Regulation of the MDR1 promoter by E2F1 and EAPP

Peter Andorfer, Hans Rotheneder*
Max F. Perutz Laboratories, Department of Medical Biochemistry, Medical University of Vienna, A-1030 Vienna, Austria

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ABSTRACT

Multidrug resistance (MDR), one of the main reasons for diminishing efficacy of prolonged chemotherapy, is frequently caused by the elevated expression of the ABCB1/MDR1 gene encoding PGP (P-glycoprotein). EAPP (E2F Associated PhosphoProtein) is a frequently overexpressed protein in human tumor cells. It inhibits apoptosis in a p21-dependent manner. We show here that EAPP stimulates the MDR1 promoter resulting in higher PGP levels. Independently of EAPP, E2F1 also increases the activity of the MDR1 promoter. Co-expression of pRb inhibits E2F1-, but not EAPP-dependent promoter activation. The upregulation of PGP might contribute to the survival of tumor cells during chemotherapy and worsen the prognosis for the patient.

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1. Introduction

The success of prolonged chemotherapy is often severely impaired by resistance to anti-cancer drugs. Two general classes of resistance exist: those that diminish delivery of the drugs to tumor cells, and those that develop in the cancer cell itself due to genetic and epigenetic modifications that reduce drug sensitivity. Poor absorption, increased metabolism or excretion and reduced diffusion from the blood into the tumor can be the reasons for impaired drug delivery [1,2]. The emergence of drug resistant cells can be easily studied by in vitro selection of cultured cells resistant to cytotoxic agents. These cells usually exhibit resistance to a variety of anticancer agents. Several different forms of such a multidrug resistance (MDR) have been identified. The most common forms are the P-glycoprotein (PGP) and the “multidrug resistance associated protein” (MRP) mediated MDR. Both kinds of proteins are members of a large family of ATP-dependent membrane proteins called ABC (ATP-binding cassette) transporters (for reviews see [3]). The principal function of PGP and the MRPs appears to be protection of the cell from xenobiotics. PGP is also essential for the maintenance of the blood–brain barrier [4]. The expression of these proteins is influenced by p53 in a conditional manner, depending on the cell type and the status of p53 (reviewed in [5]). There are indications, although not undisputed, that wild-type p53 represses and mutant p53 stimulates the expression of the ABCB1 (MDR1/PGP) [6,7] and the ABCB1 (MRP1) gene [8]. Transcriptional regulation of the human MDR1 gene seems to be rather complex and influenced by a variety of promoter binding factors but also by epigenetic mechanisms (for reviews see [9,10]).

E2F is a family of transcription factors that integrate cell-cycle progression with transcription through cyclical interactions with important regulators, such as the retinoblastoma-tumor-suppressor-gene product (pRB), cyclins and cyclin dependent kinases. E2F regulates genes, the products of which are essential for progression through the mammalian cell cycle. On the other hand, overexpression of E2F1 can induce apoptosis that is p53-dependent, but also p53-independent by direct activation of genes coding for proteins such as p73, Apaf-1 and caspases. The discovery that the expression of p14ARF, a tumor suppressor that prevents p53 degradation, and the expression of ATM and Chk2, kinases involved in the DNA damage response that phosphorylate (and stabilize) p53, is regulated by E2F provided a link between the pRB and p53 networks (reviewed in [11]). We have identified and characterized a protein that interacts with the activating E2F members and stimulates E2F-dependent transcription. Due to its strong phosphorylation we named it EAPP (E2F Associated PhosphoProtein) [12]. EAPP is enriched in the nucleus, but can also be found in the cytoplasm. Analyses of human tumor cell lines revealed that almost all express higher levels of EAPP than comparable untransformed cells [13]. EAPP is an essential...
protein, its RNAi-mediated knock down results in apoptosis. EAPP levels increase upon DNA damage and this protects cells from apoptosis in a p21-dependent manner. It turned out that EAPP binds to the p21 promoter via GC boxes in the vicinity of the TATA box and stimulates its activity [14]. Besides p21, the expression of the monoamine oxidase B gene is also regulated by EAPP [15].

Moreover, we found that EAPP selectively interacts with P-Thr68-Chk2 (checkpoint kinase 2) but not with unphosphorylated Chk2. This interaction results in enhanced dephosphorylation and thus inactivation of Chk2, indicating that EAPP plays a role in checkpoint recovery after successful damage repair [16].

We show here that independently of each other, both, EAPP and E2F1 stimulate the promoter of the MDR1 gene, resulting in increased expression and higher levels of PGP. This might contribute to the development of a multidrug resistance phenotype.

2. Materials and methods

2.1. Plasmids, reporter constructs, and reagents

pClneo-HA-EAPP, pClneo-MCS, pGal-Luc, pClneo-HA-E2F1 [12], pClneo-HA-pRb(Acdk) [17], Mdr1-Luc and Mdr1(Y-Box mutated)-Luc [18] have been described.

Etoposide, Nocodazole, MMS and Colchicine were purchased from Sigma–Aldrich and solved as recommended.

2.2. Cell culture and transfection

T98G and SAOS cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS).

Cells were transfected for 16 h using PerFectin (Genlantis). Medium was changed and cells grown for additional 24–48 h. As a control and to achieve equal amounts of DNA pClneo-MCS was used.

2.3. Western blot analysis

Whole cell extracts were prepared by using a lysis buffer (20 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). The separation was performed by SDS–polyacrylamide gel electrophoresis. Proteins were then transferred onto nitrocellulose and probed with indicated antibodies, followed by enhanced chemiluminescence and detection with X-ray films (Fuji).

The α-EAPP antibody has been described [14]. The α-Mdr1 (H-241) was purchased from Santa Cruz Biotechnology, anti-HA (16B12) from Abcam, and α-β-Actin (AC-74) from Sigma. ImageQuant (Molecular Dynamics) was used for quantification of the signals.

2.4. Luciferase reporter assay

Reporter assays were carried out as previously described [12] with a wild type and Y-Box mutated Mdr1-promoter-luciferase reporter construct. β-Galactosidase activity was assayed in parallel as a control for the transfection efficiency.

2.5. Cell cycle analysis

The cell cycle distribution was measured with DAPI (Merck). Cells were trypsinized, washed once with PBS and around 5 x 10^5 cells were taken. For the staining cells were fixed in 85% ethanol and incubated at least 30 min on ice. The DAPI concentration was 2 ng/ml and fixed cells were incubated for 20 min in the dark at 4 °C. A PARTEC PasIII was used to measure the DAPI concentration in the cells.

3. Results

3.1. EAPP stimulates the expression of MDR1 at the level of transcription

The treatment of proliferating cells with drugs like nocodazole that interfere with the assembly of the mitotic spindle results in the activation of checkpoint proteins. The following abrupt halt of cell cycle progression can be readily observed by analysis of the cell cycle profile of the respective cells. The cell cycle profile of EAPP-overexpressing cells differed radically from control cells when both were treated with nocodazole (Fig. 1 and [14]). Whereas in control cells drug treatment arrested cells in G2/M, EAPP overexpression led to a dramatic increase of the G1 fraction. Two mechanisms offered a plausible explanation for this observation. (1) EAPP induced a G1 arrest irrespective of the drug treatment, or (2) EAPP stimulated the expression and/or activity of ABC proteins that pumped the xenobiotic drugs out of the cell thus allowing continued proliferation. We followed both routes and in the end it turned out that the EAPP-stimulated upregulation of p21 caused the G1 arrest [14]. Nevertheless, we also found an influence of EAPP on the expression of MDR1/PGP, one of the ABC transporters. Fig. 2A shows the increase of MDR1 levels upon overexpression of EAPP. To find out whether this upregulation occurs at the level of transcription we carried out reporter gene assays with the human MDR1 promoter. A strong stimulation of the promoter could be observed in EAPP overexpressing cells (Fig. 2B). The MDR1 promoter exhibits a complex regulatory pattern with numerous binding sites for transcriptional regulators [9]. Fig. 2C shows a schematic drawing of the promoter region used for the reporter assays.

3.2. Drugs can modulate the activation of the MDR1 promoter by EAPP

To address the question whether drugs known to be substrates of PGP influence the activity of EAPP towards the MDR1 promoter, we overexpressed EAPP in T98G cells in the presence of colchicine, methyl-methanesulfonate (MMS), etoposide, or nocodazole. Subsequently we determined the levels of cell cycle progression can be readily observed by analysis of the cell cycle profile of the respective cells. The cell cycle profile of EAPP-overexpressing cells differed radically from control cells when both were treated with nocodazole (Fig. 1 and [14]). Whereas in control cells drug treatment arrested cells in G2/M, EAPP overexpression led to a dramatic increase of the G1 fraction. Two mechanisms offered a plausible explanation for this observation. (1) EAPP induced a G1 arrest irrespective of the drug treatment, or (2) EAPP stimulated the expression and/or activity of ABC proteins that pumped the xenobiotic drugs out of the cell thus allowing continued proliferation. We followed both routes and in the end it turned out that the EAPP-stimulated upregulation of p21 caused the G1 arrest [14]. Nevertheless, we also found an influence of EAPP on the expression of MDR1/PGP, one of the ABC transporters. Fig. 2A shows the increase of MDR1 levels upon overexpression of EAPP. To find out whether this upregulation occurs at the level of transcription we carried out reporter gene assays with the human MDR1 promoter. A strong stimulation of the promoter could be observed in EAPP overexpressing cells (Fig. 2B). The MDR1 promoter exhibits a complex regulatory pattern with numerous binding sites for transcriptional regulators [9]. Fig. 2C shows a schematic drawing of the promoter region used for the reporter assays.

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![Fig. 1. EAPP overexpression increases the G1 fraction of T98G and SAOS cells. Two series of cells were transfected with an HA-EAPP expressing or a control vector. 24 h later one series was treated for 16 h with nocodazole. Subsequently cells were harvested and analyzed for cell-cycle distribution.](image-url)
Fig. 2. EAPP induces the ABC transporter MDR1. (A) EAPP upregulates MDR1 protein levels. T98G cells were transfected with an HA-EAPP expression or a control vector, harvested and analyzed by immunostaining with the corresponding antibodies. (B) EAPP increases MDR1 promoter activity. T98G cells were either transfected with an HA-EAPP expression or a control vector and a MDR1-Luc vector harboring a truncated human MDR1-promoter in front of the luciferase gene. Reporter gene assays were carried out with these cells. (C) Chart of the MDR1 promoter used for the reporter gene assays.

Fig. 3. The EAPP-dependent upregulation of MDR1 is influenced by certain drugs. (A) Western blot showing the differential increase of MDR1 protein levels upon EAPP overexpression and drug treatment. T98G cells were transfected with the MDR1-Luc vector and a HA-EAPP expression or control vector and treated with the indicated drugs. Levels of endogenous and HA-EAPP are shown. β-Actin was used as a loading control. (B) Quantification of the signals of (A) using ImageQuant. The Actin signal was used for normalization. All mock controls were set to 1 for better comparison of the real fold increase. (C) Luciferase assays showing the stimulation of the MDR1 promoter in the presence of different drugs. T98G cells were treated as described in (A), harvested, and used for reporter assays.

Fig. 3A shows drug-specific induction levels of MDR1 upon overexpression of EAPP at the protein level. Quantification of the signals (Fig. 3B) allowed the comparison with the effects seen in reporter gene assays. The strongest effect was seen with nocodazole, followed by etoposide. Reporter gene assays (Fig. 3C) confirmed this result in principle albeit with some variations. The overall
stimulation of the promoter activity was stronger than the corresponding increase of the protein.

3.3. E2F1 also stimulates the MDR1 promoter and this is further enhanced by EAPP

Since EAPP interacts with the activating E2F proteins [12] it was conceivable that E2F proteins are involved in the activation of MDR1. Even though there are no canonical E2F binding sites within the MDR1 promoter it is not implausible that E2F plays a role in its regulation, considering the huge number of promoters where E2F is present even without an E2F binding site [19,20]. Reporter gene assays with both, the wt and the MDR1 promoter with a mutated Y-Box [18] were carried out. The Y-Box (or inverted CCAAT-box) is a ubiquitous element in eukaryotic housekeeping genes. It serves as a binding site for transcription factors like YB-1 [21] or NF-Y [22]. It turned out that E2F1 can also upregulate the Mdr1 promoter. Fig. 4A and B shows that ectopic E2F1 activates the Mdr1 promoter stronger than EAPP despite lower expression levels (Fig. 4C). Co-expression of both, E2F1 and EAPP stimulates promoter activity even further. The mutation of the Y-Box reduced overall promoter activity but did not affect the activation by EAPP and E2F1 (Fig. 4A and B).

3.4. EAPP regulates the MDR1 promoter independently of E2F and is not affected by pRb

EAPP is able to stimulate promoter activity in at least two different manners. (1) It can associate with promoters via GC-boxes [14], presumably by direct DNA binding [15], and (2) it activates transcription via E2F factors [12]. To find out whether the activation of the MDR1 promoter by EAPP is E2F-dependent, we co-expressed the retinoblastoma tumor suppressor protein (pRb), which binds and inhibits the activating E2F factors in G1. In cycling cells, pRb becomes phosphorylated by cyclin/cdk complexes resulting in the release and activation of E2F [23,24]. To avoid the inactivation of pRb we used a non-phosphorylatable and thus, constitutively active mutant (pRbΔcdk) [17]. Whereas the co-expression of pRbΔcdk considerably reduced the E2F-dependent activation of the MDR1 promoter, it rather enhanced the EAPP-caused activation...
Fig. 5A, compare 2nd and 7th column). This suggests that EAPP stimulates the MDR1 promoter more efficiently in the absence of active E2F.

4. Discussion

The expression level of MDR1 can be an important prognostic factor in human cancers. High levels of MDR1 often correlate with a poor response to chemotherapy and low complete remission rates [25]. Elucidating the mechanisms that result in elevated expression of multi drug resistance genes in general and MDR1 in particular and the development of drugs that interfere with their activity has been a major goal of cancer biologists for many years (reviewed in [26,27]) and is ongoing [28].

The expression of the human MDR1 gene, which is still incompletely understood, seems to be influenced by a variety of promoter-binding factors [9], but also by epigenetic events [10]. Very recently it has been shown that miRNAs can also be involved in the regulation of this gene [29–31].

Our observations that both, E2F1 and EAPP can independently stimulate the expression of MDR1, adds a new facet to the intricate regulation of this gene. Increased E2F1 activity can result from the inactivation of the retinoblastoma tumor suppressor [32,33], the overexpression of c-myc [34,35], or DNA damage [36]. Since E2F1 can induce apoptosis via p53-dependent [37,38] and – independent pathways [39,40] compensatory mechanisms would be required to prevent apoptosis in a transforming cell. One of these mechanisms could be the concomitant induction of EAPP resulting in elevated p21 levels [14]. Although the prime activity of p21 is the induction of cell cycle arrest or senescence it also exhibits strong anti-apoptotic properties [41] and acts as a negative regulator of p53 [42]. If pRb is already inactive, such a cell might proliferate unabated and become the nucleus of a tumor. The ability of EAPP to stimulate E2F-dependent transcription could be an additional advantage for a transforming cell with elevated levels of both kinds of proteins. The concurrent upregulation of MDR1/PGP might be only a side effect in the early stages of cancerogenesis but might contribute to the survival of tumor cells during chemotherapy and worsen the prognosis for the patient.

The stimulation of the MDR1 promoter by EAPP was more pronounced in cells treated with etoposide or nocodazole than in untreated or MMS- and colchicine-treated cells (Fig. 3). The mechanism for this is unclear but in the case of etoposide it might be mediated by the protein kinase CK2 which relocalizes upon etoposide treatment [43] and is also strongly phosphorylating EAPP (Rotheneder, unpublished).

How could EAPP stimulate PGP expression? Although E2F1 also enhances MDR1 promoter activity (Fig. 4), this can be repressed by...
pRb which is not the case with EAPP suggesting an E2F-independent mechanism. Interestingly, the repression of E2F by pRb(Acdk) is reported to result in stronger activation by EAPP (Fig. 5A). This could mean that there is a competition between E2F1 and EAPP. The MDR1 promoter comprises GC boxes serving as binding sites for Sp1 like proteins which have been shown to stimulate MDR1 expression [44]. We and others have found earlier that E2F1 interacts with Sp1 and this seems to be required for the activity of certain promoters [45,46]. On the other hand, EAPP can compete with Sp1 for GC boxes [14,15]. Taken together this could explain our observations. According to this model E2F1 stimulates the MDR1 promoter by interacting with Sp1, which could also stabilize DNA binding of Sp1. pRb might interfere with this interaction thereby giving way for the displacement of Sp1 by EAPP. A better understanding of the mechanisms resulting in multidrug resistance of which EAPP and E2F1 seem to be part of might help to prevent its development or allow its suppression and thereby increase the efficacy of chemotherapy.

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References