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ORIGINAL RESEARCH PAPER

A potentially immunologically inert derivative of the reverse tetracycline-controlled transactivator

Arnaud Zaldumbide · Susan Weening · Steve J. Cramer · Martijn J. W. E. Rabelink · Joost Verhaagen · Rob C. Hoeben

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Abstract The archetypical system for regulating heterologous gene expression in mammalian cells involves tetracycline-activated transactivators (rtTA). Binding of such transactivators to tet-operator-controlled promoters induces transcription. Immune responses directed against the transactivator proteins may limit the applicability of this system in immune-competent hosts. To circumvent such immune responses the immune evasion mechanism of the Epstein–Barr virus Nuclear-Antigen 1 was exploited. Our data show that fusion of the rtTA with the EBNA-1 derived Gly-Ala repeat yielded an efficient transactivator with no detectable activity in absence of inducer. Antigenic peptides of the fusion protein were not presented in MHC class I.

Keywords Doxycycline · Gene therapy · Inducible expression · Promoter · Transcription

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Introduction

Tetracycline-inducible systems are among the most commonly used systems for inducible heterologous gene expression in mammalian cells. Originally a Tet-off system was described in which transgene expression was repressed in the presence of doxycycline (an analogue of tetracycline). This system was evolved to a more convenient Tet-on version (Gossen et al. 1995). In the latter version, the transcription of the gene of interest is dependent on the binding of the tetracycline/doxycycline-activated transactivator (rtTA) on tet-operator-controlled promoter. The rtTA protein, used in these systems, is a fusion between a mutated form of the tetracycline repressor/activator protein (TetR) from *Escherichia coli* and the VP16 transactivation domain of herpes simplex virus. Extensive evolution increased doxycycline sensitivity, increased stability in eukaryotic cells, and reduced leakiness in absence of inducer (Urlinger et al. 2000). However, the immunogenicity of the transactivator limits wide-spread application in immune-competent animals. Intramuscular transfer of plasmid encoding rtTA triggers cellular and humoral immune responses, leading to the destruction of the modified cells (Chenuaud et al. 2004; Favre et al. 2002; Latta-Mahieu et al. 2002).

To reduce T-cell mediated destruction of rtTA-expressing cells, we took advantage of the long Gly-Ala repeat (GAr) domain from the Epstein–Barr Nuclear-Antigen-1 protein (EBNA-1) to generate an immunologically inert version of the rtTA protein

(GAr-rtTA). EBNA-1 is essential for the maintenance of the herpes virus 4 genome as stable episome during latency. Although EBNA-1-specific cytotoxic-T lymphocytes (CTLs) circulate in patients (Blake et al. 1997), the EBNA-1-positive cells are not recognized and killed. By a inhibitory effects on its own synthesis (Yin et al. 2003), and on proteasomal degradation (Levitskaya et al. 1997), the GAr efficiently reduces cytotoxic-T lymphocyte (CTL)-epitope generation from linked proteins.

Here we demonstrate that the EBNA-I modified transactivator (GAr-rtTA) retained its transactivator function. Moreover we observed that the fusion with the Gly-Ala repeat domain reduced its activity in absence of inducer. With an ovalbumin-derived $H2K^b$-restricted CTL epitope inserted at the carboxyl terminus of the rtTA protein as probe, we establish that the Gly-Ala repeat prevents antigenic peptide generation of the rtTA.

Materials and methods

Constructs

pCMV-$rtTA_{Ova}$ was generated by introducing the codons for the Ova epitope SIINFEKL by mutagenic PCR into the open reading frame for rtTA with the following primers:

Forward 5′-GACATGCTCCCCGGGAGCATAATTAATTTCGAAAAGCTCTAACTAAGTAAGGATCC-3′

Reverse 5′-GGATCCTTACTTAGTTAGAGCTTTTCGAAATTAATTATGCTCCCGGGGAGCATGTC-3′

To allow fusion of the Gly-Ala repeat domain with the rtTA gene, we used mutagenic PCR to replace the ATG by a unique SalI restriction site using the following primers:

Forward 5′-ACGAGCTGTACAAGAGCATAATTAATTTCGAAAAGCTCTAAGCGGCCGCGTC-3′

Reverse 5′-GACGCGGCCGCTTTAGAGCTTTTCGAAATTAATTATGCTCTTGTACAGCTCGTC-3′

Finally, pCMV-GArtTA was generated by introducing a *Sal*I/*Sal*I Gly-Ala repeat containing fragment from pLV-CMV-GArGFP (Hendriks et al. 2007) into pCMV-rtTA (*Sal*I). The rtTA (or $rtTA_{Ova}$) and the GAr-rtTA (or GAr-rTA_{Ova}) fragments were then subcloned into 3rd generation lentivirus vector to generate pLV-CMV-rtTA/Ova and pLV-CMV-GAr-rtTA/Ova. All constructs are depicted in Fig. 1.

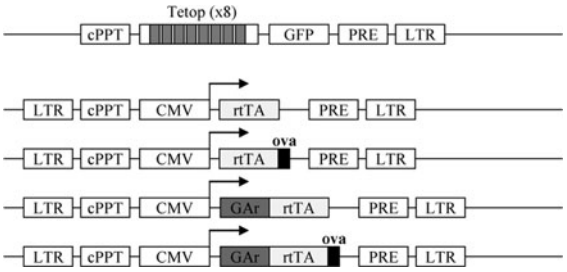

Fig. 1 Schematic representation of the vectors used. rtTA and GAr-rtTA have been cloned in the lentivirus backbone plasmid pLV described previously (Carlotti et al. 2004). *cPPT* Central polypurine tract, *PRE* post-translational responsive element, *CMV* cytomegalovirus promoter, *rtTA* reverse tetracycline-activated transactivators, *GFP* green fluorescent protein, *LTR* long-terminal repeat

Cell lines

293T were grown in high-glucose DMEM supplemented with 10% (v/v) fetal bovine serum (Gibco BRL) and penicillin/streptomycin. The B3Z indicator cells (Karttunen et al. 1992), kindly provided by Dr. Rene Toes, were grown in IMDM supplemented with 8% fetal bovine serum, 28 μM β-mercaptoethanol, and 500 μg/ml hygromycinB and 100 U penicillin/streptomycin (GlutaMAX). All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Transfections and FACS analysis

293T cells were modified at 70–80% confluency by transfection with the calcium phosphate co-precipitation technique. Sixteen hours post transfection the cells were changed to doxycycline-containing media for 24–48 h. Subsequently, the cells were trypsinized, resuspended in PBS, and GFP fluorescence was measured by FACS analysis (FACScan Becton–Dickinson). GFP fluorescence was detected using a 530/30 nm bandpass filter (FL1 channel) following excitation with an argon ion laser source at 488 nm. Data analysis was performed using FlowJo software

(Becton–Dickinson). For each sample, at least 10,000 events were collected.

Western blot

Cells were treated with RIPA lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, SDS 0.1%, 0.5% DOC, 1% NP40 + proteases inhibitors). Proteins were loaded on acrylamide/bis acrylamide SDS page gel, and after electrophoresis, transferred to Immobilon-P [Immobilon-P transfer membrane (PVDF); Millipore, Etten-Leur, The Netherlands] and treated with anti-GFP (1:1,000; Molecular Probe), anti-TetO2 (1:1,000; MoBiTec) and anti-actin (1:5,000, clone C4; ICN Biomedicals, Inc., Zoetermeer, The Netherlands).

B3Z assay

Analyses of Ova presentation were performed as previously described (Zaldumbide et al. 2007). Briefly, 293T cells transfected with an $H2K^b$ cDNA expression vector and the appropriate Ova antigen-encoding constructs, were exposed to B3Z CTL 16 h post transfection. After o/n coculture, plates were centrifuged 5 min at 1,500×*g*, and lysed in luciferase lysis buffer [125 mM Tris/HCl, pH 7.8, 10 mM CDTA, 10 mM DTT, 50% (v/v) glycerol, 5% (v/v) Triton X-100]. β-Galactosidase activity was determined by luminometry (Lumat LB9501 luminometer Berthold, Bad Wildbad, Germany) using the galactolight dual light kit (Tropix).

Results

To reduce immunogenicity of the rtTA, the long glycine-alanine rich repeat (GAr) from EBNA-1 has been fused at the *N*-terminus of the rtTA to generate an immunologically inert transactivator (Fig. 1). To test whether the GAr region affects the activity of the transactivator, 293T cells were co-transfected with a construct in which GFP expression is controlled by an 8 TA-binding-motif containing (Tet-op) CMV promoter, and with either varying amounts of the normal transactivator (rtTA)-encoding plasmid or of the EBNA-I variant (GAr-rtTA). Twenty-four hours post-transfection, cells were exposed to 1 μM doxycycline and 16 h later, analyzed for GFP expression by flow cytometry (Fig. 2a). In presence of doxycycline (black lines) the rtTA and the GAr-rtTA can efficiently activate the reporter gene, yielding 35 and 25% GFP-positive cells, respectively. The GAr significantly modified the expression kinetics. Transactivation by the GAr-rtTA was dose-dependent and with increasing amounts of transactivator plasmid expression raised gradually from 10 to 25% GFP-positive cells. A minimal amount of the rtTA plasmid is sufficient for maximal activation of the reporter (35% GFP-positive cells with 0.1 μg transfected rtTA). In the uninduced state, i.e. in absence of doxycycline, the GAr-rtTA is almost inactive (0.4% positive cells), whereas the use of the rtTA leads to background expression (20%). Thus the GAr fusion reduced the activation of transcription by the transactivator in the absence of Dox. The background activity in the absence of doxycycline is independent of the amount of EBNA-I derived transactivator, since no increase in fluorescence was noted with increased amounts of GAr-rtTA. In contrast, transcription by the rtTA was activated up to 8-fold in the absence of Dox (Fig. 2b). The GAr-rtTA is highly inducible: doxycycline treatment induces reporter gene expression up to 14-fold as measured by MFI. The presence of the Gly-Ala repeat only slightly decreased the sensitivity to doxycycline (Fig. 2c).

Western blot analyses confirmed the cytometric data and highlighted the efficiency of the GAr-rtTA. No GFP protein is detected in absence of doxycycline. Since no degradation bands were detected in GAr-rtTA transfected cells (Fig. 2d) we conclude that the activation results from binding of the intact GAr-rtTA to the Tet-op sequence and not from activity of cleaved degradation products.

To evaluate whether the GAr protects the rtTA from CTL-mediated destruction, we fused the Ova-derived CTL epitope peptide SIINFEKL at the carboxyl terminus of both transactivators and measured ova-peptide presentation by the B3Z assay (Fig. 1). Ova presentation to B3Z hybridomas, in the $H2k^b$ context induces IL2 production and activation of a heterologous IL-2 promoter driving expression of the *E. coli lacZ* gene. Thus, the beta-gal activity reflects the hybridoma's response to the antigen presentation. Only in the context of $H2K^b$, we observed a strong response of the B3Z CTLs against the $rtTA_{Ova}$ expressing cells, whereas no significant differences were detected between the GAr-$rtTA_{Ova}$

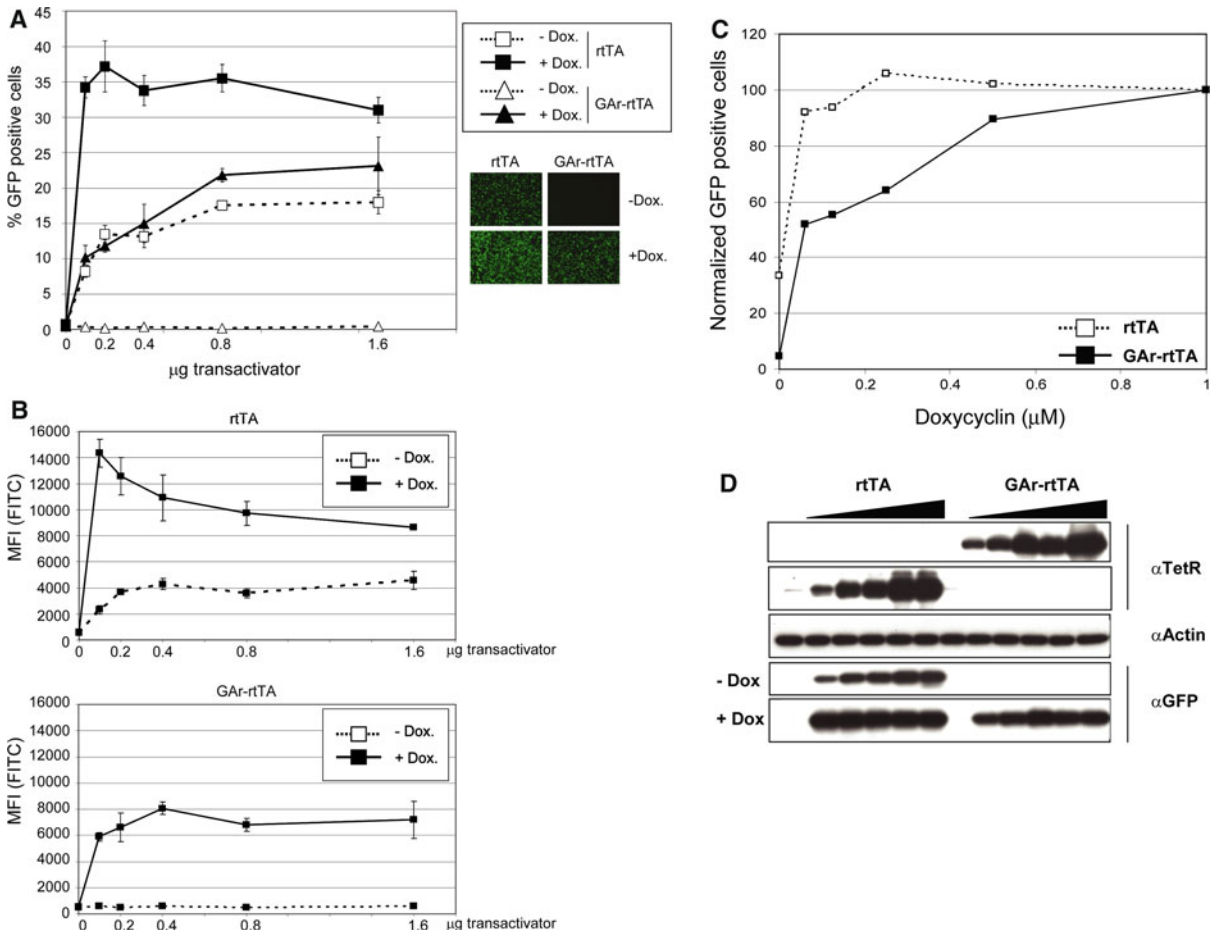

Fig. 2 Analysis of rtTA and GAr-rtTA 293T transfected cells. **a** Flow cytometric analysis and fluorescence light microscopy pictures of cells transfected with 0.1 µg of Tetop-GFP reporter and various amounts of transactivator-encoding vector, without and with 0.5 µM doxycycline. Transfected cells were treated 24 h with 0.5 µM doxycycline before analysis. The inserts show fluorescence light microscopy pictures of cells transfected with 0.8 µg of transactivator-encoding vector, without and with 0.5 µM doxycycline. **b** Mean fluorescent intensity of transfected cells. **c** Doxycycline dose response curve. 293T cells have been transfected with 0.1 µg of Tetop-GFP reporter and optimal amount of rtTA and GAr-rtTA (respectively 0.2 and 1.6 µg—as defined on Fig. 2b) and treated for 24 h with increasing amount of doxycycline. GFP-positive cells determined at 1 µM Dox. has been arbitrary set to 100%. **d** Western blot analysis of 293T cells transfected with increasing amount of rtTA or GAr-rtTA (0, 0.1, 0.2, 0.4, 0.8, 1.6 µg) and 0.1 µg of Tetop-GFP and treated with 0.5 µM doxycycline

and the negative controls lacking the Ova epitope (Fig. 3). The reduction of the B3Z reactivity confirms the inhibitory effect of the GAr domain on antigenic peptide presentation.

Discussion

Regulation of heterologous transgene expression is essential for many biomedical research applications such as gene therapy. Although regulated expression has been achieved with the tet-dependent transactivators with interesting results, i.e. in transgenic mice (Albanese et al. 2002), the residual activity in absence of inducer, and the immunogenicity of the TetR-VP16 fusion protein, thwart the widespread use of this system in many gene therapy or transplantation settings into immune-competent hosts. Here we describe the generation of a potentially immunologically inert version of the tetracycline-activated transactivator. Viruses have developed many strategies to escape the immune surveillance (Zaldumbide

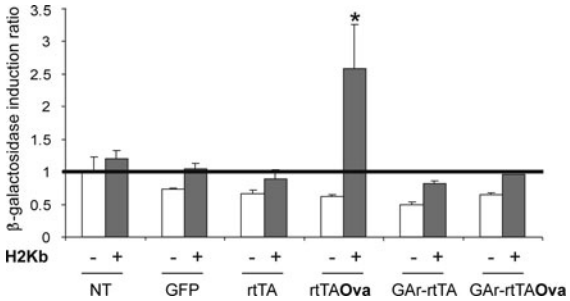

Fig. 3 The GAr sequence affects ova-peptide presentation. To measure ova-peptide presentation, 293T cells were transfected with 1 μg of GFP, rtTA or GAr-rtTA, as well as their ova variants, with or without an $H2K^b$ encoding plasmids. Twenty-four hours post transfection, the modified 293T cells were co-cultured with B3Z hybridoma cells in a 1:1 ratio for 16 h. Subsequently *β*-galactosidase activity measured. The activity measured in unmodified control cells (NT) was arbitrary set to 1. The white bars represent the *β*-galactosidase activity in absence of the appropriate MHC-I; the *dark bars* the activity in $H2K^b$ context

and Hoeben 2008). Here we exploited the GAr domain from EBNA-1. EBNA-1 is responsible for the immune evasion by inhibiting proteasomal degradation linked polypeptides. As a result it blocks presentation of transgene-derived antigens by MHC class I. This domain has been described as a specific inhibitor of the proteasomal degradation via direct interaction of glycine and alanine-containing sequences with a proteasome component (Levitskaya et al. 1997; Sharipo et al. 2001). However, the exact molecular mechanism by which EBNA-1 protects itself against CTL recognition is still unclear. Recent studies suggest an effect on the proper folding of the Gly-Ala containing protein, disrupting 26S proteasome activity (Daskalogianni et al. 2008; Hoyt et al. 2006). We and others have previously shown that insertion of the GAr region at the amino terminal part of the protein does not affect the function of *E. coli* *β*-galactosidase, HSV TK, and eGFP (Eggers et al. 2008; Hendriks et al. 2007; Ossevoort et al. 2006).

The fusion of the 430 amino acid of the EBNA-1 protein with the DNA-binding domain of the rtTA (*N*-terminus) does not affect the functionality of the transactivator. The unmodified rtTA in absence of inducer was able to activate reporter gene expression, suggesting that it binds DNA. Here it could compete with the active form in presence of doxycycline. This may explain why increasing the amount of the activator plasmid reduces the MFI from 14,000 to 8,000. In contrast, the GAr variant does not exhibit any activity in the absence of the inducer at any concentration. Altogether, our data demonstrate that the GAr-rtTA is more tightly regulated in activating Tetop-containing promoters.

The GAr has no effect on transactivator protein levels since similar amount of the two variants were detected. Nevertheless, while the Ova peptide is presented efficiently if derived from the $rtTA_{Ova}$, no presentation was detectable upon expression of the GAr-$rtTA_{Ova}$ upon co-culture with the B3Z cells, confirming the effectiveness of the GAr.

The system presented here may also provide an alternative to systems based on KRAB or Mad-1, in which the VP16 immunogenic part as been replaced by a human zinc finger protein Kox1 or by the mSin3-interacting domain of human Mad1, respectively. In the presence of doxycycline, the repressor is released and the activated rtTa can bind and activate transcription. In conclusion, our data provide a proof-of-principle and demonstrate the feasibility and the efficiency of the GAr for construction of non-immunogenic expression regulatory systems for use in mammalian systems.

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