Negative Regulation of Ros Receptor Tyrosine Kinase Signaling: An Epithelial Function of the SH2 Domain Protein Tyrosine Phosphatase SHP-1

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Abstract. Male "viable motheaten" (*me^v*) mice, with a naturally occurring mutation in the gene of the SH2 domain protein tyrosine phosphatase SHP-1, are sterile. Known defects in sperm maturation in these mice correlate with an impaired differentiation of the epididymis, which has similarities to the phenotype of mice with a targeted inactivation of the Ros receptor tyrosine kinase. Ros and SHP-1 are coexpressed in epididymal epithelium, and elevated phosphorylation of Ros in the epididymis of *me^v* mice suggests that Ros signaling is under control of SHP-1 in vivo. Phosphorylated Ros strongly and directly associates with SHP-1 in yeast two-hybrid, glutathione *S*-transferase pull-down, and coimmunoprecipitation experiments. Strong binding of SHP-1 to Ros is selective compared to six other receptor tyrosine kinases. The interaction is mediated by the SHP-1 NH_2-terminal SH2 domain and Ros phosphotyrosine 2267. Overexpression of SHP-1 results in Ros dephosphorylation and effectively downregulates Ros-dependent proliferation and transformation. We propose that SHP-1 is an important downstream regulator of Ros signaling.

Key words: protein tyrosine phosphatase • regulation • receptor tyrosine kinase • epididymis • fertility

Introduction

The transmembrane tyrosine kinase Ros, encoded by the protooncogene *c-ros*, is an "orphan" receptor with exclusive expression in specific epithelia (Sonnenberg et al., 1991; Sonnenberg-Riethmacher et al., 1996). The first discovered oncogenic variants of *c-ros* were found to encode proteins with truncated extracellular domain, and they were detected in a chick retrovirus and in human tumor cell lines (Neckameyer and Wang, 1985; Birchmeier et al., 1986). The oncogenic potential of the Ros tyrosine kinase has also been demonstrated by ligand-dependent transformation of NIH3T3 fibroblasts, which were stably transfected with a chimeric receptor consisting of the TrkA/nerve growth factor (NGF)[1] receptor extracellular domain and the Ros transmembrane and cytoplasmic domains (Riethmacher et al., 1994). The physiological function of Ros has been characterized in mice with a targeted mutation of *c-ros*. Male $Ros^{-/-}$ mice exhibit defects in differentiation and regionalization of the epididymal epithelium and, because of this defect, are sterile (Sonnenberg-Riethmacher et al., 1996).

The SH2 domain protein tyrosine phosphatase (PTP) SHP-1 (Shen et al., 1991) is expressed in hematopoietic and, at lower levels, epithelial cells. In the latter cell type, SHP-1 expression is driven by a cell type–specific promoter, which leads to the generation of an epithelial-specific SHP-1 variant (Banville et al., 1995). Multiple binding partners and substrates for SHP-1 have been identified in hematopoietic cells: SHP-1 negatively regulates the signaling of cytokine receptors, receptor tyrosine kinases, adhesion receptors, and immunoreceptors (for reviews see Feng and Pawson, 1994; Frearson and Alexander, 1997; Neel and Tonks, 1997). "Motheaten" (*me*) or "viable motheaten" (*me^v*) mice carry mutations in the SHP-1 gene, which lead to a complete loss or a 80–90% reduction of SHP-1 activity, respectively (Green and Shultz, 1975; Shultz et al., 1984, 1993). Homozygous *me* and *me^v* mice exhibit multiple abnormalities, including immunodeficiencies, increased proliferation of macrophage, neutrophil,

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[1]*Abbreviations used in this paper:* aa, amino acid; ATc, anhydrotetracycline; Erk, extracellular signal regulated kinase; GST, glutathione *S*-transferase; *me*, motheaten; *me^v*, viable motheaten; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PTP, protein tyrosine phosphatase; RTK, receptor tyrosine kinase.

Results

Ros Is Hyperphosphorylated in the Epididymis of me^v Mice

To assess whether SHP-1 might be relevant for epididymal function, we histologically examined the epididymis of male *me^v*/*me^v* mice and compared them to heterozygous controls. Macroscopically, the epididymis of *me^v*/*me^v* mice was smaller, though the overall shape appeared normal. This size reduction was proportional to the reduced body weight of the *me^v*/*me^v* mice. Histological sections revealed that the tall columnar epithelial cells, which form the tubules of the proximal segment in heterozygous animals, are replaced by considerably flatter epithelial cells in the epithelium of *me^v*/*me^v* mice (Fig. 1). In heterozygous animals, these epithelial cells are clearly structured. The nucleus is located above a clearly defined basal cytoplasmic zone. The cytoplasmic zone above the nucleus is broad and the apical surface is rough. In contrast, the nuclei in epithelial cells of *me^v*/*me^v* are often located closer to the basal membrane, and the apical surfaces appear smooth (Fig. 1). The epithelium in the more distal segments of the epididymis exhibited little differences in mutant and control animals (not shown). Histologically, the epithelium of the proximal segment in *me^v*/*me^v* mice has similarities to that of Ros$^{-/-}$ mice (Fig. 1). We also analyzed the epithelium of other organs. Epithelial cells in the intestine of *me^v*/*me^v* mice were smaller than their counterparts in heterozygous animals, but appeared fully differentiated. No differences between *me^v*/*me^v* and heterozygous animals were detectable in epithelia of stomach and pancreas (not shown). In summary, the epididymal epithelium of *me^v*/*me^v* mice exhibits signs of aberrant differentiation. This phenotype may be related to a loss of function of SHP-1 in the epididymal epithelial cells, since SHP-1 is clearly expressed in these cells (Fig. 2). In situ hybridization with an antisense RNA generated from a 1,184-bp fragment of murine SHP-1 cDNA revealed expression of SHP-1 mRNA in the epithelial cells surrounding the tubules of the initial epididymal segment (Fig. 2, A–E). More distal segments exhibit low level signals. Thus, SHP-1 expression mirrors the one of Ros in this part of the epididymis (Sonnenberg-Riethmacher et al., 1996). Strong SHP-1 expression is also seen in the corpus with highest levels in the most distal segments. These distal epithelia are devoid of Ros (Sonnenberg-Riethmacher et al., 1996). SHP-1 expression could also be detected by immunoblotting in the epididymis of mice (not shown); immunostaining failed with the available antibodies. However, in human epididymis, strong epithelial SHP-1 expression could be visualized by immunohistochemistry (Fig. 2, F and G). Thus, SHP-1 is expressed in epithelial cells of the epididymis and exhibits an overlapping expression domain with Ros.

The overlapping expression of SHP-1 and Ros prompted us to test whether impairment of SHP-1 activity in *me^v*/*me^v* mice might affect Ros signaling. We analyzed tyrosine phosphorylation of immunoprecipitated Ros from the epididymis of *me^v*/*me^v* mice. Ros from *me^v*/*me^v* mice displayed an elevated tyrosine phosphorylation compared to Ros from heterozygous animals (Fig. 3), indicating that SHP-1 is capable of downregulating Ros signaling in the epithelium of the epididymis in vivo. Intraperitoneal injection of peroxovanadate rapidly triggers tyrosine phosphorylation in multiple murine tissues (Ruff et al., 1997). After treatment with

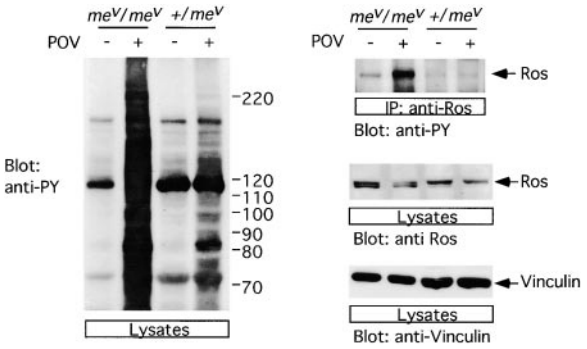

Figure 3. SHP-1 affects Ros signaling in vivo. *me^v*/*me^v* mice or heterozygous control animals were challenged with peroxovanadate (POV) or mock treated (−). The epididymis was prepared and the tissue was lysed. Lysates were used to analyze the phosphotyrosine content (left gel, anti-PY) by immunoblotting. In addition, lysates were used for immunoprecipitation (IP) of Ros and for the subsequent analysis of Ros phosphorylation, by immunoblotting (right gel). The data are representative of three independent experiments with consistent results.

peroxovanadate, a dramatic increase in overall tyrosyl phosphorylation was detected in epididymal extracts from *me^v*/*me^v* mice, which was less pronounced in heterozygous mice (Fig. 3). In particular, phosphorylation of Ros was strongly elevated in peroxovanadate-treated homozygous, but not in heterozygous *me^v* mice. Peroxovanadate is an effective inhibitor of many PTPs, but a relatively weak inhibitor of SHP-1 (Wetzker, M., and F.D. Böhmer, unpublished data). We assume that partial inhibition of SHP-1 by the pervanadate treatment eliminates the residual activity of SHP-1 in the *me^v*/*me^v* mice, but leaves substantial activity in the heterozygous mice intact, resulting in a further enhancement of the difference in Ros phosphorylation level.

Ros and SHP-1 Exhibit a Strong and Direct Interaction

To further investigate the regulation of Ros by SHP-1, we tested whether SHP-1 is a direct binding partner for Ros in a yeast two-hybrid assay. The cytoplasmic domain of Ros was fused to the DNA-binding domain of LexA. In such constructs, the tyrosine kinase of the fusion proteins is constitutively active, phosphorylated, and can interact with cognate binding partners (Weidner et al., 1996). We observed very strong binding between the SH2 domains of SHP-1 and the cytoplasmic domain of Ros (Table I).

Table I. Interaction of Various Autophosphorylated Receptor Tyrosine Kinases with SHP-1 and Other Signaling Proteins in Yeast Two-Hybrid Assays

RTK (cytoplasmic domain fusion protein as bait)	Binding strength of SHP-1 SH2 domains*	Strongly binding signaling molecules
Ros	Strong	PLCγ, c-Abl SH2
TrkA§	Not detectable	PLCγ
Insulin receptor	Not detectable	p85α
IGF-1 receptor§	Not detectable	p85α
CSF-1 receptor§	Not detectable	Grb2, Grb10
Met/HGF receptor§	Weak	Gab1, Grb2

*The interactions were tested in yeast colony growth assays as shown in Fig. 4 A. See text for further description of the signaling proteins.
§TrkA, NGF receptor; IGF-1, insulin-like growth factor-1; CSF-1, colony-stimulating factor-1; HGF, hepatocyte growth factor.