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Autoreport

of Doctoral Thesis

Transcriptional regulation of differentiation of embryonal carcinoma and malignant melanoma cells: The Role of Proteins p21(WAF1) and MITF.

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1. Introduction

p21/WAF1/Cip1 (referred to as p21 hereafter) was originally identified as a downstream effector of p53-mediated block of cell cycle progression whose physiological function is to act as an universal inhibitor of cyclin-dependent kinase (cdk) activities. Besides its function in controlling cell proliferation, p21 has been reported to function as a transcriptional regulator, and this second role appears to be independent of its cell cycle regulatory activities. p21 inhibits transcriptional activation exerted by several transactivators, including E2F (Delavaine and LaThangue, 1999), STAT3 (Coqueret and Gascan, 2000), and myc (Kitaura et al., 2000). Its role in transcriptional repression has also been described for promoters of polo-like kinase 1 and topoisomerase II〈 (Zhu et al., 2002), survivin (Lohr et al., 2003), and Wnt4 (Devgan et al., 2005), and others. In other circumstances, p21 functions as a transcriptional coactivator stimulating the transcription by estrogene receptor (Fritah et al., 2005) or NFkappaB (Poole et al., 2004). Transcription of the p21 is induced during differentiation of several cell types in the cell culture models, including oligodendrocytes (Zezula et al., 2001), chondrocytes (Negishi et al., 2001), or feochromocytoma cells (Erhardt and Pittman, 1998).

In this study, the role of p21 has been investigated in the mouse embryonal carcinoma F9 cells. Simultaneous treatment with RA and dibutyryl-cAMP or other agents that stimulate the cAMP

pathway converts F9 cells into cells with a phenotype reminiscent of parietal endoderm (PE) (Strickland et al., 1980). We examined the changes in cell cycle-related proteins during the differentiation of F9 cells into PE. We found that the p21 protein increases during differentiation, reaching high levels at the end of a 6-day differentiation period. The p21 protein transcriptionally upregulates the PE-specific marker TM, and partially localizes to the cytoplasm in a subset of differentiated cells, indicating that it may have an antiapoptotic role in differentiated cells.

In the melanocyte, the transcription of p21 seems to be governed by MITF (Carreira et al., 2005), a master regulator of melanocyte differentiation and proliferation (Goding, 2000; Vance and Goding, 2004; Levy et al., 2006). The expression of the melanocyte-specific isoform of MITF is uniformly conserved and upregulated in human malignant melanocytes and an increasing number of data support its role in melanoma cell survival and proliferation. Numerous MITF targets have been identified so far, including melanocyte markers, cdk2, c-met, HIF1, and cdk inhibitors p21 and p16. The overexpression of MITF was commonly observed in the majority of melanoma cell lines and tissue samples. The p21 protein was found to be abundantly expressed in melanoma specimens and cell lines (Trotter et al., 1997). Whether the p21 protein has any cell cycle regulatory role in malignant melanocytes and why the relatively high protein levels are tolerated by melanoma cells remains unresolved. In an attempt to determine a potential cell cycle-independent role of p21 in transformed melanocytes, we explored its functional participation in expression of pigment cell-specific genes.

The E1A 12S oncoprotein of the adenovirus is a strong transcriptional repressor. When expressed in the cell, E1A binds strongly to and inactivates the paralogous coactivators and histone acetyltransferases (HATs) p300 and CBP (Frisch and Mymryk, 2002). Apart from neutralizing p300/CBP, transcription inhibition can also be accomplished via targeting additional HATs and coactivators such as PCAF, hGCN5, p400 and TRRAP (Deleu et al., 2001; Frisch and Mymryk, 2002; Lang and Hearing, 2003; Shuen et al., 2002). The E1A's ability to repress transcription generally requires the N-terminus and the conserved region 1 (CR1). Mouse melanocytes, as well as several other cell types, can be immortalized by the adenoviral E1A 12S oncoprotein. In E1A-expressing melanocytes, MITF and its target genes are fully extinguished (Halaban et al., 1996; Yavuzer et al., 1995). These dedifferentiated melanocytes do not require MITF for survival, presumably due to the immortalizing activity of E1A. The E1A protein is also known to exert an antitumour activity, which is associated with its transcription repression function and the ability to induce apoptosis, and is a potential antimelanoma agent (reviewed by Frisch and Mymryk, 2002). In this study, the effect of E1A on the MITF promoter and MITF target promoters was investigated.

2. Aims of the study

- The role of the p21(WAF1) protein in F9 cells which differentiate into parietal endoderm:
  - expression of the protein, role in cell cycle regulation
  - transcriptional effects of p21 independent of cell proliferation, regulation of PE marker thrombomodulin
- Investigation of a role of p21 protein in transcription of melanocyte-specific genes:
  - functional role in the regulation of MITF promoter
  - mechanisms of transcriptional regulation by the p21 protein
- The effect of adenoviral protein E1A on the expression of MITF and its downstream targets – an effective way to down-regulate MITF function and inhibit proliferation of melanoma cells:
  - inhibition of the MITF promoter
  - repression of MITF target gene tyrosinase
  - repression of MITF target gene by E1A subregions
- Construction and functional analysis of dominant-negative MITF-mutant which inhibits the activity of endogenous MITF:
  - analysis of the two transcription activation domains of MITF on the endogenous target promoter
  - construction of a strong dominant-negative MITF mutant

3. Methods

The following methods were used in the study and are described in detail in the thesis and the cited published papers: Construction of expression plasmids and promoter-reporters, analysis of gene expression by Northern blotting, RT-PCR, and Western blotting, gene transfer into cultured cells, immunofluorescence, flow cytometry, protein-protein interactions (pull-down and immunoprecipitation), DNA-protein interaction by gel-shift, siRNA- and shRNA-mediated knock-down of gene expression, gene transfer (transfections), gene reporter assays, protein kinase assays.

4. Summary of results

4.1 The role of p21(WAF1/Cip1) protein in differentiation of F9 embryonal carcinoma cells into parietal endoderm.

It is shown that the levels of cyclin-dependent kinase inhibitor p21/WAF1/Cip1 (p21) protein and mRNA are dramatically elevated at the end of differentiation into parietal endoderm (PE), concomitantly with the appearance of p21 in the

immunoprecipitated CDK2–cyclin E complex. The induction of differentiation markers could not be achieved by expression of ectopic p21 alone and still required treatment with differentiation agents. Clones of F9 cells transfected with sense or antisense p21 cDNA constructs revealed, upon differentiation, upregulated levels of mRNA for thrombomodulin, a parietal endoderm-specific marker, or increased fraction of cells in sub-G1 phase of the cell cycle, respectively. Consistent with this observation, whereas p21 was strictly nuclear in undifferentiated cells, a large proportion of differentiated cells had p21 localized also in the cytoplasm, a site associated with the antiapoptotic function of p21. Furthermore, p21 activated the thrombomodulin promoter in transient reporter assays and the p21 mutant defective in binding to cyclin E was equally efficient in activation. The promoter activity in differentiated cells was reduced by cotransfection of p21-specific siRNA or antisense cDNA. Coexpression of p21 increased the activity of the GAL-p300(1–1303) fusion protein on the GAL sites-containing TM promoter. This implies that p21 might act through a derepression of the p300 N-terminal-residing repression domain, thereby enhancing the p300 coactivator function. As differentiation of F9 cells into parietal endoderm-like cells requires the cAMP signaling, the results together suggest that the cyclin-dependent kinase inhibitor p21 may promote specifically this pathway in F9 cells.

4.2 p21(WAF1/Cip1) is expressed in melanoma cells and activates the promoter of MITF, a master transcription regulator of melanocytes

This part of the study investigated the expression and transcriptional activity of p21/WAF1/Cip1 (p21) in human melanoma cells in culture. We found no correlation between proliferation and p21 protein level in unsynchronized cell populations of melanoma cells. p21 was expressed also in proliferating epidermal melanocytes and clones of melanoma cells with p21 knocked-down by siRNA had even decreased growth rates. We further found that promoters of several genes related to differentiation were stimulated by p21 overexpression, including the MITF promoter. The N-terminal portion and intact cyclin-binding site was essential for these transcription activities, suggesting that mechanisms other than cell cycle inhibition, PCNA binding, and derepression of p300/CBP repression domain CRD1 underlie the transcriptional effects. Since activation of the p21 gene expression by MITF was described previously, the reciprocal stimulation suggested here might represent a positive-feedback loop reinforcing the presumed prosurvival activity of MITF in melanoma cells. The results further suggest that p21 may have a role in MITF expression rather than in cell cycle regulation in melanoma cells.

4.3 Adenoviral E1A protein inhibits both MITF expression and MITF-dependent transcription

We tested a direct repression of the melanocyte-specific MITF promoter by E1A and its mutants. We found that the extreme N-terminus and conserved region 1 are required for repression. In contrast, the motif in conserved region 2, as well as amino acids 26-35 at the N-terminus, are not necessary. As these two later motifs mediate E1A binding to the retinoblastoma protein or to the transcriptional co-activator TRRAP, respectively, and are important for transformation by E1A in cooperation with other oncogenes, the results suggest that the transformation-defective E1A can still efficiently repress the MITF promoter. The CREB binding motif-mutated promoter had lower activity, but was also repressed by the same E1A mutants in human melanoma cells. Since recent data suggest that MITF may be a survival factor for melanoma cells, the E1A mutants described here might constitute a good targeting agent for antimelanoma therapy.

We further used the E1A oncoprotein and its mutants as repressors of both the transiently transfected and endogenous tyrosinase promoter, which is a well-established MITF target. It is shown that the requirement of the E1A N-terminus for repression of the MITF-activated tyrosinase promoter and the sensitivity to derepression by the histone deacetylase inhibitor trichostatin A are distinct when the activity of the transiently transfected or the endogenous promoter is analysed in U2-OS cells. Thus, for transiently transfected versus chromatin-embedded promoter, the activity of obligatory MITF seems to be executed through different mechanisms of transcriptional coactivation.

4.4 Inhibition of MITF function independent of targeting p300/CBP coactivators - CR1 E1A domain as an autonomous repressor of MITF-directed transcription

Although numerous MITF-dependent downstream genes have been identified, the mechanisms by which the MITF activity is coregulated remain elusive. We used a non-melanocytic cell line U2-OS as a model in which MITF evokes transcription of a MITF target tyrosinase and show that the adenoviral E1A protein represses the MITF-driven transcription in these cells. The E1A CR1 domain (which alone is insufficient to bind p300) was sufficient for repression, while the N-terminus, through which E1A binds the p300/CBP proteins and other coactivators, was unable to repress. Correspondingly, CR1 inhibited colony formation of MITF-positive, but not MITF-negative, melanoma cells. The repression by CR1 was largely independent of the PCAF-binding motif, previously recognized to be necessary for suppression of muscle-specific enhancer. Interestingly, CR1 conferred transcriptional competence to the MITF-CR1 chimera in which the MITF portion was rendered transcription-deficient. Moreover, MITF mutants defective in binding to p300/CBP in vivo still activated transcription, further supporting a p300/CBP-independent

coactivation of MITF targets. MITF is amplified in a subset of melanomas and is thought to be required for sustained proliferation of malignant melanocytes. Our results suggest that understanding how CR1 represses Mitf activity may reveal a route to melanoma therapy.

4.5 Dominant-negative MITF mutant - identification of a second transcription activation domain in MITF

A dominant negative mutant of the melanocyte-specific isoform of MITF is described carrying deletions of both N- and C-terminal transactivation domains. Cotransfection of this mutant resulted in a complete inhibition of the wild type MITF function as tested on both the reporter-linked tyrosinase promoter and an endogenous, ectopic MITF-triggered tyrosinase gene in U2-OS cells. The dominant negative construct also strongly repressed the activity of a hyperactive MITF-Vp16 chimera. Importantly, deletion of both activation domains was necessary to eliminate the residual transcription activity observed when only the N-terminal domain was removed and to achieve the repressive effect in human melanoma cells. If the activity of MITF plays a role in the long-term survival of malignant melanocytes, overexpression of a strong dominant negative MITF mutant might be another useful strategy to suppress its transactivation function.

5. Conclusions

Transcriptional activities of p21:

- The p21 protein has a role in the differentiation of F9 cells into PE. This role is independent of its function to inhibit cell cycle progression and proliferation. p21 activated the thrombomodulin promoter-reporter (a specific PE marker), stimulated expression of endogenous thrombomodulin, and partly relocalized to the cytoplasm in the differentiated cells, likely having an antiapototic role.
- We showed the positive action of p21 on the MITF promoter in melanoma cells, suggesting the existence of a positive-feedback loop constituted by reciprocal transcriptional activation between MITF and p21 in melanoma cells. The mechanism of coactivation of transcription is, for the MITF promoter, independent of its activity to derepress the p300/CBP CRD1 domain.

Mechanism of MITF function:

- The activity of MITF promoter and MITF target promoters is repressed by the adenoviral E1A protein. MITF transcriptional activity on the transfected versus an endogenous, chromatin-organized target promoter is coactivated by different mechanisms.
- We showed that unlike the N-terminus of E1A, the CR1 domain alone is surprisingly sufficient to autonomously repress transcription of the MITF-evoked target gene and inhibit colony

formation of MITF-positive melanoma cells. The CR1 might target as yet unidentified cofactor for MITF.

- We describe construction of a dominant negative mutant of the melanocyte-specific isoform of MITF carrying deletions of both N- and C-terminal transactivation domains. This mutant has a strong inhibitory effect on the activity of endogenous MITF in human melanoma cells.

6. Summary

F9 cells (embryonal carcinoma) can be induced to differentiate with retinoic acid and dibutyryl-cAMP into cells with a phenotype resembling parietal endoderm. We show that the levels of cyclin-dependent kinase inhibitor p21/WAF1/Cip1 (p21) protein and mRNA are elevated at the end of this differentiation. Clones of F9 cells stably expressing ectopic p21 revealed, upon differentiation, upregulated levels of mRNA for thrombomodulin, a parietal endoderm-specific marker. Furthermore, p21 activated the thrombomodulin promoter in transient reporter assays. The promoter activity in differentiated cells was reduced by cotransfection of p21-specific siRNA or antisense cDNA. Further experiments suggested that p21 might act through a derepression of the p300 N-terminal-residing repression domain, thereby enhancing the p300 coactivator function. Whereas p21 was strictly nuclear in undifferentiated cells, a large proportion of differentiated cells had p21 localized also in the cytoplasm. As differentiation of F9 cells into parietal endoderm-like cells requires the cAMP signaling, the results together suggest that the cyclin-dependent kinase inhibitor p21 may promote specifically this pathway in F9 cells and may have an antiapoptotic role in these cells.

Further study investigated the expression and transcriptional activity of p21 in human melanoma cells in culture. p21 was abundantly expressed in melanoma cells and was expressed also in proliferating epidermal melanocytes. We further found that promoters of several genes related to differentiation were stimulated by p21 overexpression, including the MITF promoter. The N-terminal portion and intact cyclin-binding site was essential for these transcriptional activities, suggesting that mechanisms other than cell cycle inhibition, PCNA binding, and derepression of p300/CBP repression domain CRD1 underlie the transcriptional effects. Since activation of the p21 gene expression by MITF was described previously, the reciprocal stimulation suggested here might represent a positive-feedback loop reinforcing the presumed prosurvival activity of MITF in melanoma cells.

Since recent data implicate MITF as a survival factor for melanoma cells, we further tested whether the melanocyte-specific MITF promoter activity can be inhibited directly by an adenoviral protein E1A and its mutants. We found strong repression by the

wild type E1A protein. The two motifs which mediate E1A binding to the retinoblastoma protein or to the transcriptional co-activator TRRAP, and are important for transformation by E1A in cooperation with other oncogenes, were dispensable for repression. Thus, this transformation-defective E1A mutant might constitute a good targeting agent for antimelanoma therapy. We further used the E1A oncoprotein and its mutants as repressors of both the transiently transfected and endogenous tyrosinase promoter. We have shown that the requirement of the E1A N-terminus for repression of the MITF-activated tyrosinase promoter and the sensitivity to derepression by the histone deacetylase inhibitor trichostatin are distinct when the activity of the transiently transfected or the endogenous promoter is analysed. Thus, for these two promoter versions, the activity of obligatory MITF seems to be executed through different mechanisms of transcriptional coactivation.

We have shown that the adenoviral E1A protein represses the MITF-driven transcription. The E1A CR1 domain (which alone is insufficient to bind p300) was sufficient for repression, while the N-terminus, through which E1A binds the p300/CBP proteins and other coactivators, was unable to repress. Correspondingly, CR1 inhibited colony formation of MITF-positive, but not MITF-negative, melanoma cells. The repression by CR1 was largely independent of the PCAF-binding motif. Interestingly, CR1 conferred transcriptional competence to the MITF-CR1 chimera in which the MITF portion was rendered transcription-deficient. Moreover, MITF mutants defective in binding to p300/CBP in vivo still activated transcription, further supporting a p300/CBP-independent coactivation of MITF targets. Our results suggest that CR1 may target an unknown cofactor which is required for Mitf activity.

Also, a dominant negative mutant of the melanocyte-specific isoform of MITF is described carrying deletions of both N- and C-terminal transactivation domains. Cotransfection of this mutant resulted in a complete inhibition of the wild type MITF function as tested on both the reporter-linked tyrosinase promoter and an endogenous promoter. The dominant negative construct also strongly repressed the activity of a hyperactive MITF-Vp16 chimera. Importantly, deletion of both activation domains was necessary to eliminate the residual transcription activity observed when only the N-terminal domain was removed and to achieve the repressive effect in human melanoma cells. If the activity of MITF plays a role in the long-term survival of malignant melanocytes, overexpression of a strong dominant negative MITF mutant might be a useful strategy to suppress its transactivation function.

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Drdová B. and Vachtenheim J. A role of the p21(WAF1/Cip1) protein in the differentiation of F9 mouse embryonal carcinoma cells into parietal endoderm. Acta Univ Palacki Olomouc Fac Rerum Natur Chemica 2004;43S:82. (XIX. Congress of the Cyech Society for biochemistry and molecular biology and Slovak Society for biochemistry and molecular biology, Olomouc, August 31 – Sept. 3., 2004)

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