IN HCMV-EXPOSED HUVEC, P52/RELB REGULATORY FACTORS MEDIATE ACTIVATION OF THE HUMAN PAR1 GENE PROMOTER

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Abstract — We studied the effect of HCMV (AD-169) on the regulation of PAR1 reporter activity in HUVEC. In HUVEC, HCMV significantly induced activation of PAR1 reporter constructs carrying AP-2-like and two complexes of AP-2/Sp1-binding elements. Shorter constructs with no AP-2-like binding elements had lower activity upon virus inoculation. Moreover, activity of the shortest construct without binding elements was not significantly increased in virus-exposed cells. Furthermore, in HCMV-exposed cells, activity of the PAR1 reporter was significantly augmented upon p52/RelB transfection. Taken together, the data indicate that activity of the PAR1 promoter is highly dependent on AP-2/Sp1 binding elements and mediated by p52/RelB regulatory factors.

Key words: HCMV, PAR1 gene reporter, HUVEC

INTRODUCTION

Human cytomegalovirus (HCMV) is a member of the Betaherpesviridae family and subfamily Betaherpesvirinae that establishes a life-long persistent infection (Huang and Kowalik, 1993). In immunocompromised patients, such as individuals with AIDS and patients taking immunosuppressive drugs following transplantation, HCMV may cause severe disease (Britt and Alford, 1996). This virus is a cause of the most important congenital infection and may lead to developmental damage of the central nervous system including hearing loss, mental retardation, and visual impairment (Alford et al., 1990).

Protease-activated receptors (PAR) 1, 3, and 4 are responsible for the cellular response to thrombin, the key effector protease of the coagulation cascade (Coughlin, 2005). These receptors belong to a family of 7-transmembrane G protein-linked receptors proteolytically and irreversibly activated by thrombin (Brass et al., 1994; Hein et al., 1994). Described as the prototypical thrombin receptor, PAR-1 is rapidly desensitized after activation, followed by internalization and trafficking in the lysosomes. Recovery of thrombin responsiveness is due to replenishment of the cell surface with new receptors (Hein et al., 1994). The presence of a protected intracellular PAR-1 pool in many cell types (such as ECs, smooth muscle cells, and fibroblasts) with at least as many receptors as are present initially on the cell surface provides a mechanism for quick recovery after activation, independent of protein synthesis (Woolkalis et al., 1995; Ellis et al., 1999). In the absence of an agonist, PAR-1 cycles at very low rates between the cell surface and the intracellular pool (Shapiro et al., 1996; Shapiro and Coughlin, 1998) and the time required for new PAR-1 synthesis after cleavage by thrombin is 24 hours (Yan et al., 1996).

The regulatory region of the human thrombin receptor gene has been cloned, and DNA sequence analysis indicates the presence of many regulatory
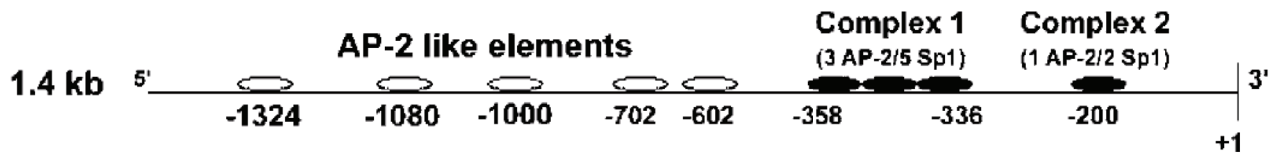
Fig. 1. Putative transcriptional regulatory sequences of the PAR1 gene (Tellez, C., Oncogene 2003; 22: 3130-3137).

factor-binding elements, including two AP-2/Sp1 complexes in the proximal 3' region of the promoter. Overall, these complexes contain four putative AP-2-binding elements (Tellez and Bar-Eli, 2003) and seven Sp1-binding elements (Wu et al., 1998) as shown in Fig. 1. The regulatory region of the thrombin receptor gene shares remarkable similarities with some other AP-2 target genes, such as a GC-rich sequence, lack of conventional TATA sequences, and multiple AP-2-binding elements (Tellez et al., 2003; Tellez and Bar-Eli, 2003).

Here we sought to determine the minimal HCMV-responsive element within the PAR1 promoter and investigate if promoter activity could be mediated by NF-κB transcription machinery (Ghosh et al., 1998), in particular p52/RelB, upon HCMV exposure.

MATERIAL AND METHODS

Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated and maintained as previously described (Laumonnier et al., 2000). In all experiments, HUVEC were used between passages 2 and 6.

Virus culture and determination of virus titer

Human cytomegalovirus, laboratory strain AD-169 (HCMV-AD169), was purchased from ATCC and propagated in MRC-5 cells. A confluent monolayer of MRC-5 cells was inoculated with virus [0.01 cytopathic effects (CPE) per cell] in serum-free medium. Cells were washed after 90 min and further cultured in growth medium until cytopathic effects were apparent in approximately 100% of cells. Infected cells were then replenished by fresh culture medium. After 5 days, the supernatant containing viral particles was collected and centrifuged at 1000x rcf at 4°C for 15 min. To sediment virus

particles, debris-free supernatant was then subjected to ultracentrifugation at 20000 rcf at 4°C for 1 h. The supernatant was carefully discarded and the virus pellet resuspended in 500 μl of growth medium, aliquoted, and stored at -80°C for further use. For each independent experiment, fresh aliquots from the same preparation were used.

The virus titer was determined by virus plaque assay. Virus samples were serially diluted in serum-free medium and adsorbed to MRC-5 cells plated in 24-well plates at a density of 10^5 cells per well. Inoculum was replaced with culture medium after 90 min. After 10 days, the cells were stained with Giemsa solution. White plaques were counted and the virus titer was presented as the number of cytopathic effects (CPE) per volume of infected medium, also known as the multiplicity of infection (MOI).

PAR1 promoter deletions and expression plasmids

We made PAR1 promoter deletions by PCR amplification using the Advantage GC Genomic PCR kit having GC melt in order to break strong guanine-cytosine bonds in the genomic DNA. Primers were designed to carry restriction sites for SacI and XhoI restriction enzymes, providing so-called "sticky ends". Further, constructs were cloned into the pGL-3Enhancer luciferase reporter vector (Promega GmbH, Mannheim, Germany) in the multiple cloning region between the aforementioned restriction sites. Primer sequences of constructs and PCR condition are given in Table 1.

The p52 and RelB expression plasmids were kindly provided by the Papillomavirus Research Unit, University of Quensland, Australia. Expression plasmids were cloned into the EcoRV and NotI restriction sites of the pcDNA3 vector (Promega GmbH, Mannheim, Germany).

Table 1. Summary of PAR1 promoter deletion constructs. Deletion constructs were generated by PCR amplification. Reaction conditions were as follows: initial denaturation at 94oC for 5 min, 35 cycles of denaturation at 94oC for 30 sec, annealing at 64oC for 30 sec, and elongation at 72oC for 90 sec. All PCR reactions were run on GeneAmp PCR System 2400 PE and GeneAmp PCR System 9700.

Construct	5' primer	3' primer
PAR1-1106 (1,2kb)	ATGCGAGCTCCCAGTGGCAA AGCAACTT	ATTACTCGAGCCTCCGGCGT GCAGTGAGAGTCTCTGC
PAR1-863 (957bp)	ATGCGAGCTCTTCTGTGGTC CCAGGTAATCCG	ATTACTCGAGCCTCCGGCGT GCAGTGAGAGTCTCTGC
PAR1-526 (620bp)	AGCTGAGCTCGCCGCTCTTC CTATTCCACTCGCA	ATTACTCGAGCCTCCGGCGT GCAGTGAGAGTCTCTGC
PAR1-298 (392bp)	GATCGAGCTCACTGAACTTT GCCGGTGTCCCA	ATTACTCGAGCCTCCGGCGT GCAGTGAGAGTCTCTGC
PAR1-87 (181bp)	ACTAGAGCTCCGAATCAACG GTGCCCAGAGGA	ATTACTCGAGCCTCCGGCGT GCAGTGAGAGTCTCTGC

Gene reporter assay

The HUVEC used were either mock- or HCMV-exposed (MOI 5) in serum-free medium for 1 h, washed, and transiently transfected with PAR1 pGL-3Enhancer luciferase reporter constructs containing full-length and progressively shorter promoter sequences of the human PAR1 gene alone or together with p52 + RelB (p52/RelB) expression plasmids using the SuperFect transfection reagent according to instructions provided by the manufacturer (Quiagen GmbH, Hilden, Germany). After 48 h, cells were harvested and lyzates were assayed for luciferase activity using the Luciferase Reporter Assay System kit (Promega, Madison, WI, USA) in a plate luminometer (Stratec Biomedicine Systems AG, Birkenfeld, Germany). We used HUVEC tansfected with a vector containing the luciferase gene to control non-specific viral effects. Results were normalized to the luciferase activity in mock-transfected HUVEC.

Statistical analysis

Values shown represent means ± SEM. Statistical significances were calculated with the Newman-Keuls test for multi-group comparisons.

RESULTS

Human cytomegalovirus significantly increases activity of PAR1 reporter deletion constructs

The regulatory region of the human thrombin receptor gene has been cloned. Analysis of DNA sequences indicates the presence of many transcription factor-binding elements, including 2 AP-2/Sp1 complexes in the proximal 3´ region of the promoter (Schmidt et al., 1996; Wu et al., 1998; Tellez and Bar-Eli, 2003). In HUVEC, basal activities of transfected reporter constructs did not differ significantly when compared to the full-length PAR-1106 construct, though activity of the shortest one, PAR1-87, was approximately 30% lower compared to PAR1-1106 (Fig. 2). However, the virus did significantly induce activity of all constructs as compared to their respective basal activities with the exception of the PAR1-87 construct. Moreover, activity of PAR1-863 was significantly higher compared to PAR1-1106, suggesting the presence of a DNA sequence in the full-length promoter that interferes with HCMV activation of the PAR1 promoter.

Human cytomegalovirus activates the PAR1 promoter via p52/RelB
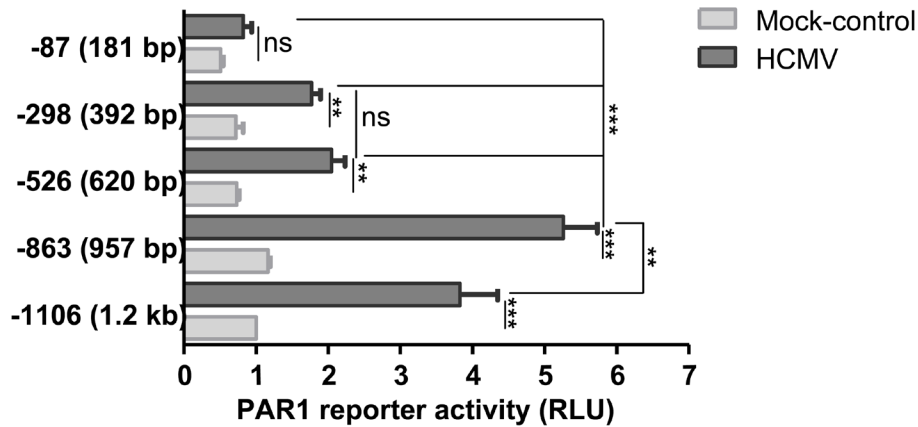
To investigate if p52/RelB transcription factors

Fig. 2. Human cytomegalovirus increases activity of PAR1 reporter constructs. Gene reporter assay: HUVEC, HMCV-(5 MOI) or mock-infected, were transfected with vectors encoding PAR1 reporter gene constructs. Cells were analyzed for luciferase activity after 48 h. Data were normalized to total protein concentration and presented as MEANS ± SEM of n = 6 independent experiments. ns – not significant, *p < 0.05, **p < 001, ***p < 0.0001.
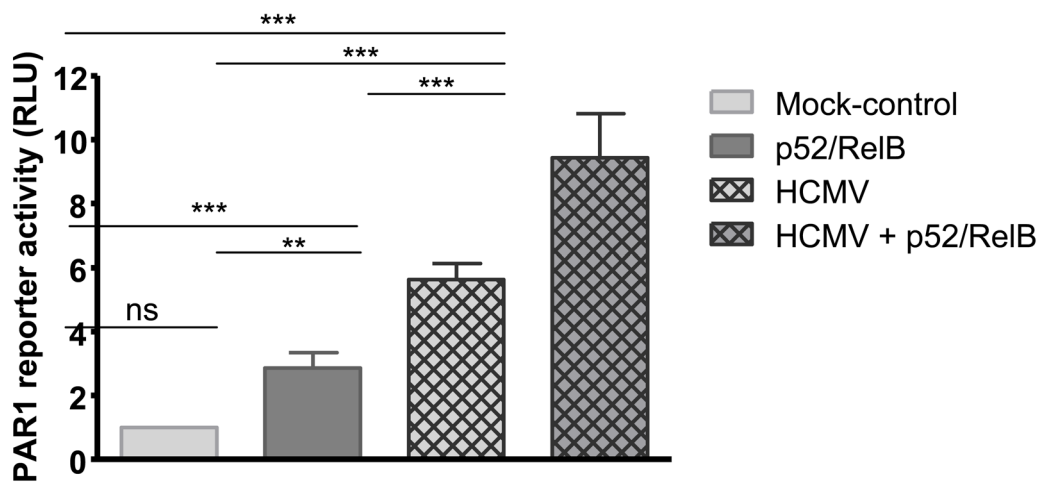

Fig. 3. The p52/RelB expression plasmids significantly increase PAR1 reporter activity in HCMV-exposed HUVEC. Gene reporter assay: HUVEC, HMCV-(5 MOI) or mock-infected, were transfected with vectors encoding either the PAR1 reporter gene alone or together with p52/RelB plasmids. Cells were analyzed for luciferase activity after 48 h. Data were normalized to total protein concentration and presented as MEANS ± SEM of independent experiments: n = 14 for the PAR1 promoter and n = 8 for p52/RelB. ns – not significant, *p < 0.05, **p <.001, ***p < 0.0001.

could mediate PAR1 reporter activation, mock- or HCMV-exposed HUVEC were transiently transfected with either the PAR1 reporter gene alone or together with p52/RelB. As shown in Fig. 3, transfection of p52/RelB increased basal activity of the PAR1 reporter gene threefold, but not significantly. However, in virus-inoculated cells, promoter activity was significantly over-activated upon p52/RelB transfection compared to either p52/RelB transfected- or HCMV-inoculated cells.

DISCUSSION

In the present study, we showed that exposure to HCMV significantly increases activity of all PAR1 deletion constructs (Schmidt et al., 1996), with the exception of the shortest one, PAR1-87, when compared to their basal activities. Progressively shorter fragments carrying none of the AP-2-like binding elements (Schmidt et al., 1996) had markedly lower activities upon virus exposure when compared to

either PAR1-1106 or PAR1-863 constructs. This suggests an important (but not crucial) role for AP-2-like binding elements, since their activities in virus-exposed cells were still markedly higher than their corresponding basal activities. Interestingly, activity of PAR1-863 was higher than that of PAR1-1106, suggesting the presence of potentially inhibitory factors in the full-length promoter. Furthermore, analysis of PAR1-526-carrying complexes 1 (3 AP-2/5 Sp1) and 2 (1 AP-2/2 Sp1) and PAR1-298 carrying only complex 2 revealed comparable levels of activity upon virus exposure, but still significantly higher when compared to their basal activities, suggesting an important role for complex 2. Moreover, activity of the PAR1-87 construct, carrying none of the regulatory binding elements and none of the complexes, was only marginally increased when exposed to the virus, and its basal activity was approximately 30% lower than activity of PAR1-1106, pointing to a crucial role for complex 2 (Wu et al., 1998; Tellez and Bar-Eli, 2003).

Taking all the data into account, we anticipated a crucial role for AP-2/Sp1 regulatory elements in concert with p52/RelB (Ghosh et al., 1998). However, it remains to be elucidated whether p52/RelB mediate PAR1 promoter activation directly or indirectly by influencing AP-2/Sp1 regulatory elements to bind to their corresponding binding sites within the PAR1 promoter.

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У HUVEC, ТРАНСКРИПЦИОНИ ФАКТОРИ P52/RELB ПОСРЕДУЈУ У АКТИВАЦИЈИ ЉУДСКОГ PAR1 ПРОМОТОРА ПОСЛЕ ИЗЛАГАЊА ВИРУСУ (HCMV)

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[4]*Gen-Trend Ltd.,* 370 04 Чешке Буђејовице, Чешка Република

Аутори су у овој студији анализирали утицај HCMV (AD-169) на регулацију активности PAR1 промотора у HUVEC. У овим ћелијама, излагање вирусу је значајно повећало активност PAR1 репортер конструката који носе AP-2-сличне везујуће елементе заједно са AP-2/Sp1-везујућим елементима организованим у два комплекса. Активност прогресивно краћих конструкти који немају AP-2-сличне везујуће елементе је била знатно смањена после излагања вирусу, али и даље значајно већа од њихових основних активности измерених у ћелијама које нису биле у додиру са вирусом. Међутим, активност најкраћег конструкта, који не поседује нити један везујући елемент, није била значајно повећана у ћелијама изложеним вирусу нити је активност била значајно повећана у односу на основну активност самог конструкта. Аутори су још показали да у ћелијама изложеним вирусу, транскрипциони фактори p52/RelB значајно повећавају активност PAR1 репортера. Аутори на крају дискусије закључују да је активност PAR1 промотора у великој мери зависна од присуства AP-2/Sp1-везујућих елемената, али и да транскрипциони фактори p52/RelB значајно регулишу активност промотора у ћелијама изложеним вирусу.