The Complement Inhibitor CD59 and the Lymphocyte Function-associated Antigen-3 (LFA-3, CD58) Genes Possess Functional Binding Sites for the p53 Tumor Suppressor Protein

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Abstract. *p53 is an oncosuppressor protein, which acts via transcriptional and non-transcriptional mechanisms. The transcriptional function of p53 is mediated by specific responsive elements. In the present study we found active responsive elements, specific for the p53 within the 5'flanking region and within the first intron of the gene encoding for the CD59 membrane inhibitor of reactive lysis, and within the first intron of the gene encoding for the CD58 membrane protein (LFA-3). The results suggest that p53 may enhance the transcription of both CD59 and CD58 and imply a novel role for p53 as a direct regulator of the immune response.*

p53 is a tumor suppressor protein involved in vital aspects of cell life, such as control of the cell cycle checkpoints G1 and G2, maintenance of genomic integrity, DNA repair, replication, transcription, programmed cell death (apoptosis) and differentiation. The majority of stimuli causing p53 activation such as hypoxia/oxidative stress (1, 2), or UV radiation (3) are accompanied by inflammatory reactions. Additionally, a variety of cytokines secreted as a result of inflammation, such as IL-1 and TNFa, induce p53 (4,5).

p53 protein is assumed to exert its effect through its ability to function as a sequence-specific DNA-binding transcription factor. Wild-type p53 binds to double-stranded DNA homologous or identical to consensus sequence containing two decamers of the type: Pu Pu Pu C A/T T/A G Py Py Py, separated by 0 to 13 bp (6). The list of genes that possess these DNA sites is rapidly increasing and includes: p21/WAF1, MDM2, GADD45, BAX, cyclin G, cyclin D, IGF-BP3, PCDNA, TGF-a, Ras and p53 itself (7-17). The genes noted above contain p53-binding sites either in their promoter regions or within their introns. Recently p53 has been reported to enhance the transcription of a GPI-linked membrane protein GML (18) which is related to apoptosis and participates in the sensitization of malignant cells to chemotherapy and inflammation (19, 20). GML exhibits a great homology with the membrane inhibitor of the reactive lysis CD59 (21). CD59 is a 18-25 kDa GPI-linked protein with a broad tissue distribution that prevents the formation of the complement membrane attack complex (MAC) on the surface of homologous cells (22). Furthermore, CD59, along with the lymphocyte function-associated antigen-3 (LFA-3, CD58), participates in the activation of T lymphocytes and NK cells by binding to CD2. Recently, CD59 and CD58 have been identified as genes which possess potential p53-responsive elements (28). Given that inflammation is related to high levels of p53 (4, 5), as well as to high levels of CD59 (23-25) and CD58 (26, 27), a possible participation of p53 in the immune response by affecting the levels of CD59 and CD58, has been hypothesized (28).

In the present study, we have examined the possibility of the consensus sequences of p53 in the promoter area of the CD59 gene and in the intron 1 of the CD59 and CD58 genes regulating promoter activity in a p53-dependent manner.

Materials and Methods

Cell culture. Saos2 osteosarcoma cells, were cultured in Dulbecco's modified Eagle medium (DMEM) containing 4 mM L-glutamine, 50 μg/ml penicillin and 50 μg/ml streptomycin supplemented with 10% fetal calf serum. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Plasmids. The 39 bp fragment (5' TAAGGAGACATGCTTTAA-ATATCAAAGCAAG-TCATCCTG 3') which contains the potential p53-responsive element CD59a, the 42 bp fragment (5'TACTTAGGC-

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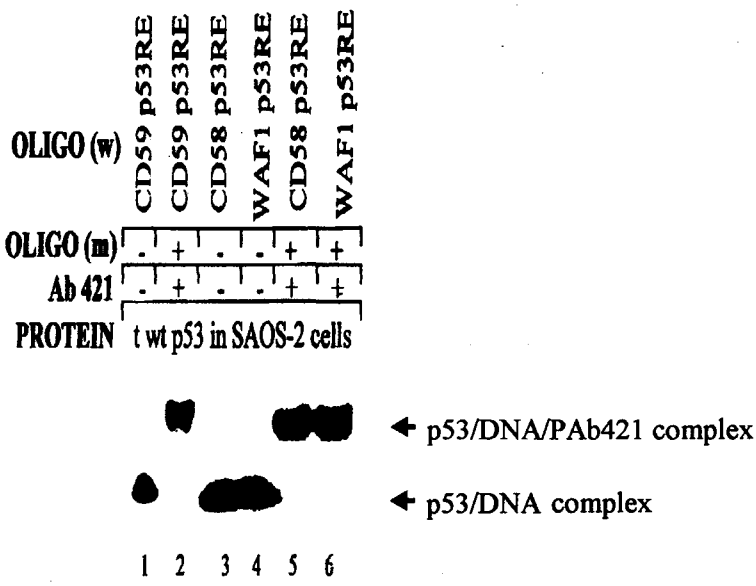

Figure 1. *Electrophoretic mobility shift assay showing sequence-specific binding of the p53 to the putative p53 responsive elements of the CD59 and CD58 genes. Lane 1: in vitro translated wild-type p53 protein, in the presence of nuclear extracts from Saos2 cells, binds with the CD59a end-labeled oligonucleotide and forms a retarded band (lower arrow). Lane 2: in vitro translated wild-type p53 protein ,in the presence of nuclear extracts from Saos2 cells, binds with the CD59a end-labeled oligonucleotide in the presence of the monoclonal antibody PAb421 and forms a super-shifted retard band indicating the specific binding of the p53 protein (upper arrow). Addition of mutant oligonucleotide to the reaction did not compete with the formation of the specific complex. Lanes 3, 4: in vitro translated wild-type p53 protein ,in the presence of nuclear extracts from Saos2 cells, binds respectively with the CD58 and WAF1 end-labeled oligonucleotides and form a retarded band (lower arrow). Lanes 5, 6: in vitro translated wild-type p53 protein, in the presence of nuclear extracts from Saos2 cells, binds with CD58 and WAF1 end-labeled oligonucleotides in the presence of the monoclonal antibody PAb421 and forms a super-shifted retard band indicating the specific binding of the p53 protein. Addition of mutant oligonucleotide to the reaction did not compete with the formation of the specific complex (upper arrow).*

AAGTTCAAAACCTCCCTAAGCTTTGGCTTCTTT 3') that contains the potential p53-responsive element CD59b, the 39 bp fragment (5'TTAAAGAGTAAGCTCCAC-ATGGTCAAACATGTTCTTACT 3') which contains the potential p53-responsive element CD58 and the 20 bp fragment (5' GAACATGTCCCAACATGTTC 3') containing the p53-responsive element of the WAF1 gene (29), were synthesized and cloned upstream of a minimal HSV promoter and a firefly human secreted placental alkaline phosphatase (SEAP) reporter gene of the mammalian expression vector pTKSeap (30). The resulting constructs were designated pTKSeapCD59a, pTKSeapCD59b, pTKSeapCD58 and pEp21TKSeap, respectively.

EMSA. The electrophoretic mobility shift assay (EMSA) probes were the 39 bp (CD59a), the 42 bp (CD59b), the 39 bp (CD58) and the 20 bp (WAF1) oligonucleotides containing a potential p53-responsive element in the CD59, CD58 and WAF1 genes, respectively. End-labelling was performed using T4 polynucleotide kinase (Boehringer, Mannheim, Germany) and [α^{32}P]ATP according to standard protocol (31). The end-labeling oligonucleotides were mixed in 0.1M sodium chloride, heated at 95°C for 5 minutes and then gradually cooled to room temperature to allow annealing. Ten μg of nuclear extracts were pre-incubated for 10 minutes with 100 ng of purified monoclonal antibody PAb421 to activate specific DNA binding of the p53 protein. It has been shown that p53 binds non-specifically to DNA through the carboxy-terminal domain, locking p53 in a conformation that cannot bind its specific element. By adding PAb421, non-specific binding is inhibited and p53 adopts the specific DNA conformation (32). Furthermore, nuclear extracts from p53-null cells (Saos2) were added to the reaction, in order to get *in vitro* translated p53 to bind the p53-responsive element (33). Subsequently, 1 ng of labelled oligonucleotide in the presence of 500 ng of poly(dI)-poly(dC) as non-specific DNA competitor was added. All incubations took place on ice with the following binding conditions: 50 mM potassium chloride, 25 mM Hepes pH 7.6, 5 mM DTT, 10 μg of leupeptin per ml, 0.05% Triton X-100 and 20% glycerol. Competitior experiments were performed by preincubated PAb421-activated extracts with 50-fold molar excess of unlabeled oligonucleotides or using mutant labeled oligonucleotide. *In vitro* translated p53 protein was used as a positive control in the DNA binding experiments (34). Protein-DNa binding reactions were analysed on a 4% polyacrylamide gel. The gels were dried and exposed on radiograph film (RX Fuji) at -70°C.

Transient transfections and Seap assay. For each transfection 1 μg of the corresponding reporter plasmid (pTKSeap-CD59a, pTKSeapCD59b, pTKSeapCD58, pEp21TKSeap

Table I. *Comparison of p53 consensus sequences.*

Gene of origin / promoter position	p53 consensus site
consensus	PuPuPuC (A/T) (T/A) GPyPyPy
WAF1 / -2235/-2215	GAACATGTCC CAACATGTTG
CD59a / -1675/-1626	AGACATGCTT TAAATATCA AAGCAAGTCA
CD59b / -680/-640	AGGCAAGTTC A AAACCTCCCT AAGCTTTGG
CD58 / -156/-108	GAGTAAGCTC CACATGGTC AAACATGTTC

Underline indicates deviations from consensus. Bold letters indicate center bases of consensus sequence.

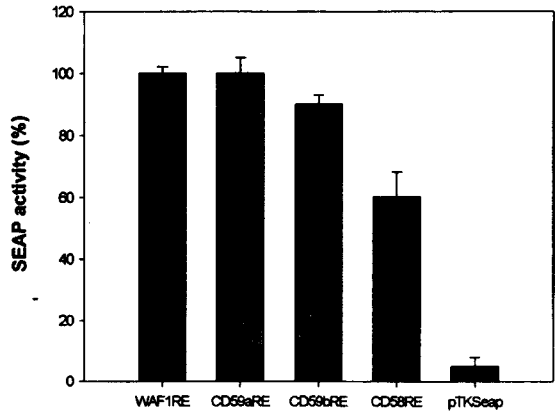

Figure 2. *Effects of the p53 responsive elements on the transcriptional activity of the SEAP reporter gene of the pTKSeap plasmid in the presence of wild-type p53. The total amount of DNA transfected in each experiment was 2 μg (1 μg of wild-type p53 and 1 μg of the reporter plasmid). Activity was expressed as a percentage of that induced by 1 μg of the reporter gene downstream of the WAF1 responsive element in the presence of wild-type p53. The results shown are an average of three independent transfection experiments.*

and empty pTKSeap) was mixed with 1 μg of the expression vector encoding wt p53 (pSV2hp53BSwt), and with 1 ng pSV promoter-β-galactosidase (Stratagene, San Luis Ohipso, CA, USA) in DMEM medium. Ten μl of diluted in DMEM (1:100) Transfectam reagent (Promega, La Jolla, CA, USA) was immediately added to the plasmid mixture. The Tranfectam/plasmid mixture was then added directly to 5 x 10^5 exponentially growing Saos2 cells in DMEM medium (without serum). The cells were incubated at 37°C with 5% CO_2 for 48 hours. The transcriptional activity was examined 48 hours post-transfection by measuring the levels of SEAP activity using the Great EscAPe SEAP Genetic Reporter System (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Successful transfection was verified by measuring the β-galactosidase activity.

Results and Discussion

Many of the biological functions of p53 are believed to be regulated by downstream genes so that understanding of the function of p53 will require examination of these downstream genes. The CD59 and CD58 genes have been identified as candidate targets of p53 (28). *Via* computer search, using the BLAST program (NHI), we revealed the presence of: a) a perfect half-binding site followed by one imperfect half- binding site with the interference of 9 nucleotides for p53, located in the 5'flanking region (ranging from nucleotide –1665 to –1637 upstream of exon 1) but not within the promoter limits of the CD59 gene (CD59a responsive element); b) a perfect half-binding site followed by two imperfect half-binding sites for p53 located in intron 1 of the CD59 gene (ranging from nucleotide –675 to –645 upstream of exon 2) but not within the enhancer limits (CD59b responsive element) and; c) a perfect half-binding site followed by one imperfect half-binding site for p53 with the interference of 9 nucleotides, located in the first intron of the CD58 gene (ranging from nucleotide –146 to –118 upstream of exon 2) (CD58 responsive element). The imperfect half-binding site of the CD59a-responsive element displays a 95% homology with the Pu Pu Pu C A/T T/A G Py Py Py motif whereas, the first imperfect half-binding site of CD59b displays an 80% homology with the motif and the second half-binding site a 70% homology. The CD58-responsive element displays a 95% homology with the motif (Table I, 28).

To determine whether the p53 protein could bind specifically to the CD59a, CD59b and CD58 putative-responsive elements, EMSAs were performed. The results presented in Figure 1 reveal a specific retard band, which was abolished in the presence of a mutated-responsive element as well as in the presence of excess unlabelled p53-responsive element.

To explore the functional significance of this new potential p53-binding site, SEAP reporter gene plasmids containing the CD59a, CD59b and CD58-responsive sequences were constructed. Contransfection assays were then performed into Saos2 cells (which are deficient in p53) using the above constructs, or the empty vector, and a plasmid coding for wild-type p53 (pSV2hp53BSwt). The results were compared with those obtained with the reporter plasmid pEp21TKSeap containing the p53-responsive element of WAF1 gene. Contransfection of

pTKSeapCD59a with the plasmid encoding the wild-type p53 resulted in the 100% activation of the SEAP activity as compared to results obtained with the WAF1 p53-responsive element (Figure 2). Under the same conditions the CD59b and the CD58 resulted in 98% and 60% activation of the SEAP activity, respectively (Figure 2). No stimulation was observed when cells were transfected with the pTKSeap vector in the presence of the pSV2hp53BSwt plasmid. On the basis of these results, the sequences identified by computer could confer p53 responsiveness to the CD59 and CD58 gene promoters.

Taking into consideration that inflammation is related to high levels of p53 (4, 5, 35) and that in an inflammatory environment the elevated levels of activated-complement render cells susceptible to complement-mediated lysis, it is possible that the CD59 up-regulation by activated-p53 may contribute to the protection of host cells from complement attack. The CD58 up-regulation by activated-p53 may also function as part of a regulatory pathway for the enhancement of the inflammatory process. Additionally, the p53-mediated CD58 up-regulation might contribute to the immune surveillance against cancer since the up-regulated wild-type p53 and a consequently CD58 might lead to the recognition of cancerous cells by NK cells *via* the CD58-CD2 interaction (36-38). The mutant p53 may disrupt this mechanism and thus constitute a way of tumour immune evasion.

Our data collectively manifest two new potential "target" genes, CD59 and CD58, for p53 and raise a new role for p53, in immune regulation.

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