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Retina-derived POU domain factor 1 coordinates expression of genes relevant to renal and neuronal development

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ABSTRACT

Retina-derived POU domain Factor 1 (RPF-1), a member of POU transcription factor family, is encoded by *POU6F2* gene, addressed by interstitial deletions at chromosome 7p14 in Wilms tumor (WT). Its expression has been detected in developing kidney and nervous system, suggesting an early role for this gene in regulating development of these organs. To investigate into its functions and determine its role in transcriptional regulation, we generated an inducible stable transfectant from HEK293 cells. RPF-1 showed nuclear localization, elevated stability, and transactivation of promoters featuring POU consensus sites, and led to reduced cell proliferation and *in vivo* tumor growth. By addressing the whole transcriptome regulated by its induction, we could detect a gross alteration of gene expression that is consistent with promoter occupancy predicted by genome-wide Chip-chip analysis. Comparison of bound regulatory regions with differentially expressed genes allowed identification of 217 candidate targets. Enrichment of divergent octamers in predicted regulatory regions revealed promiscuous binding to bipartite POU_S and POU_H consensus half-sites with intervening spacers. Gel-shift competition assay confirmed the specificity of RPF-1 binding to consensus motifs, and demonstrated that the Ser-rich region upstream of the POU domain is indispensable to achieve DNA-binding. Promoter-reporter activity addressing a few target genes indicated a dependence by RPF-1 on transcriptional response. In agreement with its expression in developing kidney and nervous system, the induced transcriptome appears to indicate a function for this protein in early renal differentiation and neuronal cell fate, providing a resource for understanding its role in the processes thereby regulated.

1. Introduction

POU transcriptional regulators, essential to developmental processes, feature a high affinity bipartite DNA binding domain composed of a POU-specific (POU_S) and a homeo (POU_H) subdomain joined through a non-conserved linker peptide (L) (Andersen and Rosenfeld, 2001). The Retina-derived POU domain Factor 1 (RPF-1), encoded by *POU6F2*, was first identified and isolated from retina, and based on the homology of its POU and L regions to the Brn-5 protein, it was classified into the POU class VI group (Zhou et al., 1996). However, while Brn-5 is highly expressed in the developing CNS and distributed in multiple tissues (Andersen et al., 1993), RPF-1 appears expressed in the retina, the developing midbrain (Zhou et al., 1996; Burbach et al., 2001), kidney (Perotti et al.,

Abbreviations: c-dbAMP, N62′-O-dibutyryl-cAMP; Dxy, doxycycline; FC, fold change; G4, GAL4 DNA binding and dimerizing domain; GO, gene ontology; HSV, herpes simplex virus; MDCK, Madin-Darby canine kidney; MM, metanephric mesenchyme; NPC, neuronal progenitor cells; WT, Wilms tumor; PAA, polyacrylamide; qRT, quantitative real-time; RT-PCR, reverse-transcriptase PCR; TAD, transactivation domain; TF, transcription factor; tRA, all-trans retinoic acid; Tet, tetracycline; TSS, transcription start site; WB, Western blotting.

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2004), and adult podocytes (Di Renzo et al., 2006), suggesting that this factor may be required for neuronal and renal development. Its full-length sequence is highly conserved in its mouse ortholog, but there is a lack of homology in its N-terminal portion and its short C-terminal tail when compared to its paralogs, which is consistent with a non-redundant function of this gene.

The coding region of *POU6F2* generates two main isoforms resulting from alternative splices occurring within exon 11. These two isoforms encode the RPF-1^{ins36} protein, with a 36-aa insertion within the POU_S, and the RPF-1 with intact POU domain. Accordingly, the latter possesses intrinsic affinity to oligomers with a core WAAT sequence (Zhou et al., 1996), indicating disruption of the DNA binding capacity by the 36-aa insertion. Nevertheless, the full identity of this sequence with the mouse, chimpanzee, and xenopus orthologs, and the abundance of this isoform in the retina, suggest a biological function for RPF-1^{ins36}.

Interstitial deletions located at the 7p14 were reported in patients with WT, with the minimal overlapping segment (390-kb) (Perotti et al., 2001; Perotti et al., 2004) encompassing a large portion of the 486-kb *POU6F2*. Furthermore, germline variants associated with allelic losses were also found in tumor DNA (Perotti et al., 2004). These findings, and the relative abundance of *POU6F2* transcripts in early fetal kidney (Di Renzo et al., 2006), might indicate a role for this transcription factor (TF) in the genesis of WT, originating through perturbed renal development. Despite these findings, no studies on candidate transcriptional targets have been reported to date, and the role of RPF-1 in organogenesis and tumorigenesis is still to be defined.

Here, we pursue a strategy aimed at exploring the impact that RPF-1 has on cell phenotype. We selected tetracycline-regulated RPF-1 transfectants from HEK293, a broadly used low-differentiated embryonic kidney cell line (Torban and Goodyer, 1998), and addressed its potential effect on cell viability and tumorigenicity. Therefore, genome-wide localization analysis coupled to global transcriptional profiling allowed us to identify candidate RPF-1 targets implicated in several biological functions, including transcription and developmental processes entailed with early kidney embryogenesis and neuronal specification.

2. Material and methods

2.1. Cloning of full-length RPF-1

RPF-1 cDNA clones were obtained from the human fetal brain 5′-stretch cDNA library (Clontech) by amplification with the Phusion high-fidelity polymerase (Fynnzymes) and with primers recognizing the 5′ and 3′ of the coding region: sense, 5′-atgatagctggacaagtcagtaagccc-3′; antisense, 5′-ttaggagttttcttccagagagtctgttaag-3′. Amplicons were cloned into the PCR2.1 vector with the TOPO TA cloning kit (Invitrogen) and fully sequenced with both universal M13 and RPF-1 internal sequence primers.

2.2. Construction of expression and reporter vectors

To generate vectors expressing the GST-fused POU domains, the HA- and the Myc-tagged RPF-1, cDNAs were inserted into the pGEX4T1, the pcDNA3HA, and the pcDNA3myc-His(−)A vectors (Invitrogen). The pTRE2/RPF-1 and/Oct1 based constructs were obtained by amplification of the HA-tagged proteins from the pcDNA3HA/RPF-1 and pcDNA3HA/Oct1 (the latter from Dr. Harinder Singh, HHMI, Chicago, IL) through *Bam*HI/*Eco*RV restriction into the pTRE2puro (Clontech). The p*Brn5*^{3}luc was generated by inserting the 5′-phosphorylated 52mer into the *Hind*III/*Bam*HI-restricted p*TATAtk*luc plasmid. The p*HSV*luc and p*FXO*^{3}luc were from Dr. Michael Wegner (Friedrich-Alexander University, Enrlagen-Nvrnberg, DE); the p*VGF*luc was from Dr. Roberta Possenti (Tor Vergata University, Rome, I). Additional primers and promoter-reporter constructs generated to measure transactivation by RPF-1 are described in Supplementary Methods. All amplicons were obtained using the Phusion polymerase and confirmed by sequencing.

2.3. Antibodies and other reagents

Anti-HA cl. 12CA5 (Roche) and HA.11 (Covance); anti-Myc cl. 9E10, anti-α-tubulin, MTT (Sigma); anti-lamin B, Matrigel (BD); and luciferase assay kits (Promega).

2.4. Generation of stable-transfectants and proliferation assay

HEK293 cells were cultured in DMEM/10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37 °C in 5% CO2 atmosphere. Tet^{OFF}-based HEK293 transfectants were generated by a two-step transfection protocol by following the manufacturer's procedure. Briefly, cells seeded in 6-cm dishes were transfected with 1 μg pTet^{OFF} plasmid (Clontech) via Fugene HD (Roche). After 24 h, cells were split, diluted into 96-wells plates and cultured in presence of 0.4 mg/ml G418 for 2 weeks. Isolated clones, assayed for the ability to transactivate the pTREluc plasmid, were transfected with the pTRE2puro-based constructs, and cells maintained in complete medium with G418, 1 μg/ml puromycin, and 1 μg/ml tetracycline (Tet). The isolate clones were tested for construct induction by Western blotting (WB) with anti-HA. Clones with the lowest background and highest induction were selected and regularly checked.

Anchorage-dependent colony assays were performed by plating cells in 6-well plates (500 cells/well) followed by 8 days culture and medium replacement every 2 days. Clones were fixed with methanol and stained with 0.1% Giemsa. Cell viability was assessed on cells seeded in 96-wells plates (500 cells/well; n = 8), cultured for 4 days in presence of Tet or not and continued to grow in Tet+ medium. Cells were then incubated 3 h with 0.1 ml MTT (0.1 mg/ml growth medium), 0.1 ml of 0.1% SDS in 10 mM HCl was added, plates incubated 18 h at 37 °C, and absorbance was read at 570 nm. Proliferation assay was performed on cells seeded in triplicate in 10-cm plates (40,000 cells/plate). Trypsinized cells were stained with Trypan blue, and counted with a hemocytometer.

2.5. In vivo tumor growth

All procedures were approved by the institutional ethical committee for animal use at the Istituto Nazionale dei Tumori (INT) Foundation and accordingly performed. Experiments and euthanasia were executed by appropriately trained personnel in accordance with INT guidelines for the use and care of animals. CD1 nude mice, 4–5 weeks age (Charles River), following 1 week stabilization were administered with doxycycline (Dxy; 2 mg/ml) in 1% sucrose drinking water for 2 days before xenografting. Mice were injected with 2.5×10^6 HEK/Mock transfectants to the right flank, and 2.5×10^6 HEK/RPF-1 to the left flank. Before injection, cells were grown in Tet$^+$-complete medium to 70% confluency and harvested. Cell viability was assessed by Trypan blue exclusion, and single cell suspensions of 0.2 ml saline/Matrigel 1:1 subcutaneously injected (6 mice/group). Dxy administration schedule was as follows: 1) Dxy supplemented; 2) Dxy supplemented in the first 2 days only; 3) no Dxy supplemented. Tumor growth was monitored weekly through tridimensional measurement with a caliper. Mice were anesthetized with a brief isoflurane exposure and euthanized by cervical dislocation 90 days following injection.