cDNA and genomic cloning and expression of the P48 monocytic differentiation/activation factor, a *Mycoplasma fermentans* gene product

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P48 is a 48 kDa monocytic differentiation/activation factor previously purified from the conditioned medium of the Reh human pre-B cell leukaemia cell line. It induces growth arrest and differentiation of HL-60 human promyelocytic leukaemia cells along the monocytic pathway and the production of the cytokines interleukin 1, tumour necrosis factor- α and interleukin 6 in human monocytes and monocytic cell lines. The cDNA for P48 was cloned from Reh cellular RNA using 3' reverse amplification of cDNA ends. Southern blot probing with P48 cDNA revealed hybridization with DNA from Reh and Molt-4 cells, but not with DNA from human peripheral blood mononuclear cells. Subsequent studies using PCR and Southern analysis revealed P48 sequences in DNA isolated from *Mycoplasma fermentans* but not *M. hominis*, *M. iowae*, *M. synoviae* or *M. lymphophilum*. Although initial studies using *Mycoplasma* culture and hybridization techniques had failed to reveal *Mycoplasma* infection in our Reh and Molt-4 cell lines, subsequent PCR studies using *Mycoplasma* genus-specific rRNA primers revealed *Mycoplasma* sequences in these cell lines. Using the P48 cDNA probe, we isolated a genomic clone from *M. fermentans* DNA which was found to be 98.5% identical with the P48 cDNA clone, and the deduced amino acid sequence agreed with N-terminal microsequencing data for P48 protein purified from

the Reh cell line conditioned medium. The 5' end of the gene has a number of consensus sequences characteristic of prokaryotic genes, and the deduced amino acid sequence has a number of features suggesting that P48 is a lipoprotein. The P48 cDNA was expressed in pMAL in *Escherichia coli*, and the 60 kDa expressed fusion protein was found to react with anti-P48 antibodies on Western blots. This is consistent with a pMAL fusion protein representing the sum of the 42 kDa maltose-binding protein and 18 kDa of P48 recombinant protein, suggesting that native P48 has significant post-translational modification. Consistent with this, Northern blot studies revealed a single 1 kb transcript. The recombinant fusion protein was found to possess anti-proliferative activity against HL-60 cells, and antibodies against recombinant P48 were found to block the biological activity of native P48 isolated from conditioned medium. These studies demonstrate that P48, a molecule with immunomodulatory and haematopoietic differentiation activities, is derived from *M. fermentans* or a closely related species. P48 may be important in the pathophysiology of *Mycoplasma* infections and may be useful in dissecting the mechanisms involved in mammalian haematopoietic cell differentiation, immune function and cytokine biosynthesis.

INTRODUCTION

Haematopoietic cell differentiation is an area of intense scientific investigation, and a number of cytokines and other factors have now been identified that modulate growth, differentiation and the functional activity of haematopoietic and lymphoid cells. Cytokines known to stimulate haematopoietic differentiation include granulocyte and granulocyte/macrophage colony-stimulating factors, tumour necrosis factor (TNF)- α and - β , interferon- γ , interleukin-6 (IL-6), transforming growth factor- β and leukaemia inhibitory factor [1–7]. Other non-cytokine factors known to modulate differentiation *in vitro* include phorbol diesters, DMSO, vitamin A (retinoic acid) and vitamin D₃ [8–11]. Other factors are known to activate leucocytes but have not been shown to induce differentiation; these include a number of microbial products such as lipopolysaccharide (LPS) [12], toxic-shock-syndrome toxin-1 [13], erythrogenic toxins A and C [14], and a variety of poorly characterized *Mycoplasma* products [15–19].

In the process of examining tumour cell conditioned media for cytokines and factors with differentiation activity, several years ago we identified and began characterizing a 48 kDa factor termed P48. The biochemical characteristics and biological activities of this factor, which have been reported previously [20–24], include induction of monocytic differentiation and cytolytic activity in the HL-60 (human promyelocytic leukaemia) cell line, growth inhibition (but not non-specific cytotoxicity) towards a large panel of other human and mouse tumour cell lines, induction of secretion of IL-1 and TNF- α by human peripheral blood monocytes and induction of the accumulation of mRNAs for several cytokines, including IL-1 α and -1 β , IL-6 and TNF- α .

We now report the cDNA cloning, starting with RNA isolated from the Reh (human pre-B cell leukaemia) cell line, of a gene which, when expressed as a pMAL fusion protein, reacts with anti-P48 antibodies [25]. We present further evidence showing that the gene is not detectable in genomic DNA from human peripheral blood mononuclear cells (PBMC), but is strongly

Abbreviations used: CM, conditioned medium; DEPC-water; diethylpyrocarbonate-treated water; IL-6, interleukin-6; IPTG, isopropyl β -D-thiogalactoside; LPS, lipopolysaccharide; MBP, maltose-binding protein; MBP-rP48, MBP-recombinant P48 fusion protein; MF-P48, P48 derived from *M. fermentans*; MF-rP48, recombinant P48 expressed from the *M. fermentans* P48 gene; PBMC, peripheral blood mononuclear cell; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TNF, tumour necrosis factor.

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detected in genomic DNA from *Mycoplasma fermentans*. Although *Mycoplasma* culture and probe hybridization techniques previously had failed to detect *Mycoplasma* in our Reh cell line, a more sensitive PCR method revealed *Mycoplasma* contamination. In addition, using *M. fermentans* genomic DNA we have cloned a gene that is 98.5% identical to the P48 cDNA cloned from Reh cells. The 5' end of this genomic clone contains a number of sequences characteristic of prokaryotic genes, and the deduced amino acid sequence has features characteristic of a lipoprotein. These studies present strong evidence that P48 is a *M. fermentans* gene product that has a number of important immunomodulatory activities which may be important in the pathophysiology of *Mycoplasma* infections. Thus P48 may be useful in dissecting the mechanisms involved in mammalian haematopoietic cell differentiation and cytokine biosynthesis.

EXPERIMENTAL

Reagents

Tissue culture media, antibiotics and Trizol® RNA extraction reagents were obtained from Gibco/BRL (Grand Island, NY, U.S.A.). Reagents for cDNA synthesis were purchased from Promega (Madison, WI, U.S.A.), and PCR reagents were purchased from Gibco and Promega.

Cells and cell lines

Reh (human pre-B cell leukaemia), Molt-4 (human T cell leukaemia), Jurkat (human T cell leukaemia), K562 (human erythroleukaemia), HSB-2 (human T lymphoblast), CEM (human T lymphoblastoid), HL-60 (human promyelocytic leukaemia), THP-1 (human monocytic leukaemia) and 5637 (human bladder carcinoma) cell lines were maintained in RPMI-1640 tissue culture medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. These cell lines have been maintained in our lab for several years and were originally obtained as gifts from a variety of investigators. In addition, for comparison in some experiments we used Molt-4 and Reh cell lines purchased from the American Type Culture Collection (A.T.C.C., Rockville, MD, U.S.A.). PBMCs were prepared by Ficoll-Hypaque sedimentation as described [22]. *Mycoplasma fermentans* (strain PG18) and other *Mycoplasma* cultures were purchased from A.T.C.C.

Oligonucleotide primers

Oligonucleotide primers were synthesized on an Applied Biosystems (Foster City, CA, U.S.A.) Model 391 DNA synthesizer as follows: BH1, forward (40-mer) primer, 5' end of the P48 cDNA sequence (see the Results section) (5' GATGAATTCA-ATAATTCATTCAAAGAGAAAGATATTAGTA 3'); BH2, reverse (35-mer) primer for 3' rapid amplification of cDNA ends (RACE) [26] containing oligo(dT) and an adapter sequence (5' GACTCGAGTCGACATCGAT₁₇ 3'); BH3, reverse (18-mer) primer for 3' RACE containing the adapter sequence (5' GACTCGAGTCGACATCGA 3'); BH35, reverse (21-mer) primer spanning nucleotides 660–680 of the P48 cDNA sequence or nucleotides 744–764 of the MF-P48 (P48 derived from *M. fermentans*) genomic sequence (5' AACATTATTAATAACAGTGTT 3'). *Mycoplasma* genus-specific rRNA primers [27] used in this work were: GP01, forward (22-mer), 5' ACTCCTACGGAGGCAGCAGTA 3'; MGS0, reverse (27-mer), 5' TGCA-CATCTGTCACTCTGTAAACCTC 3'.

RNA extraction and cDNA synthesis

In order to prepare RNA for 3' RACE, the guanidinium isothiocyanate/caesium chloride method was used [28]. Cells (10⁸) were lysed with 4 M guanidinium isothiocyanate buffer, layered on 5.7 M caesium chloride and centrifuged at 110 000 *g*_{av} for 18 h at 18 °C. Supernatants were aspirated and RNA pellets were dissolved in diethylpyrocarbonate-treated water (DEPC-water) followed by two ethanol precipitations. RNA was washed twice in 75% cold ethanol and dissolved in DEPC-water.

Prior to cDNA synthesis, the RNA was incubated at 65 °C for 5 min followed by chilling on ice. First-strand cDNA was synthesized (42 °C, 90 min) in a reaction mixture containing 1 µg of whole cellular Reh RNA, 6 µl of 5× reverse transcriptase (RT) buffer (250 mM Tris/HCl, pH 8.3, 250 mM KCl, 50 mM MgCl₂, 2.5 mM spermidine, 50 mM dithiothreitol), 40 units of RNasin, 1.25 mM dNTPs, 2 µg of primer BH2 (for 3' RACE) or oligo(dT) primer, and 10 units of avian myeloblastosis virus RT, made up to a final volume of 32 µl with DEPC-water.

For RT-PCR and Northern blots, RNA was extracted from cells and *Mycoplasma* using the guanidinium isothiocyanate/acid phenol-chloroform method [24], with all solutions made up in DEPC-water. The extracted RNA was dissolved in 0.2 M sodium acetate (pH 6.0), precipitated with 2.5 vol. of ethanol at –80 °C, washed several times with cold 80% ethanol, dried at room temperature, and dissolved in 1 mM dithiothreitol containing 1 unit/µl placental RNase inhibitor (Promega).

Northern analysis

Procedures were performed according to established methods [28]. Briefly, RNA samples (7.5 µg) were dissolved to a final concentration of 250 µg/ml in 20 mM Mops (pH 8.0), 1 mM EDTA, 50% formamide and 0.4% xylene cyanole/Bromophenol Blue. RNA samples were heated (55 °C, 15 min), cooled rapidly on ice, and then electrophoresed on 1.2% agarose gels in 20 mM Mops (pH 8.0), 1 mM EDTA and 1.1% formaldehyde. Lanes containing an RNA ladder (BRL) and rRNA for size reference were stained with ethidium bromide (0.5 µg/ml) and photographed with a ruler under UV trans-illumination. Gel portions to be transferred were washed twice (30 min each) in DEPC-water and once in 10×SSC, and then transferred (16 h, room temperature) by capillary action on to Biotrans nylon membranes employing a wick and 10×SSC. The blots were rinsed in 4×SSC, baked at 80 °C for 90 min under vacuum, and prehybridized (41 °C, 16 h) in 5×SSC, 5×Denhardt's solution, 40% formamide, 0.5% SDS, 0.1% sodium pyrophosphate and 150 µg/ml denatured herring sperm DNA. Hybridization was carried out at 41 °C for 16 h with ³²P-labelled heat denatured cDNA probe (10⁶ c.p.m./ml; specific radioactivity > 10⁸ c.p.m./µg of DNA) in prehybridization buffer containing 10% dextran sulphate, followed by washing twice (15 min each, room temperature) in 3×SSC and then at 55 °C to a stringency of 0.1×SSC. The blots were then exposed to film at –80 °C and developed as described below.

3' RACE, RT/PCR and PCR

The 3' RACE cDNA reaction mixtures were heated at 99 °C for 10 min and then submitted to 3' RACE [26]. Reaction mixtures contained 5 µl of cDNA reaction product, 1.6 µM each of forward (BH1) and reverse (BH3) primers, 2.5 µl of 10×PCR buffer (100 mM Tris/HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin), 0.2 mM dNTPs and 1 unit of *Taq* DNA polymerase, brought to a final volume of 25 µl with sterile water. PCR

reactions (with DNA) and RT-PCR reactions (with cDNA) were otherwise identical except for different primers as described in the Figure legends. RT-PCR has been described previously [24], and parallel samples consisting of cDNA amplified with β -actin-specific primers were run as controls for RNA integrity and lane loading. The amplification cycle profiles for both 3' RACE and PCR consisted of 25 cycles of denaturation at 94 °C (1 min), annealing at 55 °C (1 min) and extension at 72 °C (1 min). No ramp times were set between temperatures, and following the last cycle a final extension (72 °C, 15 min) was carried out. PCR reactions with *Mycoplasma*-specific primers were run under the same reaction conditions described above with up to 10 mg/ml DNA template. PCR products (10 μ l aliquots) were electrophoresed on 1 % or 1.5 % agarose gels in TBE or TAE buffer [28] followed by staining with ethidium bromide. *Hae*III restriction endonuclease-digested Phi-X 174 replicative-form DNA (Gibco/BRL) was used as DNA size markers.

Cloning and subcloning

The 3'-RACE-generated fragment (see the Results section) was purified by GeneClean (BIO 101, Vista, CA, U.S.A.), ligated into the pCR II vector (Cloning Kit II; Invitrogen, San Diego, CA, U.S.A.) as specified by the manufacturer, and transformed into *Escherichia coli* INV α F'. Alkaline lysed single colony miniprep DNA was submitted to *Eco*RI digestion in order to identify recombinant clones. The P48 gene was excised from one clone (P48 Clone 22) using *Eco*RI, submitted to GeneClean and subcloned into pMAL-C2 (New England Biolabs, Boston, MA, U.S.A.) for expression experiments. Positive clones were identified by colony hybridization [28], and screened for correct orientation by PCR using the 5' (forward) pMAL primer and P48 primer BH35 as reverse primer.

DNA extraction and Southern analysis

Mammalian cell DNA was extracted using the perchlorate/chloroform method [29], and *Mycoplasma* DNA was extracted using the SDS/phenol/proteinase K method [30]. Southern analysis was performed according to established methods [28]. After restriction endonuclease digestion or PCR amplification, DNA samples were electrophoresed on 0.8 % (genomic DNA) or 1.5 % (PCR products) agarose gels, which were then denatured in 0.2 M NaOH/1.5 M NaCl for 45 min followed by neutralizing for 1 h in 500 mM Tris/HCl (pH 7.2) and 1.5 M NaCl. Gels were transferred by capillary action on to Biotrans nylon membranes (ICN, Irvine, CA, U.S.A.) in 6 $\times$ SSC for 16 h, rinsed in 4 $\times$ SSC and baked at 80 °C under vacuum for 90 min. Hybridization was performed for 16 h at 65 °C in 6 $\times$ SSC containing 5 $\times$ Denhardt's solution, 150 μ g/ml sonicated herring sperm DNA, 0.1 % sodium pyrophosphate and 10⁶ c.p.m./ml ³²P-labelled heat-denatured cDNA probe. The probe for the P48 gene was the *Eco*RI-excised fragment of P48 cDNA Clone 22, and for the *Mycoplasma* rRNA gene was a PCR-amplified 0.6 kb fragment excised after agarose-gel electrophoresis. Probes were labelled using random primer methods in the presence of [³²P]dATP and [³²P]dCTP (specific radioactivity 3000 Ci/mol; ICN) to a specific radio-activity of > 10⁸ c.p.m./ μ g of DNA. After hybridization the membranes were washed to a stringency of 0.1 $\times$ SSC at 65 °C, unless otherwise noted in the Figure legends. The films were then exposed to X-AR film (Eastman Kodak, Rochester, NY, U.S.A.) with Lightning Plus intensifying screens (Dupont, Wilmington, DE, U.S.A.) at -80 °C for up to 72 h, and developed.

Genomic cloning of *M. fermentans*

Approx. 16 ng of *Eco*RI-digested *M. fermentans* DNA was ligated with 2–8 ng of *Eco*RI-digested pGEM-7Zf vector (Promega) in a series of ligations. The ligations were transformed into *E. coli* strain DH5- α , and recombinant clones were detected by blue/white screening on LB agar containing 50 μ g/ml ampicillin, 40 μ g/ml 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside (X-Gal) and 0.5 mM isopropyl β -D-thiogalactoside (IPTG). Putative white recombinants were collected on to LB/ampicillin plates, lifted and hybridized with ³²P-labelled P48 cDNA probe [28]. Colonies which gave positive signals on repeated screening were analysed by both Southern analysis of *Eco*RI-digested plasmid DNA and PCR analysis using P48 cDNA probe and primers respectively. Positive clones were further analysed by dideoxy sequencing as described below.

DNA sequencing and computer analysis

Recombinant plasmids were purified by alkaline lysis and poly(ethylene glycol) precipitation [31], and both strands were sequenced by dideoxy chain termination using the Sequenase II Kit (United States Biochemical, Cleveland, OH, U.S.A.). The sequence was deposited in Genbank. Computer analyses of nucleotide sequences were done using the GCG package, and sequence similarities were checked using FAST-A and T-FASTA [32]. Peptide sequences were analysed using Translate and Peptidesort [33].

Purification of P48 from Reh cell conditioned medium (CM), preparation of rabbit anti-P48, and Western blots

Purification of P48 from the CM of the Reh cell line, preparation of monospecific rabbit anti-P48 antisera and SDS/PAGE analysis have been described [20,22,23]. In brief, Reh cells were adapted to grow in RPMI-1640 medium containing 2 % fetal calf serum, and P48 in the CM was then partially purified by 20 % ammonium sulphate precipitation. Final purification was achieved by gradient SDS/PAGE and electroelution. For Western blot analysis, proteins from bacterial cell lysates (equivalent to 200–800 μ l of bacterial cell culture), affinity-purified fusion proteins (approx. 0.3 μ g) or partially purified P48 (1 μ g) were electrophoresed on 0.1 % SDS/9.5 % PAGE Laemmli gels [34], transferred on to 0.2 μ m nitrocellulose (Micon Separation Inc., Westboro, MA, U.S.A.), and blocked with 1 % BSA. Blots were then probed with 1:200-diluted rabbit anti-P48 antiserum or normal rabbit serum, followed by 1:5000-diluted alkaline phosphatase-conjugated goat anti-rabbit Fc-IgG (Promega), as previously described [35]. Filters were developed using the Protoblot AP system (Promega) with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium according to the manufacturer's instructions.

Expression in the pMAL system, purification of maltose-binding protein-recombinant P48 fusion protein (MBP-rP48) and production of mouse antibodies

P48 cDNA Clone 22 was ligated into the pMAL-C2 expression vector, transformed into *E. coli*, grown to exponential phase and induced with 1 mM IPTG, and bacterial lysates were prepared as described [36]. Aliquots (4–8 μ l) of lysates (equivalent to 200 μ l of culture) were analysed by 0.1 % SDS/9.5 % PAGE followed by staining with Coomassie Brilliant Blue R-250 or Western blot analysis. MBP-rP48 was purified by amylose affinity chromatography in the presence of 0.25 % Tween-20 [36], and the purified fusion protein was estimated to be greater than 95 % pure by SDS/PAGE and Coomassie Blue staining. Control pMAL vector

(without insert) was also expressed in *E. coli*, and affinity-purified protein (MBP) was similarly prepared. Balb/c mice were immunized intraperitoneally with 3 µg of MBP-rP48 in complete Freund's adjuvant followed by boosting in incomplete Freund's adjuvant three times, at 10–14-day intervals, followed by bleeding. In some experiments, the MBP-rP48 fusion protein was cleaved with Factor Xa protease as described [36].

Induction of TNF- α in HL-60 cells and blocking with anti-P48 antibodies

This assay has been described previously in detail [24]. In brief, HL-60 cells were split to $(2-3) \times 10^5$ cells/ml and 2 days later washed and plated at 1.5×10^6 cells/ml per well in 24-well (16 mm diameter) tissue culture plates (Costar, Cambridge, MA, U.S.A.). Stimulants added to various wells consisted of 2 µg/ml LPS or 2.5 µg/ml partially purified P48 (20% ammonium sulphate precipitate of Reh 2% FCS CM) [20,21] in the presence or absence of a 1:50 dilution of rabbit anti-P48, mouse anti-MBP-rP48, or control normal mouse or rabbit serum. After 5 h culture, cells were centrifuged at 250 *g* for 7.5 min, and RNA was immediately extracted for RT-PCR studies using the guanidinium isothiocyanate/acid phenol-chloroform method [28]. RNA was analysed for TNF- α mRNA using RT-PCR and TNF- α - or aldolase-specific primers as described [24,37]. We have previously shown that this RT-PCR assay is approximately linear up to at least 25 PCR cycles [24].

Proliferation assay ($[^3\text{H}]$ thymidine incorporation)

This assay has been previously described [20]. In brief, 50 µl of HL-60 human promyelocytic leukaemia cells (2×10^5 /ml) in RPMI tissue culture medium were placed in flat-bottom 96-well microtitre wells in triplicate, and incubated (37 °C, 5% CO₂/95% air) with 50 µl of test material for 3 days, followed by addition of 50 µl (0.5 µCi) of $[^3\text{H}]$ thymidine (64 Ci/mmol; ICN). After further incubation for 6 h, plates were harvested with a Dynatech Minimash 2000 cell harvester (Dynatech Labs, Chantilly, VA, U.S.A.), filters were placed in scintillation vials with aqueous-based scintillation fluid (Ecolume; ICN), and radioactivity was counted in a Beckman scintillation counter (Beckman Instruments, Palo Alto, CA, U.S.A.).

Protein microsequencing

P48 was purified from the conditioned medium of the Reh cell line as described above. For microsequencing, 10–50 µg was submitted to a second SDS/PAGE on a pre-electrophoresed 8 cm $\times$ 10 cm gel, trans-blotted on to an Immobilon membrane (Millipore, Bedford, MA, U.S.A.) and the band identified with Ponceau S and excised. Conventional Edman degradation was performed using an automated Porton/Beckman PI 2090 protein/peptide sequencer (Beckman Instruments, Fullerton, CA, U.S.A.) equipped with an on-line HPLC with a Hewlett Packard diode-array detector as previously described [38].

RESULTS

Cloning and expression of the P48 gene

P48 purified from the CM of the Reh human leukaemia cell line by ammonium sulphate precipitation followed by preparative SDS/PAGE [20] was submitted to amino acid microsequencing, with 23 residues identified. In initial cDNA cloning experiments we screened phage and plasmid cDNA libraries with anti-P48 antibodies and with degenerate oligonucleotide probes deduced

Figure 1 Cloning and RT/PCR analysis of the P48 gene

(A) Agarose gel analysis of 3' RACE and other PCR amplifications. Reh (lane 1) and Molt-4 (lane 2) RNAs were used to prepare cDNA and were then submitted to 3' RACE using BH1, BH2 and BH3 primers as described in the Experimental section. The Reh 3' RACE cDNA was cloned into the pCRII vector, and DNA from one clone (#22) was submitted to PCR amplification using the BH1 and BH3 primer (lane 3), or digested with *EcoRI* to release the insert (lane 4). Note the two bands released from *EcoRI*, indicating an internal *EcoRI* site within the cloned insert. For comparison, DNA from Clone 22 (lane 5) and pCRII vector without insert (lane 6) were also subjected to PCR with SP6 and T7 primers. SP6 and T7 promoters flank the *EcoRI* cloning site in the pCRII vector, 99 and 87 bp away respectively. (B) Agarose gel RT-PCR analysis of P48 expression in various human cell lines. All cell lines were our lab sublines except for those in lanes 5 and 11, which are Molt-4 cells freshly obtained from A.T.C.C. RNA was extracted from cell lines and submitted to RT-PCR using oligo(dT) and P48-specific BH1 and BH35 primers (lanes 1–6) or β -actin-specific primers (lanes 7–12) as a control for lane loading and integrity of RNA. Lanes: 1 and 7, Reh; 2 and 8, Molt-4; 3 and 9, CEM; 4 and 10, 5637 cells; 5 and 11, Molt-4 cells from A.T.C.C.; 6 and 12, THP-1. DNA marker sizes are indicated in kilobase pairs (kbp) at the right margin. The predicted sizes of PCR products are 680 bp for P48 and 548 bp for β -actin.

from the N-terminal amino acid sequence, but were unable to detect any positive clones. Therefore, from the 23-residue N-terminal sequence, mixed oligonucleotide probes (25-mers) corresponding to both ends of the protein sequence were designed and used in preliminary PCR experiments. Using whole cellular RNA from Molt-4 cells, a 69 bp fragment was PCR-amplified, cloned and its DNA sequenced. The DNA sequence obtained corresponded exactly to the N-terminal amino acid sequence, and was presumed to represent a portion of the P48 gene near the 5' end. From this sequence, a 5' forward primer (termed BH1; see the Experimental section) and two additional primers [BH2 and BH3, representing an adapter sequence with and without oligo(dT) respectively] were used in 3' RACE experiments in order to amplify the 3' end of the cDNA. Using total cellular RNA isolated from Reh and Molt-4 cells, cDNA was prepared using the BH2 primer, and then PCR amplification was performed using BH1 and BH3. These experiments (Figure 1) yielded a prominent DNA fragment of 1.0 kb in Reh, Molt-4 and HSB (results not shown) cells, but not in CEM, U937 or HL-60 cells (results not shown). The amplified cDNA fragment from Reh cells was then cloned into the pCRII vector and dideoxy sequenced (Figure 2, top). The resulting cDNA sequence was found to agree exactly with N-terminal amino acid sequence data, and was also found to be unique when searched in Genbank. This cDNA clone (#22) defined a 5' open reading frame of 471 bp and a possible second open reading frame of 200 bp. The clone was found to have an internal *EcoRI* site, since *EcoRI* digestion released two fragments of approx. 830 and 170 bp (Figure 1A, lane 4). Using the BH1 forward primer and the BH35 reverse primer (a P48-specific 3' reverse primer constructed from sequence analysis of Clone 22) we used RT-PCR to analyse expression of P48 in a series of human cell lines (Figure 1B). A 680 bp band of the predicted size was observed in Reh and Molt-

Figure 2 Nucleotide sequence of the 5' regulatory and coding regions of P48 cDNA Clone 22 (CL22) and *M. fermentans* genomic (5MF) clones, (top), and deduced amino acid sequence of the P48 open reading frame (bottom)

Top: the sequence of 5MF was obtained from an *EcoRI*–*HindIII* fragment as described in the Experimental section. The 5MF sequence contains the entire coding region from the ATG to the TAA (stop) codon, which are double-underlined. The P48 cDNA Clone 22 sequence starts at nucleotide +84 as a result of primers used in 3' RACE cloning (see the text). The stars above bases denote differences between the cDNA Clone 22 and 5MF sequences. Consensus sequences for '–35' and '–10' promoter regions (relative to the mRNA start site), the mRNA start site, and Shine–Dalgarno (SD) ribosomal binding regions are underlined. Bottom: the underlined amino acids represent the consensus sequence for prokaryotic polypeptide modification [44], and the arrow shows the proposed signal peptidase II cleavage site of the 24-residue signal peptide. The amino acids in bold correspond to possible glycosylation sites, and the stars indicate divergent sites between the sequences. CM, protein microsequence of P48 purified from Reh conditioned medium.

Figure 3 Southern analysis of the P48 gene

Genomic DNAs (6 µg/lane) from human cell lines and PBMCs were digested with *EcoRI*, electrophoresed on an agarose gel and probed with ³²P-labelled P48 cDNA Clone 22. Lanes: 1, PBMCs; 2, CEM cell line; 3, Molt-4 cell line; 4, Reh cell line. Cell lines were from stocks in our laboratory. DNA marker sizes in kbp are at the right margin.

4 cells but not in CEM, HL-60, THP-1, 5637 or Molt-4 cells obtained fresh from A.T.C.C. Control RT-PCR amplifications using β -actin-specific primers revealed equivalent sample integrity and lane loading.

Southern analysis with cloned P48 cDNA reveals hybridization with genomic DNA from Reh and Molt cell lines but not human PBMCs

In order to characterize further the putative P48 cDNA Clone 22, Southern analysis was performed using DNA extracted from human PBMCs and Reh and Molt-4 cell lines. *EcoRI* digests were electrophoresed on agarose gels, blotted on to nylon membranes and probed with ³²P-labelled P48 cDNA (Clone 22) (Figure 3). Whereas strongly hybridizing bands were observed in Reh and Molt-4 digested DNA, no hybridizing bands were observed in PBMC DNA. This suggested that the P48 cDNA sequence was derived from an exogenous agent that had infected the Reh and Molt-4 cell lines.

PCR analysis reveals P48 gene sequences in *Mycoplasma* species

To test the hypothesis that P48 might represent a *Mycoplasma* gene product that resulted from *Mycoplasma* infection of the Reh and Molt-4 cell lines existing in our lab, we used PCR (Figure 4) to analyse DNA from various *Mycoplasma* species and cell lines using the P48 specific primers BH1 and BH35 and *Mycoplasma* genus-specific rRNA primers [27]. The use of P48-specific primers resulted in amplification of a prominent band at 0.68 kb in *Mycoplasma fermentans* and Reh cells, but not in *M. hominis*, *M. iowae*, *M. synoviae* or *M. lipophilum* (results not shown). When these PCR products were blotted on to nylon membranes and probed with ³²P-labelled P48 cDNA Clone 22 probe, strong hybridization was observed for Reh and *M. fermentans* but not for the others. In contrast, all *Mycoplasma* species tested, as well as Reh cells and Molt-4 cells (results not shown), revealed a strong 0.6 kb band when submitted to PCR amplification with *Mycoplasma* genus-specific rRNA primers. Several other cell lines tested with these *Mycoplasma* rRNA primers, including CEM, HL-60, THP-1, U937 and Jurkat, revealed no amplified 0.6 kb band. Although *Mycoplasma* culture

Figure 4 RT/PCR analysis of P48 expression in *Mycoplasma* species

(A) Ethidium bromide-stained RT-PCR products analysed on a 1.5% agarose gel. Lanes 1–5 contain RT-PCR products obtained using *Mycoplasma* genus-specific rRNA primers [27], and lanes 6–10 contain products obtained using the P48-specific primers BH1 and BH35. Lanes: 1 and 6, *M. hominis*; 2 and 7, *M. iowae*; 3 and 8, *M. synoviae*; 4 and 9, *M. fermentans*; 5 and 10, Reh cell line. DNA marker sizes in kbp are indicated at the right margin. (B) Southern analysis of the gel in (A) hybridized with ^{32}P -labelled P48 cDNA Clone 22 probe. The lane assignments are the same as in (A).

and a commercially available probe hybridization technique had previously failed to detect *Mycoplasma* in these Reh and Molt-4 cell lines (see the Discussion section), this more sensitive PCR technique clearly demonstrated *Mycoplasma* sequences in these cell lines.

Isolation and analysis of *M. fermentans* genomic clone encoding P48

A genomic DNA library was prepared from *M. fermentans* DNA in the pGEM-7Zf vector and screened by colony hybridization with ^{32}P -labelled P48 cDNA, and positive clones were confirmed using PCR, restriction endonuclease analysis and Southern blot hybridization. One *M. fermentans* genomic clone (5MF) and the P48 cDNA Clone 22 (cloned from Reh cellular RNA) were submitted to complete DNA sequencing by the dideoxy chain termination method, and other clones were partially sequenced for confirmation. In Figure 2 (top) the sequence of the 5' regulatory and coding regions of the *M. fermentans* P48 genomic clone is compared with the sequence of the P48 cDNA clone. Sequence analysis revealed 98.5% identity between the cDNA and genomic clones, thus confirming that P48 is a *M. fermentans* gene product. On searching the Genbank database some similarities were noted, most significantly with the *M. arginini* gene for the Ag243-5 protein (60% over 208 bp), the *M. mycoides* SRPM54 gene (57% over 220 bp), the *M. capricolum* phosphocarrier protein (ptsH) gene (51% over 880 bp) and the *M. hyorhinis* p115 gene (51% over 960 bp).

The cDNA sequence revealed a 471 bp open reading frame terminated by a series of non-UGA stop codons; UGA is known to be translated as a tryptophan codon in *Mycoplasma* [39]. The genomic sequence includes an additional 84 bp of open reading frame at the 5' end, beginning with an ATG codon, suggesting the presence of a signal peptide in the precursor protein. As discussed above, the cDNA was cloned by 3' RACE using sequence information deduced from N-terminal protein microsequencing of P48 protein purified from Reh cell line CM. In the 3' RACE we used a 5' forward primer (BH1) which began with a position corresponding to microsequencing amino acid residue

no. 5, which further truncated the 5' region of the cDNA isolated. Thus the 5' sequence corresponding to the putative signal peptide plus four additional residues are not present in our P48 cDNA clone. The sequences of both the cDNA and genomic clones reveal a high percentage (69%) of A and T, which is characteristic of *Mycoplasma* genes [40]. Also characteristic of prokaryotic genes is the absence of introns in the genomic sequence.

A number of other interesting features are apparent at the 5' end of the genomic sequence. First, in the promoter region there are matches to the consensus sequences for the –35 and –10 (Pribnow box) regions where RNA polymerase binds. In the P48 genomic clone the –35 sequence is TTGAGA, with a G substituted for C in the fifth position in approximately half of the promoters analysed [41]. The Pribnow box region exactly matches the TATAAT consensus sequence. Approx. 5–9 bp downstream from the Pribnow box, prokaryotic mRNAs characteristically start at a purine which is frequently preceded by a C [42], suggesting that the P48 mRNA start site is at nucleotide –28. Approx. 15 bp further downstream there is a Shine–Dalgarno ribosomal binding site (TAAGGAGA), and 5 bp further downstream is the ATG translation start codon.

Figure 2 (bottom) presents the deduced amino acid sequence from the P48 cDNA and genomic DNA sequences, as well as N-terminal microsequencing data of P48 purified from Reh CM. There is agreement between the MF-P48 deduced amino acid sequence and protein microsequencing data in all except for one residue. The N-terminal amino acid identified by protein microsequencing (Ala) did not agree with the corresponding amino acid deduced from the MF-P48 DNA sequence (Cys), but cysteine residues are known to be difficult to detect by Edman degradation microsequencing [43]. Following the ATG translational start codon in the P48 genomic clone is an open reading frame coding for a 185-amino-acid protein. This sequence begins with a number of features characteristic of a signal sequence for a lipoprotein [44]. These features include a positively charged N-terminus (four out of the first six amino acids in the P48 sequence are lysines), followed by a hydrophobic region (in P48 there are 18 hydrophobic amino acids after the lysine at residue +6) and a potential cleavage site at cysteine. The putative signal sequence for P48 would be 24 amino acids long. The 7-amino-acid sequence (VAVSCGN) conforms to the consensus sequence of the cleavage site for prokaryotic signal peptidase II, which is known to cleave precursor polypeptides upstream of a cysteine residue to which a lipid moiety is then attached [44,45]. For example, the 16 kDa lipoprotein of *Sirpulina hyodysenteriae* has been shown to have a similar signal sequence (FAVSCNN) in this region [46]. Amino acid residues +26 and +27 (Gly and Asn) predict a β -turn secondary structure immediately following the cysteine residue, which appears to be important in prolipoprotein modification [44,45]. The MF-P48 gene would encode a polypeptide of predicted molecular mass 20406 Da for the 185-amino-acid precursor polypeptide, and 18123 Da for the 161-amino-acid mature polypeptide lacking the signal sequence.

A hydrophobicity plot was constructed from the deduced amino acid sequence, according to the algorithm of Kyte and Doolittle [47]. This analysis (results not shown) revealed several areas of hydrophobicity, with the N-terminal region comprising residues 7–11 (within the signal peptide) being the most hydrophobic.

Northern analysis

In order to compare further nucleic acids from Reh cells and *M. fermentans*, as well as to gain initial information about P48

Figure 5 Northern analysis of RNA from Reh and Molt-4 cell lines and *Mycoplasma fermentans*, probed with ^{32}P -labelled P48 cDNA Clone 22

Lanes (7.5 μg of RNA each): 1, *M. fermentans*; 2 and 5, CEM cell line; 3 and 6, Reh cell line; 4 and 7, Molt-4 cell line. Lanes 1–4 are from one blot exposed for 2.5 h; lanes 5–7 represent lanes 2–4 from the same blot, exposed for 16 h in order to demonstrate the hybridization signal from Molt-4 RNA. RNA markers in kb are shown at the right margin, and rRNA markers are shown at the left margin in Svedberg units (S).

Figure 6 Expression of P48 in the pMAL expression system

P48 cDNA Clone 22 was cloned into the pMAL-C2 vector and expressed in *E. coli* followed by 0.1% SDS/9.5% PAGE and Western blot analysis of bacterial cell lysates in comparison with cell line lysates. Lanes 1–3 and 7–10 were probed with rabbit anti-P48 antiserum, and lanes 4–6 with normal rabbit serum (control). Samples in lanes are: 1 and 4, pMAL-C2 containing P48 cDNA Clone 22, transformed into *E. coli* and induced with IPTG; 2 and 5, pMAL-C2 vector alone (without insert) transformed into *E. coli* and induced with IPTG; 3 and 6, partially purified P48 (20% ammonium sulphate precipitate of conditioned medium from the Reh cell line) [20]; 7, Jurkat cells; 8, K562 cells; 9, Reh cells; 10, Molt-4 cells. Samples in each lane represent 200 μl of bacterial culture or 1000 cell line cells. Protein molecular mass markers in kDa are indicated at the right margin.

transcript(s), Northern blots were performed using total cellular RNA extracted from *Mycoplasma* and human cell lines, and probed with ^{32}P -labelled P48 cDNA Clone 22 (Figure 5). A single 1.0 kb transcript was observed in RNA from *M. fermentans*, Reh and Molt-4 cells, consistent with the size of the P48 cDNA isolated.

Expression of P48 using the pMAL vector

The P48 cDNA Clone 22 was digested with *EcoRI*, and the 833 bp fragment was cloned into the *EcoRI* site of the MBP fusion vector pMAL-C2 and then transformed into *E. coli* and induced with IPTG. Bacterial cell lysates were then submitted to SDS/PAGE and Western blot analysis using rabbit anti-P48 antiserum (Figure 6). pMAL clones with the insert in the correct orientation expressed an anti-P48 immunoreactive protein of 60 kDa (lane 1), in contrast to pMAL-C2 vector alone (without insert), which had no anti-P48 reactivity. The MBP-rP48 fusion protein was purified by amylose affinity chromatography and then cleaved with Factor Xa protease, releasing an 18 kDa

Table 1 Inhibition of HL-60 cell proliferation (^3H)thymidine incorporation by MBP-rP48

HL-60 cells were cultured as described in the Experimental section for 3 days in the presence of various additions diluted in RPMI tissue culture medium, followed by assay of ^3H thymidine incorporation over 6 h. Results are from a representative experiment of three separate experiments, and are means $\pm$ ranges of triplicate determinations.

Addition	^3H Thymidine incorporation (c.p.m.)	Inhibition (%)
Medium	109258 $\pm$ 4168	0
MBP (1 $\mu\text{g}/\text{ml}$)	139110 $\pm$ 7557	0
MBP (2 $\mu\text{g}/\text{ml}$)	105906 $\pm$ 6567	3
MBP-rP48 (1 $\mu\text{g}/\text{ml}$)	58616 $\pm$ 3182	46
MBP-rP48 (2 $\mu\text{g}/\text{ml}$)	2701 $\pm$ 1326	98

protein which reacted with anti-P48 (results not shown). These data are consistent with a pMAL fusion protein representing the sum of the 42 kDa MBP and 18 kDa of P48 recombinant protein, suggesting that native P48 from Reh CM contains significant post-translational modification. In parallel experiments, cell lysates from Molt-4, Reh and HSB cells (results not shown) revealed a 48 kDa immunoreactive protein (native P48) which we have previously observed [23] and is now known to be due to *Mycoplasma* contamination; Jurkat, K562 and CEM cells (results not shown) did not contain this protein.

MBP-rP48 fusion protein induces growth arrest in HL-60 cells

We have previously shown that P48 purified from Reh cell line CM induces growth arrest and differentiation in a series of human and mouse tumour cell lines [20,21]. In order to demonstrate that the cloned P48 cDNA was authentic, we tested amylose-affinity-chromatography-purified MBP-rP48 fusion protein and control MBP for similar biological activity (Table 1). We found that MBP-rP48 fusion protein was strikingly anti-proliferative towards HL-60 (human promyelocytic leukaemia) cells in a 3-day assay of ^3H thymidine incorporation. In contrast, control MBP alone at the same concentrations induced no appreciable inhibition of cell proliferation. MBP and MBP-rP48 were not non-specifically toxic to cells, as assessed by Trypan Blue dye exclusion (results not shown).

TNF- α -inducing activity of P48 from Reh CM is blocked by antibodies to recombinant P48

We have previously shown that P48 purified from Reh CM induces mRNA accumulation for several inflammatory cytokines [24]. Since MBP-rP48 was purified from *E. coli* lysates, we were concerned about trace amounts of contaminating LPS which might interfere with experiments testing the activity of the fusion protein in inducing inflammatory cytokine mRNAs [12]. In order to further demonstrate that the P48 cDNA clone encodes P48 protein, the purified recombinant MBP-rP48 fusion protein was used to prepare mouse antiserum, which was then used to block the biological activity of 'native' P48 partially purified from Reh CM. As shown in Figure 7, both partially purified P48 and control LPS induced accumulation of TNF- α mRNA, as measured by a previously reported RT-PCR assay which we have shown to be approximately linear for up to at least 25 cycles of PCR [24]. This TNF- α -inducing activity was blocked by a 1:50 dilution of rabbit anti-('native' P48) or mouse anti-MBP-rP48, whereas there was no appreciable effect of the same dilution of normal rabbit serum or normal mouse serum.

Figure 7 Blocking of P48-induced TNF- α mRNA by mouse anti-MBP-rP48

HL-60 cells were incubated at 1.5×10^6 cells/ml for 5 h with LPS or partially purified P48 from Reh CM with and without antisera or control normal sera. Cellular RNAs were then analysed for TNF- α mRNA (lanes 1–7) or aldolase mRNA (control; lanes 8–14) using RT-PCR and agarose gel electrophoresis with ethidium bromide staining [24]. Lanes: 1 and 8, no treatment; 2 and 9, 2 μ g/ml LPS; 3 and 10, 2.5 μ g/ml P48; 4 and 11, 2.5 μ g/ml P48 and 1:50-diluted normal mouse serum; 5 and 12, 2.5 μ g/ml P48 and 1:50 normal rabbit serum; 6 and 13, 2.5 μ g/ml P48 and 1:50 mouse anti-MBP-rP48; 7 and 14, 2.5 μ g/ml P48 and 1:50 rabbit anti-P48. DNA marker sizes in kbp are indicated at the right margin. Rabbit anti-P48 was prepared by immunizing rabbits with 'native' P48 purified from Reh CM [20], and mouse anti-MBP-rP48 was prepared by immunizing mice with purified recombinant MBP-rP48 fusion protein.

DISCUSSION

We have extensively characterized a 48 kDa protein termed P48 which possesses a number of monocytic differentiation and activation properties, including induction of differentiation of HL-60 human promyelocytic leukaemia cells and production of inflammatory cytokines and their mRNAs by human monocytes [20–24]. P48 was originally identified in the CM of the Reh (human pre-B cell leukaemia) cell line and a few other human leukaemic cell lines, and later shown to be present as a membrane-associated form [23].

The cDNA cloning of P48 was carried out using N-terminal amino acid microsequencing data from purified P48 protein, with PCR and 3' RACE amplification starting with cellular RNA derived from Reh cells. When the cDNA was used to probe Southern blots of genomic DNA from human PBMCs and tumour cell lines, hybridizing bands were detected in Reh and Molt-4 cell lines but not in PBMCs or CEM cells. This suggested that the P48 cDNA represented an infectious agent that had infected our Reh and Molt-4 cell lines.

As part of our initial studies (R. E. Hall, unpublished work), we had previously examined P48-producing cell lines for the presence of *Mycoplasma* using *Mycoplasma* culture and commercially available probe-hybridization techniques, and found the cell lines to be negative. In addition, we examined several *Mycoplasma*-infected cell lines and found no detectable P48 differentiation-inducing activity.

Using PCR, we subsequently examined DNA from several *Mycoplasma* species and detected P48-specific sequences in *M. fermentans* but not other *Mycoplasma* species examined. Using a sensitive PCR technique with *Mycoplasma* rRNA gene primers reported by others [27], we then detected *Mycoplasma* sequences in our Reh and Molt-4 cell lines but not in other cell lines in our lab. Finally, we report the cloning of a gene from the genomic DNA of *M. fermentans* which was 98.5% identical with the P48 cDNA cloned from Reh cells. Taken together, these data strongly support the conclusion that P48 represents an important new immunomodulatory molecule derived from *M. fermentans*.

When the cDNA clone was expressed as a MBP-rP48 fusion protein (60 kDa) in pMAL-transformed *E. coli*, it reacted strongly with anti-P48 antibodies on Western blots. Cleavage of the MBP fusion protein with Factor Xa protease released an 18 kDa P48 polypeptide component (results not shown). Amylose-affinity-chromatography-purified MBP-rP48 fusion protein was found to be biologically active and was strikingly anti-proliferative (but not non-specifically cytotoxic) towards HL-60 human promyelocytic leukaemia cells. In addition, antibodies to recombinant P48 were demonstrated to block the TNF- α -mRNA-inducing activity of 'native' P48 from Reh cell line CM.

Analysis of the deduced amino acid sequence revealed a typical signal sequence of a lipoprotein and a characteristic signal peptidase II cleavage site [44,45]. Sequence analysis further predicts a 20.4 kDa precursor polypeptide from which a 2.3 kDa signal peptide is cleaved to produce a 18.1 kDa mature P48 polypeptide, which is in agreement with the molecular mass of the expressed recombinant protein. As discussed above, since the molecular mass of P48 isolated from Reh cell line CM is 48 kDa, this would imply that CM P48 contains significant post-translational modification. The DNA sequence and deduced amino acid analysis suggest that this is, at least in part, due to lipidation, which is consistent with the hydrophobic nature of Reh-cell-line-associated P48 [20,23]. However, it may be that the final secreted molecule has additional post-translational modifications such as glycosylation, acetylation or other additions, or that a multimeric protein is secreted by cells, and this is currently under investigation in our laboratory. Of interest is that *Mycoplasma* proteins are known to be both lipidated [48] and glycosylated [49]. A number of laboratories have reported that several *Mycoplasma* membrane proteins exhibit immunomodulatory activities towards human leucocytes, including induction of the cytokines IL-1, TNF α and IL-6 [16–19], expression of MHC antigens [50], stimulation of oxidative radicals [51] and macrophage activation [52]. To date, very few of these molecules have been purified or cloned. The *M. arthritidis* superantigen, a T cell mitogen with cytokine-inducing activities, has recently been purified and partially sequenced, but shows no similarity to P48 [53]. In addition, P48 possesses strong haematopoietic differentiation activity, which has not been reported in other *Mycoplasma* membrane preparations. It may be that some of the immunomodulatory activities in partially purified *Mycoplasma* membrane preparations reported by other investigators are due to P48 cross-reacting material.

It is likely that cytokine induction and other immunological activities of these and other *Mycoplasma* molecules are important in the pathogenesis of *Mycoplasma* infections. Our studies have shown that *M. fermentans*, or a closely related species, appears to be the source of the P48 gene and protein. Of interest is that substantial data have been published implicating *M. fermentans* in the morbidity, and also possibly in the pathogenesis, of AIDS as well as other human disease states [54–56]. Further characterization of P48 and other immunomodulatory *Mycoplasma* proteins and their genes should contribute to a better understanding of the mechanisms controlling cytokine expression in mammalian cells, haematopoietic differentiation and the pathophysiology of *Mycoplasma* infections.

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