Ehp53, an *Entamoeba histolytica* protein, ancestor of the mammalian tumour suppressor p53

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This paper reports the identification of Ehp53, a p53-like *Entamoeba histolytica* protein, which binds to the human p53 DNA consensus sequence (oli-p53). Monoclonal antibodies against p53 (Ab-1 and Ab-2) recognized a single 53 kDa spot in two-dimensional gels and inhibited the formation of complexes produced by *E. histolytica* nuclear extracts and oli-p53. Additionally, *E. histolytica* gene promoter sequences with high homology to oli-p53 formed complexes with nuclear proteins that were abolished by oli-p53. Ehp53 protein levels increased in UV-irradiated trophozoites. This protein was also detected in *Entamoeba moshkovskii* and *Entamoeba invadens*.

By confocal microscopy, Ehp53 was located in the nuclei, EhkO organelles and cytoplasm. The Ehp53-encoding gene was cloned and its predicted amino acid sequence showed 30–54 % and 50–57 % homology with important domains of the human and the *Drosophila melanogaster* p53 proteins, respectively. This homology included the tetramerization domain, the nuclear export signal and a nuclear localization signal. Ehp53 also contains seven of the eight DNA-binding residues and two of the four Zn²⁺-binding sites described for p53. A recombinant Ehp53 was recognized by Ab-2. Ehp53 is believed to be the first p53-like protein found in protozoa and may be the evolutionary ancestor of the mammalian p53.

INTRODUCTION

The protozoon *Entamoeba histolytica* infects more than 10% of the world’s population and kills approximately 100 000 people each year (WHO, 1997). The trophozoites show a high variation in virulence, which may be explained in part by the quantity and quality of certain proteins synthesized by them. For example, blocking the translation of cysteine protease by antisense mRNA produces a dramatic drop in the rate of phagocytosis and the ability of the trophozoites to produce liver abscesses in experimental animals (Ankri et al., 1998, 1999). DNA mutations might be responsible in part for the genome variability shown by the different strains and clones, which may produce altered proteins in the parasite. However, the parasite might possess genome-safeguarding mechanisms to maintain the genome integrity. These mechanisms may be evolutionarily related to those described in mammalian cells (El-Deiry et al., 1992).

Few DNA-binding proteins involved in *E. histolytica* transcription have been identified (Petri et al., 1987; Gómez et al., 1998; Pérez et al., 1998; Schaenman et al., 2001). The *E. histolytica* TATA-box-binding protein (EhTBP) has 55 % homology with the human TBP (Luna-Arias et al., 1999) and is located in the nuclei and kinetoplast-like organelles (EhkO), which contain DNA (Orozco et al., 1997). However, no studies have been done on the genome protection events that occur in *E. histolytica*, and DNA-binding proteins involved in these processes are unknown. In mammals, the product of the p53 tumour-suppressor gene is a transcriptional factor that has been implicated in safeguarding genomic stability. This protein is located in the cytoplasm of cells during G1-phase, then at the onset of the S-phase it is translocated into the nucleus and accumulated later in the cytoplasm during the G2/M interphase (Shaulsky et al., 1990a). Genotoxic treatments induce an increase of cellular p53 levels (Cox & Lane, 1995) which is related to G1-phase arrest (Smith et al., 1994) and DNA repair, but a high expression of p53 leads to apoptosis (Enoch & Norbury, 1995). The mechanisms by which p53 specifies its distinct functional options remain unclear. p53-like proteins and their DNA-binding consensus sequences have been found in mammals (Kumaravel & Wafik, 2000), clams (Van Beneden et al., 1997), squid (Schmale & Bamberger, 1997) and *Drosophila melanogaster* (Ollman et al., 2000;
Brodsky et al., 2000), among others, but not in lower eukaryotes. D. melanogaster p53 (Dmp53) has 21–50% identity to the human p53 main motifs and shows many, but not all, of the functions discovered for the human p53 (Ollman et al., 2000; Brodsky et al., 2000). The study of p53-like proteins in primitive organisms might help to elucidate the primitive functions of this multifunctional protein.

Here, we report the identification and cloning of an E. histolytica protein (Ehp53), which to our knowledge is the first p53-like protozoan protein described so far. Ehp53 binds to oligonucleotides containing the mammalian p53 consensus sequence (oli-p53), is recognized by antibodies against the human p53, accumulates in the cells after UV irradiation, and is mainly located in nuclei and EkkOs. The Ehp53-encoding gene was cloned and the corresponding mRNA samples by two-dimensional gel electrophoresis in 10% SDS-PAGE or 30% PAGE. Gels were dried and exposed to X-O-MAT film at 270˚C. Oligonucleotides were synthesized by BioSynthesis USA. The Ehp53 gene and expression of the recombi-
et al., 1992) (Fig. 1a) and trophozoite nuclear extracts (NE). These assays revealed a specific complex in the slowest migration region of the gel, whereas in the fastest migration region, we distinguished two or three unspecific bands (Fig. 1b, lane 2). Ab-1 and Ab-2, directed against the C-terminus and the N-terminus of the human p53 protein, respectively, as well as the non-labelled oli-p53, competed with the slowest migration complex (Fig. 1b, lanes 3, 4 and 5), but it remained in the presence of the non-specific competitor poly dl : dC (Fig. 1b, lane 6) or in the presence of irrelevant antibodies (not shown). These results evidenced the presence of an E. histolytica nuclear protein with affinity to the p53 DNA consensus binding sequence.

A 20 bp sequence located at −315 to −335 bp of the Ehadh112 gene (oli-Eh112) (Garcı´a-Rivera et al., 2000), with 75 % identity to the mammalian p53-consensus site (Fig. 1a), formed at least three specific complexes with E. histolytica NE (Fig. 1c, lane 2). These complexes were competed by non-labelled oli-p53 (Fig. 1c, lane 3) or by oli-Eh112 (Fig. 1c, lane 4), but remained in the presence of poly dl : dC (Fig. 1c, lane 5). Complexes were slightly diminished when Ab-2 antibody directed against p53 was present in the reaction mixture (Fig. 1c, lane 6). Additionally, complexes formed by nuclear proteins of COS cells were competed by non-labelled oli-Eh112, although the slowest migration band remained unchanged (Fig. 1d, lane 4). All complexes disappeared in the presence of non-labelled oli-p53, but remained when we included poly dl : dC in the reaction mixture (Fig. 1d, lanes 3 and 5, respectively).

### Detection of Ehp53 in total proteins of E. histolytica

To identify the E. histolytica proteins that are recognized by the antibodies against human p53 and that may form complexes with oli-p53, we carried out Western blot assays of total E. histolytica proteins separated by one- and two-dimensional PAGE. In one-dimensional PAGE Ab-1 and Ab-2 recognized a 53 kDa band (Ehp53) in the trophozoites of clone A (strain HMI IMSS) and strain 462, which co-migrated with the p53 protein of COS cells (Fig. 2a, b).

We also performed Western blot assays using proteins obtained from trophozoites of E. moshkovskii and E. invadens. The Ab-2 antibodies reacted with a 53 kDa band in the lanes corresponding to E. moshkovskii (Fig. 2c, lanes 2 and 3) and to E. invadens (Fig. 2c, lane 4). In some experiments, the antibodies detected an additional 50 kDa band, which could be an Ehp53 degradation product.

The number of proteins recognized by Ab-1 and Ab-2 was investigated by two-dimensional PAGE (Fig. 2d). Ab-1 (not...
shown) and Ab-2 revealed a single 53 kDa spot in the basic region of the gel, between pI 7-5 and 8-0 (Fig. 2e).

The results presented in Fig. 2 show that Ehp53 is antigenically related to the amino- and carboxy-terminus of the human p53 and it seems to be a single protein according to the two-dimensional gel results. The electrophoretic migration of Ehp53 predicted a molecular mass similar to those found for members of the p53 family in many species.

UV irradiation increases the amount of Ehp53 in *E. histolytica* trophozoites

In mammalian cells, UV irradiation produces an accumulation of p53 (Cox & Lane, 1995). The effect of UV irradiation upon live trophozoites on Ehp53 exposed to UV light (80 J m$^{-2}$) was determined as described in Methods. In Western blot analysis, the Ab-2 antibodies revealed a stronger Ehp53 band in proteins obtained from trophozoites

![Fig. 2. Detection of Ehp53 by Western blot assays using monoclonal antibodies against human p53. (a, b) Samples (15 μg) of total proteins from *E. histolytica* trophozoites of clones A (lane 1) or 462 (lane 2) were analysed by Western blotting using the Ab-1 (a) or Ab-2 (b) antibodies. Lane 3, mammalian COS cells. (c) Samples (15 μg) of proteins from *E. histolytica* clone A (lane 1) trophozoites, *E. moshkovskii* strains Laredo (lane 2) and FIC (lane 3), *E. invadens* strain PZ (lane 4), and C33 cells (lane 5) were analysed by Western blotting using Ab-2 antibody. (d, e) Samples (30 μg) of total proteins of *E. histolytica* trophozoites (clone A) were submitted to two-dimensional PAGE. (d) Gels were Coomassie blue stained. The rectangle shows the region recognized by Ab2. (e) Gels were transferred to nitrocellulose and incubated with the Ab-2 antibody. Antigen–antibody reactions were revealed by ECL.

![Fig. 3. Ehp53 in UV-irradiated *E. histolytica* trophozoites.](https://example.com/fig3)

Samples (15 μg) of total proteins of non-irradiated trophozoites (0) or trophozoites irradiated for 4 s and then incubated for 30, 60 and 120 min were separated by 10% SDS-PAGE; gels were transferred to nitrocellulose filters and incubated with (a) Ab-2 or (b) anti-actin antibodies.
incubated for 30 min after UV irradiation. The band increased in intensity at 60 min and started to diminish in trophozoites incubated for 120 min after UV irradiation (Fig. 3a). Anti-actin antibodies showed that all lanes were loaded with a similar amount of protein (Fig. 3b). The increase of Ehp53 may be related to DNA repair, suggesting a putative functional similarity between Ehp53 and the mammalian p53.

**Cellular location of Ehp53 by confocal microscopy**

p53 performs most of its functions in the nucleus, but it is frequently trapped in the cytoplasm (Scheffner et al., 1990; Middeler et al., 1997; Stommel et al., 1999; Lu et al., 2000). We investigated the location of Ehp53 in the trophozoites of clone A by confocal microscopy. The Ab-2 antibodies

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**Fig. 4.** Immunodetection of Ehp53 by confocal laser microscopy. Trophozoites (a–i), or COS cells (j–l), were fixed, permeabilized, incubated with the Ab-2 antibody and with a fluoresceinated anti-mouse secondary antibody (green; b, e, h, k), then counterstained with propidium iodide (red: a, d, g, j) to visualize DNA. Yellow fluorescence indicates overlapping DNA and Ehp53 staining (c, f, i, l). Arrows show some EhKO5s in the trophozoites; n, nucleus. Bars: (a–i) 10 μm; (j–l) 50 μm.
revealed that Ehp53 appeared in the nuclei, EhkOs (Orozco et al., 1997) and the cytoplasm in the majority of the permeabilized trophozoites (Fig. 4a–c, g–i). About 95% of the nuclei showed green fluorescence. Some of the nuclei showed small spots of fluorescence (Fig. 4a–c), whereas others were completely stained by the antibody (Fig. 4d–i). About 90% of the trophozoites showed Ehp53 in the cytoplasm, and Ehp53 was visible in 80% of EhkOs (Fig. 4a–c, g–i). Ehp53 found in EhkOs and nuclei co-localized with the propidium-iodide-stained DNA (Fig. 4c, f, i, yellow fluorescence), whereas Ehp53 located in the cytoplasm, outside the organelles, was not associated with DNA (Fig. 4a–c). As expected, p53 was detected in the nuclei of 99% of the mammalian cells used as a control, although it also appeared in the cytoplasm of some cells (Fig. 4j–l).

The predicted Ehp53 protein has p53 protein domains and the DNA-binding residues

The Ehp53-encoding gene was obtained from an E. histolytica ZAP cDNA library (Sánchez et al., 1994) using Ab-2. First, we obtained a 1300 bp cDNA clone lacking the first 10 bp at the 5’ end of the gene and containing the poly A tail at the 3’ end. Then, we used the cDNA clone to screen an E. histolytica Zap genomic DNA library (Descoteaux et al., 1992). Sequences of the cDNA and DNA clones were identical. A fragment of the predicted Ehp53 amino acid sequence had 92% identity to the clone ESHKK15TR reported in the E. histolytica genome project database (http://www.tigr.org). The Ehp53 gene has a 1290 bp open reading frame and encodes a 50-7 kDa predicted protein, which is in the range of the molecular masses reported for other p53 proteins (Fig. 5). The predicted pl of Ehp53 was 8-2, but post-translational modifications, such as phosphorylation, might change this pl to a more acidic one, as shown in the two-dimensional gels (Fig. 2e).

As in other p53 proteins, Ehp53 has an acidic amino-terminal transactivation domain and a basic carboxy-terminal domain (Fig. 5). The amino acid residues recognized by the Ab-2 antibody (Banks et al., 1986) were found between residues 36 and 48 (Fig. 5). Ehp53 showed the highly conserved regions I, II, III, IV and V reported for p53 proteins with 30–54% homology to the same regions of the human p53 and 50–57% to those of Dmp53 (Fig. 5). The tetramerization domain was also found in Ehp53 and it has 48% homology to the human p53, but only 19% to Dmp53 (Fig. 5). Ehp53 also contains amino acid sequences (residues 360–374 and 419–425) with 58 and 66% homology to p53 nuclear export signal (Stommel et al., 1999) and a nuclear localization signal (Shaulsky et al., 1990b), respectively (Fig. 5). Ehp53 has seven of the eight DNA-binding residues.

Fig. 5. Predicted Ehp53 amino acid sequence (Eh p53) compared with the human (Hs p53) and the D. melanogaster p53 (Dm p53) predicted sequences. Black boxes indicate identical amino acids. Grey boxes show the conserved changes. Ab2 shows the epitope recognized by Ab2 antibodies. I, II, III, IV and V indicate the most conserved domains in p53 family proteins. TET, tetramerization domain; NES, nuclear export signal; NLS, nuclear localization signal; D, amino acid residues for DNA binding; Z, amino acid residues for Zn$^{2+}$ binding. Asterisks mark the most important mutation sites in human cancer. Numbers on the right correspond to the amino acid number in the protein sequences.
residues in the same positions (residues 95, 217, 224, 251, 254, 266 and 268) as those reported for other p53 proteins. Four of them are identical to the human p53 and three were conserved changes (Fig. 5). We also found two of the four Zn$^{2+}$-binding residues (positions 148 and 217) in the same positions as reported for the human p53 protein (Ollman et al., 2000).

**A recombinant polypeptide (Ehp53293) is recognized by Ab-2**

Western blot assays using a recombinant polypeptide (Ehp53293) obtained by the expression of the first 293 amino acid residues of Ehp53 showed that the anti-histidine and the Ab-2 antibodies recognized Ehp53293 (Fig. 6a), indicating that it is an Ehp53 recombinant protein containing the histidine tag. Given that Ab-2 recognized a single spot in two-dimensional gels, we assume that the 53 kDa band recognized in total proteins and the recombinant polypeptide Ehp53293 correspond to the same protein, except that Ehp53293 lacks the Ehp53 carboxy-terminus.

**DISCUSSION**

Using oli-p53 and monoclonal antibodies against the human tumour suppressor p53 we have identified, characterized and cloned Ehp53, which we believe to be the first p53-like protein found in protozoa. Its predicted amino acid sequence showed 30–54 % homology with the DNA-binding domains of the human p53 and 50–57 % to the Dmp53 protein. Ehp53 has seven of the eight DNA-binding residues described for p53, and two of the four Zn$^{2+}$-binding sites. p53-like proteins have been reported in mammals (Kumarovel & Wafik, 2000), amphibians (Cox et al., 1994), plants, fungi (Loidl & Loidl, 1996) and recently in *D. melanogaster* (Ollman et al., 2000; Brodsky et al., 2000), but not, hitherto, in protozoa. Ehp53 was recognized by two different monoclonal antibodies against the carboxy- (Ab-1) and amino- (Ab-2) terminus of the human tumour suppressor p53. The epitope recognized by the Ab-2 antibody in the human p53 has been reported (Banks et al., 1986); Ehp53 has a sequence homologous to this epitope (Fig. 5), explaining why, in spite of the low general identity between the human p53 and Ehp53, Ab-2 was able to recognize Ehp53. As in mammalian cells, Ehp53 may be a transcription factor and it could be also be involved in DNA repair because its amount increased in UV-irradiated trophozoites.

As the most evolutionarily distant member of the p53 gene family to be identified, Ehp53 may shed some light on the selective pressures that have maintained p53-like molecules through evolution. It is likely that a protein with important and multiple functions may have arisen very early and may be conserved through evolution. The possibility of horizontal gene transfer of the p53 gene from the human host to the parasite is discarded because of the high divergence in sequence between human p53 and Ehp53. The fact that Ab-2 recognized 53 kDa proteins in *E. invadens*, a snake parasite, and *E. moshkovskii*, a free-living Entamoeba species, supports the hypothesis that p53 arose earlier than was thought.

An interesting finding was the presence of higher amounts of Ehp53 in the cytoplasm and EhKOs than in the nuclei of the trophozoites, shown by confocal microscopy. In mammalian cells, mutations in the nuclear localization signal of p53 and p53 proteins complexed with viral or cellular proteins prevent the efficient passage of p53 through the nuclear membrane (Shaulsky et al., 1990b). EhTBP (Luna-Arias et al., 1999) and the C/EBP-like transcription factor (Marchat et al., 2002) are also present in nuclei and EhKOs. It would be of interest to investigate whether nuclear and extra-nuclear Ehp53 have similar structure and function.

The results of our experiments strongly support the hypothesis that Ehp53 and the human p53 correspond to homologous proteins. (i) Ehp53 and p53 have been identified by two antibodies directed against different regions of p53, and both proteins showed the same relative mobility in electrophoresis, as was shown by comparison with the COS or C33 cells used throughout this work as controls. (ii) The concentration of both mammalian p53 and Ehp53 protein increases after UV irradiation. In mammals, p53 has a short half-life (15–25 min), which increases after UV irradiation (Maltzman & Zyzyk, 1984). Ehp53 may also show an extended half-life after UV-irradiation of the
trophozoites. (iii) EhP53 also binds to specific DNA sequences; it is present in the nucleus and the EhkO organelle, co-localizing with DNA. Altogether, the results presented here suggest that EhP53 is a part of the DNA-protein complexes detected by gel-shift assays, supporting the idea that this protein is a transcription factor. (iv) oli-Eh112, a 20 bp sequence homologous to the consensus p53-binding sequence present in the EhAdh112 gene promoter, formed complexes with NE of E. histolytica trophozoites and with NE of COS cells. Complexes were competed by non-labelled oli-Eh112 and oli-p53, indicating their specificity and confirming the high homology between oli-Eh112 and oli-p53. (v) The amino acid sequence predicted from the EhP53 gene showed the presence of conserved domains reported for p53. Additionally, the recombinant EhP53 protein was recognized by Ab-2.

Questions on the functions of EhP53 in E. histolytica remain open. In mammalian cells, p53 participates in apoptosis, DNA repair and cell cycle regulation, preventing uncontrolled cellular division, which produces cancer. EhP53 shows changes in three of the more frequently mutated positions described for p53 in transformed cells and in two of the residues involved in Zn$^{2+}$ binding (Fig. 5, asterisks). However, at this time, we cannot speculate on the significance of these changes. The functional characterization of EhP53 is currently under study.

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