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Characterization of P55, a Multidrug Efflux Pump in *Mycobacterium bovis* and *Mycobacterium tuberculosis*

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The *Mycobacterium bovis P55* gene, located downstream from the gene that encodes the immunogenic lipoprotein P27, has been characterized. The gene was identical to the open reading frame of the Rv1410c gene in the genome of *Mycobacterium tuberculosis* H37Rv, annotated as a probable drug efflux protein. Genes similar to *P55* were present in all species of the *M. tuberculosis* complex and other mycobacteria such as *Mycobacterium leprae* and *Mycobacterium avium*. By Western blotting, P55 was located in the membrane fraction of *M. bovis*. When transformed into *Mycobacterium smegmatis* after cloning, P55 conferred aminoglycoside and tetracycline resistance. The levels of resistance to streptomycin and tetracycline conferred by P55 were decreased in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone and the pump inhibitors verapamil and reserpine. *M. smegmatis* cells expressing the plasmid-encoded P55 accumulated less tetracycline than the control cells. We conclude that P55 is a membrane protein implicated in aminoglycoside and tetracycline efflux in mycobacteria.

Tuberculosis is the world's leading cause of mortality owing to an infectious bacterial agent, *Mycobacterium tuberculosis*. The estimated 8.8 millions new cases every year have an extraordinary impact on the economies of the developing world, where most of the cases occur (30). Short-course chemotherapy (with rifampin, isoniazid, pyrazinamide, ethambutol, and streptomycin being the backbones of treatment) is the most powerful weapon available against infection with susceptible strains of *M. tuberculosis*, breaking the chain of transmission and limiting contagion.

Recently, dramatic outbreaks caused by multidrug-resistant strains (defined as those resistant to at least isoniazid and rifampin) of *M. tuberculosis* and *Mycobacterium bovis* have focused international attention (25, 30). These cases are extremely difficult to cure, and the necessary treatment is much more toxic and expensive.

In recent years, considerable work has been done on the characterization of drug-resistant mycobacteria. That work has identified structural or metabolic genes (encoding either the enzymes that activate antimycobacterial drugs or the protein targets of drug action) that lead to a high level of resistance to a single drug when the genes are altered by mutation. In most cases, multidrug-resistant isolates have accumulated independent mutations in several genes (21, 22, 26). However, these mutations do not account for all resistant strains, indicating that other mechanisms confer resistance in mycobacteria.

In bacteria, the permeability of the membrane and the actions of active transport mechanisms prevent access of certain drugs to the intracellular targets. These constitute a general mechanism of drug resistance capable of conferring resistance to a variety of structurally unrelated drugs and toxic compounds (12, 16, 17, 19, 24). The resistance efflux systems are characteristically energy dependent, either from the proton motive force or through the hydrolysis of ATP.

Recently, efflux-mediated resistance and efflux pumps that confer resistance to one or several compounds have been described in mycobacteria (2, 4, 7, 9, 14, 29). The genome of *M. tuberculosis* strain H37Rv has 20 open reading frames encoding putative efflux proteins (8), although most of them have not yet been characterized.

In the work described here, we functionally characterized the putative multidrug efflux pump P55 from *M. bovis* (in which it was initially described [5, 6]) and *M. tuberculosis* (since P55 is identical to the product of the Rv1410c gene of the *M. tuberculosis* H37Rv genome [8]). We have found that P55 confers resistance to tetracycline and aminoglycosides such as streptomycin and gentamicin. The effect of pump inhibitors on the resistance levels conferred by P55 has been also studied. *P55* forms a operon with *P27*, which we have previously identified and characterized as a gene that encodes a lipoprotein antigen from *M. bovis* (5, 6).

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. *M. tuberculosis* H37Rv, *M. bovis* BCG, *Mycobacterium smegmatis* mc^2 155 (27), *Escherichia coli* DH5α, and derivatives of these strains were used (Table 1). Media were obtained from Difco Laboratories (Detroit, Mich.). Luria-Bertani (LB) broth was used to culture *E. coli* and was supplemented with 0.05% Tween 80 to culture the *M. smegmatis* strains. Kanamycin A (Sigma) was added at 20 μg/ml to maintain the plasmids for *E. coli* and mycobacterial species, and ampicillin was added at 100 μg/ml for *E. coli*. Mueller-Hinton agar plates were used for antibiotic susceptibility testing, and LB broth was used for microdilution tests. All the cultures were incubated at 37°C.

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TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in the present work

Strain, plasmid, or oligonucleotide	Characteristic	Reference or source
Strain		
M. smegmatis mc^{2}155	Efficient plasmid transformation mutant	27
M. smegmatis PAZ22	*M. smegmatis* mc^{2}155 carrying plasmid pPAZ22	This work
M. smegmatis PAZ23	*M. smegmatis* mc^{2}155 carrying plasmid pPAZ23	This work
M. smegmatis PAZ24	*M. smegmatis* mc^{2}155 carrying plasmid pPAZ24	This work
M. smegmatis PAZ100	*M. smegmatis* mc^{2}155 carrying plasmid pSUM41	This work
M. smegmatis PAZ101	*M. smegmatis* mc^{2}155 carrying plasmid pMV261	This work
Plasmid		
pMV261	Hyg^r *E. coli-Mycobacterium* shuttle vector	28
pSUM41	Km^r *E. coli-Mycobacterium* shuttle vector	1
pPAZ22	pMV261 with *P55* gene	This work
pPAZ23	pSUM41 with *P27-P55* operon	This work
pPAZ24	pPAZ23 with omega cassette Sm^r in *Bam*HI site	This work
pGEM-T	*E. coli* cloning vector	Promega
pRSET-A	*E. coli* expression vector	Invitrogene
pRSET-vec	pRSET-A with *P55* gene	This work
pMAL-c	*E. coli* expression vector	New England Biolabs
pMAL-vec	pMAL-c with *P55* gene	This work
Oligonucleotide		
vec21-up	CC**GGATCC**CGAGCAGGACGTCGAGTCGCGAT[a]	This work
vec21-low	GC**GAATTC**GGCTCGTTAGAGCGGCTCCACTTG[b]	This work
2-1 dir	CCTCACAGACACCCTCTACG	This work
U292	CGTTCCTCAACAATTCCG	This work

[a] The boldface indicates the *Bam*HI restriction site.
[b] The boldface indicates the *Eco*RI restriction site.

DNA manipulations. Standard methods were used for DNA manipulations (3). Plasmid DNA isolation was performed with a Wizard Minipreps SV kit (Promega) according to the manufacturer's instructions. Both *E. coli* and *M. smegmatis* mc^2 155 were transformed by electroporation (18) with a Gene Pulser (Bio-Rad Laboratories Inc. Richmond, Calif.).

Plasmid construction. To clone *P55* under the control of the *hsp60* promoter, the gene was amplified by PCR with chromosomal DNA from *M. bovis* BCG as a template with primers 2-1Dir and vec21-low (Table 1). The PCR product was digested with *Bam*HI and *Eco*RI and was cloned into the vector pMV261 (28), resulting in plasmid pPAZ22. The region containing the *P27-P55* operon was amplified by PCR with primers U292 and vec21-low. The resulting 2.2-kb fragment was cloned in the pGEM-T vector (Promega), excised with *Eco*RI, blunt ended with the Klenow enzyme, and inserted in the blunt-ended *Bam*HI site of pSUM41 (1), resulting in plasmid pPAZ23. The streptomycin resistance omega cassette (20) was inserted in the *Bam*HI site of pPAZ23 (internal to *P27* gene), resulting in pPAZ24.

To construct a plasmid for expression of the *P55* gene in *E. coli*, the coding sequence was amplified with primers vec21-up and vec21-low, which provide *Bam*HI and *Eco*RI restriction sites, respectively (Table 1). The resulting 1.6-kb fragment was cloned between the *Bam*HI and *Eco*RI sites of plasmid pRSET-A (Invitrogene), generating pRSET-vec in which *P55* is fused at the N terminus with a polyhistidine tag. The insert of pRSET-vec was excised with *Bam*HI and *Hin*dIII and was cloned into pMAL-c (New England Biolabs), generating pMAL-vec, in which *P55* has an N-terminal fusion with *malE*.

Preparation of anti-P55 sera in rabbits. Since the production of recombinant P55 from pMAL-vec was stronger than that from pRSET-vec, we used *E. coli* pMAL-vec crude extracts as the source of recombinant P55. A total of 100 mg of *E. coli* pMAL-vec crude extract was loaded in a 10-cm-wide well and developed on a sodium dodecyl sulfate-polyacrylamide gel. Using Western blotting, we determined the region of the gel where the recombinant protein was located. The gel strip containing P55 was excised, mashed, mixed with Freund incomplete adjuvant, and injected in three doses into one rabbit in order to obtain antibodies against P55. The doses were given at 2-week intervals.

Preparation of crude extracts, fractionation, and Western blotting. Crude extract preparations from mycobacteria, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blotting were performed as described previously (6). A peroxidase-conjugated secondary antibody was used for Western blotting, and bands were developed with the chemiluminescence ECL plus Western blotting detection system kit (Amersham). Sonication and centrifugation were used to fractionate *M. bovis* crude extracts into the membrane and cytosolic fractions. Membrane fractions were further fractionated by Triton X-114 extraction.

Antibiotic susceptibility testing. The susceptibilities of *M. smegmatis* mc^{2}155 derivatives containing the plasmids described above to the following drugs were tested: tetracycline, aminoglycosides (2′-*N*-ethylnetilmicin, 6′-*N*-ethylnetilmicin, netilmicin, tobramycin, gentamicin), quinolones (nalidixic acid, ciprofloxacin), sulfadiazine, chloramphenicol, cefoxitin, erythromycin, minocycline, sulfamethoxazole, ethidium bromide, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and the antituberculosis drugs streptomycin, isoniazid, ethionamide, rifampin, and ethambutol.

Initially, the antibiotic diffusion method was used to screen the drugs. Then, the MICs of those drugs for the strains that contained *P55* and that presented any resistance were determined by the Alamar Blue assay (11), which was repeated at least three times.

The MICs of streptomycin and tetracycline were also determined under the same conditions in the presence of the following inhibitors of efflux pumps: CCCP (5 mM), verapamil (100 mM), and reserpine (20 mM).

Assay of tetracycline accumulation. The accumulation of tritiated tetracycline (American Radiolabelled Chemicals Inc., St. Louis, Mo.) was monitored as described previously (2, 14) in a liquid scintillation counter (LS-6000 IC; Beckman).

Computer analysis. Information on Rv1410c was obtained from the TubercuList database (http://genolist.pasteur.fr/TubercuList/). Sequence databases were searched by using the program BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Unfinished genomes of *Mycobacterium leprae* and *Mycobacterium avium* were searched at The Sanger Centre (http://www.sanger.ac.uk/Projects/M_leprae/) and The Institute for Genomic Research (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism=m_avium), respectively.

RESULTS AND DISCUSSION

Database searches and motifs in P55. The *P55* gene from *M. bovis* was identical to the Rv1410c gene from *M. tuberculosis* (8). Database searches showed that the P55 protein had a high level of similarity to membrane efflux proteins of the major

TABLE 2. Amino acid sequence identity between the deduced product of the *P55* gene and other proteins

Protein	Organism	% Identity[a]	Description	Accession no. (reference)
Rv1410c	*M. tuberculosis*	100	Probable drug resistance protein	CAB02189 (8)
SC9C7.19	*Streptomyces coelicolor*	40	Probable efflux protein	CAA22731
DRA0061	*Deinococcus radiodurans*	38	Putative drug transport protein	AAF12254
Mdr	*Bacillus subtilis*	36	Multidrug-efflux transporter	CAB12101
Otrb	*Streptomyces rimosus*	35	Tetracycline efflux protein	AAD04032
Rv1877	*M. tuberculosis*	32	Similar to drug efflux protein	CAB10049 (8)
Rv0783c	*M. tuberculosis*	24	Similar to multidrug resistance protein	CAB02373 (8)
LfrA	*M. smegmatis*	23	Proton antiporter efflux pump	AAC43550 (14, 29)

[a] The degree of identity was determined with the program BLAST.

facilitator superfamily (MFS) of proteins (19) involved in antibiotic transport or resistance from other bacteria (Table 2). Several motifs characteristic of the drug transporters of the MFS (17, 19) are present in the sequence of P55. A motif present in all members of the MFS was found between residues 66 and 78 of P55 (GRASDRFGRKLML). Another motif was found between residues 104 and 116 (LIAGRTIQGVASG). Between residues 148 and 162 (LGSVLGPLYGIFIVW) there was a motif specific for all drug-proton antiporters; other motifs characteristic of the drug-antiporters with 14 transmembrane segments were present from residues 21 to 31 (LDT YVVVTIMR), 167 to 178 (WRDVFWINVPLT), and 202 to 208 (DLVGGLL).

Subcellular localization of the P55 protein. According to a computer-based prediction included in the TubercuList database at the Institut Pasteur generated with the TMMF program, the product of Rv1410c (which is identical to *M. bovis* P55) contains 14 membrane-spanning domains (data not shown), strongly suggesting a membrane localization for this protein. In order to check this possibility, cell fractions from *M. bovis* crude extracts were prepared and probed by Western blotting with a specific anti-P55 rabbit serum. In the membrane fraction, a band of approximately 55 kDa was detected, which corresponds with the expected size of the P55 protein (Fig. 1A), whereas no band was detected in the cytoplasmic fraction. When the membrane fraction was extracted with the detergent Triton X-114, P55 remained with the detergent fraction. These results suggest that P55 is an integral membrane protein, in agreement with the computer-predicted membrane localization of P55.

Presence of P55 in other mycobacterial species. The anti-P55 rabbit serum was used in Western blotting assays against cell extracts from several mycobacteria. Bands of approximately 55 kDa were detected in unfractionated cell extracts of *M. smegmatis, M. avium, Mycobacterium chelonae, Mycobacterium phlei, Mycobacterium gordonae, Mycobacterium microti, Mycobacterium fortuitum*, and *M. tuberculosis* (Fig. 1B). By PCR with oligonucleotides vec21-low and 2-1dir, genes similar to *P55* were detected in *M. microti* (in agreement with the results of Western blotting) and *Mycobacterium africanum* (data not shown). Also, genes similar to *P55* were detected in the unfinished genomes of *M. avium* (in agreement with the results of Western blotting) and *M. leprae* through database searches. Therefore, since *P55*-like genes or P55-like proteins

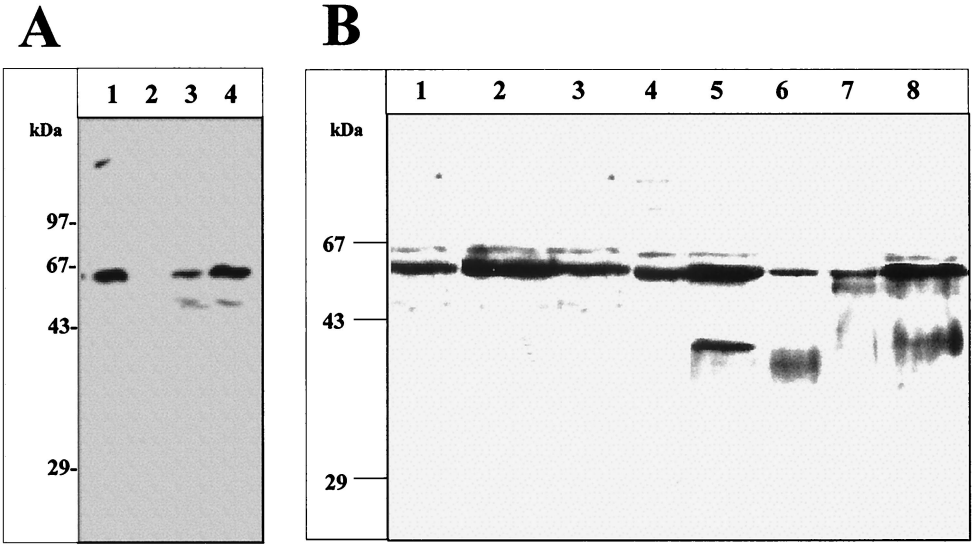

FIG. 1. Detection of P55 protein by Western blotting. (A) Different *M. bovis* BCG preparations: lane 1, total cell extract; lane 2, supernatant obtained after centrifugation at 100,000 × *g*; lane 3, detergent phase of Triton X-114 fractionation; lane 4, pellet obtained after centrifugation at 100,000 × *g*. (B) Cell extracts from different mycobacterial species: lane 1, *M. smegmatis*; lane 2, *M. avium*: lane 3, *M. chelonae*; lane 4, *M. phlei*; lane 5, *M. gordonae*; lane 6, *M. microti*; lane 7, *M. fortuitum*; lane 8, *M. tuberculosis*. The sizes of the proteins (in kilodaltons) are indicated on the left.

TABLE 3. MICs of different antibiotics for *M. smegmatis* strains

Compound	MIC (μg/ml) for *M. smegmatis* strain:		
	PAZ101	PAZ22	PAZ23
2′-*N*-Ethylnetilmicin	4	64	64
6′-*N*-Ethylnetilmicin	4	64	64
Gentamicin	2	8	8
Streptomycin	0.5	4	4
Tetracycline	0.5	4	4

have been detected in fast and slow growers and in pathogenic and nonpathogenic species, it is likely that all species of the genus *Mycobcterium* have similar genes.

Resistance levels and substrate profile. To study the involvement of P55 in antibiotic efflux, two plasmids containing the *P55* gene were analyzed. In pPAZ22, *P55* was cloned in pMV261 under the control of the *hsp60* promoter; in pPAZ23, the operon (*P27* and *P55*) with its natural promoter was cloned in pSUM41. *M. smegmatis* carrying each construct (and the vectors as controls) was tested against a series of antibiotics and chemicals. Both plasmid pPAZ22 and plasmid pPAZ23 produced 8-fold increases in the MICs of tetracycline and streptomycin, 4-fold increases in the MICs of gentamicin, and 16-fold increases in the MICs of 2′- and 6′-*N*-ethylnetilmicin compared with the MICs for the control strains containing pMV261 and pSUM41 (Table 3). (The levels of resistance to the other compounds tested were unaffected by the presence of the plasmid-encoded *P55* gene. We cannot exclude the possibility that P55 may also transport other substances apart from antibiotics.) Therefore, these increases in the resistance to antibiotics reflect the expression of the *P55* gene from both plasmids, suggesting that the P55 protein may act as a drug efflux pump that confers resistance to multiple drugs in *M. smegmatis*. Active efflux involving antituberculosis drugs has not been unambiguosly shown to cause resistance in *M. tuberculosis* and *M. bovis*, as it has been demonstrated in fast-growing mycobacteria (2, 4, 7, 9, 14, 29). Previously described putative efflux pumps from *M. tuberculosis* failed to confer resistance to any particular drug (10), or only low-level resistance to tetracycline could be detected (2).

It is worth noting that P55 conferred resistance to streptomycin, one of the first-line drugs used in the treatment of tuberculosis. It has been reported that 30% of the streptomycin-resistant *M. tuberculosis* clinical isolates do not carry any mutation in the *rpsL* and the *rrs* genes (see references 21, 22, and 26 and references therein). A possible explanation for the levels of resistance to streptomycin in these strains could be an increase in the activity of *M. tuberculosis* P55 or any other streptomycin transporter. Future work will be carried out in order to test the activity of P55 in the streptomycin-resistant *M. tuberculosis* strains carrying wild-type *rrs* and *rpsL* genes.

Apart from resistance to streptomycin, P55 also confers resistance to other aminoglycosides, such as gentamicin and 2′- and 6′-*N*-ethylnetilmycin. The *M. fortuitum* Tap efflux pump was also associated with aminoglycoside resistance (2). Both proteins are members of the MFS family of transporters. Other efflux pumps related to aminoglycoside resistance have been identified in the resistance-nodulation-division family (RDN) of proteins, for example, *E. coli* AcrD (23).

TABLE 4. MICs for *M. smegmatis* strains in the presence of various inhibitors

Compound	MIC (μg/ml) for *M. smegmatis:*	
	PAZ101	PAZ22
Streptomycin	0.5	4
Streptomycin + CCCP	0.5	1
Streptomycin + verapamil	0.5	1
Streptomycin + reserpine	1	2
Tetracycline	0.5	4
Tetracycline + CCCP	0.5	1
Tetracycline + verapamil	0.5	2
Tetracycline + reserpine	0.5	2

Effect of pump inhibitors on resistance. Because of the sequence similarity to proton-drug antiporters and the associated phenotype of multidrug resistance, we considered the possibility that P55 may act as a proton-dependent efflux pump. In order to test this hypothesis, we used the energy uncoupler CCCP, which disperses the proton gradient across the bacterial membrane, thus affecting the activities of the proton-dependent efflux pumps (12). Specific inhibitors of efflux pumps, verapamil and reserpine, were also tested since it has been shown that exposure of bacteria to substances that inhibit efflux systems produces an increase in susceptibility to antibiotics (7, 15). The MICs of streptomycin and tetracycline were determined in the presence and in the absence of these compounds (Table 4). The use of CCCP, verapamil, and reserpine produced a decrease in the MICs of both streptomycin and tetracycline for strain PAZ22 (which expresses P55 from plasmid pPAZ22), whereas the resistance levels of the control strain, PAZ101 (which contains the vector pMV261), were not changed (Table 4). These results indicate that the resistance levels produced by P55 are sensitive to both inhibitors of efflux pumps and substances that eliminate the proton gradient across membranes, suggesting that it is quite likely that P55 uses the energy from the proton gradient to drive the transport of the antibiotics.

Reserpine and verapamil reduced the MICs for PAZ22 to levels similar to those detected in the presence of CCCP. This is an important fact, suggesting that substances other than ionophores could be used to inhibit efflux pumps and improve the activities of antibiotics in the treatment of resistant clinical isolates, as has been proposed elsewhere (15).

Tetracycline accumulation assays. Since tetracycline is one of the substrates of the P55 efflux pump, we studied tetracycline accumulation in *M. smegmatis* PAZ22 (in which P55 is expressed under the control of the *hsp60* promoter) and *M. smegmatis* PAZ101 as a control. The time course of tetracycline accumulation (Fig. 2) showed that PAZ101 accumulated more tetracycline than PAZ22, indicating that P55 is capable of extruding tetracycline from *M. smegmatis*.

Is P55 modulated by P27? In the genomes of *M. tuberculosis* and *M. bovis*, the *P27* and *P55* genes form an operon and both genes are transcribed from the operon promoter (5). We tested plasmid pPAZ24, which has the streptomycin resistance omega cassette inserted in the *P27* gene, therefore preventing transcription of *P55* from the operon promoter. pPAZ24 conferred to *M. smegmatis* the same levels of resistance to 2′-*N*-ethylnetilmicin, 6′-*N*-ethylnetilmicin, and gentamicin as the paren-

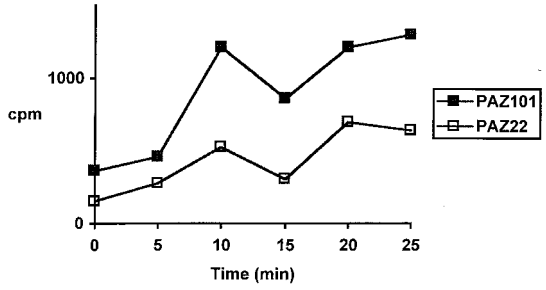

FIG. 2. Time course of tetracycline accumulation for *M. smegmatis* PAZ101 control cells (■) and *M. smegmatis* PAZ22 cells (□) expressing plasmid-encoded *P55* gene.

tal pPAZ23 did, although the levels of resistance to tetracycline were slightly lower (data not shown). This finding suggests that *P55* may have a promoter in the ca. 390 nucleotides between the cassette and the *P55* start codon.

Some bacteria have drug-sensor proteins that induce the expression of an associated efflux pump. In these cases, genes encoding the drug sensor and the efflux pump are located adjacent to each other (13). Since lipoproteins have been suggested to have a role in signal transduction (A. J. C. Steyn, J. Joseph, and B. R. Bloom, Abstr. ASM Conference on Tuberculosis: Past, Present and Future, abstr. 119, 2000), an interesting hypothesis suggests that the P27 protein could be a kind of sensor of specific signals (i.e., the presence of drugs) that would activate, either directly or indirectly, the expression of the *P55* gene. Further experiments will be carried out in order to test the role of P27 in the putative modulation of P55 activity.

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