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Induction of the Ras activator Son of Sevenless 1 by environmental pollutants mediates their effects on cellular proliferation

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ABSTRACT

TCDD (2,3,7,8-tetrachlorodibenzodioxin), a highly persistent environmental pollutant and a human carcinogen, is the ligand with the highest affinity for the Aryl Hydrocarbon Receptor (AhR) that induces via the AhR, xenobiotic metabolizing enzyme genes as well as several other genes. This pollutant elicits a variety of systemic toxic effects, which include cancer promotion and diverse cellular alterations that modify cell cycle progression and cell proliferation. Large-scale studies have shown that the expression of Son of Sevenless 1 (SOS1), the main mediator of Ras activation, is one of the targets of dioxin in human cultured cells. In this study, we investigated the regulation of the previously uncharacterized SOS1 gene promoter by the AhR and its ligands in the human hepatocarcinoma cell line, HepG2. We found that several environmental pollutants (AhR ligands) induce SOS1 gene expression by increasing its transcription. Chromatin immunoprecipitation experiments demonstrated that the AhR binds directly and activates the SOS1 gene promoter. We also showed that dioxin treatment leads to an activated Ras-GTP state, to ERK activation and to accelerated cellular proliferation. All these effects were mediated by SOS1 induction as shown by knock down experiments. Our data indicate that dioxin-induced cellular proliferation is mediated, at least partially, by SOS1 induction. Remarkably, our studies also suggest that SOS1 induction leads to functional effects similar to those elicited by the well-characterized oncogenic Ras mutations.

1. Introduction

The Ras signaling pathway is involved in cell growth and differentiation. Mutations in the Ras proto-oncogene have been described in a large variety of human malignancies [1]. These mutations result in a constitutively active Ras protein, which is one of the most common Ras dysregulations in cancers [1,2]. Three different Ras genes encoding the H-Ras, K-Ras and N-Ras 21 kDa proteins were discovered 30 years ago [1,3]. The activated Ras proteins physically associate with and activate Raf-1, a serine/threonine kinase which triggers a kinase cascade that results in the activation of the extracellular signal-regulated protein kinases (ERKs of the MAPK family). Ras acts as a functional hub by activating multiple downstream pathways that participate in cell growth and differentiation. The activity of Ras is regulated by two sets of proteins: guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs stimulate the release of the Ras bound GDP which then allows GTP to bind and activate Ras. Conversely, GAPs stimulate Ras GTPase activity and lead to the basal GDP-bound state.

Several pathways, which lead to the active Ras state, have been described in mammalian cells. The best-characterized pathway employs the activation of tyrosine kinase receptors to elicit binding of various proteins to the phosphorylated receptor, most notably the growth factor receptor bound 2 (Grb2) protein bound to the guanine nucleotide exchange factor SOS1. The complex is targeted to the plasma membrane, allowing the GEF function of SOS1 to activate Ras by GDP/GTP exchange [4–6]. SOS1 can activate other GTP-binding proteins [4] and other effectors also control Ras activity. The most extensively characterized non-physiological conditions under which increased SOS activitiy has been observed result from gain of function mutations. For example, SOS1

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mutations have been described in the Noonan syndrome, a developmental disorder that is characterized by short stature, facial dysmorphia, congenital heart defects and skeletal anomalies [7,8]. These gain of function mutations, which lead to the activation of Ras and were the first examples of activating GEF mutations associated with human disease. Surprisingly, the implications of increased expression of SOS proteins have not been addressed and little is known about the regulation of the gene promoter.

Dioxin is a member of the polychlorinated dibenzo dioxins (PCDD) family which is a class of highly persistent environmental contaminants. 2,3,7,8-tetrachlorodibenzodioxin (TCDD), the most extensively characterized dioxin, has been classified in 1997 as a ''human carcinogen'' by the International Agency for Research on Cancer (IARC) [9]. This pollutant is the ligand with the greatest affinity for the Aryl Hydrocarbon Receptor (AhR). TCDD via the AhR, induces various toxicities including chloracne, wasting syndrome, teratogenicity, immunotoxicity, neurotoxicity, tumor promotion and carcinogenesis [10]. The AhR is a cytosolic, ligand-activated transcription factor which, upon activation, translocates to the nucleus where it forms a heterodimer complex with AhR nuclear translocator (ARNT). This complex binds to specific xenobiotic responsive elements (XRE) which are characterized by a 5′-GCGTG-3′ consensus core and induces specific target genes which include xenobiotic metabolizing enzymes (XME) such as the cytochrome P450 (CYP) 1 family [11]. In addition to detoxification functions, the activation of this receptor has been shown to elicit diverse cellular effects including cell proliferation [12,13]. These cellular alterations might mediate part of the long-term toxicity of the AhR ligands in animals and humans [14]. Indeed, AhR-null mice display several defects of development and proliferation including thymus and liver hypotrophy [15]. Numerous studies using AhR defective cells or AhR invalidating strategies have clearly established a role for this receptor in cell cycle regulation and proliferation [16]. The dysregulation of these cellular pathways may disrupt normal fetal development and favor cancer progression. The AhR gene targets that are involved in the proliferative effects of this receptor are not completely identified. To this end, we have examined large-scale toxicogenomic analyses of dioxin effects and focused on regulatory proteins that control cellular growth, in particular, the main mediator of Ras activation, SOS1.

In the present work, we investigated, for the first time, the regulation of the previously uncharacterized SOS1 gene promoter by the AhR and its ligands in the human hepatocarcinoma cell line, HepG2. We show that several environmental pollutants (AhR ligands) induce SOS1 gene expression via increased transcription. Moreover, considering the critical role of SOS1 in controlling Ras activity, we also demonstrate that induction of SOS1 by AhR ligands leads to a predominant Ras-GTP state, to MAPK activation and to cell growth. The results point towards SOS1 gene induction as being a critical step for the carcinogenic effects mediated by dioxins.

2. Materials and methods

2.1. Cell culture

Human hepatocarcinoma HepG2 cells were cultured in Dulbecco's minimal essential medium (DMEM, Invitrogen, Cergy-Pontoise, France) supplemented with nonessential amino acids and containing 10% fetal bovine serum, 200 U/mL penicillin, 50 μg/mL streptomycin (Invitrogen) and 0.5 U/mL amphotericin B (Bristol-Myers Squibb Co., Stamford, CT) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The day before with the addition of various concentrations of TCDD, cells were cultured in DMEM without phenol red and supplemented with 3% charcoal-treated (desteroidized) calf serum. Cells were maintained in this medium during all the treatments except for the proliferation studies. TCDD was purchased from LCG Promochem (Molsheim, France). Benzo(a)pyrene, and quercetin were purchased from Sigma–Aldrich (St Louis, United States).

2.2. RNA extraction, reverse transcription and quantitative RT-PCR

Total RNAs were extracted using the RNeasy mini kit (Qiagen, Les Ulis France) and reverse transcription was performed with each RNA sample using the cDNA high-capacity archive kit (Applied Biosystems, Courtaboeuf, France) as previously described [17]. Specific oligonucleotides were designed using the OLIGO Explorer software (Molecular Biology Insights, Inc., Cascade, CO). Gene specific primers used for the real time PCR were: *SOS1*: forward 5′-TGAGAGGCAACAGAAAGAGC-3′ and reverse 5′-GAGAAGGGAAATGAAATGGG-3′; *AhR*: forward 5′-ACATCACCTACGCCAGTCGC-3′ and reverse 5′-TCTATGCCGCTTGGAAGGAT-3′; *RPL13A*: forward 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′ and reverse 5′-GAGGACCTCTGTGTATTTGTCAA-3′. Oligonucleotides were obtained from (Qiagen). Quantitative RT-PCR was carried out in a 10 μL reaction volume containing 40 ng of cDNA, 300 nM of each primer and ABsolute™ QPCR SYBR® Green (Abgene, Villebon sur Yvette, France). Quantitative RT-PCR measurements were performed on an ABI Prism 7900 Sequence Detector system (Applied Biosystems). PCR cycles consisted of the following steps: Taq activation (15 min, 95 °C), denaturation (15 s, 95 °C) and annealing and extension (1 min, 60 °C). The threshold cycle (Ct), which is inversely correlated to the amount of target mRNA, was measured as the number of cycles for which the reporter fluorescent emission first exceeds the background. The relative amounts of mRNA were estimated using the ΔΔCt method with RPL13A as reference.

2.3. Immunoblotting

Whole cell lysates were prepared as previously described [17] from HepG2 cells with M-PER®, Mammalian Protein Extraction Reagent, containing protease and phosphatase inhibitor cocktails (Sigma–Aldrich, St Louis, United States). Equal amounts of total protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, Orsay, France). The membranes were probed with primary antibodies recognizing SOS1 (Upstate, Molsheim, France, 07-337, 1:2000), Actin (Abcam, Paris, France ab37063, 1:10000), AhR (Abcam, ab2770, 1:1000), ERK-Phosphorylated (Cell signaling, Saint Quentin en Yvelines, France 9101, 1:2000), ERK (Cell signaling, 4695, 1:2000) and Ras (Upstate 05-516, 1:10000). Immunoreactive bands were detected with X-ray film using alkaline phosphatase-linked secondary antibody (Applied biosystems, T2191 or T2192, 1:20000) and an enhanced chemiluminescence system CDP-Star® (Applied biosystems). Relative Quantification of the amount of immunreactive material was performed with ImageJ freeware (http://rsb.info.nih.gov/ij/).

2.4. SOS1 mRNA half-life measurement

On day 1, HepG2 cells were seeded at 400,000 cells/well (in 6-well plates) in complete DMEM medium (see above). On day 2, cells were washed with PBS and the medium was replaced by red phenol-free DMEM supplemented with 3% desteroidized fetal bovine serum, 200 U/mL penicillin, 50 μg/mL streptomycin and 0.5 mg/mL amphotericin B. Then TCDD dissolved in medium (1 μM). Fifty microlitre was added to the cells medium to give a final concentration of 25 nM. Twenty-four hours later (on Day 3), cells were treated with 100 μM DRB (5,6-dichloro-beta-D ribofur-