Novel immunogenic antigen homologous to hyaluronidase in meningioma

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By screening a meningioma expression library with autologous serum we identified four cDNA clones representing a novel gene with striking homology to Caenorhabditis elegans hyaluronidase as indicated by BLASTP analysis. In humans hyaluronidase has been implicated in cancer development and three human genes are known to encode proteins with hyaluronidase activity. None of the human genes, however, showed any homology at the nucleotide or amino acid sequence level to the newly isolated antigen we termed meningioma expressed antigen 5 (MGEA5). Somatic cell hybrid mapping and fluorescence in situ hybridization mapped the gene for MGEA5 to chromosomal band 10q24.1–q24.3. Reverse transcription (RT)–PCR and northern blot hybridization revealed expression of the gene encoding MGEA5 in several meningioma and additional human tissues. Expression analysis also indicated an alternative splicing event giving rise to a shorter and altered transcript termed MGEA5s. The expression of MGEA5 and MGEA5s as fusion proteins revealed an approximate molecular weight of 92 and 54 kDa, respectively. Using heterologous sera we found antibodies against MGEA5s in five out of 23 meningioma patients, whereas no immune reaction was detected in 12 control sera from healthy individuals. Confirmation of hyaluronidase activity was independently achieved by turbidometric analysis and a gel matrix assay. A model for involvement of the novel hyaluronidase gene in meningioma development is proposed.

INTRODUCTION

Meningioma that arise from the meningeal coverings of the brain and spinal cord account for ~20% of adult brain tumours. Meningiomas are generally slow growing and well-circumscribed (MI), corresponding to grade I in the World Health Organization (WHO) classification (1). Atypical meningioma, which are characterized by increased cellularity and increased mitotic activity, account for ~8% of this tumour type (WHO grade II, MII). Anaplastic meningioma account for ~2% of this tumour type (WHO grade III, MIII). MII and MIII are characterized by an increased risk of local tumour recurrence even after complete resection (1). Current clinical and histopathological parameters do not allow prediction of malignant progression of meningioma.

Meningiomas have been extensively studied by cytogenetic and molecular genetic means. Loss of chromosome 22 has been identified as a consistent chromosomal alteration in these tumours (2–4). Loss of heterozygosity (LOH) at the NF2 locus that maps at 22q12.2 and mutational inactivation of the second allele has been found in up to 60% of all meningiomas and has been suggested as an early event in tumour progression (5). Further tumour suppressor genes which might be involved in the progression of meningioma have been implicated on chromosomes 1p, 10q and 14 (6). However, in summary, only very limited data are available on molecular lesions associated with the initiation and progression of meningioma.

Most recently, we reported cDNA cloning of a predicted coiled-coil protein, immunogenic in meningioma. The study provided the first evidence that immunogenic antigens are expressed in benign tumours such as meningioma. Here we report cloning of a novel human gene encoding a protein with striking homology to Caenorhabditis elegans hyaluronidase. Hyaluronidase has been implicated in the development and metastatic spread of several human tumours (7,8). As of yet, however, there has been no evidence for an involvement of hyaluronidase in meningioma.

RESULTS

We established an expression library of a cytogenetically normal meningioma as previously described (9). Escherichia coli host cells were transfected with the expression library, protein expression was induced and expressed polypeptides were immobilized on nitrocellulose membranes and incubated with the autologous patient serum. A secondary antibody directed towards the human IgG constant region and conjugated to alkaline phosphatase detected antigen–antibody complexes which indicated immunogenic clones. Positive clones were subjected to a second round of screening to verify specificity and to ensure

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monoclonal enrichment. An example of a secondary screening is shown in Figure 1. Confirmed phage clones were excised \textit{in vivo}, and phagemids with the insert of interest were propagated in \textit{E.coli} XLOR cells. Sequencing revealed four clones (33-20, 45-26, 23-13 and 8-5) which share nearly identical sequences. The corresponding antigen was termed meningioma expressed antigen 5 (MGEA5), and the clones are referred to as \textit{mgea5} sequences. A schematic overview of clone lengths and homology is given in Figure 2.

The consensus sequence of the four clones consists of 5147 bp and contains an open reading frame (ORF) spanning bases 396–3143, resulting in a predicted protein of 916 amino acids. With the exception of clone 33-20, the clones share the same 3’-end with a polyadenylation signal at bases 5131–5136.
followed by adenine stretches. The different insert lengths of mgea5 clones 45-26, 23-13 and 8-5 reflect different efficiencies in mRNA reverse transcription during construction of the library, leading to varying 5′-ends of the sequences. Clone 45-26 contains the complete coding sequence of mgea5. Clone 33-20, which begins at position 1 of the consensus sequence, showed a different 3′-end starting at nt 2381 and resulting in a shorter ORF of 677 amino acids. This clone, which is termed mgea5s, most likely represents a splice variant using an alternative polyadenylation signal at nt 3251. An alignment of the consensus sequence of mgea5 and its splice variant (mgea5s) is depicted in Figure 3. The mgea5 sequences have been deposited in the GenBank database under accession nos gb036144 and gb036145.

Sequence comparison with public databases using the BLASTN algorithm showed no significant homology to any known gene. On the protein level, however, BLASTP and BEAUTY revealed a significant homology to the gene 'similar to hyaluronoglucosaminidase' in *C. elegans* (gi861300), with 31.2%
identical and 45.8% conservatively substituted residues. Figure 4 shows amino acid sequence alignment of the MGEA5 protein and the protein sequence of the gene product ‘similar to hyaluronoglucosaminidase’ carried out with the multiple alignment program Clustal W 1.5. A further database search revealed three different human proteins that possess the activity of a hyaluronic acid (HA) degrading enzyme. The human proteins include a lysosomal variant (HYAL2) encoded by a gene on human chromosome 3, an enzyme (HYAL1) isolated from plasma and the sperm expressed gene PH-20 (10–12). The genes for the latter proteins have not been mapped according to GenBank. Homologies between the human variants, hyaluronidase enzymes of other species and the mgea5 sequence were utilized to construct a phylogenetic tree using the Phylogeny Inference Package (PHYLIP v.3.5) as described in Materials and Methods. The results demonstrate that the mgea5 gene belongs to an evolutionarily different group in comparison with the known human genes with hyaluronidase activity (Fig. 5).

To further demonstrate that the mgea5 gene can be distinguished from known human hyaluronoglucosaminidases, we...

![Figure 3. Continued](https://academic.oup.com/hmg/article-abstract/7/12/1859/579901)
determined its chromosomal location. The gene for MGEA5 was mapped to chromosomal band 10q24.1–q24.3 by means of somatic cell hybrid mapping and fluorescence in situ hybridization (Fig. 6).

Expression of mgea5 was analyzed by reverse transcription PCR (RT–PCR) and northern blotting using a 482 bp in vitro transcribed RNA probe that included nt 1633–2102 of the mgea5 consensus sequence. Twenty-two meningioma samples and nine normal tissues, including brain, cranial skin, skeletal muscle, stomach, liver, placenta and heart, were analysed for mgea5 expression by RT–PCR (Fig. 7). The presence of mgea5 transcripts was demonstrated in all cases. Northern blot hybridization revealed mgea5 messages of 5300 and 3500 bp. These results are consistent with the sizes of mgea5 clones 45-26 and 33-20 isolated from the meningioma cDNA library (Fig. 8 A). Following an extended exposure time, a third smaller mRNA of ∼1450 bp was detected in all tissues analysed. In total, 18 meningiomas of different histological grades, eight normal tissues on a commercially available blot (Clontech) and four fetal tissues, including brain, lung, liver and kidney, were analysed by northern blot hybridization using a mgea5-specific RNA probe. Meningiomas did not show any significant differences in

Figure 3. Continued
expression levels of mgea5. In normal tissue samples the expression levels of mgea5 varied, with the strongest signals found in brain, skeletal muscle and pancreas (Fig. 8). In all four human fetal tissues northern analysis revealed a strong expression of the largest message (Fig. 8C).

To further analyse the immune response against mgea5, clones 45-26 and 33-20 were expressed as fusion proteins and analysed by western blot hybridization. In brief, mgea5 and mgea5s sequences were excised in vivo using helper phages, transfected into E.coli XLOLR host cells, and expression was induced by IPTG. Cell extracts were separated by SDS–PAGE, electroblotted and incubated with autologous patient serum. As shown in Figure 9, western blot analysis revealed two bands of ≈73 and ≈100 kDa. The molecular weight of the β-galactosidase fusion portion is 42 kDa. The molecular weight of the polypeptide encoded by the first 96 bp of clone 45-26 is 3.5 kDa and the molecular weight of the polypeptide encoded by the 395 bp up to the first AUG in a Kozak consensus sequence in clone 33-20 is 14.5 kDa. Taking these data into account, MGEA5 and MGEA5s have molecular weights of ≈92 and ≈54 kDa, respectively.

To clarify how frequent an immune response against MGEA5 occurs in meningioma patients, sera from 23 patients were analysed by western blot analysis. Sera from 12 healthy persons were used as controls. As shown in Figure 10A and B, we detected antibodies against MGEA5s in five of 23 meningioma patient sera (22%) but in none of the control sera. There was no correlation between immune response and tumour grade. Interestingly, one patient with a malignant meningioma which recurred three times in a period of 11 months did not possess MGEA5 antibodies at the time of the first and second surgeries. In contrast, the sera collected before the third and fourth surgeries were positive for MGEA5s antibodies, as shown by western blot studies (Fig. 10C).

As for the function of MGEA5, we performed two independent assays to evaluate its potential to degrade hyaluronic acid, which is the substrate of hyaluronidases. In a gel matrix assay, we demonstrated depolymerization of hyaluronic acid co-polymerized within a native polyacrylamide gel. In detail, extracts of MGEA5-expressing bacterial cells were run on the matrix gel along with bacterial cells containing the expression vector without an insert. Purified hyaluronidase from bovine sperm served as a positive control. After overnight incubation in hyaluronidase buffer at 37°C, hyaluronan degrading activity of MGEA5 and the bovine hyaluronidase was visualized by cleared areas on the alcian blue stained gel (Fig. 11 A). Alternatively, in a microtitre plate turbidometric assay, hyaluronidase activity was confirmed and quantitatively evaluated. In detail, hyaluronic acid was combined with bovine serum albumin (BSA) in agarose and applied to a microtitre plate at different pHs. The agarose plugs were overlaid with native extracts...
of cells expressing MGEA5 or MGEA5s. Extracts of non-recombinant cells served as a negative control. After incubation at 37°C cell extracts were removed and substituted by acetic acid. Residual hyaluronan was precipitated with BSA and measured in a microplate reader. MGEA5s-expressing extracts were found to degrade up to 32% of the hyaluronan at pH 7.0 in a dose-dependent manner, whereas MGEA5 depolymerizes ~50% of the substrate under the same conditions (Fig. 11B).

**DISCUSSION**

Screening a meningioma expression library with autologous serum identified four cDNA clones with high homology to *C. elegans* hyaluronidase. Sequencing and chromosomal mapping indicate that the meningioma-expressed antigen is likely to represent a novel member of the human hyaluronidase encoding genes.

RT–PCR and northern blot hybridization revealed expression of the gene for MGEA5 in several meningioma and additional human tissues. Since the first AUG codon is in a Kozak consensus sequence (13) at position 396, the ORF consists of 2748 nt encoding a putative MGEA5 protein of 916 amino acids. Notably, one of the four mgea5 clones displayed a different nucleotide sequence at the 3′-end, most likely reflecting an alternative splicing event. Northern blot hybridizations using *in vitro* transcribed RNA probes specific for mgea5 identified three mRNA molecules of 5300, 3500 and 1450 bp in all analysed RNA samples. These results indicate that clones 33-20 and 45-26 contain full-length cDNA inserts. In addition, the northern blot results are consistent with the idea of an alternative splicing event for mgea5s. The shorter transcript encodes an altered amino acid sequence and is characterized by a different 3′-untranslated region. These changes are likely to influence protein function and expression control. As recently shown, the 3′-untranslated region of an mRNA may impact on its stability, its cellular localization and its translational and transcriptional regulation (14,15).

Sequence comparison of the predicted amino acid sequence of MGEA5 revealed a striking homology to a *C. elegans* protein designated ‘similar to hyaluronoglucosaminidase’ (16). The *C. elegans* protein possesses strong similarity to *Clostridium perfringens* hyaluronidase, an enzyme that is part of *C. perfringens* endotoxin (17). Hyaluronidase (HAse) is characterized by the ability to depolymerase hyaluronic acid (HA), which is a major polysaccharide component of the extracellular matrix. HAse represents a group of evolutionarily conserved enzymes that have been found in the venom of snakes and insects and in mammalian tissues. Our phylogenetic analysis of members of the HAse family suggest that MGEA5 together with the *C. elegans* and *C. perfringens* enzymes developed from a different evolutionary branch than the human proteins HYAL1, HYAL2 and PH-20. Both the enzymes with HAse activity and their substrate HA have been suggested to play a pivotal role in tumour development. HA belongs to the family of glycosaminoglycans and is typically found in the connective tissues of vertebrates (18). An elevated level of HA was found in several tumour cells, including melanoma, colon carcinoma, glioblastoma cell lines and breast carcinoma (19,20). It has been suggested that tumour cells expressing high amounts of HA are protected against immune cell attack (21). Degradation products of HA, termed angiogenic oligosaccharides, stimulate endothelial cell proliferation and promote neovascularization associated with angiogenesis. In addition, angiogenic oligosaccharides up-regulate expression of immediate early response genes, including *c-fos*, *c-jun*, *jun-B*, *Krox-20* and *Krox-24*. High molecular weight HA is a counter player of these breakdown products (22). The receptor that binds HA on the cell surface is CD44, whose cytoplasmic domain is directly linked to the cytoskeleton via ankyrin (23). These...
interactions may play a pivotal role in regulation of cell morphology and gene expression. Furthermore, CD44 is expressed in various alternatively spliced forms, each of which exhibits specific binding properties with regard to HA (24–26). For example, specific CD44 splice variants are expressed in prostate and breast cancer cell lines, suggestive of a specific influence on development and metastasis of these tumours (23).

Notably, HA plays a unique role in the brain extracellular matrix. While matrix proteins common in other human tissues are almost absent in brain, HA and HA-binding lectins constitute the brain extracellular matrix. This specific composition is thought to play a pivotal role in the resistance of brain tissue against invasion by tumours of non-neuronal origin (27). In meningiomas HA expression is predominantly found in the stromal connective tissue but also in perivascular sites involved in neovascularization (28).

In humans, three different members of the HAse protein family have been cloned, including a 48 kDa plasma-derived protein designated HYAL1 (10), a 48 kDa lysosomal variant (HYAL2) and the sperm membrane protein PH-20, which is involved in the fertilization of oocytes (11,12). The gene for HYAL2 has been mapped to human chromosome 3p21. In tumour cells zymographic analysis of HAse activity revealed three proteins of different molecular weights (20). Specifically, a 55 kDa HAse is overexpressed in prostate cancer cells (29), and a dominant band of 65 kDa is described in the vast majority of ovarian and endometrial cancer cells (8). The genes for the latter two proteins bearing HAse activity have not yet been cloned. The newly isolated mgea5 gene is a possible candidate gene. This hypothesis is supported both by the function and the molecular weight of the MGEA protein. The function of MGEA5 and MGEA5s as hyaluronan degrading enzymes was confirmed by two independent assays. The expression of MGEA5 and MGEA5s as fusion proteins revealed two bands of ∼100 and ∼73 kDa by western blot hybridization, indicating an approximate molecular weight of 92 and 54 kDa for MGEA5 and MGEA5s, respectively.

The immune response against MGEA5 in 22% of meningioma patients and the lack of immunity against MGEA5 in healthy persons provides the first evidence for a possible involvement of HAse in meningioma development. This speculation is consistent with the finding that a patient with a recurring meningioma developed an immune response against meningioma-expressed HAse in the course of the disease.

There is at least one report of trisomy 10 in five of 34 meningiomas as determined by CISH analysis (30). Gain of chromosome 10 sequences may impact on expression of mgea5 located at 10q24.1–q24.3 and may be related to the immune response against MGEA5. However, there was no evidence for cytogenetic abnormalities in the meningioma used for library construction.

Autoantibodies against MGEA5 may result from overexpression at the protein level. An elevated level of HAse may in turn cause increased depolymerization of HA molecules in the stroma of meningioma tissue accompanied by induction of angiogenesis and up-regulation of genes involved in the HA–CD44 network. Alternatively, the immune response may be induced by an altered MGEA5 enzyme which may in turn result in over-representation of HA, protecting the tumour cells against immunological attack and facilitating tumour outgrowth. This idea is consistent with data provided by some other groups who detected LOH at chromosome 10 loci preferentially in atypical and malignant meningiomas (31,32). Loss of the long arm of chromosome 10 has recently been described in 44% of grade II and grade III meningiomas (33). Nevertheless, there is no evidence for a correlation between tumour stage and immunoreactivity for MGEA5 in our studies.
Both scenarios imply a severe disruption of the sensitive equilibrium of extracellular matrix composition.

MATERIALS AND METHODS

RNA isolation

RNA isolation was according to the manufacturer’s instructions (Stratagene). Frozen tissue was homogenized, proteins were phenol/chloroform extracted, and RNA was precipitated twice with isopropanol and resuspended in RNase-free water. Integrity and concentration of RNA were evaluated using formaldehyde agarose gels.

In situ hybridization

Clone DNA (10 ng) was labelled with biotin-16-dUTP by nick translation according to the manufacturer’s instructions (Nick Translation System; Gibco BRL). Biotinylated DNA was hybridized against metaphase chromosome spreads of a normal karyotype and visualized using avidin conjugated to fluorescein isothiocyanate. After three rounds of amplification using goat anti-avidin antibodies fluorescent signals were analysed in a Zeiss microscope and documented with the program ISIS3 of MetaSystems.

ZAP Express meningioma expression library

A meningioma expression library was established from a common meningioma type without cytogenetically detectable chromosomal alterations as previously described (9).

Immunoscreening of fusion proteins

Escherichia coli XL1blue MRF’ cells were transfected with the meningioma phage expression library and plated at a density of 10 000 p.f.u./plate on NZCYM agar plates in the presence of 12.5 µg/ml tetracycline. After 4 h incubation at 42°C, fusion protein
expression was induced by applying DuraloseUV membranes (Stratagene) soaked in 10 mM IPTG. Subsequent to a second incubation for 4 h at 37°C, the plates with the filters were stored overnight at 4°C. Membranes were removed, washed twice for 15 min in 1×TBS, 0.5% (v/v) Tween 20 and blocked with 5% dry milk in 1×TBS, for 1 h. Following three additional wash steps of 10 min in 1×TBS the membranes were incubated for 3.5–4 h in 1:100 diluted autologous serum preabsorbed for E.coli and phage proteins as described previously (9). Membranes were washed three times for 10 min in 1×TBS and incubated with goat anti-human IgG Fcγ antibody conjugated to alkaline phosphatase (Dianova) for 1 h. Antigen–antibody complexes were detected by 0.005% (w/v) BCIP prediluted in 100% (v/v) DMF and 0.01% (w/v) NBT prediluted in 70% (v/v) DMF in 1× color developing solution.

**PCR**

PCR was carried out in a thermal cycler (PTC100, MJ Research) for 26–28 cycles. Initial denaturation was at 94°C for 5 min, cycling was with 1 min denaturation at 94°C, 30–60 s annealing at 58°C and 30–60 s extension at 72°C depending on product length.

**RT–PCR**

RNA was treated with 20 U DNase I per 2 µg RNA for 15 min. Absence of DNA was evaluated by Alu–PCR using A1S primer. Reverse transcription was performed using either oligo(dT) or random primers for 1 h at 37°C with MMLV reverse transcriptase (Stratagene). PCR was performed as described above.

**Sequencing**

Sequencing was performed according to the manufacturer’s instructions using the Perkin Elmer ABI Prism Cycle Sequencing kit. Clone inserts were sequenced with an automated sequencer (373A DNA sequencer; Applied Biosystems). Sequence alignment was with the BLASTN and BLASTX algorithms.

**Western blot analysis**

Single colonies of E.coli XLOLR cells containing pBKCMV phagemids with cDNA inserts were grown overnight in LB medium with 50 µg/ml kanamycin at 37°C, and subsequently diluted 1:20 in fresh LB medium (50 µg/ml kanamycin) to a final volume of 100 ml. Fusion protein expression was induced with IPTG at a final concentration of 10 mM at the time when the cultures reached an OD600 of 0.6. Protein expression was allowed to proceed for 30–300 min, and bacterial cells were then harvested by centrifugation at 3000 r.p.m. for 10 min. Bacterial pellets were frozen at −70°C. Subsequently the bacterial pellets were sonicated in Laemmli buffer (5 × 10 s). Cell extracts were separated by electrophoresis on 10% SDS–polyacrylamide gels at 25 mA for 3 h. The proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by electroblotting at 330 mA at 14°C for 75 min. The filters were blocked with 5% dry milk in 1×PBS and hybridized with patient serum at a dilution of 1:100 overnight at 4°C. After washing three times for 10 min in 1×PBS, the blots were incubated in secondary goat anti-human IgG Fcγ antibody conjugated to horseradish peroxidase (Dianova) diluted 1:16 000 in 1× PBST, 5% dry milk for 1 h at room temperature. Detection was carried out by ECL with detection reagents (Amersham) according to the manufacturer’s instructions.
Figure 10. Western blot analysis using recombinantly expressed MGEA5s protein (clone 33-20). (A) Western blot stained with autologous patient serum (H4, left) and serum of meningioma patient H15 (right). NR, bacterial cell extract containing non-recombinant pBKCMV vector as negative control; 5s, cell extract of MGEA5s-expressing bacterial culture. The arrow indicates the size of recombinant MGEA5s protein at ~75 kDa. (B) Western blot of the same bacterial extracts probed with the sera of three different healthy individuals (102, 103 and 107). No antibodies against MGEA5s were detected. (C) Western blot of a patient serum (H46) with a recurrent meningioma. Sera were collected at the time of the tumour surgery. The serum collected at surgery of the first recurrent meningioma contained no antibodies against MGEA5s, as did the serum collected at surgery of the primary meningioma (not shown). In contrast, sera from the third and fourth surgeries were positive for MGEA5s (arrow).

**Phylogenetic tree**

The phylogenetic tree was constructed using amino acid sequences of proteins with HAse activity deposited in GenBank. Sequences were subjected to a multiple alignment using the Clustal W 1.5 program. The output file was produced in a PHYLIP (Phylogeny Inference Package v.3.5) compatible format. The multiple alignment output file was then used to compute a distance matrix employing the PROTDIST program of the PHYLIP package. This matrix was converted into a phylogenetic tree by the NEIGHBOR module of PHYLIP.

**Northern blot**

Northern blot analysis was performed according to the instruction manual of the Northern Max Kit (Ambion). In brief, total RNA samples (5 µg) and 3 µg RNA ladder (Gibco BRL) were each mixed with 3 vols northern sample loading dye and incubated for 15 min at 65°C to melt secondary structures. Electrophoresis was performed at 5 V/cm on 1% agarose RNA gels prepared with 1x denaturing gel buffer included in the kit. The lane containing the molecular weight marker was subsequently cut off and stained in 1x gel running buffer containing 0.5 µg/ml ethidium bromide. Capillary transfer to the BrightStar-Plus membrane was performed by downward transfer according to the manufacturer's instructions. Transfer was allowed to proceed for 1 h using a 6 mm thick gel. The membrane was briefly rinsed in 1x gel running buffer and crosslinked in a UV crosslinker. The membranes were stored at -20°C. Normal tissue and fetal tissue northern blots containing 2 µg/lane poly(A) mRNA were purchased from Clontech Laboratories. Prehybridization and hybridization were carried out with prehybridization/hybridization buffer at 65°C. Hybridization using the 32P-labelled antisense RNA probe was carried out overnight at 65°C. The membranes were washed for twice for 5 min with wash solution 1 at room temperature, once for 15 min with wash solution 2 at 65°C and once for 2-15 min with wash solution 2 at 65°C according to the manufacturer's instructions. Autoradiography was performed using Kodak BioMax TransScreen-HE intensifying screens.

**In vitro transcription**

To generate a DNA template for *in vitro* transcription, we utilized a two-step PCR strategy to add a T7 phage promoter to the 3’-end
Figure 11. Functional assay to demonstrate hyaluronidase activity of MGEA5/MGEA5s protein. (A) Gel matrix assay for hyaluronidase activity. Hyaluronic acid (HA) was co-polymerized within a native polyacrylamide gel. Cell extracts of MGEA5-expressing and non-recombinant bacteria (NR) were separated on the matrix gel together with purified bovine hyaluronidase (bHAse) as a positive control. The gel was washed and incubated for 16 h in HAse buffer at 37°C. The HA matrix was stained with alcian blue and destained with 3% acetic acid. Brackets indicate cleared areas indicative of enzymatic digestion of HA. (B) Turbidometric microtitre plate assay for hyaluronidase activity. HA–BSA–agarose plugs in microtitre plate cavities were overlaid with 15 and 30 µl extracts of bacterial cultures expressing MGEA5 and MGEA5s, respectively, as well as extracts of non-recombinant control cells. The agarose plugs were kept at different pH values for 20 h at 37°C. The microtitre plate was washed with PBS and residual HA was precipitated using 3% acetic acid. Optical density (OD) was measured at 595 nm. The relative amounts of enzymatically depolymerized HA were determined by comparing the OD values from non-recombinant extracts with the OD values of the MGEA5/MGEA5s-expressing cultures.

of the PCR product. In the first step a downstream antisense PCR primer including a linker sequence (in bold) at its 5′-end (mgea5-IT, 5′-GGA GGT GGA GGT AAG GAA GGT AGA ATA GAT-3′) was used together with a sense upstream primer (mgea5.for, 5′-ATG GGA CTC CTT TAG TTG CAG C-3′) for amplification of a 482 bp product. PCR was for 10 cycles at 61°C annealing temperature, 20 cycles at 54°C annealing temperature with Taq buffer containing 1.5 mM MgCl₂ and 2% DMSO. Aliquot of 1 µl of the 50 µl PCR reactions were diluted 1:100 and 1 µl of the dilution was used for the second amplification. A universal promoter primer composed of the T7 promoter consensus sequence (italic) and a linker sequence (bold) (uni-T7-promoter primer, 5′-GCC AGC TCT AAT ACG ACT CAC TAT AGG GAG GTG GAG-3′) was utilized in the second PCR with the mgea5.for primer to generate the template for in vitro transcription. PCR was for 10 cycles at 58°C annealing
temperature, 20 cycles at 55°C annealing temperature with 1.5 mM MgCl₂ and 2% DMSO. PCR reactions were quantitatively loaded on a 1.5% low melting point agarose gel and stained with ethidium bromide after electrophoresis. PCR fragments were isolated with the Nucleotrap Extraction Kit (Macherey-Nagel).

*In vitro* transcription was performed using the MAXIscript Kit (Ambion) according to the manufacturer’s instructions. In brief, 150 ng purified template was used in the transcription reaction with 20 U T7 RNA polymerase, 0.5 mM each ATP, UTP and GTP, 6.25 μM [32P]CTP and 6.25 μM unlabelled CTP in a final volume of 20 μl. The reaction was allowed to proceed for 1 h at room temperature. The sample was heat denatured at 95°C for 2 min and incubated with 1 μl DNase I (2 U/μl) for 15 min at 37°C. Prior to hybridization unincorporated nucleotides were removed from the radiolabelled RNA probe with NucTrop push columns (Stratagene).

**Gel Matrix assay for hyaluronidase activity**

A sample of 0.17 mg/ml HA from human umbilical cord (Sigma) was completely dissolved in sterile water and co-polymerized in a native polyacrylamide gel. Proteins of bacterial cell extracts were separated in the gel at 20 mA, 4°C for 3 h. The gel was washed twice in hyaluronidase buffer (100 mM NaCOOH, 100 mM NaCl, pH 4.5) and incubated for 16 h in the same buffer at 37°C to allow for enzymatic digestion of HA. The gel was washed three times for 15 min in 25% (v/v) EtOH, 10% (v/v) acetic acid prior to staining with alcian blue solution (25% v/v methanol, 10% v/v acetic acid, 0.5% w/v alcian blue).

**Microtitre plate assay for hyaluronidase activity**

Hyaluronic acid forms a white precipitate with BSA in the presence of 3% acetic acid. To determine the HAase activity quantitatively, we developed a microtitre plate assay. An aliquot of 0.1 g agarose was melted in 10 ml phosphate buffer at pH values of 4.5, 5.0, 6.0 and 7.0 and subsequently cooled to 55°C. Two separate stock solutions of 4 mg/ml HA and 5% (w/v) BSA in phosphate buffer of the same pH values were prepared at 55°C. Samples of 1.5 ml agarose solution were combined with 0.5 ml each HA solution and BSA solution and 100 μl aliquots were placed in microplate wells.

The agarose plugs were overlaid with the protein extracts and incubated at 37°C for 20 h. After washing the wells twice with 200 μl 1x PBS, the plate was developed with 3% (v/v) acetic acid for 15 min. The optical density was determined in a microplate reader at 595 nm.

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