plasmid DNA as directed by the manufacturer (Qiagen). AKR-2B mouse embryonic fibroblasts (MEFs) or rat A7r5 VSMCs were cultured and transiently transfected as previously described (42). Briefly, subconfluent AKR-2B or A7r5 cells seeded in 60-mm dishes were transfected with the use of GenePORTER™ reagent (Gene Therapy Systems) at a ratio of 3 µl/µg of plasmid DNA. After 48-h incubation in growth medium, cells were washed with phosphate-buffered saline and extracted using 1X reporter lysis buffer (Roche Applied Science) supplemented with protease inhibitors. In some cases, transfected cells were growth-arrested by incubation in serum-free medium for 48 h then re-stimulated with either 20% FBS or 5 ng/ml TGFβ1 (R&D Systems) for 6 h prior to harvesting cell lysates. Total protein in transfected cell lysates was determined by bicinchoninic acid assay (Sigma) using bovine serum albumin (BSA) as a protein standard. Commercial immunoassays were used to measure chloramphenicol acetyltransferase (CAT) or β-galactosidase reporter proteins as directed by the manufacturer (Roche Applied Science). Reporter values were corrected for total protein content. Transfections were typically performed in triplicate and repeated two to three times to ensure reproducibility. Data sets were subjected to one-way analysis of variance to identify differences among group means at the p < 0.05 significance level. Post-hoc comparisons were performed using the Bonferroni adjustment for multiple comparisons.

**Construction of Promoter-Reporter Plasmids and Expression Vectors—**Mouse SMαA promoter-reporters and mammalian expression plasmids encoding His-epitope-tagged versions of mouse Purα and Purβ were described previously (18). A methodological description of the design and construction of plasmid- and lentiviral-based short hairpin RNA (shRNA) expression vectors targeting mouse Purα or Purβ can be found in the supplemental material. All plasmids used for transfection were purified from *Escherichia coli* cultures by double cation chloride gradient centrifugation.

**Western Blotting of Transgene-expressed and Endogenous Proteins—**Ectopic expression of His-tagged Purα or Purβ was monitored by Western blotting of lysed cell protein with an RGS(H)4 monoclonal antibody (Qiagen) as previously described (25). Expression of endogenous Pur proteins was similarly assessed with a rabbit polyclonal antibody that recognizes a conserved sequence present in both mouse Purα and Purβ (19). Commercial monoclonal antibodies were used for detection of SMαA (clone 1A4, Sigma) and glyceraldehyde-3-phosphate dehydrogenase (clone 6C5, Research Diagnostics Inc.).

**Purification of His-Pura and His-Purβ Expressed in E. coli—**Bacterial expression constructs encoding mouse Purα and Purβ with C-terminal His epitope tag were described previously (19). Detailed methods used for the expression, purification, and quality control of His-tagged Purα and Purβ are presented in the supplemental materials. His-tagged MSY1 expressed in *E. coli* was purified and quantified as previously described (19).

**Biochemical Assays for DNA Binding and Protein-Protein Interaction—**A discontinuous solid-phase DNA binding assay was performed as previously described with some minor modifications (43). Recombinant His-Purα or His-Purβ was diluted to a final concentration of 50 nM in coating buffer consisting of 20 mM HEPES, pH 7.5, 150 mM NaCl, 5 µg/ml BSA. Proteins were passively adsorbed to microtiter wells (Costar® ELA/RIA plate, 96-well Easypath™ high binding) by applying 100 µl of coating solutions and incubating overnight at 4 °C for 16–24 h. Coating solution was removed by aspiration, and wells were rinsed twice with wash buffer consisting of 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.05% v/v Tween 20. Blocking buffer containing 2% w/v BSA (Sigma, ELA grade) dissolved in HEPES buffer was then applied to each well. After 1-h incubation at room temperature, blocking buffer was removed and 100 µl of fluid phase competitor (Purα or Purβ or unlabeled oligonucleotide) diluted in binding buffer (0.2% w/v BSA dissolved in wash buffer) was applied in triplicate. After 10-min incubation at 37 °C, 100 µl of 2 nM 3'-biotinylated ssDNA probe diluted in binding buffer was added. Plates were incubated for 1 h at 37 °C. Binding solutions were removed by aspiration, and wells were washed three times. This was followed by addition of 100 µl of ExtraAvidin-HRP (Sigma) diluted 1:2000 in wash buffer to each well and incubation for 30 min at 37 °C. Wells were washed three times, and 100 µl of ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]) substrate solution (Chemicon) was added. After 15-min incubation at room temperature, absorbance readings at 405 nm were obtained with a microplate reader. In another set of experiments, the DNA binding assay was performed the same way as described above except that the fluid phase contained dif-
Functional Consequences of Purα or Purβ Knockdown in Fibroblasts and VSMCs— To investigate the functional properties of endogenous Purα and Purβ in cultured fibroblasts and VSMCs, a loss-of-function approach utilizing RNA interference was undertaken. The effectiveness and specificity of shRNA-mediated knockdown of both exogenous and endogenous Purα and Purβ in fibroblasts or VSMCs was confirmed by transfection and immunoblot analyses (see supplemental data, Fig. S1). Given previous studies indicating that these proteins function as corepressors of the SmxA promoter (18), we hypothesized that knockdown of Purα and/or Purβ in non-differentiated fibroblasts would result in promoter activation. A series of co-transfection experiments was conducted in AKR-2B MEFs with the use of a full-length SmxA promoter-CAT reporter known as VSMP8 (Fig. 1A) and selected Purα- or Purβ-specific shRNA expression plasmids. The VSMP8 promoter construct was chosen, because it contains mouse SmxA sequence (−1074 through the first intron) required for smooth muscle-specific transcriptional activity (boxes) and binding sites (PE, THR, and SPUR) for Purα/Purβ (18, 26). VSMP4 lacks the −2.5-kbp intron 1 (triangle), 5′-flanking region from −1074 to −192, and is transcriptionally activated due to exposure of a cryptic MCAT enhancer (ΔK1) (18). B, specific and dose-dependent activation of VSMP8 by Purβ shRNA-I. AKR-2B cells were co-transfected with 2.8 μg of VSMP8, 0.2 μg of pCMVβ, selected amounts of plasmid encoding Purβ shRNA-I or scrambled control sequence, and filler DNA to 5.0 μg/dish. Whole cell extracts were prepared 48 h after transfection and assayed for total protein and CAT reporter. C, extracts of AKR-2B MEFs transduced with lentiviral vectors encoding Purβ shRNA-I (lane 1) or scrambled sequence (lane 2) were analyzed by Western blotting for detection of Purβ and Purβ (10 μg of protein/lane) or SmxA (1 μg of protein/lane). Note that the faster migrating Purβ band is specifically reduced while SmxA is increased in shRNA-expressing cells. The SmxA blot was reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. Numbers on the left denote the size of prestained protein markers in kilodaltons. D, de-repression of the SmxA promoter by Purβ shRNA-I is promoter context-dependent. AKR-2B cells were co-transfected with VSMP8 or VSMP4 = 1 μg of the indicated shRNA plasmids then assayed as described above. B and D, promoter activity is expressed as CAT reporter divided by total cell protein (mean ± S.D.). *, p < 0.025; **, p < 0.01 compared with reporter only control.
FIGURE 2. De-repression of the SmαA promoter in MEFs and VSMCs in response to knockdown of Purα and/or Purβ and the requirement for Pur/Pyr element integrity. A and B, AKR-2B MEFs or primary C57BL/6J VSMCs were transiently transfected with 2.8 μg of VSMP8 or VSMP4 reporters, 0.2 μg of pCMVβ, and 1.0 μg of expression plasmid encoding the indicated shRNA or scrambled control sequence. pBLCAT3 was used as filler to equalize the amount of DNA transfected at 5.0 μg/dish. Whole cell extracts were prepared 48 h after transfection and assayed for total protein and CAT reporter. To compare the relative effect of shRNAs or scrambled controls on each promoter, corrected CAT values were normalized to values obtained in transfectants in which VSMP8 or VSMP4 were co-transfected with pBLCAT3 filler DNA only (control activity defined as 1 for each reporter). Results are expressed as -fold de-repression (mean ± S.E.). **, p < 0.01; ***, p < 0.001 compared with reporter only control.

Inset, AKR-2B MEFs were co-transfected with the indicated SmαA promoter-reporter constructs and a combination of Purα plus Purβ shRNA-I plasmids or scrambled control plasmids then assayed as described above. To assess the relative level of shRNA-mediated induction of each reporter, CAT values obtained in Purα plus Purβ shRNA-I co-transfectants were divided by CAT values measured in scrambled control co-transfectants. Results are expressed as relative promoter induction (mean ± S.E.). **, p < 0.01; ***, p < 0.001 compared with VSMP4. C and D, deletion mutants of the SmαA Pur/Pyr element were tested for their ability to compete for binding of full-length biotinylated ssDNA probe (ssPE32-bF, −195 to −164 5'-GGGAGCAGAACAGAGGAATGCAGTGGAAGAGA-3' -biotin, 1 nM) to purified recombinant Purα or Purβ in a solid-phase colorimetric ssDNA binding assay. Truncating competitor oligonucleotide to −191 reduced competition efficiency by −10-fold.
transgene expression in vivo (45) and, as such, exhibits very weak transcriptional activity in transfected AKR-2B cells due, in part, to negative regulation by endogenous Pur repressor proteins. As shown in Fig. 1B, co-transfection of Purβ shRNA-I resulted in a dose-dependent increase in VSMP8 promoter activity, whereas a scrambled control construct (Purβ Scm) had no effect on this reporter. Corroborative results were obtained in lentiviral-transduced AKR-2B MEFs where stable shRNA-mediated knockdown of Purβ augmented SmαA protein expression (Figs. 1C and S1, see supplemental data).

To evaluate whether the effect of Purβ knockdown was promoter context-dependent, a truncated SmαA reporter known as VSMP4 was also tested for responsiveness to Purβ deficiency. As previously documented (18), this mutant reporter exhibits unrestricted MCAT enhancer activity due to the absence of 5′-nucleotides required for strand-specific Pur/Pyr element recognition by endogenous Pur repressors (see Fig. 1A for schematic). Hence it was not surprising to find that expression of Purβ shRNA-I had no discernible effect on the transcriptional activity of VSMP4 in transfected MEFs (Fig. 1D). To assess whether knockdown of Purα would yield analogous results, shRNA constructs demonstrating the maximum efficiency of Purα or Purβ knockdown (Fig. S1). Purα shRNA-I and Purβ shRNA-I were transfected either individually or in combination into AKR-2B MEFs together with VSMP8 or VSMP4 reporters (Fig. 2A). Knockdown of Purα alone resulted in a modest ~2-fold enhancement in VSMP8 activity, whereas knockdown of Purβ alone induced VSMP8 by ~4-fold. Importantly, a synergistic response (~12-fold activation over reporter only control) was observed in AKR-2B MEFs expressing both Purα and Purβ shRNAs implying that endogenous Pur repressors likely function in a collaborative manner to regulate the transcriptional activity of the full-length SmαA promoter in this cell type. Transient transfection of the same Purα or Purβ shRNA constructs in primary mouse VSMCs yielded similar results, although the extent of de-repression was less pronounced than seen in AKR-2B MEFs (Fig. 2B). Relative to VSMP8, VSMP4 exhibited little or no responsiveness to combined Purα and Purβ knockdown in both cell types. Evaluation of other SmαA reporter constructs containing differing lengths of 5′-flanking region indicated that significant promoter induction in response to co-knockdown of Purα and Purβ in AKR-2B cells minimally required 5′-sequence extending to ~195 (Fig. 2A, inset, compare A195 to VSMP4). This result coincides with biochemical studies indicating that high affinity binding by purified Purα or Purβ to the purine-rich strand of a Pur/Pyr element is impaired by deleting the GGGA motif from ~195 to ~192 (Fig. 2, C and D).

Functional Consequences of Purα or Purβ Overexpression in Fibroblasts and VSMCs—In an earlier study, we reported that Purα and Purβ demonstrate vastly different repressor activity toward the full-length VSMP8 reporter when ectopically expressed in rat A7r5 VSMCs (25). To ensure that this finding was not entirely cell line-dependent, analogous transfection studies were conducted in exponentially growing primary mouse VSMCs. As shown in Fig. 3, whereas Purα showed potent repressor activity toward VSMP8, Purβ failed to significantly inhibit the promoter. This result was not due to an overt difference in transgene-mediated expression of Purα or Purβ shRNA constructs primary mouse VSMCs and Purβ in AKR-2B cells minimally required 5′-sequence extending to ~195 (Fig. 2A, inset, compare A195 to VSMP4). This result coincides with biochemical studies indicating that high affinity binding by purified Purα or Purβ to the purine-rich strand of a Pur/Pyr element is impaired by deleting the GGGA motif from ~195 to ~192 (Fig. 2, C and D).

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were performed in AKR-2B MEFs and A7r5 VSMCs. Consistent with results obtained in primary VSMCs, Purβ showed more potent VSMP4 inhibitory activity than Purα when overexpressed in A7r5 cells (Fig. 4A). However, exogenous Purα and Purβ exhibited equally strong repressor activity toward VSMP4 in AKR-2B cells (Fig. 4B). Comparable dose-dependent expression of His-Purα or His-Purβ in each cell type was confirmed by Western blotting (insets of Fig. 4, A and B). Adjusting cell culture conditions did not appear to influence the cell type-dependent activity of exogenous Purα or Purβ, because similar results were obtained in synchronized cells that were either serum-starved or stimulated with serum or TGFβ1 after growth arrest (Fig. 4, C and D). Relative to A7r5 cells, which exhibit a stable adult VSMC phenotype (46), the VSMP4 reporter predictably showed a higher degree of serum and TGFβ1 inducibility in synchronized AKR-2B cells consistent with transition to a myofibroblast-like phenotype (20). In growth factor-stimulated AKR-2B MEFs, exogenous Purα and Purβ were equally effective in attenuating VSMP4 induction (Fig. 4D). In contrast, only Purβ inhibited VSMP4 activity in synchronized A7r5 VSMCs, whereas Purα had a
modest potentiating effect on VSMP4 in serum-free and TGFβ1-treated cells (Fig. 4C).

**Binding Affinity and Specificity of Recombinant Purα and Purβ for SmMAA-derived cis-elements**—In an attempt to uncover a physical basis for the differences in transcriptional activity of Purα and Purβ observed in gain-of-function studies, quantitative nucleoprotein interaction assays were conducted in which biotinylated DNA probes in either single- or double-stranded configurations were assessed for binding to His-Purα or His-Purβ immobilized on microtiter wells. Care was taken to ensure that purified preparations of recombinant Purα and Purβ were devoid of contaminating nucleic acid and functional in terms of DNA binding in solution (supplemental Figs. S2 and S3). As shown in Fig. 5 (A and B), His-Purα and His-Purβ exhibited identical DNA-binding properties when probed with either the purine-rich strand (ssPE30-bf) or complementary pyrimidine-rich strand (ssPE30-bR) of a minimal 30-nucleotide SmMAA Pur/Pyr element. High affinity binding (apparent $K_d \sim 1.7$ nM) was observed to the purine-rich strand, whereas weaker and non-saturable binding characterized the interaction of His-Purα and His-Purβ with the pyrimidine-rich strand. The $A_{405}$ nm readings generated with the double-stranded form of the Pur/Pyr element containing the canonical MCAT motif was near or below background (BSA-only-coated wells) consistent with the notion that Pur proteins preferentially bind to single-stranded nucleic acid sequences rich in purine residues. As a control, purified His-tagged MSY1 was also tested against the same probes. In agreement with past findings (18, 19), recombinant MSY1 demonstrated specific and high affinity interaction with the pyrimidine-rich strand only (Fig. 5C).

Results of previous SmMAA promoter-reporter transfection studies coupled with in vitro DNA-binding assays using cell extract-derived proteins suggested that Pur proteins can physically and functionally interact with other cis-elements in the core enhancer region of the SmMAA gene. These sites include a TGFβ1-hypersensitive region (THR, −176 to −145) (47) and a Sp1/3 and Purα/β-interacting element (SPUR, −59 to −28) (26). To quantitatively characterize how Pur proteins associate with each of sites relative to the MCAT-containing Pur/Pyr element (−195 to −164), biotinylated DNA probes of equivalent 32 nucleotide length in either single- or double-stranded configurations were evaluated for binding to recombinant mouse Purα or Purβ by colorimetric assay (Fig. 6). Results indicated that Pur proteins preferentially interact with the purine-rich strand of each of these elements albeit with distinctly different affinities (ssSPUR32-bf ≈ ssPE32-bf > ssTHR32-bR from highest to lowest affinity, apparent $K_d \sim$1 nM to 20 nM. Purα or Purβ binding to double-stranded PE32, SPUR32, and THR32 probes in which the high affinity strand was annealed to its lower affinity biotin-labeled complement was either not detectable or very weak under the assay conditions employed. Although Purα and Purβ exhibited comparable affinities for each purine-rich ssDNA probe tested, a significant difference was noted in terms of apparent binding capacity ($A_{405}$ reading at saturation) for PE and SPUR versus the THR element. Although disparities in surface orientation of the biotin moiety in the solid-phase ssDNA-protein complex could account for such a result, an equally plausible interpretation is that the SPUR and PE interaction stoichiometry may differ from that of the THR.

**Interaction Profile of Recombinant Purα and Purβ with SmMAA Promoter-associated trans-Acting Factors**—Results of DNA binding studies utilizing purified recombinant proteins suggested that Purα and Purβ are indistinguishable in terms of affinity and specificity for relevant SmMAA cis-elements in vitro. To ask whether the same is true for known MCAT, CArG, THR, and SPUR/TCE element-binding trans-acting factors, ELISA-based profiling of Purα and Purβ interaction partners was conducted using protein extracts from proliferating Akr-2B and A7r5 cells. In this assay system, equimolar amounts of recombinant His-Purα or His-Purβ were immobilized on microtiter wells. A control ELISA performed with the use of a pan Pur polyclonal antibody verified that the coating efficiencies of His-Purα and His-Purβ were virtually identical over a broad range of concentrations (supplemental Fig. S4). A surface-saturating coating concentration of 100 nM was selected for all subsequent protein interaction studies in which a fixed amount of nuclear or cytosolic protein extract from either fibroblasts or VSMCs was incubated with wells pre-coated with His-Purα or His-Purβ. BSA-only-coated wells served as a control for binding speci-
ficity. After washing to remove unbound proteins, solid-phase transcription factor-Pur complexes were detected by ELISA with the use of primary antibodies specific for MSY1, TEF-1, SRF, Sp1, Sp3, pRB, or Smad2/3 followed by the appropriate HRP-coupled secondary antibody for colorimetric signal generation. As a further control for the fidelity of this detection system, each antibody was also independently screened for cross-reactivity with immobilized Pur/H9251 or Pur/H9252 in the absence of added cell extract. This was done so that any background signal due to nonspecific primary or secondary antibody binding could be subtracted from absorbance readings obtained in Purα- or Purβ-coated wells incubated with cell extract. To account for the variability inherent in cell extract preparation, binding profiles were determined using at least two different preparations from each cell type.

As shown in Fig. 7, protein interaction profiles for Purα or Purβ were dependent upon the cell type and subcellular compartment from which factors were derived. Striking differences between Purα and Purβ in
FIGURE 7. Transcription factor interaction profile of recombinant Purα and Purβ in different cell types. Microtiter wells coated with recombinant His-tagged Purα or Purβ were incubated with a fixed amount of nuclear protein (200 μg/ml) extracted from either AKR-2B fibroblasts (A) or A7r5 VSMCs (B). After 3-h incubation, wells were washed and relative binding of selected proteins to immobilized Purα or Purβ was determined with the use of primary rabbit or goat polyclonal antibodies against the indicated proteins by ELISA. Bars show corrected A405 readings after subtracting absorbance due to nonspecific binding to BSA-only-coated wells (mean ± S.E.). Primary rabbit polyclonal antibodies SRF (G-20), Sp1 (PEP2), and Sp3 (D-20) or goat polyclonal antibodies against Smad2/3 (E-20) and pRB (M-15) were obtained from Santa Cruz Biotechnology, Inc. Rabbit polyclonal antibodies recognizing MSY1 amino acids 242–267 and mouse TEF-1 amino acids 1–15 were generated in our laboratory (19). Insets, representative profiles generated with the use of cytosolic extracts from the same cell type are shown for comparison (mean ± S.E.).
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terms of binding capacity for certain factors were evident both within and between cell types. For example, MSY1 demonstrated a 2-fold greater preference for Purβ than for Purα in nuclear extracts from both AKR-2B MEFs and A7r5 VSMCs (Fig. 7, compare A and B). Relative to Purα, Purβ also demonstrated increased binding activity toward nuclear Sp3 in both cell types and nuclear Sp1 particularly in A7r5 cells. Curiously, these differences were less pronounced or entirely absent when cytosolic proteins were used as a source of Pur interacting factors (compare insets in Fig. 7, A and B). In fact, Purα exhibited a trend toward preferential binding to cytosol-derived TEF-1, Sp3, and Smad2/3, whereas Purβ preferred to associate with the nuclear forms of these proteins in AKR-2B cells (Fig. 7A). In contrast, Purα and Purβ were comparable in their abilities to bind TEF-1, SRF, and Smad2/3 present in the nucleus of A7r5 cells. The MSY1, TEF-1, and Sp3 interaction profiles of Purα and Purβ were virtually identical using cytosol-derived proteins from A7r5 cells (Fig. 7B). Consistent with the rapid growth status of cells at the time of extract preparation, interaction of pRB with Purα or Purβ was not detectable in this assay system.

DISCUSSION

Mature Purα and Purβ are structurally related proteins exhibiting ~70% sequence identity at the amino acid level (28). The highest level of similarity exists within the central modular region of each molecule where the minimal nucleic acid-binding domain resides (25, 29, 48). Interestingly, sequence elements within the more divergent amino- and carboxyl termini have been implicated in providing stability and/or specificity of Purα or Purβ interaction with certain protein and/or nucleic acid-binding partners (25, 29). This has led us to speculate that certain nucleoprotein interactions modulated by Pur paralog-specific sequence elements may account for the distinct transcriptional properties ascribed to Purα and/or Purβ in different cell types and promoter contexts (49–60). However, because most cell-based studies to date have focused on a single Pur paralog, we decided to conduct an in-depth comparative analysis of the physical and functional properties of Purα and Purβ in the context of SMαA gene regulation in fibroblasts and VSMCs.

Experimental evidence from multiple laboratories indicates that Purα and Purβ can associate with purine-rich ssDNA in a sequence-specific fashion as a monomer, dimer, or possibly higher oligomers depending upon the sequence context of the target element and in vitro binding conditions (19, 24, 61). Self-association of Purα (62) and interaction between Purα and Purβ in the absence of ssDNA has also been reported (19, 43), although the biophysical parameters of Pur oligomerization and relevance to nucleic acid binding remains to be determined. Nonetheless, based on these known physical properties, negative regulation of the MCAT enhancer in the SMαA promoter could conceivably occur by either the independent or combined action of Purα and Purβ. To resolve this issue, we turned to RNA interference as a means of testing whether only one or both of the endogenous Pur proteins was required to maintain the full-length promoter in a repressed state in fibroblasts as suggested by previous cis-element mutation studies (18). Results indicated that, whereas Purβ appeared to play a dominant role in repression, Purα also contributed to a significant extent under conditions in which Purβ expression was knocked down simultaneously and an intact Pur/Pyr element between −195 and −165 was present in the promoter (Figs. 1 and 2). These observations are consistent with earlier in vivo footprinting analyses that showed the genomic SMαA region spanning −210 to −150 to be a hotspot for modification by chemical agents that selectively target unpaired bases (20). In short, new findings based on RNA interference-mediated loss-of-function offer proof of principle that Purα and Purβ do indeed function as authentic repressors of the SMαA promoter despite their preferential affinity and specificity for ssDNA sequences. Independent support for the notion that Pur proteins have the intrinsic ability to recognize and/or induce formation of non-B-DNA structures in the regulatory regions of certain genes is also provided by in vitro footprinting studies of functional Pur recognition elements located in the c-myc P1 and PDGF-A gene promoters (24, 59).

Although functional cooperation between endogenous Purα and Purβ is consistent with the previously proposed model of cryptic MCAT enhancer regulation (18), synergistic activation of the full-length SMαA promoter in response to combined knockdown of both factors in AKR-2B cells implied the possible unmasking of additional cis-elements and associated trans-activators under negative Pur control in fibroblasts (26, 47). Such a scenario may explain the lesser response observed in primary VSMCs where knockdown of Purα and/or Purβ resulted in only modest induction of the full-length SMαA promoter. An alternate possibility is that the steady-state levels of endogenous Purα and Purβ were suboptimal for robust gene repression in differentiated VSMCs, a setting in which the SMαA promoter would be expected to be in a constitutively activated state. To test this idea, we performed gain-of-function experiments and found that forced expression of Purβ, but not Purα, repressed the full-length promoter and the minimal core SMαA enhancer in primary mouse VSMCs and clonal rat A7r5 cells (Figs. 3 and 4). In the case of Purα overexpression, definitive repressor activity was restricted to the core enhancer in AKR-2B MEFS in a manner consistent with gain-of-function analysis of Purα in other fibroblast cell lines (26). These results suggested that exogenous Purα and Purβ regulate transcription by cell type- and promoter context-specific mechanism(s). In support of this contention, forced expression of Purα actually increased core enhancer activity in growth-arrested or TGFβ1-treated A7r5 VSMCs but not in asynchronous or serum-treated cultures of the same cell type (Fig. 4). Moreover, in keeping with the neutral action of exogenous Purα in asynchronous VSMCs, co-expression of Purα had no substantive effect on Purβ-mediated repression of the full-length SMαA promoter in A7r5 cells (supplemental Fig. S5). This contrasted with the co-repressor activity demonstrated by MSY1 when co-expressed with Purβ in the same cell type (25). Taken together, findings based on gain-of-function and loss-of-function assays underscore the importance of taking into account cell type, culture conditions, promoter context, and cofactor requirements when attributing transcriptional regulatory activity to endogenous or exogenous Pur proteins.

To more rigorously define the molecular targets through which Pur proteins repress SMαA promoter activity, we endeavored to characterize the interaction properties of purified recombinant Purα and Purβ with the use of cell-free DNA and protein binding assays in vitro. In the case of DNA binding, experiments were conducted with biotinylated probes corresponding to specific cis-regulatory sequences present in SMαA 5′-flanking region previously shown to serve as binding sites for cell extract-derived Purα/β by qualitative band shift or pull-down assays (18, 26, 47). Pilot studies indicated that reliable measurement of binding affinity of Purα and Purβ for ssDNA in vitro requires that care be taken to prevent fortuitous nucleic acid contamination in recombinant protein preparations (supplemental Figs. S2 and S3). Although seemingly a mere technical issue, the presence of extraneous nucleic acid may explain some discrepancies in the literature regarding the reported binding affinity, specificity, and/or stoichiometry of Pur family members for certain single-stranded nucleic acid sequences (24, 25, 63, 64). Our quantitative analyses revealed that recombinant E. coli-derived
Purα and Purβ were essentially identical in terms of their strand preference and binding affinity toward each SMαA promoter-derived element tested (Figs. 5 and 6). Consistent with our previous findings (25), and those of others (24), high affinity binding to ssDNA (apparent Kd ~ 1–2 nM) was dictated by the presence of nucleotide repeats of the general form (GGN)n, where N is not a G. At least two repeats were minimally required for ssDNA binding. Annealed dsDNA probes were poor interaction partners under the assay conditions utilized. Thus, the preponderance of evidence does not support a mechanism whereby disparities in cis-element recognition could account for functional differences observed between Purα and Purβ in overexpression studies. However, these results do not exclude the possibility that one or more paralog-specific post-translational modifications such as phosphorylation (65) might significantly alter Pur DNA-binding affinity and specificity in a cellular milieu. In this regard, our report, implicating the lysine acetyltransferases cAMP-response element-binding protein (CREB)-binding protein (CBP) and p300 as co-activators of the core SMαA enhancer in fibroblasts and VSMCs (42), points to site-specific acetylation as another modification of potential relevance to modulation of Purα and/or Purβ structure-function.

Although resolution of the physiological significance of post-translational modification to the molecular biology of Pur proteins will require further investigation, the importance of protein-protein interaction as a significant contributor to the cellular effects of Purα and/or Purβ is becoming more apparent. Reported Pur interaction partners fall into several functional classes and include gene regulatory factors such as human JC polyomavirus large T-antigen (66), E2F1 (67), YY-1/MSY1 (19, 50), Sp1 (26, 51), Sp3 (18), SRF (18), TEF-1 (18), Sox10 (68), heterogeneous nuclear ribonucleoprotein K (68), and Smad2/3 (26), cell cycle regulators, including certain cyclin/cdk complexes (38, 69) and pRB (31), and proteins involved in RNA transport (70–72). Some Pur binding partners such as YY-1/MSY1 and heterogeneous nuclear ribonucleoprotein K are known to play important roles at multiple levels of RNA metabolism, including transcription, transport, and translation. Thus, it is very likely that the gene-regulatory properties of Pur proteins are not restricted to control of transcription initiation but may extend to other processes including transcription, transport, and translation. Thus, it is likely that the gene-regulatory properties of Pur proteins are not restricted to control of transcription initiation but may extend to other processes such as metabolism, including transcription, transport, and translation. Furthermore, such conditions likely existed in cells transfected to overexpress Purα or Purβ as evidenced by the fact that the core SMαA enhancer was repressed (Figs. 3 and 4) despite the absence of 5′-nucleotides required for high affinity binding by Purα and Purβ to the purine-rich strand of the MCAT-containing Pur/Pyr element. Hence, at high local concentrations, such as those achieved in gain-of-function studies, Purα or Purβ may disrupt cooperative interactions among TEF-1, SRF, and Sp1/3 thereby impairing efficient assembly of a composite enhancosome complex (42). Importantly, support for the proposition that steady-state concentrations of endogenous Purα and Purβ are adequate to allow inhibitory physical interaction with specific activators comes from a study of SMαA enhancer induction during TGFβ1-induced myofibroblast differentiation (26). In this more physiologically germane model, Pur repressors appear to play a key role in restricting enhancer activity by interacting with Sp1/3 and Smad co-activators in a manner affecting protein occupancy at the THR and SPUR elements.

In conclusion, we propose that Purα and Purβ act to limit SMαA gene transcription in fibroblasts and VSMCs by a combination of protein-DNA and protein-protein interactions typified by 1) high affinity strand-specific binding to cis-regulatory elements exhibiting distinct purine/pyrimidine asymmetry and 2) cell type- and compartment-dependent binding to certain SMαA-associated transcriptional regulatory proteins. A corollary of this two-pronged model is that the relative intracellular concentrations and localization of Purα and Purβ will necessarily affect the stability and viability of inhibitory interactions with DNA and protein targets, and perhaps, the degree of SMαA gene repression.

Purα and Purβ may thus serve as molecular rheostats allowing fibroblasts and VSMCs to meet the physiological demand for phenotypic plasticity during wound repair and arteriolar remodeling (76). Ex vivo analyses of mouse and human tissue specimens bolster this argument by providing circumstantial evidence that in vivo levels of Purα and Purβ change in a manner consistent with progressive repression of SMαA and α-myosin heavy chain gene expression in vascular and cardiac cell types during developmental or pathological remodeling of the heart (26, 27, 77). Identification of environmental cues that alter Purα and/or Purβ expression, subcellular localization, and/or structure-function will be crucial to validating this conceptual model of SMαA gene regulation.

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