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A NOVEL APPROACH TO PROTEIN-PROTEIN INTERACTION: COMPLEX FORMATION BETWEEN THE P53 TUMOR SUPPRESSOR AND THE HIV TAT PROTEINS

Fatima Longo*, Maria A. Marchetti*, Luisa Castagnoli†, Piero A. Battaglia°
and Franca Gigliani*[1]

*Dipartimento di Biopatologia Umana Sezione di Biologia Cellulare
Università di Roma La Sapienza, Rome, Italy

†Dipartimento di Biologia Università di Tor Vergata, Rome, Italy

°Laboratorio di Biologia Cellulare, Istituto Superiore di Sanità, Rome, Italy

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SUMMARY By using a novel genetic approach, based on the properties of λ cI repressor, we demonstrate that the HIV-1 Tat protein specifically interacts with the human p53 protein *via* the p53 O2 dimerization domain. By random and site-specific mutagenesis, we also identify the residues in Tat and O2 peptides which are involved in this interaction. Two alternative biological consequences are expected to result from Tat-p53 interaction: (i) Tat-O2 interaction inactivates p53 regulation function, thus producing cell transformation; (ii) Tat-O2 interaction favours the formation of p53 dimers, thus leading the cell towards apoptosis.

We have developed a bacterial genetic system to detect protein interaction and identify the amino acids involved in the interaction. The advantage of using a cell for these studies as opposed to the biochemical approach is at least twofold; (i) the rapidity and ease of identification of active sites by production and selection of mutations; (ii) the elimination of the protein purification step, normally required by biochemical approach. This work describes the application of this assays to a protein that can form homo- and hetero-dimers, the p53 protein (1, 2, 3), which represents the control system. Moreover we show for the first time, the interaction between p53 tumor suppressor protein with the Tat protein of HIV.

The rational for choosing these proteins is the following. The p53 tumor suppressor protein (4,5) can be considered as a cell "branch-point" between cellular proliferation and apoptosis and is present in the cell in different forms, namely as a homodimer or tetramer or can form complexes or interact with a number of cellular (6, 7) and viral

[1]To whom correspondence should be addressed. Fax: 39 6 4462891.

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proteins (8, 9). These interactions are responsible for opposite effects on the life of the cells. For example, the E1B protein of adenovirus forms complexes with the p53 protein by interacting with the N-terminal domain of p53, and inhibits the p53 trascriptional activity (10). While this interaction (E1B/p53) results in tumorigenesis, the expression of E1A adenovirus protein directs the cell towards apoptosis (9, 11).
Likewise, HIV-1 infected cells undergo cell transformation or apoptosis (12, 13). The HIV-1 Tat protein plays an important role in regulating the expression of viral genes (14). The Tat protein can form dimers (15, 16, 17) and we recently identified residue cys37 as one of the amino acids required for this process (17). By analogy to the adenovirus system, it is possible that Tat interacts with p53 and is involved in the processes that lead the HIV-infected cell to transformation or apoptosis.

MATERIALS AND METHODS

E.coli strains, plasmids and phages The strains used were: 71.18 (18); CSH50 (19); BW313 (19). Plasmids used are in Figure 1. Phages were: λ(ref), showing a wild type phenotype and λcY17 carrying a mutation of the cI gene.
General microbiological and DNA recombinant techniques Media and standard techiques were as described (20) .Preparation of plasmids and phages DNA was according to published procedure (18). The isolation of restricted fragments, cloning and DNA sequences were performed by standard procedures (18, 21).
Plasmids construction The maps the plasmid vectors used in this work are illustrated in Fig.1. At the top of the figure we are schematically represented the structure of the proteins that are synthetized under the control of pLac promoter. The structure of pC132 and pC135 have been described (22). pC132 directs the synthesis of a fusion between the amino-terminal domain of the λ repressor and the dimeric protein Rop. pC168 is analogous to pC132 and was obtained by inserting a PvuII fragment containing the pLac-cI-Rop gene fusion, obtained by partial PvuII digestion of pC132, into the HindIII-HincII replicon derived from plasmid pACYC184. pACYC184 replicates under the control of the replication origin of plasmid p15A and it is therefore

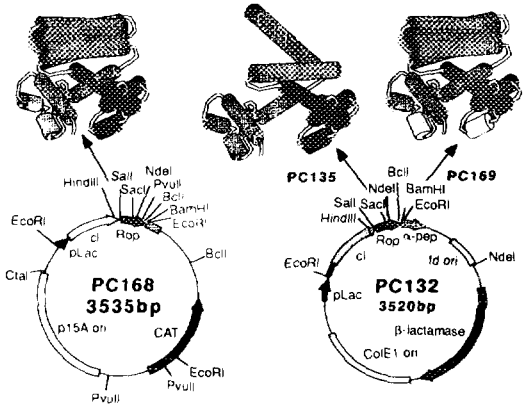

Fig. 1. Plasmid maps. Genetic structure of the plasmids used in this work. The drawings on the top of the figure are a schematic representation of the hybrid proteins synthetized by the plasmids. The white cylinders in the pC169 structure represent modified helix 3 unable to bind the operator.

compatible with pC132 and its derivatives. pC135 and pC169 derive from pC132 by site directed mutagenesis. In pC135 the codon encoding Asp32 of Rop was changed into a TAA stop codon, while in pC169 the solvent exposed surface of cI helix 3 was modified to prevent binding to λ operators. Thus, differently from pC132 and pC168, neither pC135 nor pC169 are able to direct the synthesis of an active repressor. In fact the hybrid repressor synthetized by pC135 can not dimerize, whereas the one synthetized by pC169 can not bind the operator. All these vectors can be conveniently exploited to insert DNA fragments encoding putative dimerization domain between the SalI and BamHI site in place of the Rop gene.

RESULTS AND DISCUSSION

The genetic system

This assay allows the distinction between homodimers and heterodimers formation, taking advantage of the following properties of the lambda immunity system.The N-terminal DNA-binding domain of the λ cI repressor dimerizes inefficiently and requires a separate C-terminal dimerization domain, in order to increase the binding affinity by its operator (23). Thus, the fusion of N-terminal domain to a hererologous dimerizing protein should resulting in a functional λ repressor which immunizes the bacterial cells against λ phage infection. In other words, only those sequences that mediate efficient dimerization of chimeric proteins *in vivo* will permit the survival of the host cell to infection by λ phage. This assay that we have already described (dimerization test), detects the presence of homodimers of the chimeric repressor protein (23, 22, 17).

The heterodimer formation test (negative dominance assay) is based on the same principle, but entails two different interacting protein (or protein domains). In addition, one of the two domain must produce homodimers. The homodimerizing peptide is fused to the DNA-binding domain of the cI λ repressor gene and cloned into a low copy number plasmid. The other protein domain (heterodimerizing) is fused to a cI DNA-binding domain carrying a mutation which prevent binding of the repressor to the DNA. This construct is cloned into a high copy number plasmid. Both plasmid, carrying compatible origins of replication, are transfected into a suitable bacterial strain. When interaction between the two protein domains occurs, the functional repressor is titrated out by the heterodimerizing protein and becomes inactive, making the transformed cells sensitive to λ phage infection. (Fig.2).

Homo- and hetero-dimerization of the p53 protein as control system

We have constructed two chimeric proteins called (i) cI-O1, which contains the DNA binding domain and the linker region of λ repressor fused to the p53 O1 domain (amino acids 365-386) which contains the structural elements for tetramerization and (ii) cI-O2 containing the O2 peptide (amino acids 341-355) of the p53 wich is the major determinant for p53 dimerization (1, 2, 3). The two hybrid genes were constructed by cloning the O2 (or O1) nucleotide sequence into plasmid pc135 (22) coding for the truncated form of the cI repressor which cannot dimerize and is therefore inactive unless supplemented with a dimerization domain. The CSH50 *E.coli* strain was transformed with either the recombinant plasmids (pcI-O2 and pcI-O2) or the pc135

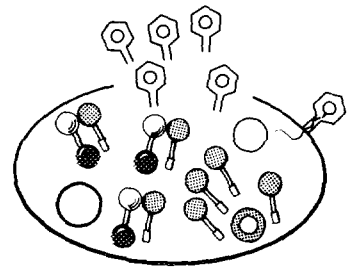

Fig. 2. Schematic drawing of the negative dominance assay. If the two protein domains expressed by the two compatible plasmids form heterodimers, the over-production of one the two domains (fused to a cI mutated repressor) should block the homodimers formation of the other domain by titrating it and makes the transformed cells sensitive to λ infection.

vector alone. The synthesis of the chimeric protein was induced by supplementing the medium with isopropyl-β-D-thiogalactopyranoside (IPTG). The immunity to λ infection of the two strains was evaluated as described in Table 1.The results show that bacterial cells harbouring the DNA-binding domain alone (pc135 plasmid) were sensitive to lambda infection, while cells containing either the cI-O2 or the cI-O1 chimeric repressors were immune to lambda phage infection. These results show homodimerization of the O1 and O2 domains of the p53 protein.

The results of the "negative dominance" genetic assay are shown in Table 2. For this assay the O1 domain, was fused to the DNA binding domain of the cI repressor of plasmid pC169 (plasmid pcA-O1) which is a high copy number plasmid and codes for a mutated cI repressor incapable of binding to λ the operator sequence (Fig.1). Correspondingly, the O2 domain was fused to the DNA binding domain of the cI repressor of the plasmid pC168 which is a low copy number plasmid and codes for a wild type cI DNA-binding domain. *E.coli* cells (CSH50 strain), cotransformed with the two recombinant plasmids were tested for their sensitivity to λ phage infection. The

Table 1. Regulatory properties of the cI-O1 and cI-O2 chimeric proteins

Strain (plasmid)	Repressor	Sensitivity to	
		λ(ref)[a]	λcY17[b]
CSH50 (pc135)	none	sensitive	sensitive
CSH50 (pcI-O1)	cI-O1	immune	immune
CSH50 (pcI-O2)	cI-O2	immune	immune

a)λ(ref) has a wild type phenotype; b) λcY17 carries a mutation in the cI gene. In order to test the sensitivity to λ phage of different chimeric repressors, 300 μl of CSH50 *E.coli* cells, carrying different plasmid constructs and 3ml of BBL-Tripticase soft agar (supplemented with 1mM IPTG) were plated on BBL-Tripticase agar plates. 5 μl each of serially diluted (from 10^{-1} to 10^{-6}) phage stocks (10^{10} phages/ml) were used to infect bacteria containing plasmids expressing chimeric repressors. The efficiency of plating was calculated using CSH50 as standard strain. The term sensitive indicates the efficiency of plating=1; immune: efficiency of plating $<10^{-7}$.

Table 2. Negative dominance assay: Sensitivity to λ infection of CSH50 strain coexpressing different chimeric proteins

Strain (plasmids[a,b])	Chimeric proteins	Sensitivity to	
		λ(ref)	λcY17
CSH50 (pcY-O1)	cY-O1	immune	immune
CSH50 (pCY-O2)	cY-O2	immune	immune
CSH50 (pcY-O1/pcA-O2)	cY-O1/cI*-O2	sensitive	sensitive
CSH50 (pcY-O2/pcA-O1)	cI*O1/cY-O2	sensitive	sensitive

a) pcY-O1, pcY-O2 : the vector used is the pC168 (cI wild-type, low copy number plasmid). b) pcA-O2 and pcA-O1: the vector is the pC169 (cI* indicates the mutated cI repressor present in the pC169 high copy number plasmid). Sensitivity *versus* immune is as explained in note of Table 1.

heterodimerization of the O1 and O2 protein domains is shown by the sensitivity to λ phage infection of the bacterial cells expressing the cI*-O1 and cY-O2 (or cI*-O2 and cY-O1) fusion proteins

In conclusion, these results show that the two assays can be utilized to study protein-protein interaction.

Interaction of Tat with the p53

Since both the p53 O1 and O2 domains are essential for p53 function (1, 2), we tested the possible interaction of both domains with Tat.

The full lenght cDNA of Tat was cloned into the pC169 plasmid (pcA-tat) and fused with the N-terminal DNA-binding domain of the cI mutated repressor, while O2 (or O1) peptide was cloned into the pC168 plasmid (pcA-02 and pcA-O1) fused to the wild-type DNA-binding domain of the λ cI repressor The CSH50 *E.coli* cells, were cotransformed with plasmids pcA-tat and pcY-O1 or pcY-O2 and assayed for their sensitivity to λ infection. The results of Table 3 show that bacterial cells containing the cY-O1 and cI*-Tat fusions are immune to lambda infection, whereas cells containing

Table 3. Negative dominance assay: Interaction between Tat and the p53 O2 domain

Strain (plasmids[a,b])	Chimeric proteins	Sensitivity to	
		λ(ref)	λcY17
CSH50 (pcY-Tat)	cI-Tat	immune	immune
CSH50 (pcY-O1/pcA-Tat)	cY-O1/cI*-Tat	immune	immune
CSH50 (pcY-O2/pcA-Tat)	cY-O2/cI*-Tat	sensitive	sensitive
CSH50 (pcA-O2/pcY-Tat)	cI*-O2/cY-Tat	sensitive	sensitive

a) The vector used to construct the plasmids pcY-O1, pcY-02 and pcY-Tat is the low copy number pC168 carrying the wild type cI DNA-binding domain. b) The vector used to construct the plasmids pcA-O2 and pcA-Tat is the pC169 high copy number plasmid carrying the mutated cI DNA-binding domain (cI* indicates the chimeric proteins carrying the cI mutation).

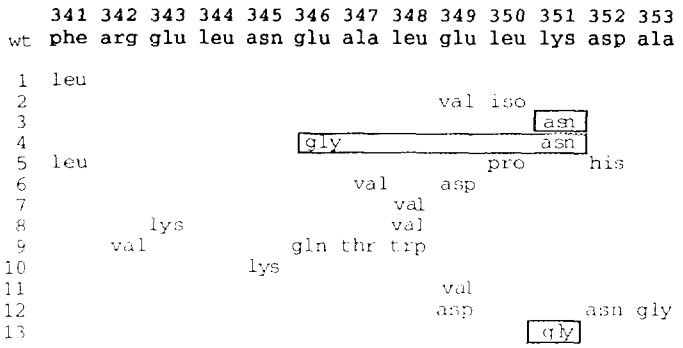

Fig. 3.p53 O2 mutants. The figure summarizes the amino acid sequence of the O2 mutants obtained by a single experiment utilizing an oligo (55 nucleotides in length) of degenerated sequence and tested by their immunity to λ infection. The wild type sequence is drawn at the top in bold characters. Only the amino acids changed in the mutagenesis procedure are indicated. Boxes identify the O2 mutants that are unable to dimerize as demonstrated by their inability to restore the function of the amino terminus of the λ repressor.

cY-O2 and cI*-Tat fusion proteins are sensitive to lambda infection. We conclude that, under these conditions, Tat interacts with the O2 domain of the p53 protein. This interaction is specific since the homodimerization of the p53 O1 domain was not quenched by the Tat protein.

Identification of the amino acids involved in the interaction between Tat and O2

In order to identify the amino acid residues of the O2 domain involved in the interaction with Tat, we constructed a series of mutated O2 peptides. Randomly mutated sequences of the O2 DNA, obtained by constructing degenerated oligonucleotides were cloned into the pc135 vector and the recombinant plasmids were used to transform CSH50 strain; 30 clones were sequenced. 13, carrying different amino acid substitutions, were first tested for formation of homodimers (Fig. 3). This experiment shows that only the lys351/asn (and lys351/gly) mutant can abolish the homodimerization of O2.

To ascertain whether the O2-Tat interaction depends on the same amino acids which are involved in homodimerization of Tat (Cys37) and O2 (lys351) respectively, two different negative dominance assays were carried out. One assay was designed to test if the cys37 residue of Tat is required for Tat-O2 interaction. The mutated form of Tat with cys37 replaced by serine was cloned into the pC169 plasmid and the O2 wild type DNA sequence in pC168 plasmid. Both recombinant plasmids were used to simultaneously transform *E.coli* CSH50 strain. While, to test whether lys351 of O2 p53 domain is responsible for Tat-O2 interaction, we cloned the O2 lys351 to gly mutation into the pC169 plasmid and wild type sequence of Tat into the pC168 plasmid. Both recombinant plasmids were used to transform CSH50 cells. The results show that

Table 4. Interaction between Tat and O2 mutated peptide tested by the negative dominance assay

Chimeric repressors expressed in the CSH50 strain	Sensitivity to λ(ref) and λcY17
cI-O2*	immune
cI-O2*/cI-01	immune
cI-Tat*	immune
cI-O2*/c-ITat	immune
cI-O2/cI-Tat*	immune

The mutated peptides (indicated by the asterisks) were obtained by site-directed mutagenesis (19). CSH50 cells were cotransformed with the pC169 plasmid containing the wild type sequence of Tat (or O1) and with the pC168 plasmid carrying the O2 peptide mutated in the acidic residues and *viceversa*. The cotransformed cells were assayed to their immunity to λ phage.

bacterial cells expressing the fusion proteins were sensitive to λ phage indicating that neither cys37 of Tat or lys351 of p53 are responsible for the Tat-O2 interaction.

Sturzbecher et al.(1992) have shown that the interaction of O2 with O1 is due to negatively charged amino acids at position 343, 346, 349 and 352 of the p53 O2 domain. We have correlated this observation with the presence in the Tat protein of a stretch of basic amino acids at position 49-57, wondering whether this could be the binding site of Tat to the O2 domain. This hypothesis was tested by constructing an O2 mutant containing lysines in place of glu343, glu346, glu349 and asp352. As expected, (1) these amino acid substitutions abolishes the interaction between the O2 and O1 domain (Table 4). In parallel we have mutagenized the Tat protein substituting arg49,lys50, lys51, arg52, arg53, gln54, arg55, arg56, arg 57 with gln49, glu50, glu51, ala52, glu53, thr54, asp55, glu56, glu57. The Tat and O2 analogues were assayed by the negative dominance assay. The results are shown in Table 4. The substitution of the acidic residues of the O2 domain abolishes the O2-Tat interaction allowing the formation of Tat homodimers. Similarly the substitution of the basic region of Tat abolishes the Tat-O2 interaction, without altering the formation of Tat homodimers.

These results are consistent with the possibility that Tat-O2 interaction depends on ionic interactions between the acid amino acids of O2 and the basic region of Tat. They also indicates that the residues involved in O2-Tat interaction differ from those required for homodimerization of the two proteins. Therefore, the Tat-O2 interaction does not hinder the ability of Tat and O2 to form homodimer.

Because of the pivotal role played by the p53 protein in regulating the cell cycle, it will be interesting to ascertain whether the Tat-p53 interaction takes also place in the course of HIV infection. If this were the case, the biological consequences of this interaction might depend on the relative concentration of the two proteins and on the affinities of the p53 and Tat domains involved in this interaction.

It is tempting to speculate that Tat-p53 heterodimers might not be able to efficiently bind to DNA as it has been described by Oren for p53 heterodimers (25, 26). The

functional consequence for the cell could then be the acquisition of a transformed phenotype since it has been shown that loss of trans-activation by p53 results in cell transformation (25). Alternatively, binding of p53 to Tat could lead to increased levels of dimeric p53 (by favouring the formation of p53 dimers) which might influence the cellular decision towards apoptosis (27). Experiments are now in progress to establish whether the Tat-p53 interaction can be detected in HIV infected cells and to determine whether changing the cellular concentration of Tat has any effect on the pathways leading to cellular transformation (12) or to apoptosis (13)

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