

Infection by *Mycoplasma hyorhinis* strongly enhances uptake of antisense oligonucleotides: a reassessment of receptor-mediated endocytosis in the HepG2 cell line

Philippe de Diesbach*, Francisca N’Kuli, Michel Delmée¹ and Pierre J. Courttoy

Cell Biology Unit, Christian de Duve Institute of Cellular Pathology and Université catholique de Louvain, UCL 7541, 75 avenue Hippocrate, B-1200 Brussels, Belgium and ¹Bacteriology Unit, Université catholique de Louvain, UCL 5490, 54 avenue Hippocrate, B-1200 Brussels, Belgium

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ABSTRACT

This paper shows that the ~66 kDa band, previously isolated from the HepG2 cell line as an oligonucleotide (ON) plasma membrane ‘receptor’, is induced by *Mycoplasma* infection. Moreover, this band has been identified as the invariant membrane protein of *Mycoplasma hyorhinis*, p70, based on ribosomal DNA sequencing combined with ON ligand blotting after p70 immunoprecipitation by a monoclonal antibody. Whereas antibiotic treatment of infected HepG2 cells strongly decreased ON capture, as measured by a biochemical assay, conversely, deliberate infection of HeLa cells with *M.hyorhinis* dramatically promoted ON uptake but did not affect receptor-mediated endocytosis of transferrin. This was confirmed by confocal microscopy of infected HepG2 cells, which also showed an indistinguishable labelling pattern after exposure of living cells to fluorescent ON and after p70 immunolabelling in permeabilised fixed cells. We propose that ON binds to p70 on *M.hyorhinis* attached at the cell surface, after which the complex is internalised by ‘piggy-back’ endocytosis.

INTRODUCTION

Oligonucleotides (ON), including small interfering RNA (si-RNA), are short nucleic acids fragments with a considerable potential for research and therapy. The ON target is located either in the cytosol or in the nucleus. With the possible exception of special uncharged derivatives, such as morpholinos, which are thought to diffuse across biological phospholipid bilayers (1), the plasma and endocytic membranes are impermeable to the negatively charged ON. These are accordingly taken up by endocytosis, a constitutive process leading to entrapment in endosomes and/or lysosomes.

A great diversity of specific ON-binding proteins with variable affinities have been reported on different cultured cells, but their role in ON capture and effect remains to be clarified. In some reports, the modality of ON capture was clearly compatible with adsorptive or receptor-mediated endocytosis, but a direct relation between the membrane ON-binding protein and accelerated ON endocytosis was not demonstrated. Furthermore, the level of ON capture in a given cell line varied considerably between experiments (2,3). Finally, a relation between the rate of ON endocytosis and the magnitude of its subsequent effect has not been established.

We (2) and others (3,4) have studied ON endocytosis in HepG2 cells, an established hepatocarcinoma cell line. ON endocytosis was found to be saturable and to approach a steady state level with time. Based on the combination of *in situ* photo-affinity labelling on intact cells as well as ligand blotting of cellular extracts with competition studies, we postulated that ON is taken up in these cells by receptor-mediated endocytosis and identified a ~66 kDa membrane ‘receptor’. This protein was purified, and partially sequenced, but these sequences could not be retrieved from human genome or expressed sequence tags (EST) databases (5).

However, established cell lines may be crippled by cryptic *Mycoplasma* or viral infection, and these could affect ON endocytosis. Indeed, Rosenblatt *et al.* (6) reported that *Mycobacterium tuberculosis* infection of macrophages strongly promotes the cellular uptake of fluorescent ON, as measured by FACS analysis. These authors could exclude ON trapping in dead cells, based on exclusion of nuclear staining by propidium iodide as a plasma membrane integrity test, specifically recommended to exclude artefacts in ON uptake experiments (7). Similarly, transfection of HepG2 cells with a plasmid containing hepatitis B virus DNA leads to a ~2-fold increase of ON uptake (8).

In the course of our studies, we noticed that both the abundance of the ‘ON receptor’ in cellular extracts, as assessed by ligand blotting, and the level of endocytic uptake of radioiodinated ON in living cells were highly consistent within a single experiment, but could vary considerably with time. Furthermore, we recently discovered that all lots of

*To whom correspondence should be addressed. Tel: +32 2 764 7541; Fax: +32 2 764 7543; Email: diesbach@cell.ucl.ac.be

HepG2 cells available to us were infected with *Mycoplasma*, these wall-free prokaryotes that are spread worldwide as contaminants of cultured cell lines. This paper demonstrates a clear causal relationship between infection by *Mycoplasma hyorhinis* and accelerated ON uptake by cultured cell lines, identifies the 'receptor' involved as an invariant bacterial membrane protein, and calls attention to the need of reinterpreting previous results published by us, and possibly by other investigators, based on this pitfall.

MATERIALS AND METHODS

Tracer source and modifications and other reagents

A phosphodiester 25mer ON derivative, fluoresceinated at its 5' end and protected at its 3' end by phosphoro-alkylamine (Eurogentec, Seraing, Belgium), was used throughout (2). For photo-cross-linking experiments, this ON was further derivatised with benzophenone, as described (9). Both products were radioiodinated with IodoBeads (Pierce, Rockford, IL, USA), as previously described (9) and will be referred to as ^{125}I -ON or ^{125}I -ON-benzophenone. ON-Alexa 488 and transferrin-Alexa 568 were synthesised as previously described (2). Unless otherwise stated, all reagents were from Sigma or Merck and were of the highest available purity.

Cell culture

Several clones of HepG2 cell line were analysed. They were either purchased (twice) from the American Type Tissue Culture Collection, or kindly provided by Dr G. Strous (Utrecht, The Netherlands) and Dr D. Hoekstra (Groningen, The Netherlands) and were propagated as described (2).

HeLa cells containing a plasmid for hygromycin resistance were kindly provided by Dr R. Kole (University of North Carolina, Chapel Hill, USA). These cells were propagated in DMEM medium with 10% FCS (both from Life Technologies, Gaithersburg, MD, USA), supplemented with 10 mM HEPES, 10 mM NaHCO_3 , 16 mM glucose, 100 mg/l streptomycin, 60 mg/l penicillin (all from Sigma, St Louis, MO, USA) and 200 mg/l hygromycin (Life Technologies), and adjusted to pH 7.4.

In an attempt to cure *Mycoplasma* infection, HepG2 cells were treated with BM cyclin® antibiotics for 3 weeks (Roche, Mannheim, Germany). This treatment is recognised as the most effective against *Mycoplasma* infection (10). Conversely, HeLa cells were intentionally infected by incubation with the medium of contaminated HepG2 cells for 24 h, followed by propagation in fresh medium that was supplemented or not with hygromycin, as indicated. At each subculture following intentional infection, cells were plated on Petri dishes for carbonate extraction, followed by ligand blotting (5) and western blotting (see below).

Mycoplasma detection and identification

Mycoplasma infection was screened either by DNA staining with Hoechst 33258 (Aventis, Strasbourg, France) (11), or by a biochemical assay using a MycoTect™ Kit (Life Technologies), as recommended by the manufacturer. The latter assay proved more sensitive and objective.

Identification of the *Mycoplasma* strain was achieved by rRNA amplification, followed by sequencing. Briefly, DNA

extraction was prepared with chelex-100 resin (BioRad, Nazareth, Belgium), as described (12). HeLa cells were lysed by boiling for 15 min in a 5% chelex suspension and centrifuged at 13 000 g for 10 min to pellet cell debris. Three microlitres of each supernatant containing extracted prokaryote DNA was used as template for PCR. The 16S rDNA gene (1500 bp) was fully amplified by PCR using 16S rDNA primers '1522R' and '27F' (13). The PCR product was resolved by electrophoresis in a 1.2% agarose gel and visualised by ethidium bromide staining under UV. A 1.5 kb fragment was excised from the gel and purified by the QIAquick Gel Extraction Kit (Westburg, Leusden, The Netherlands), according to manufacturer's instructions, and sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit, as recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA), using the universal forward and reverse 16S rDNA sequencing primers 321, 530, 685, 1100, 1242, 1392, relative to the *Escherichia coli* 16S rRNA numbering (13). The sequencing product was purified by ethanol/sodium acetate precipitation and sequenced with a 3100 automatic sequencer (Applied Biosystems). The 16S rDNA sequences obtained were compared with those available in GenBank and EMBL databases by multiple sequence alignments using the Blast program.

Cell incubations and blots

Photo-cross-linking experiments and ligand blotting were performed as described (5). For photo-cross-linking, HepG2 cells were incubated with 25 nM ^{125}I -ON-benzophenone for 1 h at 37°C, followed by eight PBS- Ca^{2+} washes, UV irradiation at 365 nm for 1 h and either immediate lysis by 0.5% (w/v) Nonidet P-40 (NP-40) supplemented with complete protease inhibitor (Roche), in 50 mM Tris, pH 7.2, or carbonate extraction followed by lysis as above (5,14). For ligand blotting, naive cell extracts were resolved by SDS-PAGE before transfer onto PVDF membrane. ON-binding proteins were evidenced using 100 nM ^{125}I -ON in the presence of 1.25 μM dextran sulfate and 6 μM suramin, as described (5). Signal was demonstrated by PhosphorImager analysis and the intensity of the ~66 kDa band was quantified using Scion software (<http://www.scioncorp.com>).

For western blotting, blotted PVDF was saturated in development solution containing 90 mM NaCl, 2 mM CaCl_2 , 50 mM Tris-HCl, pH 8.0 supplemented with 0.15% (v/v) Tween-20 and 2% (w/v) commercial milk powder for at least 2 h, then incubated for at least 2 h with a 1:50 000 dilution in the same solution of monoclonal antibody AB3 (an IgG1 κ recognising p70, an invariant membrane protein of *M.hyorhinis*) (15) that was kindly provided as ammonium sulfate precipitate by Dr K. Wise (University of Missouri-Columbia, MO, USA). The final concentration of AB3 antibody was estimated at ~100 ng/ml. After five washes in development solution without milk powder, the membrane was incubated with a 1:10 000 dilution of commercial goat anti-mouse antibodies coupled to peroxidase in development solution with milk powder for 1 h (Amersham Pharmacia Biotech, Uppsala, Sweden), prior to five washes and chemiluminescence detection (ECL Kit; NEN, Boston, MA, USA) using a Kodak X-Omat film (Eastman Kodak,

Rochester, NY, USA). Developed films were scanned and quantified using Scion software.

For uptake experiments, HeLa cells were cultured in Petri dishes to near confluency ($\sim 3 \times 10^6$ cells in a 30 cm² dish) and incubated in serum-free RPMI at 37°C for 1 h, with either 5 µg/ml ¹²⁵I-transferrin (62 nM) or 1 µg/ml ¹²⁵I-fluorescein-ON (125 nM), as described (2). After uptake, dishes were transferred to 4°C and extensively washed: thrice with PBS containing 0.44 mM Ca²⁺ (PBS-Ca²⁺), thrice with PBS-Ca²⁺ supplemented with 1% BSA, and thrice with PBS-Ca²⁺, for 5 min each. This washing procedure proved essential to minimise non-specific surface binding. After surface digestion by 0.1% (w/v) pronase (Sigma) in RPMI medium for 1 h, cells were harvested by centrifugation, briefly rinsed twice with PBS and lysed in 0.01% (v/v) Triton X-100.

Confocal microscopy of living cells was performed as previously described (2). Briefly, HepG2 cells were grown for 36 h in serum-coated LabTek chambers (Nunc, Roskilde, Denmark), then incubated with 300 nM ON-Alexa 488 together with 80 nM transferrin-Alexa 568 for 2 h at 37°C, before washes and observation without fixation. Confocal settings were the same as in our previous report. *Mycoplasma*-infected HepG2 cells were also incubated for 30 min with 1 µM CellTracker green (Molecular Probes, Eugene, OR, USA) in RPMI medium, then fixed with 4% formaldehyde (w/v) in 0.1 M phosphate buffer pH 7.4 for 15 min, permeabilised with 0.5% saponin (w/v) in PBS for 20 min and quenched with PBS supplemented with 1% BSA, 0.1% L-lysine and 0.01% saponin (all w/v). The same quenching PBS solution was used for all subsequent incubations. Cells were then incubated for 30 min with a 10 000-fold dilution of AB3 antibody, washed six times and incubated with 5 µg/ml anti-mouse antibodies-Alexa 568 (Molecular Probes) and washed six times before mounting in Mowiol. Living or fixed cells were visualised using a Zeiss Axiovert microscope coupled to an MRC-1024 confocal scanning laser imaging system (BioRad). Signals of the two fluorochromes in double labelling experiments were sequentially recorded.

Immunoprecipitation

Approximately 30 µg of protein of the pellet after carbonate extraction of membrane proteins from HepG2 cells was mixed in an Eppendorf tube with 40 µl of packed protein A-Sepharose beads (Amersham Pharmacia Biotech), together with 7 µl of rabbit antiserum against mouse immunoglobulins (Dako, Glostrup, Denmark), with or without a 500× dilution of AB3 monoclonal antibody. The final volume was adjusted to 80 µl by adding ~ 55 µl of PBS containing 0.5% NP-40. After gentle mixing for 3 h, Eppendorf tubes were centrifuged, supernatants (~ 60 µl) were saved and pelleted beads were washed four times with 60 µl of PBS supplemented with NP-40. Both pellets and supernatants were analysed by ligand blotting, immediately followed by western blotting without stripping.

Statistical analysis

The statistical significance of differences was determined using Student's *t*-test, with *P* > 0.05 regarded as non-significant (NS).

RESULTS

Evidence for latent *Mycoplasma* infection in several lots of the HepG2 cell line and its effective treatment

The four lots of HepG2 cells we obtained, but no other cell line in the laboratory, unless intentionally infected, eventually developed a florid *Mycoplasma* infection upon very prolonged culture. However, when cells had been received, *Mycoplasma* screening using DNA Hoechst 33258 staining consistently scored negative (i.e. there was no cytoplasmic or plasma membrane-associated Hoechst 33258 staining to be detected). Cells were accordingly frozen and aliquots were typically subcultured for 2–3 months, then discarded. In contrast, when culture was extended for several months, repeating the procedure consistently revealed *Mycoplasma* infection by Hoechst staining.

We thus applied Mycotect™, a more sensitive and more objective test: this yielded a positive response for all four lots, irrespective of their origin, even if they had only been passaged a few times. However, after a vigorous 3 week BM cyclin treatment, this test became negative, indicating a good response, if not a cure. BM cyclin-treated cells were thus analysed in parallel with non-treated cells, either by incubation with ¹²⁵I-ON-benzophenone for *in situ* photo-affinity labelling of intact cells, or directly lysed for ligand blotting on cell extracts. Both methods labelled a ~ 66 kDa band only in infected HepG2 cells (Fig. 1A and B, compare lanes 1 and 4). As shown by carbonate extraction, this band was membrane-associated (compare lanes 2 and 3 of each panel).

Transfer of the ~ 66 kDa 'ON receptor' to HeLa cells upon *Mycoplasma* infection

To examine whether this ~ 66 kDa band was indeed due to *Mycoplasma* infection, naive HeLa cells containing a hygromycin-resistance plasmid (see below) were intentionally infected by exposure for 1 day to medium conditioned during 2 days of culture with non-treated HepG2 cells, then further grown in fresh medium. At each subculture, an aliquot of HeLa cells was saved for ligand blotting and western blotting. After 12 passages, prokaryotic DNA was extracted, rDNA was amplified by RT-PCR and the presence of *M.hyorhina* was evidenced, demonstrating successful infection and identifying the species involved.

The kinetics of the infection was further monitored by western blotting with monoclonal antibody AB3 recognising p70, an invariant membrane protein common to all *M.hyorhina* strains tested, and considered as a marker for this *Mycoplasma* species (15). The p70 protein was indeed recovered in the membrane pellet after carbonate extraction. Figure 2 compares the kinetics of acquisition by HeLa cells of the ~ 66 kDa 'ON receptor' upon infection, as detected by ligand blotting in carbonate extracts (A), with the kinetics of infection, based on western blotting using AB3 monoclonal antibody (B). Both signals appeared and fluctuated in close parallel to one another. Interestingly, hygromycin that restrained *M.hyorhina* infection also abrogated the 66 kDa signal (Fig. 2A and B, compare lanes 2 and 7).

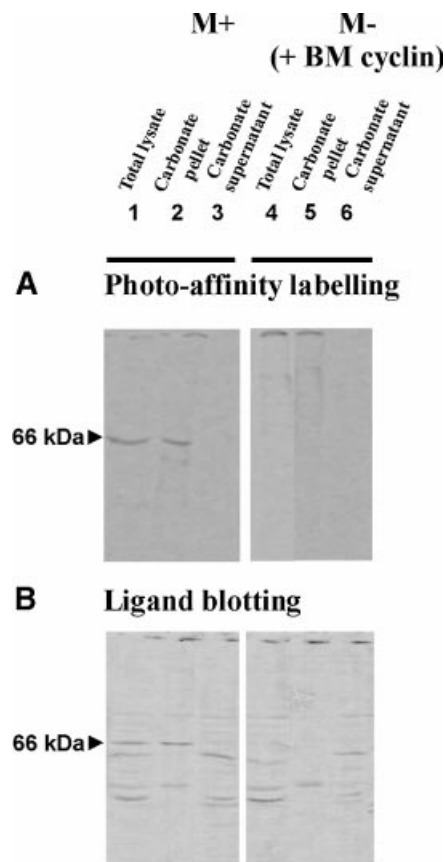


Figure 1. Role of *Mycoplasma* infection in the occurrence of the 'ON receptor' in HepG2 cells. HepG2 cells were either left untreated (M+, left) or were treated for with BM cyclin for 3 weeks (M-, right). (A) *In situ* photo-affinity labelling. Cells were further incubated with 25 nM 125 I-ON-benzophenone at 37°C for 1 h. After extensive washing, bound ligand was cross-linked to adjacent residues of the interacting protein by UV exposure for 1 h, after which cells were directly lysed (lanes 1 and 4, total lysate, 100 μ g of protein) or carbonate-extracted before lysis (lanes 2 and 5, carbonate pellet, ~20 μ g of protein; lanes 3 and 6, carbonate supernatant, ~80 μ g of protein). Proteins were resolved by SDS-PAGE and labelled bands were revealed with a PhosphorImager. (B) Ligand blotting. The same as above, except that cells were not incubated with ON-benzophenone, but ON-interacting bands were revealed in cell extracts, upon 125 I-ON incubation of the blot after SDS-PAGE. Arrows show the position of the 66 kDa band, previously identified as 'ON receptor'.

Identification of the ~66 kDa 'ON receptor' as the invariant membrane protein of *M.hyorhinis*, p70

Since the ~66 kDa membrane band observed by both photo-affinity labelling and ligand blotting was clearly linked to *M.hyorhinis* infection, and in view of the close similarity of electrophoretic mobility and parallel evolution of expression with the invariant bacterial membrane protein, p70, we speculated that the 'ON receptor' previously identified in the HepG2 cell line (5) was not of eukaryotic but of bacterial origin, and was actually p70 itself.

To test this hypothesis, carbonate extracts of infected HepG2 cells were immunoprecipitated with AB3 monoclonal antibody and blots were revealed by ligand blotting (Fig. 3A), followed by western blotting with the same AB3 antibody (Fig. 3B). With both methods, only a faint signal was recovered in the pellet when monoclonal antibody was

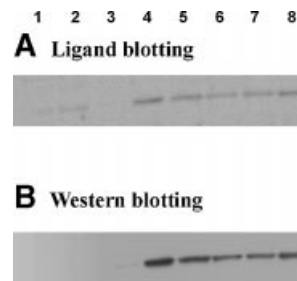


Figure 2. Parallel acquisition by HeLa cells of the 'ON receptor' and the invariant membrane protein of *M.hyorhinis*, p70. *Mycoplasma*-free HeLa cells were intentionally infected by HepG2 medium containing *M.hyorhinis* for 24 h, then cultured in fresh medium. At each subculture, aliquots were submitted to carbonate extraction; 20 μ g of protein of the pellet after carbonate extraction of each sample were analysed by SDS-PAGE and blotted. (A) Ligand blotting. Lane 1, non-infected cells after 11 days of culture; lane 2, infected cells cultured for 11 days in hygromycin-containing medium; lanes 3–8, infected cells were cultured in hygromycin-free medium for 2, 5, 7, 9, 11, 14 days, respectively. (B) Western blotting. The same blot was saturated with milk powder, re-probed with monoclonal antibody AB3, recognising the invariant membrane protein of *M.hyorhinis*, p70, and revealed by chemiluminescence.

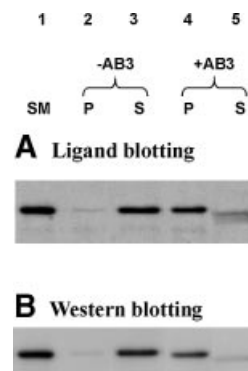


Figure 3. Identification by immunoprecipitation of the 'ON receptor' in HepG2 cells as p70. (A) Ligand blotting. Integral membrane proteins of *M.hyorhinis*-infected HepG2 cells were extracted as carbonate pellet and lysed, of which 30 μ g of protein were submitted to immunoprecipitation where monoclonal antibody AB3 was present on protein A-Sepharose (lanes 4 and 5) or omitted (lanes 2 and 3). Lane 1 is starting material (SM). Material retained on protein A-Sepharose is shown in lanes 2 and 4 (P) and the supernatant of Sepharose beads is shown in lanes 3 and 5 (S). All fractions were finally tested by ligand blotting as above. (B) Western blotting. The same blot was re-probed with AB3 monoclonal antibody, and revealed by chemiluminescence.

omitted (compare lanes 1 and 2), the bulk remaining in the supernatant (lanes 3). When the monoclonal antibody was added, the band recognised by ligand blotting was almost completely immunoprecipitated (compare lanes 4 and 5). When ligand blotting and western blotting were prepared in parallel instead of sequentially, results were identical (data not shown). Furthermore, there was an excellent linear correlation between the intensity of the ~66 kDa band revealed by ligand blotting and that of p70 demonstrated by western blotting ($r = 0.954$). Altogether, these results unambiguously identify the ~66 kDa 'ON receptor' evidenced by ligand blotting as the invariant membrane protein of *M.hyorhinis*, p70.

Mycoplasma hyorhinitis infection triggers ON uptake

Finally, to test whether acquisition of p70 upon infection would selectively accelerate ON capture, intentionally infected HeLa cells were analysed for ^{125}I -ON and ^{125}I -transferrin endocytosis [intracellular uptake was determined after surface digestion by pronase; see de Diesbach *et al.* (2)]. Transferrin, a well established tracer of receptor-mediated endocytosis, was used as a negative control. Infection of HeLa cells by *M. hyorhinitis* led to an up to ~10-fold increase of ON capture [from 0.38 ± 0.03 (mean $\pm$ SD) ng ON/mg cell protein after 1 h of uptake before infection, to 3.91 ± 0.11 ng ON/mg cell protein after infection; $n = 3$; $P < 0.001$]. In contrast, the level of transferrin endocytosis, measured strictly in parallel, was not affected by the infection ($98 \pm 3\%$ of control values; $n = 3$; NS). Incubation of HeLa cells with AB3 monoclonal antibody marginally but consistently reduced ON capture in infected HeLa cells, down to $90 \pm 1\%$ of values without antibody ($n = 3$; $P < 0.01$), but not in non-infected cells ($112 \pm 27\%$; NS). BM cyclin treatment of HepG2 cells also decreased ON capture, from 14.9 ± 0.7 to 2.8 ± 0.1 ng ON/mg cell protein ($n = 3$; $P = 0.001$).

Infected or BM cyclin-treated HepG2 cells were also incubated with submicromolar concentrations of fluorescent ON and transferrin, which allows to study their receptor-mediated endocytosis, given appropriate confocal settings (2). In these conditions, *Mycoplasma*-infected HepG2 cells showed an obvious dotted labelling for ON at the periphery of the cytoplasm (Fig. 4A, green signal), that almost completely disappeared after BM cyclin treatment (Fig. 4B). For comparison purposes, labelling of transferrin in endosomes showed no co-localisation with ON, and was not affected by the antibiotic treatment (identical red signals in Fig. 4A and B). The observation of intracellular signal is always a difficulty when it is mainly at the cell periphery (2). At low magnification as shown in Figure 4A, the vast majority of labelling appeared to be associated with the plasma membrane, thus precluding unambiguous discrimination between intracellular or membrane-adsorbed ON. However, at the higher magnification shown in Figure 4C, intracellular signals for both transferrin and ON were clearly detected.

Finally, to better evidence the association of ON capture with p70, we compared the labelling pattern of living cells by fluorescent ON with the immunolocalisation of the invariant surface protein of *M. hyorhinitis*, p70, in cells fixed after overall staining of the cytoplasm by CellTracker green. As for ON, *M. hyorhinitis* was mainly membrane-associated and showed a comparable punctuate pattern (Fig. 4D, red signal). As an additional control, there was no detectable p70 by confocal microscopy on BM cyclin-treated cells (data not shown).

DISCUSSION

This paper reports that HepG2 cells obtained from three respected sources show a cryptic infection by *Mycoplasma*, whereas all other cell lines grown in parallel in our laboratory remained negative, even when using the most sensitive biochemical test. Vigorous anti-*Mycoplasma* treatment by BM cyclin surprisingly led to the disappearance of the ~66 kDa band, previously identified as 'ON receptor' by photo-affinity labelling and ligand blotting (5). Conversely, deliberate

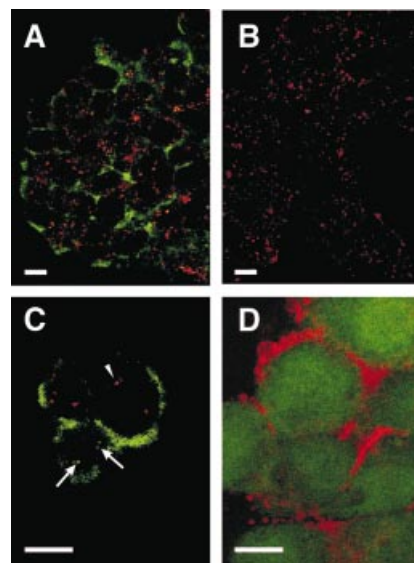


Figure 4. Effect of *M. hyorhinitis* infection on ON capture: confocal microscopy. (A–C) Life cell imaging. Infected (A and C) or BM cyclin-treated HepG2 cells (B) were incubated at 37°C for 2 h with the combination of 300 nM ON-Alexa 488 and 80 nM transferrin-Alexa 568. At (C), arrows point to intracellular ON and the arrowhead to intracellular transferrin. (D) Immunolocalisation of *M. hyorhinitis*. Infected HepG2 cells were incubated with CellTracker green before fixation and permeabilisation, then p70 was immunolocalised with AB3 antibody (red signal). Bars indicate 10 μm .

infection of naive HeLa cells by conditioned medium from contaminated HepG2 cells not only induced the ~66 kDa band but also strongly promoted ON uptake.

The other bands we evidenced by ligand blotting as signature for ON-binding proteins remained unaffected by the infection level. However, based on their full resistance to surface proteolytic digestion and/or lack of association with membrane proteins after carbonate extraction and pelleting, these proteins were regarded as intracellular and/or soluble, respectively, and thus rejected as candidate ON plasma membrane receptors (5). Nevertheless, ligand blotting using ^{125}I -ON has three serious limitations. First, despite its sensitivity, less abundant ON-binding proteins may escape detection. Secondly, binding to proteins essentially by electrostatic interactions is suppressed by suramin and dextran sulfate added together with ^{125}I -ON at the blotting step. Thirdly, it cannot reveal glycolipids or phospholipids that could also interact with ON. Some of these surface constituents could thus take part in ON binding at the plasma membrane and endocytic uptake and we do not know if their abundance is modified upon *Mycoplasma* infection. Thus, as a word of general caution, and in view of the widespread contamination of cultured cells by this bacteria, we would like to call attention to the possibility that some ON-binding proteins reported by other investigators in cultured cell lines could be linked to a cryptic *Mycoplasma* infection.

The sequence of infecting *Mycoplasma* rRNA we obtained was found to be 99.8% identical to that of *M. hyorhinitis*, strain BTS-7. Several strains of *M. hyorhinitis* bear a set of highly variable membrane lipoproteins, but all show one invariant protein referred to as p70, recognised by AB3 monoclonal

antibody (15). We used this antibody as a marker for the level of infection.

The occurrence of the ~66 kDa 'ON receptor', as evidenced by photo-cross-linking and ligand blotting, was closely associated with *M.hyorhinis* infection in HepG2 cells, based on p70 detection; conversely, antibiotic treatment to eradicate *Mycoplasma* abolished both signals. In addition, monitoring the infection on carbonate-extracts membrane proteins by western blotting using AB3 monoclonal antibody, showed that the *Mycoplasma* marker and the ~66 kDa signal by ligand blotting evolved in close parallel to one another.

This correlation did not solve the question as to whether *Mycoplasma* infection would trigger the expression of a eukaryotic gene for p66 as it does for several other genes [e.g. for cytokines, see Zhang *et al.* (16)], or whether p66 and p70 were actually one and the same protein. This identity was confirmed upon immunoprecipitation by AB3 monoclonal antibody against p70 of the ~66 kDa band revealed by ON ligand blotting. The ~66 kDa 'ON receptor' can thus be identified as p70 from *M.hyorhinis*.

As a functional line of evidence, deliberate infection of HeLa cells by *M.hyorhinis* strongly promoted ON intracellular uptake, but did not affect transferrin endocytosis examined in parallel, as shown by biochemical endocytic assays and confocal microscopy. This result is reminiscent of previous reports that bacterial and possibly viral infection selectively promote ON endocytosis (6,8).

Intracellular localisation of *M.hyorhinis* is supported by the partial sensitivity of p70 to pronase digestion on intact cells, as shown either by ligand blotting (5) or by photo-cross-linking labelling after incubation with ON-benzophenone at 37°C, whereas ON-p70 complex formed only at the cell surface after incubation at 4°C was fully sensitive to this treatment (C. Berens, personal communication). This suggests that *M.hyorhinis* can be internalised by infected cells.

We therefore propose that ON first binds to p70 of *M.hyorhinis* adherent to the plasma membrane, after which the complex is phagocytosed, a process referred to as 'piggy-back' endocytosis. This hypothesis would easily explain several non-conventional characteristics of ON endocytosis that we recently reported in the HepG2 cell line and erroneously interpreted as due to an endogenous eukaryotic cell receptor (2). First, ON uptake could be resolved into a high-affinity component (sub-micromolar) that clearly showed saturation and a low-affinity component (supra-micromolar) with very a high binding capacity. In contrast to ON, competition by tRNA suppressed only the high-affinity component, suggesting specific recognition of tRNAs by *M.hyorhinis* p70, possibly due to its secondary structure. This property might be of interest to promote RNA interference in eukaryotic cells. Secondly, the distinct localisation pattern by confocal microscopy between internalised ON and either dextran (a fluid-phase endocytosis tracer) or transferrin (a receptor-mediated endocytosis tracer) would simply reflect the distinct intracellular routing after pinocytosis and phagocytosis. Thirdly, upon analytical subcellular fractionation, most of the cell-associated ¹²⁵I-ON (data not shown) as well as 'ON receptor' (5) were found in the first postnuclear pellet obtained at low centrifugation work. This pellet is usually referred to as the 'M' fraction, because it includes the relatively large-sized mitochondria, but it should also include

M.hyorhinis-containing phagosomes, expected to be of comparable size. Fourthly, the observed bimodal distribution upon density equilibration after a 2 h uptake can now be reinterpreted as comprising a light membrane peak, possibly corresponding to low-affinity sites of the HepG2 cell plasma membrane, and a dense one, corresponding to *Mycoplasma*. In contrast, after a 24 h chase, only the high-density peak remained and was found to be different from lysosomes. Whereas active membrane recycling should favour extensive release of ON internalised upon adsorption to the plasma membrane, only ON within *Mycoplasma*-containing phagosomes would be retained, appearing as a dense peak in the gradient.

Finally, the observation that ON taken at low concentrations does not reach the lysosome would suggest that *M.hyorhinis* survives inside the cell by escaping transfer to this degrading organelle, as it is well known for virulent *M.tuberculosis* strains (17). Phagosomes containing living *Mycobacterium* fail to acquire lysosomal markers. One explanation suggests that a protein from the eukaryotic plasma membrane could remain sequestered in the phagosomal membrane, blocking its fusion with endocytic organelles (18). However, the lipid composition of the phagosomal membrane also changes during transfer towards lysosomes (19). Virulent *Mycobacterium* modifies this composition in such a way that lipids reflecting phagosomal maturation are lacking (20). Some *Mycoplasma* species (e.g. *M.penetrans*) exhibit a phospholipase A activity that could possibly alter phagosomal maturation (21). It is tempting to speculate that *M.hyorhinis* also possesses a lipid-modification activity that would similarly prevent phagosomal maturation and delivery to lysosomes of *M.hyorhinis* and its bound ON.

Surprisingly, the presence of *Mycoplasma*, including *M.hyorhinis*, was reported in several human cancers (22). Whether this infection could be exploited to specifically increase capture of ON with anti-tumoral properties in these cancer cells is speculative, but the possibility should not be neglected for the targeting of cancer therapy involving ON [for a review about antisense cancer therapy, see Jansen and Zangemeister-Wittke (23)].

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