CELL CYCLE REGULATION BY THE AH RECEPTOR

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Introduction

Most, if not all, symptoms of dioxin toxicity are mediated by the Ah receptor (1-3). The Ah receptor acts as a conditionally regulated transcription factor and binding of dioxin-like or aromatic hydrocarbon ligands enables the receptor to recognise specific sequences in target genes as well as to enhance transcription of these genes (4-6). In addition the Ah receptor, like the steroid hormone receptors, seems also to act on cellular signal transduction cascades possibly by primarily acting on the c-Src tyrosine kinase (7). Work over the last two decades has provided a detailed understanding of the Ah receptor's mode of action as a transcription factor and how the Ah receptor regulates a number of genes. Most of these genes code for xenobiotica metabolising enzymes but also protooncogenes, cytokines and cytokine receptors appear to be under control of the Ah receptor (8). The latter type of genes could account for some but not all aspects of dioxin toxicity. Stringent evidence, however, is sparse that indeed one of the candidate genes would mediate certain biological and toxic actions of dioxins.

Many symptoms of dioxin poisoning, e.g. in skin, testis and the immune system are associated with changes in cell proliferation and differentiation. Hence we used the previously defined model system of the continuously growing 5L rat hepatoma cell line (9, 10) to search for an Ah receptor dependent pathway to the control of cell proliferation. Upon treatment with 1 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) proliferation of 5L cells is dramatically slowed. The time for completion of one cell cycle increases from 20 to over 70 h. Cell cycle analysis by flow cytometric detection of cellular DNA contents indicated that proliferation is halted in the G1-phase of the cell cycle and/or that the progression from G1 to the S-phase is delayed. Thus, the analysis of TCDD effects on 5L cells presented here has been focussed on the cell cycle regulatory events in G1 (For original figures to some of the data as well as experimental procedures please refer to Kolluri et al., *Genes and Development*, in press).

Results and Discussion

A hallmark of G1-S progression in the cell cycle is the hyperphosphorylation of the retinoblastoma (Rb) protein. Hypophosphosphorylated Rb (Rb in Figure 1) sequesters proteins like the E2F transcription factors that are required for S-phase progression into inactive complexes. Rb hyperphosphorylation (P-Rb) releases these factors and by that permits cell cycle progression. Kinases which phosphorylate Rb but also other substrates essential for G1-S progression are formed by association of the cyclins D and cyclin E with their respective cyclin dependent kinases (CDKs) 4 or 6 and CDK 2, respectively (Figure 1). The biochemical analysis of the basal cell cycle machinery in TCDD-treated 5L cells showed that TCDD-treated cells accumulate at a stage where Rb is hypophosphorylated (Kolluri et al. Genes and Development, in press. For a summary see Figure 1). Cyclin E dependent kinase activity in asynchronously proliferating 5L cells is

dramatically decreased by TCDD although the protein levels of neither cyclin E nor the associated CDK2 are reduced. Likewise also the protein levels of the D-cyclins and CDKs 4 or 6 are not reduced by TCDD treatment. This observation of decreased kinase activity despite unchanged cyclin/CDK protein levels is indicative of the presence of inhibitory proteins like the members of the Cip/Kip or the Ink protein families. Indeed, the TCDD-dependent induction of p27 Kip1 (11, 12) was observed. A 4-5-fold increase in protein levels is associated with a similar increase in steady state mRNA levels. The increase in mRNA levels is the cause for the induced protein levels since TCDD treatment did not alter the rate of protein translation or protein degradation. The increase in Kip1 mRNA appears to be a bona fide transcriptional induction since the mRNA synthesis rate was found increased in a nuclear run-off analysis. Furthermore, a reporter gene comprising 1610 bp upstream of the transcriptional start site in the murine Kip1 promoter (13) suffices to confer TCDD inducibility to a luciferase reporter gene.

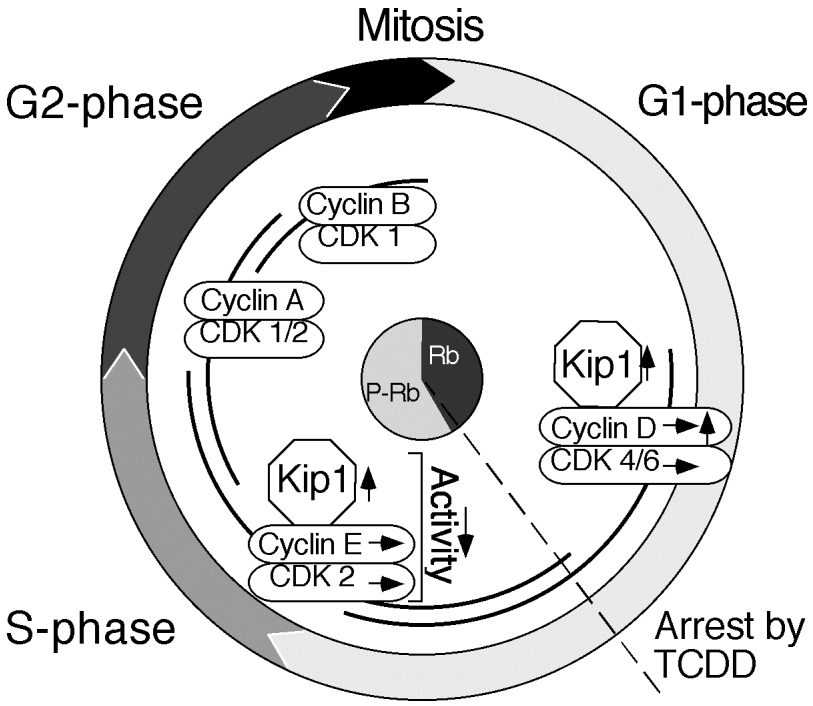

Figure 1: Schematic drawing of the basal cell cycle machinery and the effects of TCDD on the 5L hepatoma cell cycle. The arrows indicate the changes induced by TCDD treatment, e.g. induced, unchanged, repressed. For details please refer to the text.

Causal evidence that Kip1 mediates the TCDD effects on the 5L cell cycle was provided by transient Kip1 antisense RNA expression in 5L cells. The antisense RNA was coexpressed with either the green fluorescent protein or a surface protein tag so that efficiently transfected cells could be purified by fluorescence activated cell sorting or panning by the use of antibodies coupled to ferromagnetic beads. In the antisense RNA expressing cells Kip1 levels were decreased and could not be induced by TCDD. Also, TCDD had no effect on proliferation of these cells as assessed by BrdU incorporation. These data clearly show that TCDD-induced expression of Kip1 causes the inhibition of 5L cell proliferation. The effect, as expected, strictly depends on the Ah receptor since it is not found in a receptor deficient subclone of the 5L cells ($BP8^{AhR-}$) but can be reconstituted by ectopic AhR expression in these cells ($BP8^{AhR+}$).

As a model system more closely related to dioxin toxicity in vivo we chose to study the dioxin effects on the developing thymus since this organ is highly sensitive to dioxins and is accessible to an analysis in primary organ explants. In cultures of fetal thymus glands (Fetal Thymus Organ Culture, FTOC) TCDD-exposure induced Kip1 protein levels already after one day of culture. This increase in Kip1 levels was associated with a 30% reduction in proliferation rates of thymocytes after 2 days of culture. Such a relatively subtle drop in proliferation rate could well account for the dramatic reduction in thymocyte recovery, e.g. less than 10%, after numerous cell cycles during 9 days of culture. To test a role of Kip1 in this process FTOCs were derived from Kip wildtype and Kip-deficient (knockout) embryos. FTOCs from Kip1-deficient embryos were substantially less sensitive to TCDD though not completely resistant. Thymocyte recovery from Kip1-deficient TCDD treated FTOCs was 40 % of that from solvent treated glands as compared to less than 10 % in FTOCs derived from wild type embryos. Thus, induction of Kip1 upon activation of the Ah receptor by TCDD is likely to explain at least one aspect of dioxin toxicity, e.g. the thymus toxicity. Other symptoms like the effect on spermatogenesis or the inhibition of liver regeneration may follow the same pathway but other toxic actions of dioxins like carcinogenicity are not easily explained by the induction of an inhibitor of proliferation.

Dioxin actions on biological systems require quantitative descriptions by dose-response relationships, particularly, when effects of low dioxin levels should be considered. In this study we compared the dose-dependency of Kip1 mRNA induction to the inducibility of CYP450 1A1 mRNA (Figure 2) of which the latter codes for a xenobiotica metabolising enzyme which has been widely used as a marker of biologically relevant dioxin activities. In the 5L rat hepatoma cells dose-response relationships are very similar for the induction of both genes if intermediate to high concentrations of dioxins, e.g. greater than 5 pM are used. Considerable differences are found below 5 pM of TCDD. Inducibility of the CYP450 1A1 mRNA at 1 pM follows within the range of assay accuracy the expected relationships of Michaelis Menten's kinetics. Kip1 inducibility, however differs substantially, because even without dioxins a substantial basal expression is found which is not significantly changed by the presence of 1 pM TCDD and only slightly increased by 6 pM of TCDD. Thus, dioxin effects on Kip1 expression become small or non-observable compared to the endogenous level of gene expression already in the range of 1-5 pM TCDD concentrations whereas this is not the case even at 1 pM concentrations with respect to CYP450 1A1 induction.

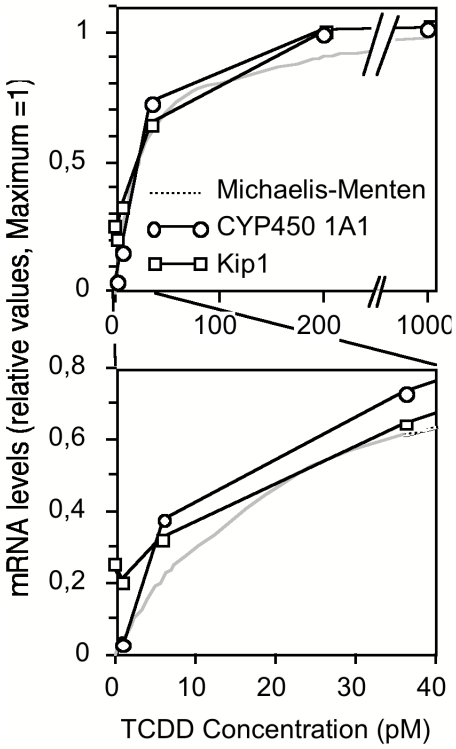

Figure 2: Dose-response relationships for the induction of CYP450 1A1 and Kip1 mRNAs. mRNA expression levels were determined by Northern blot hybridisation of RNA prepared from 5L cells which had been treated for 24h with the indicated concentrations of TCDD or the solvent (0.1% DMSO) only. Quantification was performed by phosphoimager analysis. Data are shown in comparison to theoretically calculated Michaelis Menten's kinetics.

In summary we show that TCDD in an Ah receptor dependent fashion induces the cell cycle inhibitor Kip1 through an increase in mRNA expression. Kip1 is required for the cell cycle delay in 5L cells and also the major mediator of dioxin toxicity in the developing thymus. Comparison of dose-response curves indicates that different genes follow different relationships particularly in the low dioxin-level range. Thus, predicting the activity of low dioxin levels with respect to a specific phenomenon requires the knowledge of the molecular mechanism(s) underlying this particular phenomenon as well as the quantitative description of the relevant molecular and cellular events.

Acknowledgements
Andrew Koff (New York) is gratefully acknowledged for the collaboration on the Kip1-deficient mice. C.A. Bradfield and A.A. Nordin provided essential plasmids to this work. The study was supported by a fellowship of the Forschungszentrum Karlsruhe (SKK) and by the Deutsche Forschungsgemeinschaft (DFG GO 473/3, MG).

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